ISSN 1677-5538

INTERNATIONAL BRAZ J UROL

OFFICIAL JOURNAL OF THE BRAZILIAN SOCIETY OF UROLOGY
VOLUME 41, NUMBER 5, SEPTEMBER - OCTOBER, 2015

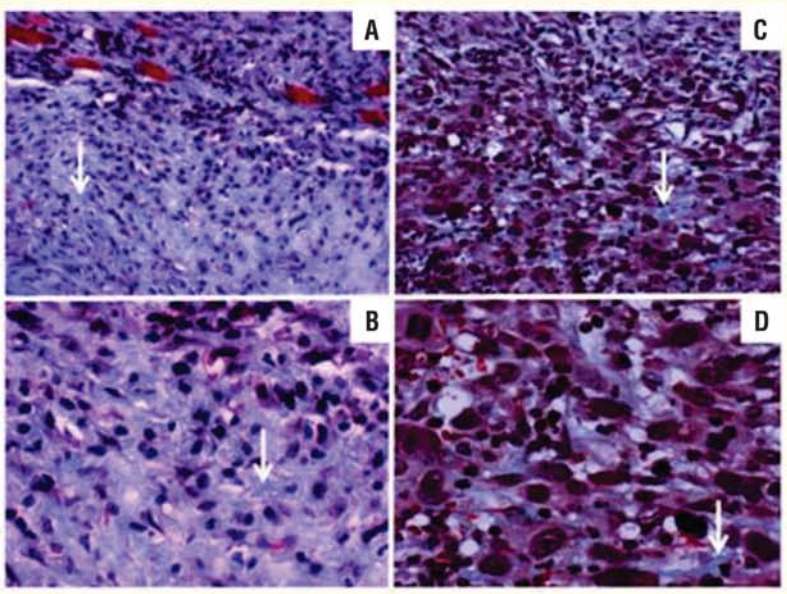

Figure 1 - Histological slices representative of PC-3 prostatic tumors stained with Masson's trichrome. Collagen fibers are blue, as signaled by arrows. Tumors treated with meclofenamic acid (A: X200 and B: X400) present with fibrosis (blue color) clearly covering a large portion of the tumor, whereas tumors treated with PBS (controls) only present with isolated collagen fibers (C: X200 and D: X400). Clear differences in cellularity and nuclear polymorphism are also observed. (Page 1004)

XXXV Brazilian Congress of Urology
October 31 - November 4, 2015 - Rio de Janeiro - RJ - Brazil

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The paper on which the International Braz J Urol is printed meets the requirements of ANSI/NISO Z39, 48-1992 (Permanence of Paper). Printed on acid-free paper.
The International Braz J Urol is partially supported by the Ministry of Science and Technology. National Council for Scientific and Technological Development.

Editorial and Graphic Composition
DRQ Gráfica e Editora Ltd.

The International Braz J Urol, ISSN: 1677-5538 (printed version) and ISSN: 1677-6119 (electronic version) is the Official Journal of the Brazilian Society of Urology–SBU, has a circulation of 6,000 copies per issue and is published 6 times a year (bimonthly, starting in January - February).
The issue date is up to 2 weeks after the month of issuefor the hard copy and up to 1 week after the month of issue for the electronic version. Intellectual Property: All content of the journal, except where identified, is licensed under a Creative Commons attribution-type BY-NC.

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doi: 10.1590/S1677-5538.IBJU.2015.05.01

Prostate Biopsy: What is the future of this old Procedure? The Multiparametric Magnetic Resonance Imaging MRI/TRUS fusion prostate biopsy

The September-October 2015 issue of the International Braz J Urol presents original contributions with a lot of interesting papers in different fields: Infertility, Kidney Stones, Pediatric Urology, Uro-Gynecology, BPH, Prostate Cancer, Renal Cancer, Sexual Dysfunction and basic research. The papers come from many different countries such as Brazil, USA, Italy, Korea, Thailand, Turkey, Iran, Mexico, Spain and India as usual the editor´s comment highlights some papers. We decided to comment about prostate biopsy, because we had 4 papers about this topic in this issue.

Doctor Pepe and collegues, from Italy performed on page 844 an interesting study about the anterior prostate biopsy. They studied 400 patients with negative digital rectal examination and PSA values > 10 ng/mL and PSA between 4.1-10 or 2.6-4 ng/mL with free/total PSA ≤ 25% and ≤ 20%, respectively. They concluded that Anterior zone (AZ) biopsies increased detection rate for prostate cancer (PCa) (10% of the cases), the majority of AZ PCa with histological findings predictive of clinically significant cancer were found at repeat biopsy (about 70% of the cases).

Doctor Temiz and collegues, from Turkey performed on page 859 an elegant study about local anesthesia and the detection rates of prostate cancer in Transrectal prostate biopsy. The authors studied 422 patients underwent 10 core-TRUS-Bx because of elevated serum prostate specific antigen (PSA) level (>2.5ng/mL) and/or suspicious digital rectal examination findings. Patients were divided into two groups according to the applied anesthesia technique: intrarectal lidocaine gel anesthesia (IRLA) and periprostatic nerve blockade (PNB) techniques. They concluded that PNB is superior to IRLA in terms of cancer detection rates.

Doctor Ucer and collegues, from Turkey performed on page 864 a study about PSA-Age volume score in predicting positive prostate biopsy findings. The authors performed 4717 prostate biopsies. PSA-age volume was calculated by multiplying the patient's age by the prostate volume and dividing it by the PSA level. Sensitivities and specificities of the PSA-AV were assessed by retrospective analysis of findings from 4,717 prostate biopsies. The authors concluded that the sensitivity and specificity of a PSA-AV of 700 for predicting positive biopsy findings were similar to a PSA of 4ng/mL. They suggested the PSA-AV cut-off of 700 should only be used in patients younger than 60 with low prostate volumes (<20cm3).

Doctor Bulut and collegues, from Turkey performed on page 906 an interesting study about the efficacy of duration of prophylactic antibiotics in transrectal

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ultrasound guided prostate biopsy. The authors studied 367 patients undergoing a prostate biopsy and divided into 2 groups according to prophilaxy: oral ciprofloxacin (750 mg every 12 hours) for 3 or more days in Group-1 and single day in Group-2 and concluded that in a selected patient population single dose prophylaxis with ciprofloxacin can be safely administered compared to other regimens of 3 or more days. Increasing the duration of antibiotic prophylaxis does not decrease infectious complications.

The 4 papers are very interesting but what is the future of the prostate biopsy? The answer is Multiparametric Magnetic Resonance Imaging MRI/TRUS fusion prostate biopsy.

Multiparametric Magnetic Resonance Imaging (MRI) is emerging as a powerful test to diagnose and stage prostate cancer (PCa) (1). Prostate Imaging Reporting and Data System version 2 (PI-RADSv2) correctly identified 95 % of PCa foci ≥ 0.5 mL, but the PI-RADSv2 was limited for the assessment of GS ≥ 4+3 tumours ≤ 0.5 mL (2). Quantitative MRI parameters can predict malignant histology on MRI/TRUS fusion prostate biopsy, which is a valuable technique to ensure adequate sampling of MRI-visible suspicious lesions under TRUS guidance and may impact patient management (3).

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Luciano A. Favorito

Professor Associado da Unidade Urogenital, Universidade Estatual do Rio de Janeiro, Urologista de Lagoa Hospital Federal, Editor associado do International Braz J Urol

DIFFERENCE OF OPINION

Vol. 41 (5): 830-831, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2015.05.02

Is There a Space to Improve the Treatment of Erectile Dysfunction in the Next Years?

Opinion: YES

Stanley E. Althof

Executive Director, Center for Marital and Sexual Health of South Florida
Professor Emeritus, Case Western Reserve University School of Medicine

Keywords: Erectile Dysfunction; Therapeutics; PDE-5 Inhibitors

Prior to the US approval of sildenafil in 1998, the available treatment options for erectile dyfunction (ED) included: intraurethral alprostadil (MUSE), intracavernosal injections (tri mix, alprostadil), vacuum pump therapy, placement of a penile prosthesis, hormone replacement therapy and individual or couples psychotherapy (1-7). The approval of sildenafil, a phosphodiesterase type 5 inhibitor (PDE5i), dramatically changed the treatment and research landscape. By utilizing sildenafil millions of men with ED could reliably and safely restore their erectile function. In addition to sildenafil, three other PDE5i medications have been approved in the US for the treatment of ED; they are: tadalafil (daily and as needed), vardenafil and avanafil.

While the PDE5i medications are successful in restoring erectile function in the majority of men, they are not as effective in men whose cavernous nerve has been damaged from a radical prostatectomy or in men with diabetes mellitus. Additionally, some men may not respond to PDE5i's because their vascular disease is too severe, they take concomitant medications that interfere with ED restoration, or they harbor severe psychological and/or interpersonal issues that overwhelm the prosexual effect of the drug. For men who utilize nitrate medications, PDE5i's are contraindicated because of their synergistic hypotensive effects, therefore these men must find other treatment options. For all the above reasons clinicians would welcome new agents that could overcome the limitations of the current PDE5i drugs.

I am certain that we will see new and better options for men suffering from ED. The introduction of the PDE5i's drugs revolutionized the manner in which we currently treat ED patients and opened the pathway for further research into the biological underpinnings of ED. One important limitation of the PED5i's is that they only provide short--term solutions to the chronic vascular issues that cause the ED. Treatments that would cure or reverse the underlying precipitating and maintaining factors would move us beyond the current standard of care.

Stem cell therapy is an exciting new treatment option that in theory offers the potential to reverse the underlying causes of ED and reduce patient reliance on the tran-

sient effects of the PDE5i drugs. It is also targeted at men with cavernous nerve injury or diabetic men whose response to PDE5i drugs is suboptimal.

REVIEW OF STEM CELL THERAPY FOR ED

Stem cell therapy was initially based on the theoretical rationale that stem cells can differentiate into a range of cell types such as endothelial, smooth muscle, Schwann cells, and neurons8. Stem cells were delivered via intracorporal penile injections to replenish the depleted endothelial cells and/or caveronous smooth muscle cells. A different theoretical understanding is that stem cell therapy results in the host's regeneration, as opposed to simply replenishment, of endothelial and cavernous smooth muscle cells and is able to restore the interactions between these structures (8).

The vast majority of published studies focus on animal models with only one study in humans and one ongoing clinical trial in humans. Lin reports that intracavernous injected stem cells can escape the penis and hone into the bone marrow possibly accounting for systemic antidiabetic effects and prolonged restoration of erectile function (8).

CONCLUSION

New and promising therapies for ED continue to evolve. The PDE5i's significantly advanced our understanding and ability to treat men suffering from ED. However, stem cell therapy may become the next generation of ED treatment offering the field of sexual medicine and our patients new possibilities. While it will take time to conduct the necessary human trials and obtain regulatory and ethical approvals, stem cell therapy may move us into the next wave of treatment options for ED.

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Stanley E. Althof
Executive Director, Center for Marital and Sexual Health of South Florida
Professor Emeritus, Case Western Reserve University School of Medicine
1515 North Flagler Drive, Suite 540
West Palm Beach, Florida 33401
Fax: + 1 561 822-5458
E-mail: sxa6@case.edu

DIFFERENCE OF OPINION

Vol. 41 (5): 832-834, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2015.05.03

Is There a Space to Improve the Treatment of Erectile Dysfunction in the Next Years?

Opinion: No. Ten reasons that there will be no new pharmacologic therapies for erectile dysfunctionin the foreseeable future

Ira D. Sharlip

University of California San Francisco School of Medicine, San Francisco, CA.

Keywords: Erectile Dysfunction; Therapeutics; PDE-5 Inhibitors

BACKGROUND

From the 1960s to the 1990s, breakthrough developments in sexual medicine occurred about once every ten years. In the decade of the 1960s, there was the launch of birth control pills for women and the resulting sexual revolution. This was followed by penile implant surgery in the 1970s, intracavernous injection therapy in the 1980s and oral therapy with phosphodiesterase type 5 (PDE5) inhibitors in the late 1990s. Landmark developments since then have been the approval in 2013 of collagenase clostridium histolyticum for intralesional treatment of Peyronie's disease and the very recent approval in 2015 of flibanserin for the treatment of hypoactive sexual desire in pre-menopausal women. However, no landmark development has occurred for the treatment of erectile dysfunction since the introduction of PDE5 inhibitors starting in 1998. Moreover, there are no clinically relevant innovations on the horizon for erectile dysfunction. It is likely that current PDE5 inhibitors and intracavernous agents will be the last pharmacologic therapies for erectile dysfunction in the foreseeable future. There are ten reasons for this.

1. Absence of innovative therapies using new molecular targets

The first reason is that no innovative therapy using a molecular target other than PDE5 has reached any stage of clinical application. Since the molecular biology and physiology of erectile function and dysfunction have been clearly defined, there has been plenty of time for development of strategies to treat erectile dysfunction other than PDE5 inhibition, yet no new therapeutic options have emerged. Among other therapeutic targets, perhaps the most promising has been the Rho kinase system. While this system seemed to hold some potential for treatment of erectile dysfunction, it has not borne fruit for clinical development. Moreover, there are no other innovative treatment options on the clinical horizon.

2. Saturation of PDE5 inhibitor market

At this point, the market for PDE5 inhibitors is saturated with widely known and recognized products. Further development of PDE5 inhibitors is unlikely to occur because of this saturation. Currently there four PDE5 inhibitors which are available on many continents, two others which are available only in Asia and one other in Brazil. Two of these PDE5 inhibitors, sildenafil and tadalfil, control the great majority of the worldwide market for oral therapy of erectile dysfunction. Each of the other five products its own unique characteristics but, in reality, they do not differ greatly from the market leaders in their clinical effects. Unless a new PDE5 inhibitor can deliver distinctly advantageous characteristics, no new oral therapy for erectile dysfunction based on PDE5 inhibition will enter this saturated market.

3. High degree of clinical efficacy of PDE5 inhibitors

The third reason that PDE5 inhibition is likely the last oral therapy for erectile dysfunction is the clinical efficacy of PDE5 inhibitors. While currently available PDE5 inhibitors are effective for about two-thirds of men with erectile dysfunction, it is unlikely that any new PDE5 inhibitor or any other oral therapy for ED will successfully treat the one-third of men who have failed PDE5 inhibitor therapy. This is because most of the failures occur in men who have either (1) advanced fibrosis of the corpora cavernosa, for which no oral therapy can deliver sufficient PDE5 inhibitor serum levels to be effective, or (2) psychogenic problems, for which oral PDE5 inhibition or intracavernous therapy are not the therapies of choice.

4. High cost of drug development

The fourth reason that there will not be new drug therapy for erectile dysfunction is the huge cost in the United States to develop a new drug, which now approaches one billion US dollars. With entrenched, successful , safe and proven PDE5 inhibitors already in place and the PDE5 inhibitor market saturated, the possibility of recouping the cost of drug development is small to non-existent.

5. Limited size of erectile dysfunction market

The fifth factor inhibiting development of innovative therapies is the unexpectedly limited size of the market for treatment of ED. When Viagra was first launched in 1998, clinicians and pharmaceutical executives expected that all men with ED would desire treatment. Using epidemiologic evidence from the 1990s, it was estimated that there were about 30 million men in the United States alone and at least 150 million worldwide who had erectile dysfunction. It was expected that most if not all of them would want to use Viagra. While the estimates of the number of men who have erectile dysfunction are probably correct, the concept that all men with erectile dysfunction want treatment turned out not to be true. Clinical experience over the last 17 years since Viagra was launched in 1998 has shown that perhaps only one-third of men with erectile dysfunction use PDE5 inhibitors for treatment. Men who have erectile dysfunction are often curious and may try a PDE5 inhibitor once or twice but the majority of men with erectile dysfunction eschew ongoing treatment because of decreased interest in sex, absence of an interested sexual partner, contraindications due to medical co-morbidities, cost of treatment and/or embarrassment at having to identify themselves as having erectile dysfunction in order to obtain and fill a prescription.

6. Limited prospects for increase in market size

A corollary to the limited size of the PDE5 inhibitor market is that there are no factors which indicate that the market size will expand in a significant way. After fifteen-plus years of availability of PDE5 inhibitor therapy, large pharmaceutical companies have had extensive experience with advertising and publicity for PDE5 inhibitors. There appear to be no new techniques which promise to increase PDE5 inhibitor sales in developed regions of the world. In developing regions, widespread poverty, the cost of treatment and the need to allocate medical assets to life-threatening conditions put market expansion out of reach.

7. Impending transition of Viagra to generic status

The seventh factor is the end of patent protection and the impending transition of Viagra and perhaps other PDE5 inhibtors to generic status. When this happens, it is likely that the cost of sildenafil and possibly other PDE5 inhibitors to the consumer will fall. This means that if a new drug for erectile dysfunction were to be developed, it would have to compete with the reduced cost of generic PDE5 inhibitors. This will be a powerful inhibitor of new drug development. The only way a new drug might be able to compete successfully with generic sildenafil or other generic PDE5 inhibitors is to provide a unique characteristic such as very rapid onset, much stronger effect, reduced side effects or significantly less cost compared to generic sildenafil. These problems are likely to eliminate development of a new competitor for generic PDE5 inhibitors.

8. Possible transition of PDE5 inhibitors to over-the-counter status

The eigth reason that a new drug for erectile dysfunction will not be developed is the possibility that one or more of the current PDE5 inhibitors will become over-the-counter products not requiring a prescription in the next few years. Lack of anonymity in purchasing prescription drugs is a very strong inhibitor of PDE5 inhibitor purchase. Many men are too embarrassed to go to a doctor and/or to their local pharmacy and be revealed as a man with erectile dysfunction. An over-the-counter PDE5 inhibitor might be sold online or can be packaged in an anonymous method for presentation at the point of purchase, eliminating the steps of a costly visit to a doctor and submission of a prescription to a pharmacy. New therapies will have to be prescription drugs while the availability of generic and/or over-the counter PDE5 inhibitors will inhibit sales of prescription drugs.

9. Limited market size for intracavernous injection therapy

These same arguments can be advanced for development of new intracavernous agents for treatment of erectile dysfunction. Papaverine, phentolamine and especially prostaglandin E1 are sufficiently effective that new intracavernous agents are unnecessary. The best possibilities for success of a new intracavernous agent would be a drug that would be signficantly less expensive than current products and/or a drug that would not need to be refrigerated. However, the size of the market for intracavernous drugs is small enough that there is little incentive for development of a new intracavernous agent.

10. Cost of compounded intracavernous injections is much less than cost of PDE5 inhibitors

An additional inhibitor to new oral drug development in the United States is the relatively low cost of intracavernous injection agents such as prostaglandin E1, phentolamine and/or papaverine that can be obtained from compounding pharmacies. Lesser expensive drugs such as these compounded agents provide another element of price competition which will discourage prospective developers of new oral or intracavernous agents for the treatment of erectile dysfunction.

SUMMARY

In summary, the process of developing a new oral or intracavernous agent to treat erectile dysfunction is fraught with such overwhelming impediments that it is very unlikely, if not impossible, for a new drug to emerge in the foreseeable future. What is available now is the end of the line for pharmacologic treatment of erectile dysfunction.

Ira D. Sharlip, MD
Department of Urology
University of California San Francisco School of Medicine
San Francisco, CA 94143-0916, USA
E-mail: isharlip@aol.com

REVIEW ARTICLE

Vol. 41 (5): 835-843, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0651

Current management and future directions in the treatment of advanced renal cell carcinoma–a latin american perspective: 10 years in review

Oren Smaletz [1]

[1] *Departamento de Oncologia, Hospital Israelita Albert Einstein, São Paulo, Brasil*

ABSTRACT

The worldwide incidence of kidney cancer is estimated at 337,860 new cases per year in the International Agency for Research on Cancer's GLOBOCAN 2012 update, with an estimated 143,369 deaths annually. Over the past 10 years, there have been significant advances in the treatment of advanced/metastatic renal cell carcinoma, including the development of targeted therapies. Currently recommended first-line treatments include sunitinib, temsirolimus, bevacizumab plus interferon, and pazopanib, or high-dose interleukin-2 or sorafenib for selected patients. Recommended second-line treatments include all of the above agents, as well as everolimus and axitinib. Unfortunately, combination therapies have generally resulted in increased toxicity and little improvement in efficacy. Recent studies focused on identification of predictive biomarkers for responses to specific targeted therapies and have not been successful to date. Despite recent advances in targeted treatment for metastatic renal cell carcinoma, important questions regarding biomarkers of efficacy, and optimal combination and sequencing of agents remain to be answered. This paper reviews literature concerned with first-and second-line treatment of metastatic renal cell carcinoma and will discuss key issues in Latin America.

ARTICLE INFO

Key words:
Kidney Neoplasms; Molecular Targeted Therapy; Angiogenesis Inhibitors; Vascular Endothelial Growth Factors

Int Braz J Urol. 2015; 41: 835-43

Submitted for publication:
December 11, 2014

Accepted after revision:
March 19, 2015

INTRODUCTION

Renal cell carcinoma (RCC) arises primarily from the proximal tubular epithelium and accounts for ~85% of all kidney cancers, with the remainder consisting of renal pelvis cancer and other rare malignancies (1). Many RCCs are asymptomatic and cannot be diagnosed until relatively late in the course of the disease. It has been estimated that more than 50% of RCCs are detected incidentally as a result of imaging tests carried out for other reasons, and that 25–30% of all patients with RCC are initially diagnosed due to symptoms of metastases (2, 3).

The age-standardized rates (ASRs) for kidney cancer incidence are similar in Latin America and the Caribbean (estimated ASR 3.5 per 100,000 population) to those in North America and Europe (ASR 3.6 and 3.3, respectively), while the ASR for mortality is slightly lower in Latin America and the Caribbean (estimated ASR 1.8 per 100,000 population) compared with that in North America and Europe (ASR 2.4 and 2.8, respectively) (4).

Due to the late stage at which many RCC patients are diagnosed, survival is often poor. The estimated average 5-year survival rate in the US is 91.7% for patients with localized disease, but only 12.3% for those diagnosed with distant metastases (5).

Patient risk assessment and prognosis

In the cytokine treatment era, investigators at the Memorial Sloan-Kettering Cancer Center (MSKCC) developed a model for dividing patients with advanced disease in low-, intermediate-, and high-risk categories (6). Patients were assigned to one of three groups: those with zero risk factors (favorable risk), with one/two (intermediate risk), and with three or more (poor risk). Median overall survival (OS) for patients in these groups was 30, 14, and 5 months, respectively (6). Assessment of prognostic factors in patients with metastatic RCC (mRCC) treated with anti-vascular endothelial growth factor (VEGF) therapies led to a slightly different model (7), known as the International Metastatic Renal Cell Carcinoma Database Consortium model, in which neutrophilia and thrombocytosis are also considered independent prognostic factors, and has been recently validated; patients in the favorable, intermediate, and poor-risk groups had a median OS of 43.2, 22.5, and 7.8 months, respectively (8).

Biomarkers

Despite considerable research, there are currently no validated biomarkers for use in the clinical management of mRCC, and only histology, staging, and clinical/laboratory characteristics can guide physicians in defining therapy and predicting patients' outcomes. Nevertheless, biomarkers related to the VHL tumor suppressor gene, hypoxia-inducible factor (HIF), tumor-promoting genes responsive to HIF (e.g. those for VEGF, platelet-derived growth factor [PDGF], cyclin D1, glucose transporter 1), the mammalian target of rapamycin kinase (mTOR) pathway, the tumor suppressor gene phosphatase and tensin homolog, Akt, and phosphorylated S6K are all being explored (9). Single nucleotide polymorphism (SNP) genotyping is also being employed to identify significant polymorphisms in RCC-related genes related to prognosis; results to date suggest that polymorphisms in the interleukin (IL)-4 and VEGF genes are correlated with prognosis (9). A number of other biomarkers have shown prognostic value in clinical studies of targeted therapies for mRCC (10).

Treatment of RCC

This review is focused on patients with advanced or mRCC. Stages I–III kidney cancers are managed with partial or radical nephrectomy, active surveillance or ablative techniques for non-surgical candidates (5). At this time, adjuvant strategies are not validated for the treatment of these stages.

First-line treatment for advanced kidney cancer

Currently, the National Comprehensive Cancer Network (NCCN) guidelines' recommended first-line treatments for metastatic and surgically unresectable RCC, supported by category 1 evidence, including sunitinib, temsirolimus (for poor-prognosis patients), bevacizumab plus IFN-α, and pazopanib (Figure1; 5). Sunitinib and pazopanib are both tyrosine kinase inhibitors; targets of sunitinib include VEGF receptors (VEGFRs), PDGF receptors (PDGFRs), cKIT, and other kinases, while pazopanib also inhibits VEGFR, PDGFR, and cKIT. Bevacizumab is a monoclonal antibody that inhibits VEGF, and temsirolimus is an inhibitor of mTOR. With these agents, median progression-free survival (PFS) ranged from approximately 9 to 11 months in phase III studies, and median OS from 23 to 26 months; both parameters were shorter with temsirolimus, which was investigated in primarily poor-risk patients expected to have shorter survival (Table-1) (12-18).

Despite differences in mechanism of action, the safety profiles of all four treatments share some similarities, with asthenia/fatigue (20-63%), nausea (26-52%), diarrhea (20-63%), and anorexia (22-37%) among the most commonly reported adverse events (AEs) (12, 13, 15, 17, 18). Hematologic toxicities, including leukopenia (37-78%), neutropenia (34-77%), lymphopenia (31-68%), and thrombocytopenia (32-78%), are also common with sunitinib and pazopanib. More unusual AEs include hand-foot syndrome (29-50%) and hypothyroidism (14-24%) reported with sunitinib, bleeding events (33%) with bevacizumab, rash (47%) and pneumonitis with temsirolimus (19), and hypertension with all three VEGF inhibitors (any grade, 26-46%; grade 3/4, 3-15%). With each treatment, most AEs are mild to moderate (grade 1 or 2) and manageable with standard medical intervention or dosing modifications.

Figure 1 - Therapeutic biological pathways for targeted therapies in mRCC (11).4E-BP1=4E binding protein-1; AKT=protein kinase B; FKBP=FK binding protein; eIF-4E=eukaryotic initiation factor-4 subunit E; FGF=fibroblast growth factor; HIF=hypoxia-inducible factor; IL-8=interleukin-8; mLST8=mammalian lethal with SEC13 protein 8; mTORC1=mammalian target of rapamycin complex 1; P70S6K=P70S6 kinase; PDGFR=platelet-derived growth factor receptor; P=phosphorous; PI3K=phosphoinositide 3-kinase; Pro=proline; PTEN=phosphatase and tensin homologue; Ub=Ubiquitin; VEGFR=vascular endothelial growth factor receptor; VHL=Von Hippel-Lindau.

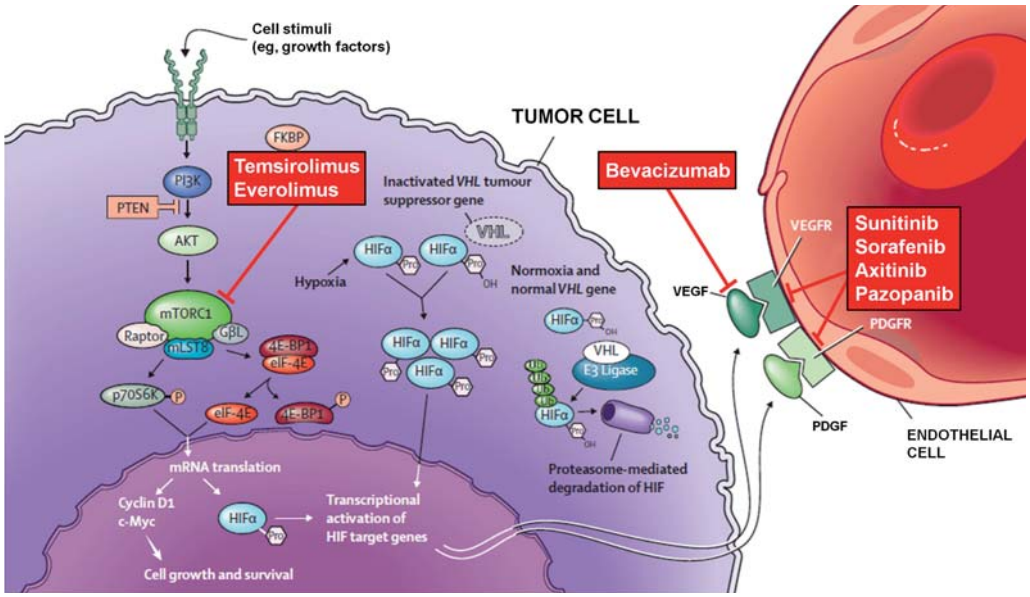

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Table 1 - Efficacy results from phase III studies with NCCN recommended first-line therapies for advanced RCC.

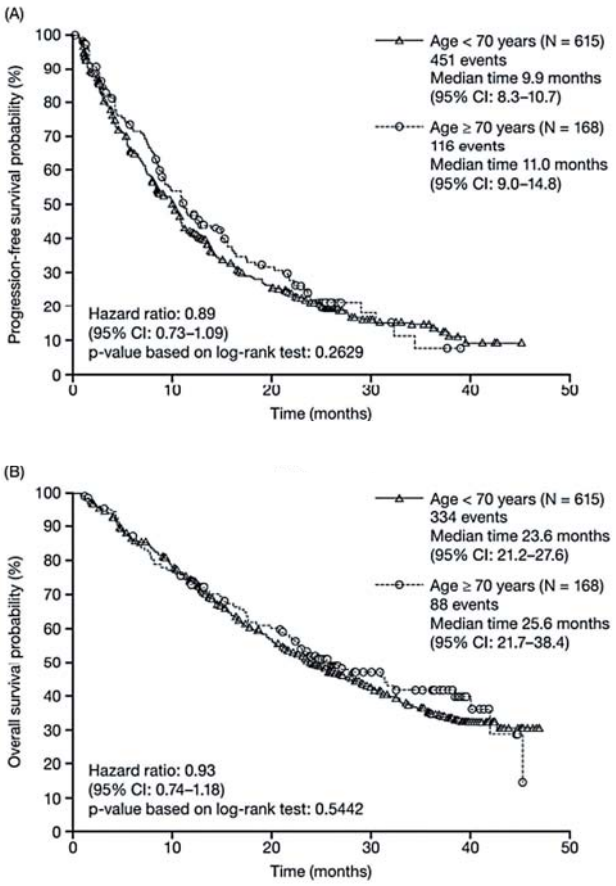
Test agent	Comparator	Progression-free survival				Overall survival			
		Median (months)		HR (95% CI)	p-value	Median (months)		HR (95% CI)	p-value
		Test	Comparator			Test	Comparator		
Sunitinib (12)	IFN-α	11.0	5.0	0.539 (0.451-0.643)	<0.001	26.4	21.8	0.821 (0.673-1.001)	0.051
Bevacizumab+IFN-α (13, 14)	IFN-α+placebo	10.2	5.4	0.63 (0.52-0.75)	0.0001	23.3	21.3	0.91 (0.76-1.10)	0.3360
Pazopanib (15, 16)	Placebo	9.2	4.2	0.46 (0.34-0.62)	<0.0001	22.9	20.5*	0.91 (0.71-1.16)	0.224
Pazopanib (17)	Sunitinib	8.4	9.5	1.05 (0.90-1.22)	NR	28.4†	29.3†	0.91 (0.76-1.08)	0.28
Temsirolimus (18)	IFN-α	5.5	3.1	NR	NR	10.9	7.3	0.73 (0.58-0.92)	0.008

*Overall survival analysis confounded by the early, high rate (54%) of crossover to placebo from pazopanib.
†Interim analysis of overall survival.
CI=confidence interval; **HR=** hazard ratio; **IFN-α=** interferon-α; **NCCN=** National Comprehensive Cancer Network; **NR=** not reported; **RCC=** renal cell carcinoma.

An expanded-access program was established to provide sunitinib to patients with mRCC who were ineligible for ongoing sunitinib clinical trials and/or before regulatory approval in their countries (20, 21). The program included 4564 patients from 246 sites in 52 countries, with 348 patients treated from Latin America. Overall efficacy and tolerability were similar among patients in this broader population to those participating in a phase III pivotal trial. Among Latin American patients, median PFS and OS were 12.1 and 16.9 months, respectively, 17% of patients had an objective response, and the clinical benefit rate (objective response plus stable disease≥3 months) was 57% (22). Responses were seen across all subgroups analyzed, including those with poor performance status, non-clear cell histology, or brain metastases (22). Results from a larger study found no significant effect of age on efficacy in patients receiving first-line or cytokine refractory sunitinib monotherapy for advanced RCC (20). For example, in first-line patients aged<70 and≥70 years, median PFS was 9.9 versus 11.0 months, respectively (hazard ratio (HR), 0.89; 95% confidence interval (CI): 0.73-1.09; p=0.2629) and median OS was 23.6 versus 25.6 months, respectively (HR, 0.93; 95% CI: 0.74-1.18; p=0.5442) (Figure-2) (23).

To date, there are few direct comparisons of the safety and efficacy of currently recommended first-line treatments for advanced RCC. The COMPARZ trial recently showed that pazopanib was non-inferior to sunitinib with respect to PFS, with an HR of 1.05 (95% CI: 0.90-1.22), and OS was similar (HR, 0.91; 95% CI: 0.76-1.08) (Table-1) (17). There were differences in the safety profile in patients treated with sunitinib compared with pazopanib, including a higher incidence of fatigue (63% vs. 55%, respectively), hand-foot syndrome (50% vs. 29%), and thrombocytopenia (78% vs. 41%), but a lower incidence of increased levels of alanine aminotransferase (43% vs. 60%). Similar proportions of patients needed dose interruptions or reductions because of toxicity, or discontinued treatment because of AEs. During the first 6 months of treatment, the mean change from baseline in 11 of 14 health-related quality of life domains favored pazopanib, particularly those related to fatigue or soreness in the mouth, throat, hands or feet (p<0.05 for all 11 comparisons).

Figure 2 - Progression-free survival (A) and overall survival (B) in sunitinib-treated patients by age (<70 vs. ≥70 years) in the first-line setting (23).



There are still unanswered questions related to treatment selection for patients with advanced RCC (see below).

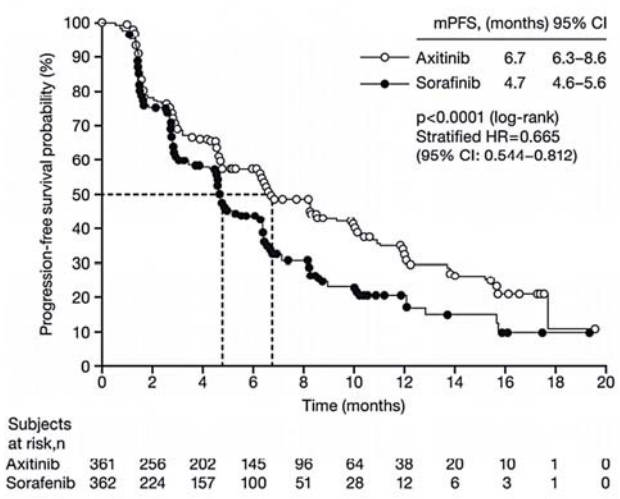
Second-line treatment for advanced RCC

Current recommendations for second-line treatment of advanced RCC following a prior tyrosine kinase inhibitor include everolimus and axitinib, and following prior cytokine therapy include axitinib, sorafenib, sunitinib, and pazopanib (Figure-1; 5). The most recently approved of these agents, axitinib (approved in the US and Europe in 2012 and now also approved in several Latin American countries), is a selective and potent oral inhibitor of VEGFR-1,-2, and-3.

In a phase III study comparing axitinib with sorafenib as second-line treatment in 723 patients

with clear-cell mRCC, median PFS was 6.7 months for axitinib and 4.7 months for sorafenib (p<0.0001) (Figure-3) (24) and the objective response rate (ORR) was 19.4% versus 9.4% (p=0.0001) (24). Updated results showed that OS did not differ between the two groups (median OS 20.1 months with axitinib vs 19.2 months with sorafenib; HR, 0.969; 95% CI: 0.800-1.174; one-sided p=0.3744), but that investigator--assessed PFS remained longer with axitinib (median PFS 8.3 months) than with sorafenib (median PFS 5.7 months) (25). Common AEs occurring more frequently with axitinib than sorafenib were hypertension (40% vs. 29%, all grades), nausea (32% vs. 22%), dysphonia (31% vs. 14%), and hypothyroidism (19% vs. 8%); those occurring more frequently with sorafenib were hand-foot syndrome (27% vs. 51%), rash (13% vs. 32%), and alopecia (4% vs. 32%) (24).

Figure 3 - Progression-free survival with axitinib versus sorafenib as second-line therapy (24).



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To explore the efficacy of temsirolimus after VEGF inhibitor therapy, the INTORSECT trial compared temsirolimus with sorafenib as second--line treatment for patients with disease progression after sunitinib (26). PFS did not differ significantly between treatment arms (HR, 0.87; 95% CI: 0.71-1.07; two-sided p=0.19), but OS favored sorafenib (HR, 1.31; 95% CI: 1.05-1.63; two-sided p=0.01); median OS was 12.3 and 16.6 months in the temsirolimus and sorafenib arms, respectively. Safety data were as expected based on previous trials with each agent.

COMBINATION TREATMENT

Many of the established and emerging treatments for mRCC have similar or overlapping biologic actions and we need more information about how they might influence each other's efficacy and the mechanisms underlying resistance to each (27). At present, it is unclear whether combination therapy aimed at vertical or horizontal inhibition is the best approach to second-line treatment. Vertical inhibition aims to block the same pathway at two points, as attempted by combining bevacizumab with sorafenib (28) or with sunitinib (29). These approaches were associated with improved activity, but also increased toxicity (28, 29). Horizontal inhibition combines agents of different mechanisms of action and non-overlapping toxicities with the goal of an additive or synergistic antitumor effect, as tested in the INTORACT trial which compared the combination of temsirolimus plus bevacizumab with interferon plus bevacizumab as first-line therapy in 791 patients with mRCC (30). Efficacy did not differ significantly between the treatment arms; median PFS was 9.1 and 9.3 months in the temsirolimus and interferon combinations arms, respectively (HR, 1.1; 95% CI: 0.9-1.3; p=0.8), and median OS was 25.8 and 25.5 months, respectively (HR, 1.0; p=0.6). Safety was consistent with the known profiles for the three agents. A recent randomized phase II trial in 361 treatment-naïve patients with advanced RCC compared single-agent bevacizumab with both vertical and horizontal combinations, namely temsirolimus plus bevacizumab, bevacizumab plus sorafenib, or sorafenib plus temsirolimus (31). However, none of the combinations tested were superior to single-agent bevacizumab with respect to PFS, and severe toxicity was increased with combination therapy. At this point, no combination has been shown to be superior to the approved combination of bevacizumab and interferon, and several combinations explored have been limited by excessive toxicity.

CONTINUING EVOLUTION IN THE TREATMENT OF ADVANCED RCC

Predicting treatment response

Identifying the optimum treatment for advanced RCC requires increased understanding of the tumor biology and patient characteristics predictive of response to specific treatments. A retrospective analysis based on pooled efficacy (n=544) and safety (n=4917) data from four clinical trials showed that sunitinib-induced hypertension was associated with significantly improved clinical outcomes (32). For patients with versus without hypertension, median PFS was 12.5 vs. 2.5 months, median OS was 30.9 vs. 7.2 months, and ORR was 54.8% vs. 8.7% (all $p<0.001$). In a similar study, using pooled data from 770 patients, patients who developed hand--foot syndrome had significantly better ORR (66.5% vs. 31.8%), median PFS (14.3 vs. 8.3 months), and median OS (38.2 vs. 18.9 months) than those not developing this AE (all $p<0.0001$) (33). Although confirmation in prospective studies is needed, one or more of these AEs may possibly serve as a predictive biomarker of sunitinib efficacy.

A gene expression profiling study identified a 20-gene signature predicting response to sunitinib with 68.5% accuracy (34), and microRNA profiling showed that 29 microRNAs were differentially expressed in patients with mRCC experiencing early progression on sunitinib (35). Analysis of potential soluble protein biomarkers found that lower angiopoietin-2 and higher matrix metalloproteinase-2 baseline levels were significantly associated with better overall response in patients treated with sunitinib, while higher tumor expression levels of HIF-1-α were associated with longer PFS (36). In patients treated with pazopanib, higher baseline plasma levels (relative to the median) of hepatocyte growth factor (HGF), IL-8, tissue inhibitor of metalloproteinases (TIMP)-1, and osteopontin were associated with shorter PFS (37). In the same study, high concentrations of IL-6 were predictive of improved PFS benefit from pazopanib compared with placebo. Single nucleotide polymorphisms in the genes IL8, FGFR2, NR1I2, and ABCB1 have all been associated with OS in patients with advanced RCC receiving pazopanib monotherapy (38).

THE LATIN AMERICAN PERSPECTIVE

One consideration when using targeted agents to treat Latin American patients with mRCC is their different racial/ethnic mix compared with patients from North America. Results from a survey of 508 patients with RCC in Brazil indicated that 78.9% of patients were white, 6.5% were black, and 14.0% were mixed race (39). In contrast, results from a survey of 27,304 patients with RCC in the United States showed that 69.2% were white, 7.0% were black, 18.1% were Hispanic, and 5.0% were Asian/Pacific Islander (40). These small differences may influence the distribution of prognostic biomarkers as well as treatment efficacy and safety. On the other hand, a subpopulation analysis indicated that the efficacy and safety profile of sunitinib in patients with mRCC from Latin America who participated in a global expanded access program was comparable to that observed in the entire population. For example, in the Latin American and total populations, median PFS was 12.1 and 9.4 months, respectively, and median OS was 16.9 and 18.7 months, respectively (21, 22).

Drug availability is one of the key points to improved survival in patients with mRCC, as has been shown by the impact of post-progression therapy on OS in the clinical trial setting. In the AVOREN phase III trial of bevacizumab plus IFN-α, 63% of patients in the control arm (i.e. those randomized to IFN-α only) received at least one post-protocol therapy, comprising either sunitinib or sorafenib in 37% of cases (14). In this arm, median OS was 21.3 months, which is considerably longer than the median OS of approximately 13 months assumed for a patient population treated with IFN-α (6) when the trial was designed. In addition, a post-hoc exploratory analysis showed that median OS in patients randomized to bevacizumab plus IFN-α who received post-study tyrosine kinase inhibitors was 38.6 months, compared with a median OS of 23.3 months in the same treatment arm of the intent-to-treat population (14). The CALGB phase III study of bevacizumab plus IFN-α also showed that median OS was greater for patients receiving further treatment after stopping trial therapy than for those receiving no subsequent therapy, regardless of treatment arm (28.2 months

vs. 10.2 months) (41); however, in both arms patients receiving subsequent therapy had more favorable baseline prognostic features than those who were untreated.

These data show the positive impact of targeted therapy on survival in patients with advanced RCC. Nonetheless, with the exception of temsirolimus in poor risk patients, median OS, unlike PFS, was not significantly increased with targeted therapies compared with standard therapies or placebo in the phase III trials of first-line therapy (Table-1); however, as noted above, this is potentially due to post-protocol therapy received by patients in both treatment arms and, as discussed elsewhere (42), the confounding effect of crossover trial design. In addition, in some cases, postponing treatment with targeted therapy until a patient shows signs of disease progression may be prudent, due to the side effects associated with these treatments, which can potentially impact quality of life. Finally, these agents (and in particular sorafenib, sunitinib, and bevacizumab) are generally widely approved in Latin America (Table-2). However, regulatory approval does not guarantee widespread use of a drug. Clinicians from Latin American countries need to be more active in taking part in clinical trials of new drugs, which is an effective way of providing patient access to these agents, and also of making their benefits known to a wider population.

ACKNOWLEDGEMENTS

Support was provided by Emily Seidman at ACUMED®, part of the KnowledgePoint360 Group,

Table 2 - Approval of targeted therapies by country in Latin America.

	Therapy							
Country	Sorafenib	Sunitinib	Pazopanib	Temsirolimus	Everolimus	Bevacizumab†	Axitinib	Observations
Argentina	√	√	√	√	√	√	√	Broad indication (everolimus after TKI failure only)
Brazil	√	√	√	√	√	√		Sorafenib second-line after cytokines; everolimus second-line after VEGFR TKI-based treatment
Chile	√	√	√	√	√	√	√	
Colombia	√	√	√	√	√	√	√	Sorafenib second-line after cytokines; everolimus second-line after VEGFR TKI-based treatment; temsirolimus in poor-prognosis patients
Venezuela	√	√	√	√	√	√		Broad indication
Ecuador	√	√	√			√	√	Broad indication
Peru	√	√	√			√	√	Broad indication
Mexico	√	√	√	√	√	√	√	Broad indication (everolimus after TKI failure only; pazopanib awaiting final approval)
Central-American countries*	√	√	√		√	√	√	Sorafenib second-line; sunitinib first- or second-line; everolimus second-line after TKI failure

*Panama, Costa Rica, Nicaragua, El Salvador, Guatemala, Honduras, and Dominican Republic.
†Approved in combination with interferon-alpha.
TKI=tyrosine kinase inhibitor; **VEGFR=**vascular endothelial growth factor receptor.

an Ashfield company (New York, NY, USA) with funding from Pfizer Inc.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Oren Smaletz, MD
Médico Oncologista do Escritório
de Estudos Clínicos sobre Câncer
Departamento de Oncologia do Hospital
Israelita Albert Einstein
Av. Albert Einstein, 627/721 Bloco A 2oSS
São Paulo, SP, Brasil
Fax: + 55 11 2151-1486
E-mail: osmaletz@einstein.br

ORIGINAL ARTICLE

Vol. 41 (5): 844-848, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0234

Anterior prostate biopsy at initial and repeat evaluation: is it useful to detect significant prostate cancer?

Pietro Pepe [1], Michele Pennisi [1], Filippo Fraggetta [2]

[1]Unità Urologia & [2]Unità Patologia, Ospedale Cannizzaro, Catania, Italy

ABSTRACT

Purpose: Detection rate for anterior prostate cancer (PCa) in men who underwent initial and repeat biopsy has been prospectively evaluated.
Materials and Methods: From January 2013 to March 2014, 400 patients all of Caucasian origin (median age 63.5 years) underwent initial (285 cases) and repeat (115 cases) prostate biopsy; all the men had negative digital rectal examination and the indications to biopsy were: PSA values > 10 ng/mL, PSA between 4.1-10 or 2.6-4 ng/mL with free/total PSA≤25% and ≤20%, respectively. A median of 22 (initial biopsy) and 31 cores (repeat biopsy) were transperineally performed including 4 cores of the anterior zone (AZ) and 4 cores of the AZ plus 2 cores of the transition zone (TZ), respectively.
Results: Median PSA was 7.9 ng/mL; overall, a PCa was found in 180 (45%) patients: in 135 (47.4%) and 45 (36%) of the men who underwent initial and repeat biopsy, respectively. An exclusive PCa of the anterior zone was found in the 8.9 (initial biopsy) vs 13.3% (repeat biopsy) of the men: a single microfocus of cancer was found in the 61.2% of the cases; moreover, in 7 out 18 AZ PCa the biopsy histology was predictive of significant cancer in 2 (28.5%) and 5 (71.5%) men who underwent initial and repeat biopsy, respectively.
Conclusions: However AZ biopsies increased detection rate for PCa (10% of the cases), the majority of AZ PCa with histological findings predictive of clinically significant cancer were found at repeat biopsy (about 70% of the cases).

ARTICLE INFO

Key words:
Prostatic Neoplasms; Prostate; Biopsy

Int Braz J Urol. 2015; 41: 844-8

Submitted for publication:
May 11, 3014

Accepted after revision:
May 07, 2015

INTRODUCTION

Prostate cancer (PCa) has become the most common tumor in men (1), but in about 50% of the cases diagnosed by a screening protocol an indolent PCa can be found increasing the risk of overtreatment (2); active surveillance program (AS) has been introduced in clinical practice (3) to reduce the number of unnecessary definitive treatment, but certain criteria to select aggressive PCa in early stages and in the presence of minimal PCa biopsy histological findings are still missing (4). Recently, multiparametric magnetic resonance imaging (mMRI) (5) and targeted MRI imaging/ultrasound fusion-guided biopsy (6, 7) have dramatically improved biopsy accuracy to detect significant PCa (8); however, the majority of urological Centers are still no Table to perform mMRI. In definitive, hoping that mMRI could be introduced routinely in clinical practice, the extended (EPBx) and saturation biopsy (SPBx) schemes performed at initial and repeat prostate biopsy should be improved; in this respect, repeat biopsy mMRI has found a significant percentage of cancers located in the anterior zone of the gland (about 20-30% of the cases) that would have been missed by standard SPBx (8, 9).

In our study, detection rate for PCa with a prostate biopsy scheme that included anterior zone cores in men who underwent initial and repeat procedure has been prospectively evaluated.

MATERIALS AND METHODS

From January 2013 to March 2014, 400 patients all of Caucasian origin and between the ages of 47 and 75 years (median 63.5 years) underwent initial (285 cases) and repeat (115 cases) prostate biopsy; all men had negative digital rectal examination and the indications to biopsy were (10): PSA values>10ng/mL, PSA between 4.1-10 or 2.6-4ng/mL with free/total PSA≤25% and ≤20%, respectively. In case of initial EPBx and repeat SPBx a median of 22 (range: 21-23) and 31 (range: 26-40) cores were performed: 9 (EPBx) vs 12 cores (SPBx) in the peripheral zone (PZ) of each lobe (apex, median zone and base of the gland) beginning parasagittally to reach the outer edges of the gland (lateral margins); in addition 4 cores of the anterior zone (AZ) and 4 cores of the AZ plus 2 cores of the transition zone (TZ) were sampled at initial and repeat biopsy, respectively. The procedure was done transperineally (11) using a tru-cut 18 gauge needle (Bard; Covington, GA), a GE Logiq 500 PRO ecograph (General Electric; Milwaukee, WI) supplied with a biplanar transrectal probe (5-6.5 MHz) under sedation and antibiotic prophylaxis (one tablet of levofloxacin for 3 days beginning the day before biopsy). All patients signed an informed consent form which, in addition to the biopsy-related complications, explicitly reported the risk of diagnosing a clinically non significant PCa when AZ cores are taken especially in case of initial biopsy. Detection rate and histological findings of PCa located in the PZ, AZ, TZ and PZ plus AZ were prospectively evaluated; moreover, quantitative biopsy histology was recorded (12). For statistical analysis the t Student's-test was used; a p value<0.05 was considered statistically significant.

RESULTS

Median PSA was 7.9ng/mL (range: 2.8-58ng/mL): 50 (12.5%) had PSA>10ng/mL, 330 (82.5%) between 4-10 and 20 (5%) between 2.6-4ng/mL, respectively. Overall, a cT1c stage PCa was found in 180/400 (45%) patients: in 135 (47.4%) and 45 (36%) of the men who underwent initial and repeat biopsy, respectively. In the remaining 220 patients an intraepithelial prostatic neoplasia (HGPIN), an atypical small acinar proliferation (ASAP), a chronic prostatitis and a normal parenchyma was found, in 18 (4.5%), 3 (0.7%), 29 (7.2%) 170 (42.5%) cases, respectively. Overall, clinical parameters and histological findings in the presence of PCa are listed in Table-1. In detail, in case of initial biopsy the highest percentage of PCa was found in the PZ (71.2% of the cases) of the gland; moreover, the percentage of cancer detected in the PZ plus AZ increased from 13.3% (18/135 cases) to 44.5% (20/45 cases; p=0.0001) in patients submitted to initial and repeat biopsy, respectively. An exclusive PCa of the anterior zone was found in 8.9 (12/135 cases) vs 13.3% (6/45 cases) (p=0.36) of the men who underwent initial vs repeat biopsy, respectively; the histological findings of AZ cancers in comparison with the PCa located in the PZ and/or PZ plus AZ demonstrated a significantly lower value (Table-1) of positive cores (1 cores vs 6.5 and 7.5 cores; p=0.0001), GPC (greatest percentage of cancer: 14% vs 55% and 65%; p=0.0001) and TPC (total percentage of cancer: 1% vs 8% and 12%; p=0.017). The highest percentage of microfocus of cancer (one positive core with Gleason score of 6 and GPC≤5%) (12) was found in the AZ (11 patients equal to 61.2% of the cases); on the other hand, in 7 of 18 AZ PCa the biopsy histology was predictive of significant cancer (GS≥6 and GPC>50%) in 2 (28.5%) and 5 (71.5%) men who underwent initial and repeat biopsy, respectively. Finally, AZ cores found a statistically significantly higher percentage of PCa in comparison with TZ cores (6 vs 1 equal to 13.4% vs 3% of the cases, respectively; p=0.003).

Overall, side effects following prostate biopsy occurred in 36.2% (140/400) of patients: 105 (26.2%) cases of hemospermia, 42 (10.5) of acute urine retention and 36 (9%) of hematuria; none needed hospital admission; moreover, all patients had a grade I of the Clavien-Dindo complications scale (13).

Table 1 - Clinical and histological findings in the 180 patients (pts) with prostate cancer (PCa).

Clinical and histological parameters	Overall PCa	PZ PCa	AZ PCa	PZ + AZ PCa	TZ PCa
	180 pts (100%)	126 pts (71.2%)	18 pts (10%)	35 pts (19.5%)	1 pts (0.5%)
PSA ng/mL (range)	8.9 (2.8-58)	7.4 (2.8-19)	11.5 (5.3-21)	12.4 (5.2-60)	12.5 (12.5)
Initial biopsy	135 (75%)	105 (77.8%)	12 (8.9%)	18 (13.3%)	-
Repeat biopsy	45 (25%)	19 (42.2%)	6 (13.4%)	20 (44.4%)	1 (3%)
Median GS (range)	6.4 (6-8)	6.4 (6-9)	6.1 (6-7)	6.5 (6-8)	6 (6)
Median GPC (range)	56% (5-100%)	65% (5-100%)	14% (5-90%)	55% (5-100%)	5% (5%)
Median TPC (range)	8% (1-72%)	8% (1-60%)	1% (1%)	12% (1-72%)	1% (1%)
No of positive cores (range)	6.5 (1-23)	7 (1-19)	1 (1-2)	7.5 (1-23)	1 (1)
Microfocus of PCa*	34 (18.9%)	22 (17.5%)	11 (61.2%)	-	1 (100%)

PZ = Peripheral Zone; **AZ =** Anterior Zone; **TZ =** Transition Zone; **GS =** Gleason score; **GPC =** Greatest Percentage of Cancer; **TPC =** Total Percentage of Cancer;
***** Microfocus of PCa = 1 positive core with GS of 6 and GPC≤5%.

DISCUSSION

Aggressive screening and prostate needle biopsy protocols have been successful in early detection of low-volume tumors increasing the incidence of anterior-predominant prostate cancers especially in patients submitted to repeat biopsy (14-21). Anterior tumors are less likely to be palpable, are not easily visualized by imaging and may require more biopsy sessions to establish a diagnosis (14); moreover, even when diagnosed, biopsies usually yield fewer involved cores and less total tumor length, making the assessment of cancer volume difficult (18-20). However, recently Sundi et al. (9, 22) reported a higher prevalence of AZ cancers in African American men with very low-risk PCa (59% of the cases), and still today few data are available regarding the incidence and clinical relevance of the AZ PCa. Bott et al. (14) have shown that about one fifth of all index cancers were located anterior to the prostatic urethra; Hikmat et al. (15) in radical prostatectomy specimen demonstrated that 15% of PCa were located in the AZ of the gland and characterized by a superimposable grading and staging in comparison with PZ cancers. In the last years, mMRI and MRI imaging/ultrasound fusion-guided biopsy have increased the detection of PCa (5-7, 21) located in about 20-30% of the cases in the anterior zone of the gland; in this respect, transperineal prostate biopsy demonstrated a better accuracy in comparison with transrectal approach in the diagnosis of AZ cancers (23).

In our series, overall detection rate for cancer located in the anterior zone was equal to 10% (18/180 cases) in initial (8.9% of the cases) vs repeat (13.4% of the cases) prostate biopsy (p=0.36). In detail, 11/18 (61.2%) AZ PCa were characterized by the presence of a single microfocus of PCa at risk for clinically indolent PCa; on the other hand, 7/18 clinically significant AZ PCa (GS≥6 and GPC>50%) would have be missed through a standard biopsy scheme (2 vs 5 cases at initial vs repeat biopsy, respectively). In definitive, the AZ biopsies increased detection rate for PCa allowing to reduce the prostate biopsy false negative rate and to characterize the histological findings of the cancer; in addition, in case of repeat SPBx biopsy AZ cores found a statistically significantly higher percentage of significant PCa in comparison with TZ cores (13.4 vs 2% of the cases) (p=0.003).

Regarding our results, some limitations should be reported. Firstly, the true incidence of AZ PCa should be evaluated in the entire specimen of the prostate; secondly, the clinical relevance of the AZ PCa remains unknown; finally, a greater number of cases should be evaluated.

In conclusion, although AZ biopsies increased overall detection rate for PCa (10% of the cases) the majority of AZ cancers with histological findings predictive of clinically significant PCa were found in case of repeat procedures (about 70% of the cases).

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Pietro Pepe, MD
Unità Urologia, Ospedale Cannizzaro
Via Messina 829, Catania (Italy)
Fax: + 39 95 726-3259
E-mail: piepepe@hotmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 849-858, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0355

Characterization of reactive stroma in prostate cancer: involvement of growth factors, metalloproteinase matrix, sexual hormones receptors and prostatic stem cells

Maurício Moreira da Silva Júnior [1], Wagner Eduardo Matheus [1], Patrick Vianna Garcia [2], Rafael Mamprim Stopiglia [1], Athanase Billis [3], Ubirajara Ferreira [1], Wagner José Fávaro [2]

[1] Departamento de Cirurgia, Área de Urooncologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil; [2] Laboratório de Carcinogênese Urogenital e Imunoterapia (LCURGIM), Departamento de Biologia Estrutural e Funcional, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil; [3] Departamento de Patologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil

ABSTRACT

Introduction and Objectives: Reactive Stroma (RStr) is observed in many human cancers and is related to carcinogenesis. The objectives of the present study were to stablish a relationship of the RStr microenvironment with prostate cancer (Pca) through a morphological and molecular characterization, and to identify a possible relationship between RStr with worse prognosis factors and occurrence of malignant prostatic stem cells.

Materials and Methods: Forty prostatic samples were selected from men with Pca diagnosis submitted to radical prostatectomy; they were divided in two groups: Group-1 (n=20): samples without reactive stroma; Group-2 (n=20): samples of PCa with intense stroma reaction. Prostatic samples were evaluated for RStr intensity by Masson Trichromic stain and posteriorly submitted to histopathological and immunohistochemistry analysis for antigens: α-actin, vimentin, IGF-1, MMP-2, FGF-2, C-Myc, PSCA, AR, Erα and ERβ.

Results: Reactive stroma with intense desmoplastic reactivity was significantly more frequent in intermediate (Gleason 7, 3+4) and high grade tumors (Gleason 7, 4+3). The group with intense stromal reactivity showed significant higher levels of Vimentin, IGF-1, MMP-2, FGF-2, C-Myc, PSCA and ERα.

Conclusions: It can be concluded that RStr may be a predictive marker of Pca progression, since it was associated with increase of growth factors, imbalance of androgen and estrogen receptors and presence of malign prostatic stem cells.

ARTICLE INFO

Key words:
Reactive stroma, prostate cancer, growth factors, sexual hormones receptors, epithelium-stroma interaction

Int Braz J Urol. 2015; 41: 849-58

Submitted for publication:
July 17, 2014

Accepted after revision:
February 05, 2015

INTRODUCTION

Prostatic epithelium comprises three cellular types: luminal or columnar, basal and neuroendocrine (1). Luminal epithelial cells are the most frequent cell type in normal and hyperplasic epithelium and represent the exocrine compartment of prostate (2). Since tumor cells express similar characteristics to luminal cells, mutant luminal cells were considered precursors of adenocarcinoma (3, 4). These cells express androgen receptor (AR) and respond to androgen and are androgen-

-dependent (5). On the other side, basal cells are relatively undifferentiated, independent of androgens but androgen-respondent, they do not show secretory activity and form the basal compartment of the prostate (6). The neuroendocrine cells do not respond to androgens (7-9) and can modify in prostate cancer (number, histology and function) with a suggestive regulatory role in that disease (10, 11).

Reactive stroma (RStr is defined as the microenvironment closely adjacent to epithelium able to coordinate several activities as wound repair, homeostasis changes and interaction with neoplastic complexes, comprising an dynamic environment that influence directly the behavior of epithelial cells and perform tissue repair after lesion (12). Modifications of peritumor stroma start in prostatic intraepithelial neoplasia (PIN) and include phenotypic alterations of stromal cells, remodeling of extracellular matrix and induction of angiogenesis (13, 14). Reactive stroma (RStr) is defined as a new stroma environment in response to carcinoma. It follows tumor growth and is characterized by an increase of inflammatory cells, desmoplastic reaction, increase of angiogenesis and growth factors, with remodeling of extracellular matrix (15). RStr has a fibroblastic and a myofibroblastic compound associated to tumor and the origin of these cells is not clearly understood. Some authors suggest that these cells are originated from the prostatic stroma or smooth muscle or even stem cells (14, 15). Stem cells have the capacity of self-renovation and regeneration throughout adult life and are present in the epithelial and stromal compartments (16).

At the prostate several biological processes (regulation of proliferation and cellular differentiation, mitogenic activity, secretory processes and tumor growth) are regulated and/or influenced by different growth factors, such as IGF (insulin growth factor), FGF (fibroblast growth factor), VEGF (vascular endothelium growth factor), transforming growth factors, metalloproteinases and PSCA (prostate stem cell antigen) (17-22).

Additionally, testosterone is an important stimulant to prostatic cell proliferation, mainly when it's more potent form di-hydro-testosterone (DHT) bind to androgen receptors of cells from the epithelial and stromal compartments (23, 24), so those above mentioned processes are under direct influence of androgens, estrogens and their alpha (ERα) and beta (ERβ) receptors (25, 26).

Neoplastic transformation consists of a multi-causal process, where normal controls of cellular proliferation and interaction cell-to-cell are lost. Aberrant activation of proto-oncogenes along with non-regulated inhibition of tumor suppressor genes are fundamental in that process. In that context, stand out proto-oncogene C-MYC. In tumors, the scarce vascularization and the high proliferative profile lead to a hypoxic status (known as Warburg effect) that is able to induce the expression of C-MYC, that promotes an energetic reinforcement through glycolysis and that can additionally act as a suppressor of antiangiogenic factors in an attempt to oppose hypoxia and to promote adequate metabolic supply demanded by the tumor (27).

In view of the facts discussed above, it is essential to establish a correlation between the stromal microenvironment of prostate adenocarcinoma through morphologic and molecular characterization, and also to determine any association of growth factors, matrix metalloproteinases, sexual hormone and stem cell receptors with tumors with worse prognosis.

MATERIALS AND METHODS

Human Samples and Histopathological Analysis of Reactive Stroma

Forty prostatic samples of patients submitted to retropubic radical prostatectomy with 60-80 years old (median 71 years) were collected. Samples were obtained from the collection of the Department of Pathology of the Hospital de Clínicas da Universidade Estadual de Campinas (UNICAMP).

Samples were collected from the peripheral region based on the division of the posterior side, with basal to apical orientation of the organ. Next, samples were fixed in 10% buffered formaldehyde for 12 hours. After fixation, tissue samples were routinely processed (inclusion in paraffin, 5µm sections and Hematoxilin-Eosin staining).

Pca diagnosis was based in morphological criteria and classified according to Gleason system

by a senior pathologist of the Department of Pathology of the Faculdade de Ciências Médicas da Universidade Estadual de Campinas (UNICAMP).

For reactive stroma analysis, the prostatic samples were divided in two groups (20 samples per group): Group-1: PCa samples without reactive stroma (Grade-0), Group-2: Pca samples with intense stromal reactivity (Grade-3).

Stromal reactivity was determined at the Urogenital Carcinogenesis Laboratory and Immunotherapy of the Biological Institute of UNICAMP, using Masson Trichromic stain. The intensity of reactive stroma was evaluated by the frequency (in percentage) of smooth muscular fibers (stained red with Masson Trichromic) adjacent to neoplastic areas in each sample with an augment of x400. Images were captured by photomicroscope Leica DM2500 equipped with a Leica camera DFC295 and analyzed by the software Leica LAS V3.7 for image analysis. The percentage of smooth muscular fibers adjacent to neoplastic areas was graded and expressed as 0>50% of smooth muscular fibers adjacent to neoplastic ducts, 1:36-50% of smooth muscular fibers adjacent to neoplastic ducts, 2:15-35% of smooth muscular fibers adjacent to neoplastic ducts, 3: 0-14% of smooth muscular fibers adjacent to neoplastic ducts. For this study intermediate levels of reactive stroma (grades 1 and 2) were discarded.

The study was approved by the Ethical Committee of Faculdade de Ciências Médicas/UNICAMP (#0094.0146.000-08).

Immunohistochemistry for antigens: α-actin, Vimentin, IGF-1, MMP-2, FGF-2, C--Myc, PSCA, AR, ERα and Erβ

The same prostatic samples of 40 patients used for histopathological analysis ere submitted to immunohistochemistry. Antigen recovery was obtained by incubating the slices in buffered citrate (pH 6.0) at 100oC in microwave. The blockage of endogenous peroxidase was obtained with H2O2 and posterior incubation with a blocking solution with bovine serum albumin (BSA) for 1 hour at room temperature. After that, the antigens were localized with specific antibodies (Table-1), diluted in BSA and incubated overnight at 4oC. It was used the MACH 4 Universal HRP-Polymer® (Biocare Medical) kit for antigen detection, according to the manufacture instructions. Posteriorly, the slices were revealed with diaminobenzidin (DAB), counter-stained with Harris Hematoxilin and evaluated at the photomicroscope.

In order to evaluate the intensity of the antigen immunoreactions, the percentage of positive epithelial and/or stromal cells was examined in 10 fields for each antibody with an augment of 400x. The intensity of staining was grade in a 0-3

Table 1 - Characteristic of primary antibodies for immuno-staining.

Primary antibodies	Host species	Code	Soource
α-actin	Mouse (monoclonal)	sc-32251	Santa Cruz, Biotechnology, EUA
Vimentin	Mouse (monoclonal)	ab8069	Abcam, EUA
IGF-1	Rabbit (policlonal)	sc-720	Santa Cruz, Biotechnology, EUA
MMP-2	Mouse (monoclonal)	ab86607	Abcam, EUA
FGF-2	Rabbit (policlonal)	sc-79	Santa Cruz, Biotechnology, EUA
C-Myc	Rabbit (policlonal)	ab32072	Abcam, EUA
PSCA	Rabbit (policlonal)	251249	Abbiotec, EUA
AR	Rabbit (policlonal)	ab74272	Abcam, EUA
ERα	Rabbit (policlonal)	04-227	Merck-Millipore, EUA
ERβ	Mouse (monoclonal)	ab16813	Abcam, EUA

scale and expressed as 0 (no immunoreactivity), 0% of positive epithelial and/or stromal cells, 1 (weak immunoreactivity), 1-35% of positive epithelial and/or stromal cells, 2 (moderate immunoreactivity), 36-70% of positive epithelial and/or stromal cells, 3 (intense immunoreactivity), >70% of positive epithelial and/or stromal cells.

Statistical analysis

The histopathological and immunohistochemistry analysis for different antigens were evaluated with the proportion test. For these analyses, an error type-I of 5% was considered statistically significant.

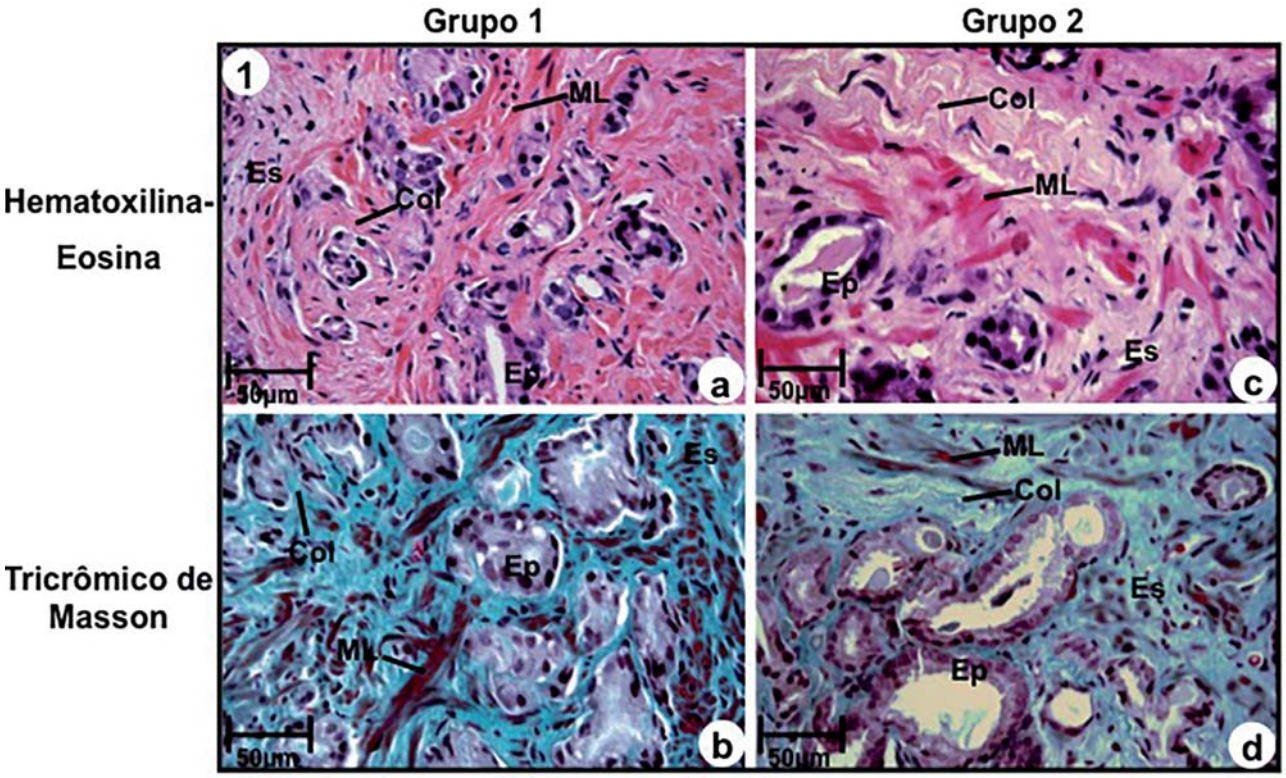
RESULTS

Histopathological Analysis of Reactive Stroma

Stroma without desmoplastic reaction (Group-1) was characterized by the presence of a great amount of smooth muscular fibers, above 50% of adjacent ducts with collagen fibers interspersed among the smooth muscular fibers (Figures 1a and 1b).

In relation to stroma with intense desmoplastic reaction (Group-2), it was characterized by an outstanding increase and thickening of collagen fibers, associated to an intense reduction (below 14%) of smooth muscular fibers (Figures 1c

Figure 1 - Photomicrography of prostatic peripheral zone of groups 1 (a, b) and 2 (c, d). (a) and (b) Stroma without desmoplastic reaction consisting of excess of smooth muscular fibers (ML) and thin collagen fibers (Col) adjacent to prostatic ducts. Stains: Hematoxilin-Eosin (a) and Masson Trichromic (b). (c) and (d) Stroma with intense desmoplastic reactivity consisting of excess of collagen fibers (Col) and rare smooth muscular fibers (ML), stains: Hematoxillin-Eosin (c) and Masson Trichromic (d).



a-d: Ep=secretory epithelium; Es-stroma. Scale of 50µm.

and 1d). Stroma without desmoplastic reaction (Group-1) was significantly more frequent in Gleason 4 (2+2), 5 (3+2) and 6 (3+3) (Table-2), and absent in high grade tumors (Gleason 7, 4+3) (Table-2).

Stroma with intense desmoplastic reactivity was significantly more frequent in intermediate (Gleason 7, 3+4), and high grade tumors (Gleason 7, 4+3) and in low grade tumors it was observed in only 3 cases with Gleason 6 (3+3) (Table-2).

(Figures 3a. and 3f, Table-3). Likewise, immunoreactivity to prostatic stem cell antigen (PSCA) was observed both in epithelial and stromal compartments, being significantly more intense in samples of Group-2, compared to Group-1, that showed moderate immunoreactivity (Figures 3b and 3g, Table-3).

Immunoreactivity for AR was moderate in both epithelium and stroma of samples of Group-2, while in Group-1 it was observed low

Table 2 - Distribution of Gleason score and stromal reactivity in prostatic adenocarcinoma without stromal reactivity (Group-1) and with intense stromal reactivity (Group-2).

Gleason score	Number of cases (%)	Group-1	Group-2
Gleason 4 (2+2)	1 (2.5%)	1 (100.0%)*	0 (0.0%)
Gleason 5 (3+2)	2 (5.0%)	2 (100.0%)*	0 (0.0%)
Gleason 6 (3+3)	19 (47.5%)	16 (84.2%)*	3 (15.8%)
Gleason 7 (3+4)	10 (25.0%)	1 (10.0%)	9 (90.0%)*
Gleason 7 (4+3)	8 (20.0%)	0 (0.0%)	8 (100.0%)*
Total	**40 (100.0%)**	**20 (50.0%)**	**20 (50.0%)**

Immunohistochemistry of antigens: α-actin, Vimentin, IGF-1, MMP-2, FGF-2, C--Myc, PSCA, AR, ERα and ERβ

Immunoreactivity to α-actin, a marker of smooth muscle, was significantly more intense in Group-1 in relation to Group-2. The last one showed moderate immune staining (Figures 2a and 2f, Table-3). On the contrary, immunoreactivity of vimentin, a fibroblast and myofibroblast marker, was significantly more intense in Group-2 in relation to Group-1, that showed moderate immunoreactivity (Figures 2b and 2g, Table-3).

Immunoreaction to IGF-1, MMP-2 and FGF-2 were significantly more intense in epithelium and stroma of samples of Group-2, compared to Group-1, that showed moderate reactivity (Figures 2c, 2d, 2e, 2h, 2i, and 2, Table-3).

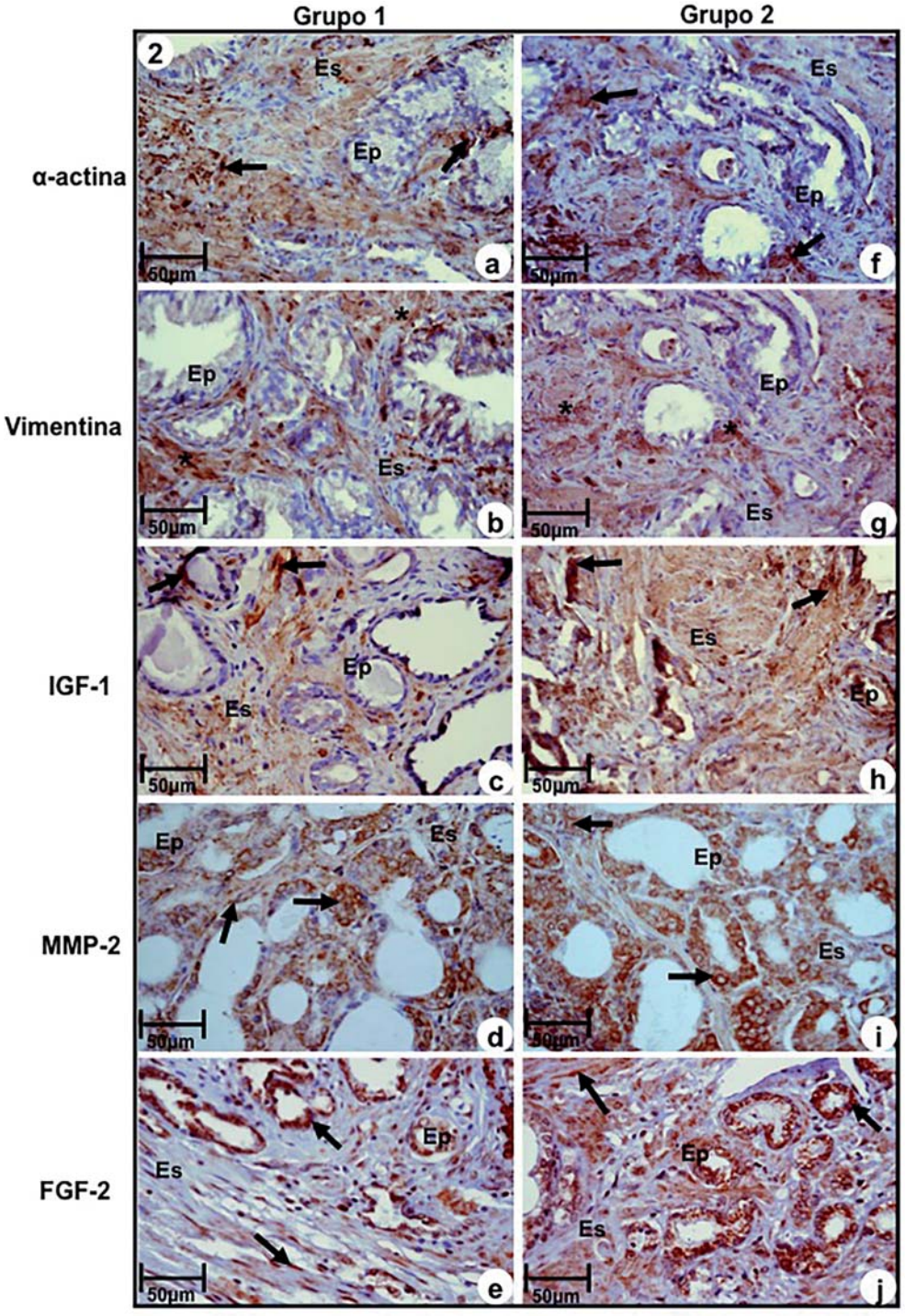
Immunoreactivity to C-Myc was significantly more intense in both epithelium and stroma in samples of Group-2, and moderate in Group-1 immunoreactivity (Figures 3c and 3h, Table-3). Immunoreactivity to ERα was predominant in stromal compartment in both groups, being intense in Group-2 and moderate in Group-1 (Figures 3d and 3i, Table-3).

In contrast, immunoreactivity for Erβ was predominant in epithelial compartment of both groups, and immune staining of stroma was present only in Group-2 (Figures 3e and 3j). Immunoreactivity of this marker was moderate in Group-1 and weak in Group-2 (Figures 3e and 3, Table-3).

DISCUSSION AND CONCLUSIONS

Interaction of epithelium-stroma has a primary role in maintenance of structure and functioning of prostate. Stromal cells associated to tumor cells respond to androgens forming growth factors that lead to interruption of epithelium-stroma homeostasis, initiating growth and

Figure 2 - Immuno-staining of antigens α-actin, Vimentin, IGF-1, MMP-2 and FGF-2 at prostatic peripheral zone of Groups-1 (a, b, c, d, e) and 2 (f, g, h, i, j). (a) and (f) Immunoreactivity to α-actin (arrows). (b) and (g) Immunoreactivity to Vimentin (asterisks) in myofibroblasts. (c) and (h) Imunoreactivity to IGF-1 (arrows) in epithelium and stromal compartments. (d) and (i) Immunoreactivity to MMP-2 (arrows) in epithelial and stromal compartments. (e) and (j) Immunoreactivity to FGF-2 (arrows) in cells of secretory epithelium and fibroblasts of stromal compartment.



a-j, Ep–secretory epithelium; **Es-**stroma. Scale of 50µm.

Table 3 - Intensity of immuno-staining of different antigens in epithelial and stromal cells of prostatic adenocarcinomas without stromal reactivity (Group-1) and with intense stromal reactivity (Group-2).

	Groups	
Antigens	Group 1 (n=20)	Group 2 (n=20)
α-actin	3 (80.3%)*	2 (69.0%)
Vimentin	2 (61.4%)	3 (92.4%)*
IGF-1	2 (57.6%)	3 (96.8%)*
MMP-2	2 (67.3%)	3 (89.7%)*
FGF-2	2 (62.7%)	3 (91.5%)*
C-Myc	2 (65.5%)	3 (93.3%)*
PSCA	2 (56.5%)	3 (85.8%)*
AR	1 (30.9%)	2 (59.5%)*
Erα	2 (39.7%)	3 (77.9%)*
Erβ	2 (38.6%)*	1 (26.5%)

0 (absence of immunoreactivity), 0% of positive epithelial and/or stromal cells; **1** (weak immunoreactivity), 1-35% of positive epithelial and/or stromal cells; **2** (moderate immunoreactivity), 36-70% of positive epithelial and/or stromal cells; **3** (intense immunoreactivity), >70% of positive epithelial and/or stromal cells.

migration processes, angiogenesis, apoptosis and tumor metastasis (28).

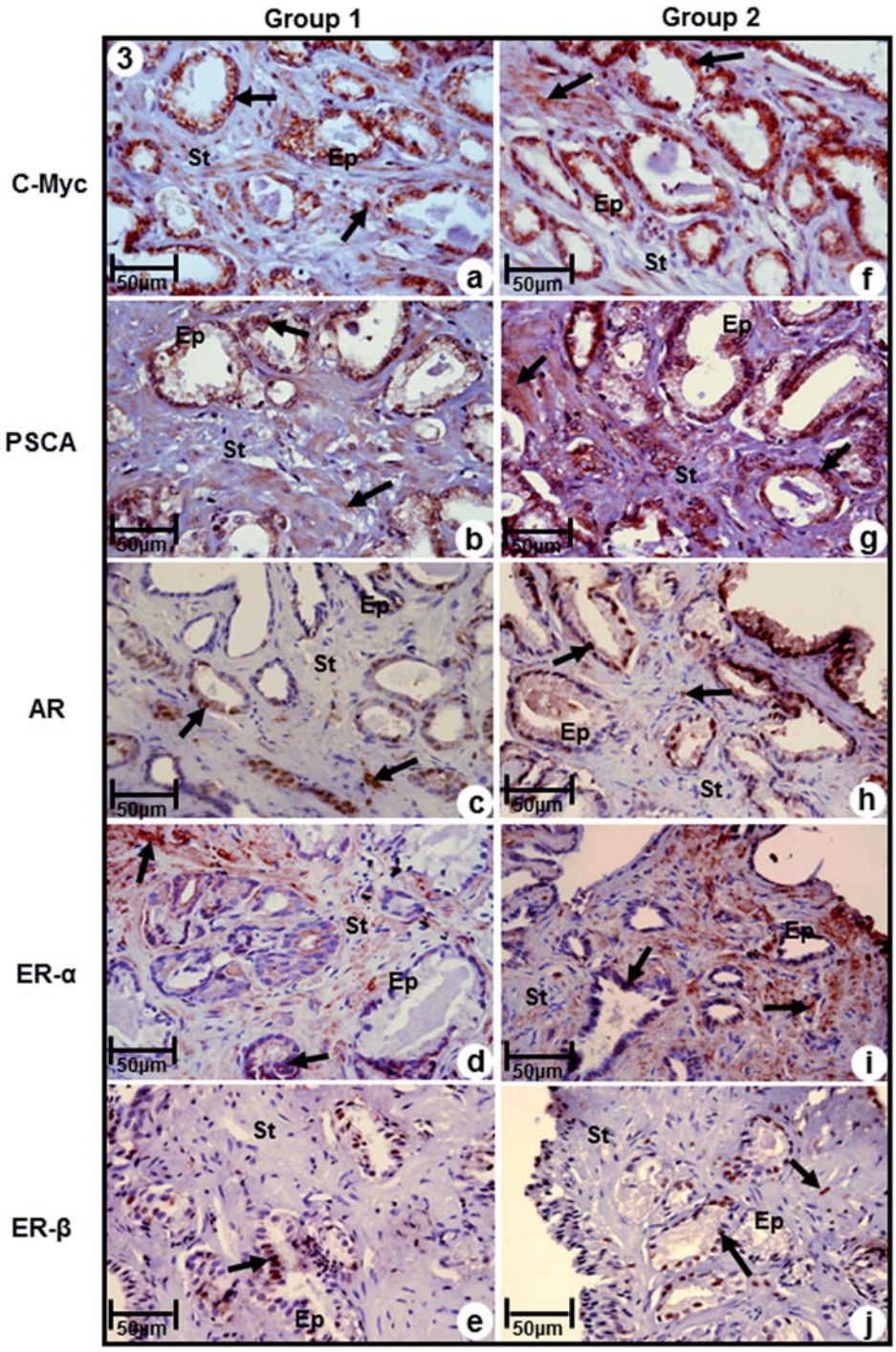
In the present study RStr was morphologically characterized by the significant reduction of smooth muscular fibers and excess of collagen fibers in stroma adjacent to neoplastic ducts. Intense stromal reactivity was observed in intermediate (Gleason 7, 3+4) and high grade tumors (Gleason 7, 4+3) and in low grade tumors it was observed in only 3 cases with Gleason 6 (3+3), pointing out that RStr may be considered a predictive marker of tumor progression.

In relation to molecular characterization of RStr, the results showed increased reactivity to vimentin, IGF-1, MMP-2, FGF-2 and C-Myc in samples with intense stromal reactivity when compared to samples without reactivity. Such markers were fundamental to activation of RSTr and made the prostatic microenvironment favorable to tumor progression due to increase of imbalance of epithelium-stroma interaction.

Several studies have demonstrated that RStr is associated with lower survival free of disease. Yanagisawa (29) analyzed prostatic biopsies of 205 patients and demonstrated a significant difference between high and low reactive RSTr and concluded that the intensity of RStr may be considered a prognostic factor independent of biochemical recurrence. Also, Ayala (15), after analyzing samples from radical prostatectomy and Billis (30), that analyzed 266 needle prostatic biopsies showed that RStr could only be considered an prognostic factor independent of biochemical recurrence when it showed intense stromal reactivity. Still, RStr with intense stromal reactivity was observed in Ayala (15), Yanagisawa (29) and Billis (30) studies in 9.0%, 6.7% and 5.3% of samples respectively, with very similar frequencies among the studies. However, RStr with low stromal reactivity frequencies were very distinct among these three papers: 6.25% (Ayala, 15), 0.5% (Yanagisawa, 29) and 53.8% (Billis, 30). That reflects that lack of uniform morphological criteria to characterize RSTr.

In conclusion, the present study shows a new approach to Pca diagnosis and the results demonstrated RStr may be considered a predictive marker of PCa progression, since increase of vimentin, IGF-1, MMP-2, FGF-2 and C-MTC are evidences of worse tumor prognosis and the occurrence of prostatic stem cells (elevation of PSCA) and the balance of AR and Erα with concurrent

Figure 3 - Immuno-staining of antigens C-Myc, PSCA, AR, ERα and ERβ in prostatic peripheral zone of Groups 1 (a, b, c, d, e) and 2 (f, g, h, i, j). (a) and (f) Immunoreactivity to C-MyC (arrows) in epithelial and stromal compartments (b) and (g) Immunoreactivity to PSCA (arrows)) in epithelial and stromal compartments (c) and (h) Immunoreactivity to AR (arrows)) in epithelial and stromal compartments (d) and (i) Immunoreactivity to ERα (arrows)) in epithelial and stromal compartments (e) and (j) Immunoreactivity to ERβ (arrows)) in epithelial and stromal compartments.



a-j, Ep=secretory epithelium; **Es=**stroma. Scale of 50µm.

inhibitory action of ERβ at RStr point to a greater malignancy of these tumors as well as an indication of recurrence. However, new studies are necessary to better understanding of this microenvironment and upgrading of available treatments of prostate cancer, and the development of new modalities that assure better clinical results and quality of life of patients.

ABBREVIATIONS

AR = androgen receptor
EstR = Reactive stroma
IGF = Growth factor Homologous Insulin
FGF = Fibroblast Growth Factors
VEGF = Vascular Endothelial Growth Factor
MMP = Matrix metalloproteinase
CaP = Prostate cancer
PSCA = Antigen for Prostate Stem Cell
DHT = dihydrotestosterone
ERα = Estrogen Receptor Alpha
ERβ = Estrogen Receptor Beta

ACKNOWLEDGE

Academic and financil support by: Fundação de Amparo À Pesquisa do Estado de São Paulo (FAPESP), process number 2009/50397-9.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Wagner José Fávaro, PhD
Laboratório de Carcinogênese
Urogenital e Imunoterapia (LCURGIM)
Departamento de Biologia Estrutural e Funcional
Universidade Estadual de Campinas (UNICAMP)
13083-865, Campinas, SP, Brasil.
Telephone: +55 19 3521-6104.
E-mail: wjfavaro@gmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 859-863, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0337

Local anesthesia type affects cancer detection rate in transrectal ultrasound guided prostate biopsy

Mustafa Zafer Temiz [1], Engin Kandirali [2], Aykut Colakerol [2], Murat Tuken [2], Atilla Semercioz [2]

[1] Department of Urology, Bitlis State Hospital, Besminare, Bitlis, Turkey; [2] Department of Urology, Bagcilar Training and Research Hospital, 6. Sokak, Bagcilar/Istanbul, Turkey

ABSTRACT

Purpose: Studies about the anesthesia techniques during transrectal ultrasound guided prostate biopsy (TRUS-Bx) are usually focused on pain relief. Although patients' tolerance is an important issue in TRUS-Bx, cancer detection rate (CDR) must not be ignored. In this study, we compared the impact of intrarectal lidocaine gel anesthesia (IRLA) and periprostatic nerve blockade (PNB) techniques on CDR.

Materials and Methods: A total of 422 patients underwent 10 core-TRUS-Bx because of elevated serum prostate specific antigen (PSA) level (>2.5ng/mL) and/or suspicious digital rectal examination findings. Patients were divided into two groups according to the applied anesthesia technique: IRLA group and PNB group. Age, serum PSA level, prostate volume, visual analogue scale (VAS) score and CDR were recorded and compared statistically with chi square and unpaired t-tests.

Results: Of the patients 126/422 (29.9%) underwent TRUS-Bx by using IRLA whereas 296/422 (70.1 %) by PNB technique. The mean, age, serum PSA level and prostate volume were similar between the two groups. CDR was 19.8% and 25.4% in IRLA and PNB groups, respectively (p=0.001). The mean VAS score of the PNB group (1.84±0.89) was significantly lower than that for IRLA group (3.62±1.06) (p=0.001).

Conclusions: Our results revealed that PNB is superior to IRLA in terms of CDR. Further studies are required to confirm our findings.

ARTICLE INFO

Key words:
Neoplasms; Anesthesia, Local; Prostate; Biopsy

Int Braz J Urol. 2015; 41: 859-63

Submitted for publication:
July 09, 2014

Accepted after revision:
October 28, 2014

INTRODUCTION

Transrectal ultrasound guided prostate biopsy (TRUS-Bx) is a widely performed procedure in the diagnosis of prostate cancer. Although it is considered a minor and well-tolerated procedure, 65% to 90% of the patients complain about pain (1, 2). Local anesthesia prior to biopsy is a crucial part of TRUS-Bx for pain control. Several methods of local anesthesia for TRUS-Bx are available, including periprostatic nerve blockade, topical rectal administration or intraprostatic injection of local anesthetics (3).

Numerous studies regarding anesthesia techniques compared the efficacy of pain management during TRUS-Bx (4-6). Although patient tolerance is an important issue in TRUS-Bx, CDR must not be ignored. To our knowledge, there is no clinical study which primarily compares the CDR with different anesthesia techniques during TRUS--Bx. Thus, the aim of this study was to determine the impact of intrarectal lidocaine gel anesthesia (IRLA) and periprostatic nerve blockade (PNB) on CDR following TRUS-Bx.

MATERIAL AND METHODS

Between February 2009 and December 2012, 526 men who underwent TRUS-Bx at our institution were included for this retrospective study. The institutional review board approved the protocol and all participants provided their informed consent for TRUS-Bx prior to the procedure. Indications for biopsy were elevated serum PSA levels (>2.5ng/mL) and/or suspicious digital rectal examination findings.

Exclusion criteria included previous prostate biopsies, lidocaine allergy, hemorrhagic diathesis, recto-anal pathology, diabetes mellitus, neurologic diseases, and inabilities to rate visual analog scale (VAS). Moreover, patients who were diagnosed with high grade prostatic intraepithelial neoplasia (HG-PIN) and/or atipic small acinar proliferation (ASAP) on pathologic evaluation of the initial TRUS-Bx were not included either.

All patients who had sterile urine culture before the procedure received an enema on the morning of the procedure. Oral levofloxacin (500mg daily, for 5 days, started the night before the biopsy) was given. All procedures were performed by an urologist of our clinic. After the patients being positioned on left lateral decubitus, either intrarectal 6mL 2% lidocaine HCl gel (Aqua Touch Jelly; Istem Medical, Turkey) was applied digitally on the anterior anal wall and prostate surface (group IRLA) or PNB was performed with 5mL 1% lidocaine which was bilaterally injected with a 18 Gauge spinal needle (Gallini Medikal Devices, Italy) into the region of the prostatic vascular pedicle on each side (group PNB). The choice of anesthetic methods was completely up to the urologist who performed the procedure. After administration of the local anesthetics, prostate volumes were measured by using the prostate ellipse formula (7) and prostate gland was evaluated sonographically (Pro Focus 2202 color, Prostate Triplane 8818, 4-12 MHz; BK Medical, Denmark). Afterwards, 10 cores systematic TRUS-Bx was performed via 25cm 18 Gauge tru-cut biopsy needle (Gallini Medikal Devices, Italy) and an automatic biopsy gun (Pro-Mag Ultra-Angiotech, Denmark). Each patient was asked to rate the severity of pain during the procedure on a 10cm visual analogue scale (VAS).

All complications such as vasovagal hypotension, hematuria, rectal bleeding, urethrorrhagia, hematospermia, lower urinary tract symptoms (LUTS), fever, and other possible complications during and after the procedure were recorded. Patients were invited for follow-up after 10 days of the procedure.

Patient characteristics, mean VAS score and CDR were compared between the two groups.

Unpaired t-test and chi-square test were used for the statistical analyses. A p value<0.05 was considered statistically significant.

RESULTS

Of the 526 patients, 422 (80.2%) who met the inclusion criteria were included to the study. The mean age, serum PSA level and prostate volume of patients were 64.5±7.9 years, 58.1±27.7cc and 12.8±17.2ng/mL, respectively. TRUS-Bx was performed with IRLA in 126/422 (29.9%) whereas 296/422 (70.1%) patients received PNB. The characteristics of the patients in IRLA and PNB groups are shown on Table-1.

There were no statistical differences on digital rectal examination findings. Suspicious examination rates were 14.9% and 15.1% in IRLA and PNB groups, respectively (p=0.8).

The groups were similar in terms of mean age, prostate volume and serum PSA levels (p>0.05 for each). Mean VAS score was statistically lower in IRLA group compared to that for the PNB group (p=0.001). CDR was 25.42% and 19.84% in IRLA and PNB groups, respectively and the difference was statistically significant (p=0.001).

Gleason scores rates were similar between the groups. Gleason score≤6 rates were 46.2% and 41.7% and Gleason score≥7 rates were 53.8% and 58.3% in IRLA and PNB groups, respectively (p=0.1).

There were only minor complications, such as vasovagal hypotension, mild hematuria, rectal bleeding, hematospermia and LUTS all of which were managed conservatively. No significant difference was found between the groups when the complications were taken into consideration (Table-2).

DISCUSSION

TRUS-Bx is the standard method for diagnosis of prostate cancer (8). Prostate biopsy has evolved from digitally guided biopsy technique to

Table 1 - Data of the patient characteristics, VAS score and CDR in IRLA and PNB groups.

	IRLA group	PNB group	P value
N	126	296	
Age (years)	64.7±7.8	64.4±7.9	0.8[a]
PSA (ng/mL)	11.6±14.3	13.4±18.3	0.06[a]
Prostate volume (cc)	60±26	57±28.4	0.6[a]
VAS score	3.62±1.06	1.84±0.89	0.001[a]
CDR (%)	19.84 %	25.42 %	0.001[b]

[a] Student t test
[b] Chi-square test
CDR = Cancer Detection Rate
VAS = Visual Analog Scale

Table 2 - Comparison of the minor complications after the procedure in IRLA and PNB groups.

	IRLA group	PNB group	P value
Vasovagal hypotension (n/%)	8/6.3	17/5.7	0.7[a]
Mild hematuria (n/%)	62/49.2	149/50.3	0.4[a]
Rectal Bleeding (n/%)	17/13.4	46/15.5	0.08[a]
Hematospermia (n/%)	12/9.5	34/11.4	0.09[a]
LUTS (n/%)	7/5.5	18/6	0.7[a]

LUTS = Lower urinary tract symptoms.
[a] Chi-square test

the standard sextant transrectal ultrasound guided method (9, 10). Although TRUS-Bx is regarded as a safe and minimal invasive technique, patients experience significant discomfort during the procedure (11). Several clinical studies demonstrated that up to 20% of patients report significant pain during TRUS-Bx and they would refuse re-biopsy without analgesia (12).

Thus, many strategies are adopted to reduce pain and enhance patient tolerance during this procedure.

Pain during biopsy is mainly caused by the introduction and manipulation of the ultrasound probe in the anal canal and penetration of the needle into the prostate capsule, which is richly innervated with autonomic sympathetic and parasympathetic fibers (13, 14). Probe manipulations are necessary to obtain samples from different regions of the prostate (e.g. far-lateral and apex) with adequate imaging in TRUS-Bx. The prostatic apex is a common site of cancer detected by traditional biopsy techniques (15). Similarly, some studies found that addition of laterally directed biopsies of the base, mid-gland, and apex resulted in a 14-17% increase in CDR (16, 17). Importantly, when repeat biopsies are considered, assurance that the far-lateral region and the apical region were sampled appears to be essential because disease in these sites is frequently missed during first biopsy (15). Therefore, manipulation of the ultrasound probe is necessary for an effective biopsy procedure in order to enhance CDR which may be associated with increased patient discomfort.

Berger et al. reported a significant reduction in the level of discomfort from prostate biopsy and transrectal probe manipulation after administration of periprostatic lidocaine. The authors stated that the

lidocaine depot had a local effect on the autonomic innervation of the rectal wall (18). In addition to this, anesthetic blockade of the capsular sensation fibers with PNB can decrease the level of patient anxiety and makes the examination more tolerable due to decrease in pelvic muscle contraction (19). Therefore, probe manipulation may be easily done with less patient discomfort as a result of decreased pelvic muscle contraction and pain during TRUS-Bx.

In our study, mean VAS score was lower in PNB group which confirms the findings of Berger et al. (18) Furthermore, CDR was also higher in the PNB group. Ameliorated patient comfort during TRUS-Bx via PNB may enable the sonographer manipulate the probe for better visualization of the gland and obtain biopsies from the apex and far lateral parts of the prostate. This advantage of PNB may play a role in improved CDR compared to IRLA.

Application of perianal-intrarectal topical anesthetic creams or gels to reduce probe related pain during TRUS-Bx is a controversial issue. Some studies reported effective pain relief during probe manipulation with perianal-intrarectal gel anesthesia, whereas others did not (20-22). Since rectal mucosa has a rich absorptive capacity, topical anesthetic gel administered intrarectally may spread into the circulation which may decrease its local effects (23, 24). In the present study, VAS score was higher in IRLA group which may be related to decreased CDR. In our opinion the reason is that, limited manipulation of ultrasound probe with IRLA may avoid sampling apical and far lateral zones where prostate cancer can commonly exists (15).

Various variables may influence the cancer detection rates and diagnostic yield of prostate biopsies such as; patient age and race, serum PSA level, prostate volume, biopsy quality and method of biopsy (e.g., random or ultrasound guided etc.), operator skill (e.g., learning curve etc.), location and number of cores, core length (25, 26). In our study, all of these parameters were similar in each group, except the core length which was not evaluated. According to core length analysis obtained with PNB, it is likely that the adequate core lengths may have improved CDR in our study. This issue must be addressed in further studies.

Nowadays in clinical practice, optimum anesthetic method for interventional procedures is general anesthesia (27). According to our findings, general anesthesia may further improve CDR theoretically because of its maximal patient compliance effect. However, we think that routine usage of general anesthesia during TRUS-Bx does not seem possible because of its invasiveness and expensiveness.

Some limitations of our study merit consideration. 1: There is a difference in number of patients between the two groups. The retrospective nature of our study design is responsible for this phenomenon. Further prospective studies may bypass this limitation. 2: Evaluation of the pain score that is especially related to probe manipulation could have strengthened the conclusions of our study. Further studies about this topic must be considered. 3: The zones where prostate cancer was detected should have also been compared. 4: Clinical significance of prostate cancers was not evaluated in this study. We have especially evaluated cancer detection rates. This issue must be evaluated with further studies in order to determine which anesthetic method is superior for diagnosis of clinical significant cancers.

CONCLUSIONS

Our results revealed that PNB is superior to IRLA in terms of CDR. We concluded that PNB is an useful anesthesia method in TRUS-Bx for effective procedure as well as for pain relief. Further studies are required to confirm our findings.

ACKNOWLEDGEMENTS

This publication has been supported by Bagcilar Training and Research Hospital, Bagcilar, Istanbul. We thank members of the department of urology and management of hospital.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Mustafa Zafer Temiz, MD
Department of Urology
Bitlis State Hospital
Besminare Mahallesi 13000
Besminare, BITLIS, Turkey
Fax: + 90 434 246-8424
E-mail: dr_mustafazafertemiz@hotmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 864-868, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0462

Assessment of PSA-Age volume score in predicting positive prostate biopsy findings in Turkey

Oktay Uçer [1], Uğur Yücetaş [2], İlker Çelen [3], Gökhan Toktaş [2], Talha Müezzinoğlu [1]

[1] Department of Urology, Faculty of Medicine, Celal Bayar University, Manisa, Turkey; [2] Department of Urology, Istanbul Teaching and Research Hospital, Istanbul, Turkey; [3] Urology Clinic, Acipayam State Hospital, Denizli, Turkey

ABSTRACT

Objectives: To evaluate PSA-age volume (AV) scores in predicting positive prostate biopsy findings in Turkey.
Materials and Methods: PSA-AV was calculated by multiplying the patient's age by the prostate volume and dividing it by the PSA level. Sensitivities and specificities of the PSA-AV were assessed by retrospective analysis of findings from 4,717 prostate biopsies.
Results: The population's average age was 63.71±7.63 years, the mean PSA level was 9.73±17.01ng/mL, the mean prostate volume was 44.46±23.88 cm^3. Of the 4,717 prostate biopsies, 1,171 biopsy specimens (24.8%) were positive for prostate cancer. A PSA-AV score of 700 had a sensitivity and specificity of 95% and 15%, respectively. These values were similar to the sensitivity and specificity for a PSA cut-off of 4ng/mL (94% and 13%, respectively). Although the sensitivity of a PSA-AV cut-off of 700 in patients over 60 years was similar to the PSA cut-off of 4ng/mL and the age-adjusted PSA, in patients <60 years, its sensitivity was higher. While the sensitivities of a PSA-AV cut-off of 700 in patients with low prostate volume was higher than a PSA cut-off of 4ng/mL, the sensitivities of both methods with moderate prostate volumes were similar.
Conclusions: Considering all the biopsies, the sensitivity and specificity of a PSA-AV of 700 for predicting positive biopsy findings were similar to a PSA of 4ng/mL. We suggest the PSA-AV cut-off of 700 should only be used in patients younger than 60 with low prostate volumes (<20cm^3).

ARTICLE INFO

Key words:
Prostate-Specific Antigen; Prostatic Neoplasms; Prostate; Biopsy; Digital Rectal Examination

Int Braz J Urol. 2015; 41: 864-8

Submitted for publication:
September 16, 2014

Accepted after revision:
March 24, 2015

INTRODUCTION

Regular serum prostate-specific antigen (PSA) evaluations and digital rectal examinations (DRE) are recommended for detecting PCa (1). However, the serum PSA level is the most widely used marker to detect this cancer in the general population. Because PSA is organ specific, but not disease specific, its use for prostate cancer screening lacks adequate sensitivity (2). Thus, due to the false-positive results obtained by the PSA test during screening, many patients are subjected to an unnecessary transrectal ultrasound-guided prostatic biopsy (TRUSPB), which is an invasive procedure that can lead to significant morbidity, and even mortality (3, 4).

Recently, various strategies were introduced to improve the sensitivity and specificity of the PSA (5). In these studies, prostate volume, PSA level and age were clinically significant predictors of positive

biopsy findings (5, 6). Patel et al. (7) developed a novel formula that incorporates age, prostate volume, race and PSA level into a single score for prostate cancer detection. The PSA-age volume (PSA--AV) score is calculated by multiplying the age and prostate volume and then dividing the total by the pre-biopsy PSA. Patel et al. noticed the formula is useful for predicting positive biopsy findings. According to their data, the PSA-AV score was more sensitive in younger patients and in patients with a small prostate volume. They also reported that the PSA-AV score was more specific in older patients and in patients with a large prostate volume. The purpose of the present retrospective study was to evaluate the novel score in predicting positive prostate biopsy findings in Turkey.

MATERIALS AND METHODS

This retrospective study was based on the data of 5,299 TRUSPB procedures performed between 2005 and 2013 at the Department of Urology of the University Hospital in Manisa and the Clinic of Urology of the Teaching and Research Hospital in Istanbul, Turkey. The indications for performing a TRUS-PB were elevated or increasing PSA levels, abnormal DRE findings or a previous abnormal TRUSPB. The database consisted of four variables including age, pre-biopsy PSA level, prostate volume and digital rectal examination information.

TRUSPBs were performed using the LOGIQ machine at both the University Hospital and the Teaching and Research Hospital by the urologists. The prostate volume was calculated using ultrasonography during the TRUSPB. The number of biopsy cores (6-12 cores) was determined by the urologists according to their preference. In patients with an abnormal DRE or ultrasound findings, additional biopsy cores were taken.

A total of 5,063 biopsy records were reviewed. We eliminated those biopsy records that did not have complete data, number of biopsy cores with less than 10, patients who were <40 years or >79 years old, and patients who had undergone repeat biopsies. A total of 4,717 biopsy specimens were analysed. The PSA-age volume (PSA-AV) score was calculated by multiplying the age and prostate volume and then dividing the total by the pre-biopsy PSA level (7).

Patel et al. (7) stratified the PSA-AV score in intervals of 400, and also analysed the score to determine an effective cut-off score for predicting prostate cancer. The sensitivities and specificities of the PSA AV of 500 and 700 were also analysed since the PSA-AV of 500 or 700 were recommended as a PSA-AV cut-off in their study. Therefore we used PSA-AV of 500 and 700 as cut-off of PSA-AV. The sensitivities and specificities of PSA-AV of 500 and 700 were calculated in the patient groups divided according to age and prostate volume. The sensitivities and specificities of the age-adjusted PSA levels and PSA cut-off of 4ng/mL were calculated. For the age-adjusted PSA data, patients were categorized into four categories, each with its own abnormal PSA value. The abnormal values were >2.5ng/mL (age 40-49 years), >3.5ng/mL (age 50-59 years), >4.5ng/mL (age 60-69 years), and >6.5ng/mL (age 70-79 years). Statistical analyses were carried out using SPSS 13.0 (SPSS Inc.). The study protocol was approved by the ethics committee of our institution.

RESULTS

The mean age of the patients in our study was 63.71±7.63 (n=4,717). The mean PSA level and mean prostate volume were 9.73±17.01ng/mL and 44.46±23.88cm^3, respectively (10% trimmed mean). Of the 4,717 prostate biopsies, 1,171 biopsy specimens (24.8%) were positive for prostate cancer.

The sensitivities and specificities of the PSA-AV scores in intervals of 400, PSA-AV of 500 and 700, and PSA cut-off of 4ng/mL are shown in Figure-1. The positive predictive value of the PSA--AV cut-off of 500 and 700 was 30% and 27%, respectively. The positive predictive value of the PSA cut-off of 4ng/mL and the age-adjusted PSA method was 26% and 25%, respectively. Although using a PSA-AV cut-off of 700 decreased the number of biopsies by 114, it led to 10 more detected cancer cases compared to using the PSA cut-off of 4ng/mL. In the same population, using a PSA cut--off of 4ng/mL increased the biopsies taken by 875 compared with a PSA-AV cut-off of 500 and led to 95 more detected cancer cases. The sensitivity, specificity, positive predictive and negative predictive value changes within the age and prostate volume groups are listed in Tables 1 and 2.

DISCUSSION

Significant research efforts are ongoing to identify the optimal PSA threshold to recommend a prostate biopsy in an asymptomatic patient (5, 8). Catalona et al. reported this PSA level or higher was appropriate as the PSA cut-off value for the screening of PCa. Since then, this value has been the most commonly used clinically. While its sensitivity is 67.5% to 80%, the specificity is only 20% to 30% (9). Although PSA is a highly organ--specific marker, it is not a cancer-specific marker. Therefore, it may also show increases with age or other benign conditions, including benign prostate hyperplasia or prostate inflammation (10). Prostate--specific antigen density (PSAD) was investigated to decrease the impact of prostate volume on the PSA level before deciding on a TRUSPB. Because studies using PSAD for prostate cancer screening have led to conflicting results, it is not widely used by clinicians (11). PSA-AV was developed by Patel et al. to correct the impact of prostate volume on PSA levels (7). They noticed that a PSA-AV score of 700 was a useful formula for predicting positive biopsy findings in patients with small prostates. According to their data, in patients with low to moderate prostate volumes (<20cm^3 and 20-60cm^3), a PSA-AV cut-off of 700 had sensitivities of 97% and 91%, respectively compared with sensitivities of 74% and 86% for a PSA cut-off of 4ng/mL. This

Table 1 - Sensitivity and specificity of various cut-off methods in different age groups.

Variable	Total biopsies (n)	Cancers detected (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Age 40-49 years						
Psa cut off 2.5ng/mL	120	12	85.7	6.1	10.0	77.8
Psa cut off 4.0ng/mL	91	9	64.3	28.7	9.9	86.8
Psaav cut off 700	123	14	100	5.2	11.4	100
Psaav cut off 500	108	13	92.9	17.4	12.0	95.2
50-59 years						
Psa cut off 3.5ng/mL	1191	199	91.7	12.0	16.7	88.2
Psa cut off 4.0ng/mL	1081	192	88.5	21.1	17.8	90.5
Psaav cut off 700	1224	213	98.2	10.3	17.4	96.7
Psaav cut off 500	1005	194	89.4	28	19.3	93.2
60-69 years						
Psa cut off 4.5ng/mL	1767	445	89.6	15.9	25.2	82.8
Psa cut off 4.0ng/mL	1908	472	95.0	8.7	24.7	84.5
Psaav cut off 700	1764	474	95.5	17.9	26.9	92.5
Psaav cut off 500	1408	424	85.3	37.4	30.1	89.0
70-79 years						
Psa cut off 6.5ng/mL	912	375	84.7	26.6	44.1	74.1
Psa cut off 4.0ng/mL	1128	430	97.1	4.6	38.1	72.3
Psaav cut off 700	983	412	93.0	22.0	41.9	83.9
Psaav cut off 500	812	377	85.1	40.6	46.4	81.8

PPV = Positive predictive value; **NPV =** Negative predictive value; **PSA =** prostate-specific antigen; **PSA-AV =** PSA-age volume.

Table 2 - Sensitivity and specificity of PSA-AV cut-off of 700 and 500, and PSA cut-off of 4ng/mL in different prostate volume groups.

Variable	Total biopsies (n)	Cancers detected (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Prostate volume <20cm³						
Psa cut off 4.0ng/mL	298	139	90.8	25.4	46.6	79.4
Psaav cut off 700	345	151	98.7	8.9	43.8	90.5
Psaav cut off 500	341	151	98.7	6.3	44.3	92.0
Prostate volume 20-60cm³						
Psa cut off 4.0ng/mL	2914	821	94.0	14.2	28.2	86.9
Psaav cut off 700	3072	849	97.3	8.8	27.6	90.0
Psaav cut off 500	2555	778	89.1	27.1	30.5	87.4
Prostate volume 60-100cm³						
Psa cut off 4.0ng/mL	786	116	100.0	5.4	14.8	100.0
Psaav cut off 700	553	91	78.4	34.7	16.5	90.8
Psaav cut off 500	358	63	54.3	58.3	17.6	88.6

PPV = Positive predictive value; **NPV =** Negative predictive value; **PSA =** prostate-specific antigen; **PSA-AV =** PSA-age volume.

makes it useful for ruling out prostate cancer. In patients with low to moderate prostate volumes (<20cm³ and 20-60cm³) in our study, a PSA-AV cut-off of 700 had sensitivities of 98% and 97%, respectively. The sensitivities of the PSA cut-off of 4ng/mL were 91% and 94%, respectively, in the same patient group. In both studies, the sensitivities of the PSA-AV cut-off of 700 were higher than the sensitivities of the PSA cut-off of 4ng/mL. However, the sensitivities of the PSA cut-off of 4ng/mL in our study were higher compared with their study. Although their study had 687 patients (prostate volume <60cm³) receiving a TRUSPB, our study had 3,417 patients. Therefore, we think that our data is statistically more reliable. Our findings suggest that, although the sensitivities of PSA-AV cut-off of 700 in patients with low prostate volumes (<20cm³) are higher than with a PSA cut-off of 4ng/mL, the sensitivities of the two methods in patients with moderate prostate volumes (20-60cm³) are similar.

Considering all of the biopsies, we found that the sensitivities of a PSA-AV cut-off of 700 and a PSA cut-off of 4ng/mL were 95% and 94%, respectively. Positive predictive values of a PSA-AV cut-off of 700 and a PSA cut-off of 4ng/mL were 27% and 26%, respectively. According to our data, the effectiveness of the PSA-AV cut-off of 700 compared with a PSA cut-off of 4ng/mL are similar for predicting positive biopsy findings, considering all age groups and prostate volumes. Compared with using the PSA cut-off of 4ng/mL, using the PSA--AV of 700 decreased the number of biopsies by 114; however, it detected 45 more cancer cases. Patel et al. (7) noticed that using a PSA-AV cut-off of 700, rather than a PSA cut-off of 4ng/mL, led to 16 fewer biopsies with seven additional cancers detected.

Our data showed that in the 60-69-year--old population, the sensitivities of an age-adjusted PSA, a PSA cut-off of 4ng/mL and a PSA-AV cut--off of 700 were 90%, 95% and 95%, respectively. In this age group, the diagnostic values of these three methods for predicting positive prostate biopsy findings are similar to each other. In patients younger than 60, the sensitivity of the PSA-AV cut--off of 700 was higher than the age-adjusted PSA and the PSA cut-off of 4ng/mL (Table-1). In the 50-59-year-old population, the sensitivities of the PSA cut-off of 4ng/mL, the PSA cut-off of 3.5ng/mL and a PSA-AV cut-off of 700 were 88%, 91% and 98%, respectively. In this group, compared with using the PSA cut-off of 4ng/mL, using PSA-AV cut-off of 700 led to 143 more biopsies and 21 more

cancer cases detected. Although the effectiveness of a PSA-AV cut-off of 700 in patients aged over 60 is similar to the other methods, in patients under 60 years old its effectiveness seems higher. Similarly, Patel et al. (7) suggest that a PSA-AV score of 700 is useful in ruling out cancer in younger patients.

US Preventive Services Task Force noticed that the amount of overdiagnosis of prostate cancer is an important concern because a man with cancer that would remain asymptomatic for the remainder of his life cannot benefit from screening or treatment (12). One of the limitations of our study was that we did not divide the patients into groups according to their Gleason score. Therefore we could not assess overdiagnosis of PCa and insignificant cancer in our study population. We suggest that further studies should evaluate the effect of PSA--AV formula on insignificant prostate cancer and overdiagnosis.

CONCLUSIONS

Our data supports the findings from the previous study that developed PSA-AV formula. However, in patients with a moderate prostate volume (20-60cm^3), we did not determine any superiority of the PSA-AV formula. Therefore, we suggest that the PSA-AV cut-off of 700 be used for predicting positive prostate biopsy findings in patients under the age of 60 and with low prostate volumes (<20cm^3). Further studies should evaluate the effectiveness of PSA and PSA-AV for predicting positive prostate biopsy findings in the patients without abnormal DRE.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Oktay Ucer, MD
Department of Urology, Faculty of Medicine,
Celal Bayar University, Manisa, Turkey
Telephone: + 90 505 211-4618
E-mail: uceroktay@yahoo.com

ORIGINAL ARTICLE

Vol. 41 (5): 869-897, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0041

Single nucleotide polymorphisms in DKK3 gene are associated with prostate cancer risk and progression

Min Su Kim [1], Ha Na Lee [2], Hae Jong Kim [3,4], Soon Chul Myung [5]

[1]Department of Urology, Seoul Medical Center, Seoul, Korea; [2]Department of Urology, Seoul Seonam Hospital, EwhaWomans University, Seoul, Korea; [3]Research Institue for Biomedical and Pharmaceutical Sciences, Chung-Ang University, Seoul, Korea; [4]Advanced Urogenital Diseas Research Center, Chung-Ang University, College of Medicine, Seoul, Korea; [5]Department of Urology, Chung-Ang University, College of Medicine, Seoul, Korea

ABSTRACT

We had investigated whether sequence variants within DKK3 gene are associated with the development of prostate cancer in a Korean study cohort. We evaluated the association between 53 single nucleotide polymorphisms (SNPs) in the DKK3 gene and prostate cancer risk as well as clinical characteristics (PSA, clinical stage, pathological stage and Gleason score) in Korean men (272 prostate cancer subjects and 173 benign prostate hyperplasia subjects) using unconditional logistic regression analysis. Of the 53 SNPs and 25 common haplotypes, 5 SNPs and 4 haplotypes were associated with prostate cancer risk (P=0.02-0.04); 3 SNPs and 2 haplotypes were significantly associated with susceptibility to prostate cancer, however 2 SNPs and 2 haplotypes exhibited a significant protective effect on prostate cancer. Logistic analyses of the DKK3 gene polymorphisms with several prostate cancer related factors showed that several SNPs were significant; three SNPs and two haplotypes to PSA level, three SNPs and two haplotypes to clinical stage, nine SNPs and two haplotype to pathological stage, one SNP and one haplotypes to Gleason score. To the author's knowledge, this is the first report documenting that DKK3 polymorphisms are not only associated with prostate cancer but also related to prostate cancer-related factors.

ARTICLE INFO

Key words:
Biological Markers; Genetic Variation; Prostatic Neoplasms; Polymorphism, Single Nucleotide

Int Braz J Urol. 2015; 41: 869-97

Submitted for publication:
January 26, 2014

Accepted after revision:
March 06, 2014

INTRODUCTION

Prostate cancer is one of the most common cancers in men. Rates of detection of prostate cancer vary widely across the world, with less frequent detection in South and East Asia than in Europe and especially the United States (1, 2). However the incidence rate of prostate cancer in Korea has rised rapidly during the last decade (3). The etiology of prostate cancer is largely unknown, although several risk factors such as diet, occupation, sexually transmitted agents were investigated epidemiologically; the only established risk factors for prostate cancer are increased age, ethnic background and familial history (4). Recently genome-wide association studies (GAWS) have identified more than 40 single-nucleotide polymorphisms (SNPs) on various genes or chromosomal loci that are significantly associated with prostate cancer susceptibility (5). There is increasing interest in investigating the potential usefulness of SNPs as diagnostic and prognostic biomarkers for prostate cancer outcomes (6, 7).

The wingless-type mouse mammary tumor virus integration site (Wnt) signaling pathway describes a complex network of proteins well known

for their roles in embryogenesis and tumorigenesis (8). Uncontrolled Wnt signaling has been recognized as an important trait of human cancer (9). Their activity is regulated by the secreted Wnt signaling inhibitors including Wnt antagonist families namely the secreted frizzled-related protein (sFRP), Wnt inhibitory factor 1 (Wif-1), and Dickkopf (DKK1-4) families (10).

Dickkopf homologue 3 (DKK3) gene which is located at 11p15, is proposed to function as a tumor suppressor gene since its expression is down-regulated in many types of cancer cells (11). Inactivation of tumor-suppressive genes by either genetic or epigenetic mechanisms contributes to cancer formation. Ectopic expression of DKK3 results in decreased proliferation and is accompanied by attenuation of the mitogen-activated protein kinase pathway (12). If DKK3 regulates the growth of normal and cancerous prostate cells, the variation in DKK3 activity may be important in the onset and progression of prostate cancer. We hypothesized that sequence variations in DKK3 are candidates for risk factors for development of prostate cancer and progression.

However to our knowledge, there have been no reports regarding DKK3 gene polymorphisms in prostate cancer. Here we investigated whether SNPs of the DKK3 gene were associated with the development of prostate cancer in a Korean cohort.

MATERIALS AND METHODS

Study Population

Blood samples were obtained from the Korean Prostate Bank (Seoul, Korea). Both prostate cancer and benign prostatic hyperplasia (BPH) groups originated from a population of older men treated at St. Mary's Hospital (Seoul, Korea). Peripheral blood leukocyte samples for genotyping were obtained from 445 men (prostate cancer, n=272; BPH, n=173) and were stored at -80°C. BPH subjects had true biopsy for confirmation for free of prostate cancer at the time when the samples were taken according to prostate-specific antigen blood tests and digital rectal prostate exams and were excluded from the study if they had a history of prostate cancer. Prostate cancer subjects with primary, incident, histologically confirmed prostate cancer were recruited within 6 months of diagnosis. The median age of the BPH cohort was 67.3 years, and the median age of the prostate cancer cohort was 68.2 years. BPH samples were used as the control group for several reasons. First, most men have evidence of BPH by the age of 70 or 80 years; thus, the presence of some degree of BPH is "normal" at the median age of diagnosis in our prostate cancer cohort (age 67.3 years). Truly "normal" samples would thus only be obtained in a much younger control cohort, which could introduce bias. Second, the collection of blood samples requires a hospital visit and a prostate cancer screening procedure, which would only be undertaken in men with evidence of symptoms of prostate enlargement. All the study participants provided written informed consent. The institutional review board of Chung--Ang University Hospital and Catholic University Hospital approved the study. Blood samples were collected in tubes containing sodium ethylene diaminetetraacetic acid from St. Mary's Hospital in Korea. The QIA amp blood extraction kit (Qiagen, Seoul, Korea) was used for DNA extraction.

The PSA level was classified as low (PSA<4), intermediate (4≤PSA<10), or high (PSA≥10). The Gleason score was designated as low (Gleason score 2-6), intermediate (Gleason score 4+3, 3+4), or high (Gleason score 8-10) grade. The clinicopathologic regional stages were categorized as localized (Stage T1N0M0 orT2N0M0), locally advanced (Stage T3N0M0 or T4N0M0), and metastatic (TxN+ or TxM+) according to the pathologic and/or radiologic reports. Clinical characteristics of the study population are listed in Table-1 and were similar to those of a previous Korean study (13).

SNP Selection and Genotyping

We selected 53 SNPs from two international databases (International HapMap and National Center for Biotechnology Information dbSNPs). SNP selection from the International HapMap database (Han Chinese and Japanese) was performed as follows: (a) extraction of all genotypes from the CHB and JPN population in DKK3 gene region using HapMart of the International HapMap database (version release 27; available from: http://

Table 1 - Study characteristics of prostate cancer cases and controls.

	Cases	Controls
N	272	173
Age (year)±SD	68.2±6.8	67.3±8.8
BMI in kg/m^2 (%)	24.1±3.3	24.0±3.0
Prostate volume (cm3)±SD	37.2±18.6	48.4±26.2
PSA, ng/mL (mean±SD)	48.2±192.8	5.2±6.7
Gleason score, n (%)		
low grade	29 (11%)	
(3+4, 4+3)	202 (75%)	
high grade	39 (14%)	
Clinical Stage, n (%)		
localized	252 (55.1%)	
locally advanced	10 (35.7%)	
metastatic	8 (8.5%)	
unknown	2 (0.7%)	
Pathologic Stage, n (%)		
Localized (T2)	152 (60.3%)	
Advanced (≥T3)	100 (39.7%)	

www.hapmap.org); (b) calculation of minor allele frequency and linkage disequilibrium using Haplo view software (Cambridge, MA; available from: http://www.broad.mit.edu/mpg/haploview), and (c) selection of SNPs having minor allele frequency >0.05 and tagging SNPs if several SNPs showed high linkage disequilibrium >0.98. Furthermore, we added the SNPs in the DKK3 gene region from the National Center for Biotechnology Information db-SNPs. The selection criteria included location (SNPs in exons were preferred) and amino acid changes (nonsynonymous SNPs were preferred). Genotyping was performed at the multiplex level using the Illumina Golden Gate genotyping system (14). In brief, approximately 250 ng genomic DNA extracted from the blood of each subject was used for genotyping by DNA activation, binding to paramagnetic particles, hybridization to oligonucleotides, washing, extension, ligation, amplification by polymerase chain reaction, and hybridization to the Bead plate in an appropriate hybridization buffer. The image intensities were scanned using the Bead Xpress Reader, and genotyped using the Genome Studio software (Illumina). The genotype quality score for retaining data was set to 0.25. A total of 53 SNPs were successfully genotyped.

Statistical analysis

The SNP genotype frequencies were examined for Hardy-Weinberg equilibrium using the chi-square test, and all were found to be consistent (P>0.05) with Hardy-Weinberg equilibrium among the Korean controls. The data were analyzed using unconditional logistic regression analysis to calculate the odds ratio (OR) as an estimate of the relative risk of prostate cancer associated with SNP genotypes (15).

To determine the association between the genotype and haplotype distributions of patients and controls, logistic analysis was performed, controlling for age (continuous value) as a covariate to eliminate or reduce any confounding influence. Significant associations were indicated (P≤0.05). Multiple comparisons were also accounted for by using per mutations to calculate the exact P values for each significant SNP (α=0.05). Lewontin's D' and the linkage disequilibrium coefficient r2 were examined to measure linkage disequilibrium between all pairs of bi-allelic loci (16). Haplotypes were inferred from the successfully genotyped SNPs using the PHASE algorithm, version 2.0 (17), and association analysis was performed using SAS, version 9.1 (SAS Institute, Cary, NC). To achieve optimal correction for multiple testing of markers, representing SNPs in linkage disequilibrium with each other, the effective number of independent marker loci (21.3) was calculated using SNP spectral decomposition software (available from: http://genepi.qimr.edu.au/general/daleN/SNPSpD/), a program that is based on the spectral decomposition of matrices of pair-wise linkage disequilibrium among markers (18).

Statistical power of single associations was calculated with false positive rate of 5%, disease

lifetime prevalence of 0.02%, given minor allele frequencies and sample sizes, and assuming a relative risk of 1.5, using PGA (Power for Genetic Association Analyses) software (19).

RESULTS

The association between DKK3 polymorphisms and the risk of prostate cancer

A total of 53 SNPs from the human DKK3 gene in 272 patients with prostate cancer and 173 control subjects were successfully genotyped to determine the potential association of the gene with the development of prostate cancer (Figure-1). The genotype distributions in the control group were in Hardy-Weinberg equilibrium (P>0.05; data not shown). The measured linkage disequilibrium among 53 SNPs was determined by calculating Lewontin's D' and r^2 values; the results showed that these SNPs were divided among five haplotype blocks (Figure-2). The allele frequencies of each of the polymorphisms and common haplotypes were compared between the patients and the normal controls using logistic regression models. The results of the analysis revealed that five SNPs showed nominal evidence of an association at a P<0.05 level of significance (Table-2, Supplementary Table-1). Of the five significantly associated SNPs, three (rs12421658, rs11022105, and rs4586138) showed a greater frequency in patients with prostate cancer than in the normal controls (OR 1.63, p=0.04; OR 1.54, p=0.04; and OR 1.89, p=0.02, respectively). In addition, a haplotype association test was performed on 25 common haplotypes (frequency >0.05) within the five haplotype blocks. Two haplotypes (Block2_ht3 and Block5_ht5) showed a marginal association with the risk of prostate cancer (P=0.04 and P=0.03, respectively). There were two SNPs (rs2087882 and rs1472190) and two haplotypes (Block3_ht6 and Block5_ht4) that exhibited a significant protective effect from prostate cancer (Table-2).

The association between DKK3 polymorphisms and PSA level in prostate cancer group

We performed analyses involving only the patients with prostate cancer. Four SNPs and two haplotypes exhibited a significant association with the PSA levels (Table-3, Supplementary Table-2). Two SNPs (rs16910308, rs7116879) and one haplotype (Block2_ht2) had a markedly significant effect on elevated PSA levels in the co-dominant and dominant model (OR 1.77, p=0.007 and OR 2.27, p=0.0007; OR 1.71, p=0.0009 and OR 2.21, p=0.0008; OR 1.77, p=0.007 and OR 2.27, p=0.0007, respectively). One SNP (rs988666) had a positive association with PSA in the recessive model (OR 1.92, p=0.04), the other SNP (rs16910295) had same result in the co-dominant model (OR 1.64, p=0.04). One haplotype (Block3_ht5) had a negative association with PSA in the co-dominant model (OR 0.59, p=0.05) and dominant model (OR 0.59, p=0.05).

The association between DKK3 polymorphisms and clinical stage in prostate cancer group

In an analysis according to the clinical stage criteria, five SNPs (rs2403557, rs12295349, rs12288230, rs4586138, and rs7480000) were significantly correlated with clinical stage in each model (OR 2.16, p=0.03 in the co-dominant model and OR 3.53, p=0.02 in the dominant; OR 2.70, p=0.05 in the dominant model; OR 2.78, p=0.04 in the dominant model; OR 2.25, p=0.04 in the co-dominant model and OR 8.71, p=0.01 in the recessive model; OR 2.72, p=0.006 in the co-dominant and OR 4.82, p=0.04 in the dominant model and OR 3.26, p=0.02 in the recessive model, respectively). In addition, two haplotypes (Block3_ht2 and Block5_ht5) were associated with clinical cancer stage (OR 2.78, p=0.04 in the dominant model; OR 2.53, p=0.02 in the co-dominant model and OR 8.71, p=0.01 in the recessive, respectively; Table-4, Supplementary Table-3).

The association between DKK3 polymorphisms and pathologic stage in prostate cancer group

The pathological cancer stage was found to be associated with 9 SNPs (rs751580, rs11544817, rs4757519, rs1552796, rs11022098, rs2087882, rs1472190, rs11022095, and rs1472189) and one haplotype (Block1_ht2) (OR2.08 , p=0.01; OR 1.82, p=0.05; OR 1.64, p=0.009; OR 1.83, p=0.02; OR 1.88, p=0.05; OR 1.74, p=0.04; OR 1.46, p=0.05; OR 1.70, p=0.05; OR 2.66, p=0.0005; OR 1.93, p=0.04, respectively; Table-5, Supplementary Table-4).

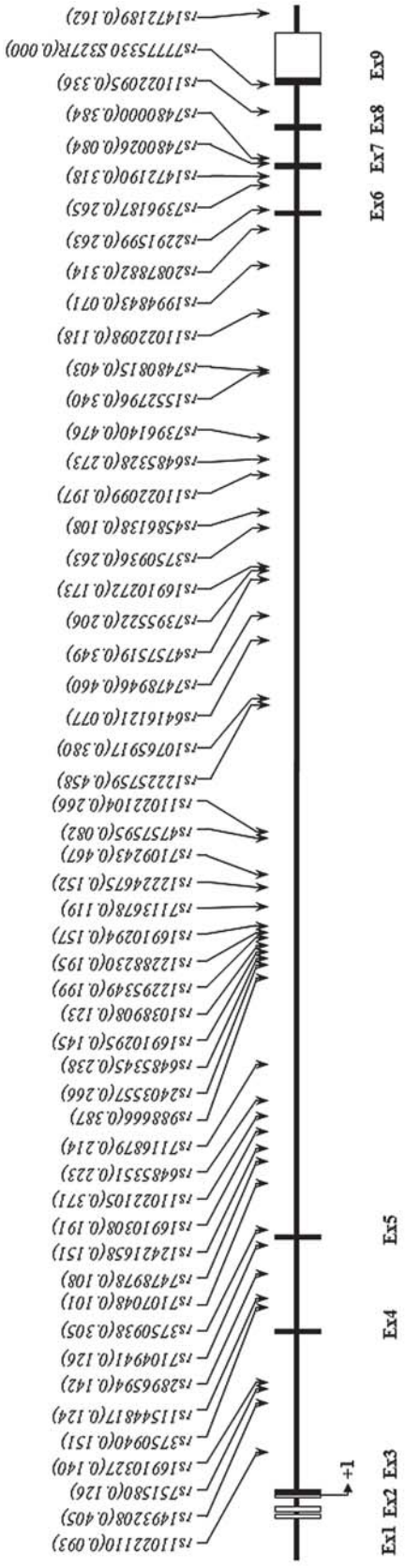

Figure 1 - Map of DKK3 (dickkopf 3 homolog) on chromosome 11p15.2 (46.38 kb).

Figure 2 - Haplotypes among DKK3 SNPs. A) Haplotypes in DKK3. "Others" category contains rare haplotypes, B) Linkage disequilibrium among DKK3 polymorphisms.

A

Block 1

Hap.	*rs1493208*	*rs751580*	*rs16910327*	*rs3750940*	*rs11544817*	*rs2896594*	*rs7104941*	*rs3750938*	Freq.
ht1	C	T	C	A	G	T	C	A	0.580
ht2	T	C	C	A	A	T	C	C	0.109
ht3	T	T	T	G	G	A	T	C	0.075
ht4	T	T	C	A	G	T	C	C	0.059
ht5	T	T	C	G	G	A	T	C	0.043
ht6	T	T	T	A	G	T	C	A	0.037
ht7	T	T	C	A	G	T	C	A	0.036
ht8	T	T	T	G	G	A	C	A	0.018
ht9	T	C	C	A	A	T	C	A	0.011
ht10	T	T	T	G	G	T	C	A	0.011
Others.	.	.	.	.	.	.	.	.	0.022

Block 2

Hap.	*rs7478978*	*rs12421658*	*rs16910308*	*rs11022105*	*rs6485351*	*rs7116879*	Freq.
ht1	A	G	T	T	A	T	0.474
ht2	A	G	G	C	A	C	0.191
ht3	A	A	T	C	G	T	0.151
ht4	T	G	T	T	A	T	0.108
ht5	A	G	T	T	G	T	0.046
ht6	A	G	T	C	G	C	0.023
ht7	A	G	T	C	G	T	0.003
ht8	A	G	T	C	A	T	0.003
ht9	A	G	T	C	A	C	0.001

Block 3

Hap.	*rs16910295*	*rs1038908*	*rs12295349*	*rs12288230*	*rs16910294*	*rs7113678*	*rs12224675*	*rs7109243*	Freq.
ht1	C	C	T	C	A	C	C	T	0.252
ht2	C	C	C	T	A	C	C	C	0.195
ht3	C	C	T	C	A	C	T	C	0.148
ht4	T	C	T	C	G	C	C	T	0.137
ht5	C	T	T	C	A	C	C	C	0.113
ht6	C	C	T	C	A	T	C	T	0.108
ht7	C	C	T	C	G	C	C	T	0.017
Others.	.	.	.	.	.	.	.	.	0.031

Block4

Hap.	*rs4757595*	*rs11022104*	*rs12225759*	*rs10765917*	*rs6416121*	Freq.
ht1	A	A	G	C	T	0.375
ht2	A	A	A	T	T	0.355
ht3	A	C	A	T	T	0.185
ht4	G	C	G	T	A	0.075
ht5	G	A	G	C	T	0.004
ht6	A	C	G	T	A	0.002
ht7	G	C	A	T	T	0.002
ht8	A	C	G	C	T	0.001
ht9	G	C	G	T	T	0.001

Block5

Hap.	*rs4757519*	*rs7395522*	*rs16910272*	*rs3750936*	*rs4586138*	*rs11022099*	*rs6485328*	*rs7396140*	*rs1552796*	*rs7480815*	*rs11022098*	*rs1994843*	*rs2087882*	*rs2291599*	*rs7396187*	*rs1472190*	*rs7480026*	*rs7480000*	*rs11022095*	*rs77775330*	*rs1472189*	Freq.
ht1	G	T	G	T	T	A	C	G	C	C	C	C	G	T	C	C	G	G	A	G	C	0.181
ht2	G	C	G	C	T	G	G	G	C	C	C	C	G	C	G	C	G	C	G	G	C	0.157
ht3	A	C	G	T	T	A	G	A	T	T	C	C	A	C	G	T	G	C	A	G	C	0.137
ht4	A	C	G	T	T	A	G	A	T	T	T	C	A	C	G	T	G	C	G	G	T	0.097
ht5	G	C	A	T	C	A	G	A	C	C	C	C	G	C	G	C	G	G	A	G	C	0.096
ht6	G	C	A	T	T	A	C	G	C	C	C	C	G	T	C	C	G	G	A	G	C	0.059
ht7	G	C	G	C	T	A	G	A	C	T	C	T	G	C	G	C	A	C	A	G	C	0.058
ht8	A	C	G	T	T	A	G	A	T	T	C	C	A	C	G	T	G	C	A	G	T	0.043
Others.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.173

B

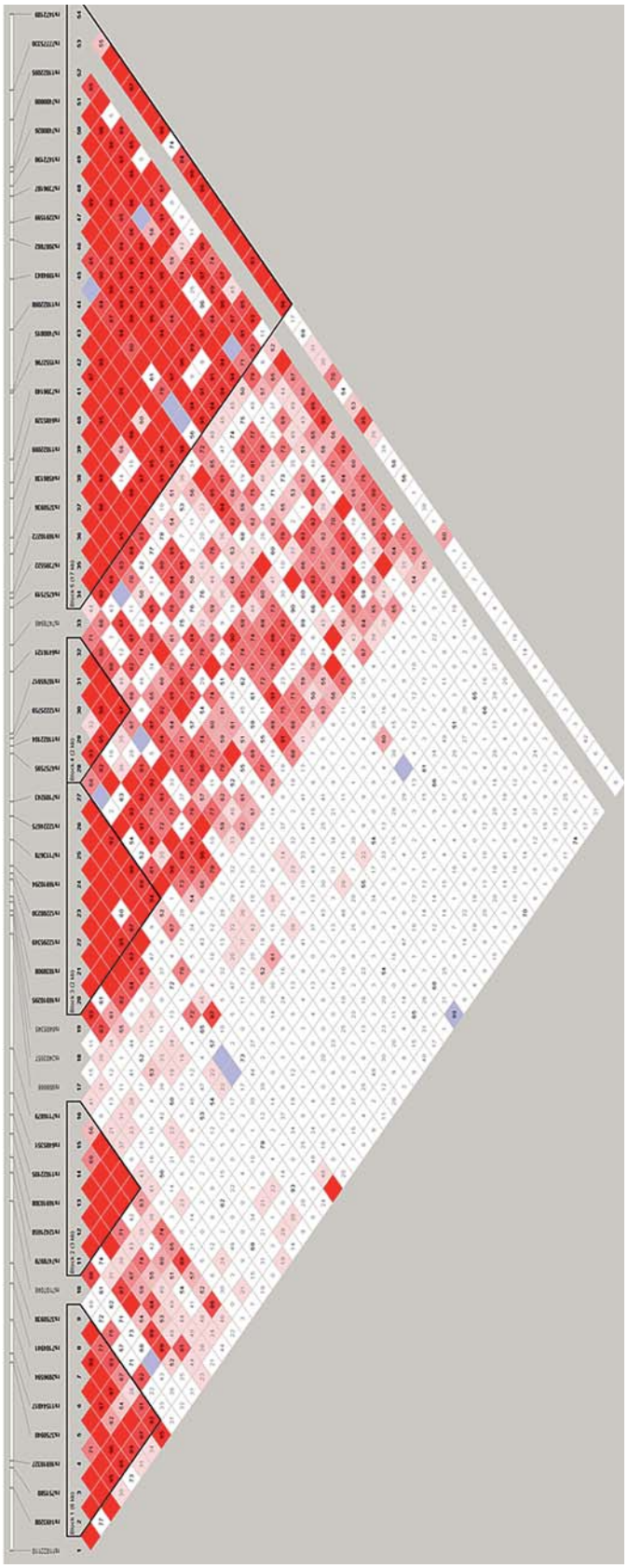

Table 2 - Logistic regression analysis of DKK3 SNPs with the risk of prostate cancer in Korean Population.

SNPID	Chr	Position	Alleles	Minor Allele Frequency		Co-dominant		Dominant		Recessive	
				Pca (n=272)	BPH (n=173)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs12421658	11	Intron	G>A	0.167	0.113	1.59(1.05-2.41)	**0.03***	1.63(1.03-2.59)	**0.04***	2.44(0.50-11.93)	0.27
rs11022105	11	Intron	T>C	0.395	0.318	1.46(1.08-1.99)	**0.02***	1.54(1.03-2.30)	**0.04***	1.83(0.95-3.52)	0.07
rs4586138	11	Intron	T>C	0.136	0.078	1.75(1.09-2.80)	**0.02***	1.89(1.12-3.17)	**0.02***	1.85(0.34-9.97)	0.47
rs2087882	11	Intron	G>A	0.287	0.358	0.73(0.55-0.97)	**0.03***	0.67(0.45-0.99)	**0.04***	0.64(0.36-1.17)	0.15
rs1472190	11	Intron	C>T	0.290	0.361	0.74(0.56-0.98)	**0.04***	0.69(0.47-1.03)	**0.07**	0.61(0.34-1.10)	0.10
DKK3_B2_ht3	11	.	.	0.167	0.113	1.59(1.05-2.41)	**0.03***	1.63(1.03-2.59)	**0.04***	2.44(0.50-11.93)	0.27
DKK3_B3_ht6	11	.	.	0.092	0.142	0.62(0.40-0.96)	**0.03***	0.58(0.36-0.94)	**0.03***	0.65(0.12-3.47)	0.62
DKK3_B5_ht4	11	.	.	0.077	0.127	0.55(0.34-0.88)	**0.01***	0.57(0.35-0.95)	**0.03***	.	.
DKK3_B5_ht5	11	.	.	0.119	0.069	1.67(1.02-2.73)	**0.04***	1.80(1.04-3.12)	**0.03***	1.85(0.34-9.97)	0.47

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown. OR: odd ratio, CI: confidence interval * Statistically significant at $P \leq 0.05$

Supplementary Table 1 - Logistic regression analysis of DKK3 SNPs with the risk of prostate cancer in Korean Population.

SNPID	Chr	Position	AA Change	Alleles	Minor Allele Frequency		Co-dominant		Dominant		Recessive	
					Pca (n=272)	BPH (n=173)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs11022110	11	Intron	.	G>A	0.097	0.081	1.26(0.76-2.09)	0.36	1.21(0.72-2.04)	0.48	.	.
rs1493208	11	Intron	.	C>T	0.412	0.395	1.13(0.84-1.54)	0.42	1.31(0.86-2.00)	0.21	0.95(0.54-1.68)	0.86
rs751580	11	Intron	.	T>C	0.134	0.113	1.34(0.86-2.08)	0.19	1.26(0.79-2.00)	0.34	.	.
rs16910327	11	Intron	.	C>T	0.136	0.156	0.82(0.55-1.22)	0.32	0.85(0.55-1.32)	0.47	0.38(0.08-1.76)	0.21
rs3750940	11	Intron	.	A>G	0.145	0.162	0.89(0.60-1.32)	0.55	0.90(0.58-1.38)	0.62	0.63(0.13-2.98)	0.56
rs11544817	11	Intron	.	G>A	0.131	0.113	1.30(0.84-2.01)	0.24	1.20(0.75-1.92)	0.45	.	.
rs2896594	11	Intron	.	T>A	0.140	0.147	0.94(0.63-1.41)	0.76	0.96(0.62-1.49)	0.86	0.65(0.14-3.05)	0.58
rs7104941	11	Intron	.	C>T	0.126	0.124	1.04(0.68-1.58)	0.87	1.08(0.68-1.72)	0.76	0.71(0.15-3.32)	0.66
rs3750938	11	Intron	.	A>C	0.315	0.283	1.29(0.93-1.77)	0.13	1.25(0.84-1.87)	0.27	1.87(0.84-4.19)	0.13
rs7107048	11	Intron	.	C>T	0.101	0.099	1.04(0.67-1.61)	0.88	1.02(0.61-1.69)	0.96	1.29(0.30-5.51)	0.73
rs7478978	11	Intron	.	A>T	0.110	0.107	1.02(0.65-1.61)	0.94	1.02(0.63-1.65)	0.95	1.09(0.11-10.79)	0.94
rs12421658	11	Intron	.	G>A	0.167	0.113	1.59(1.05-2.41)	**0.03**	1.63(1.03-2.59)	**0.04**	2.44(0.50-11.93)	0.27
rs16910308	11	Intron	.	T>G	0.205	0.165	1.29(0.89-1.87)	0.18	1.39(0.91-2.11)	0.13	1.02(0.33-3.20)	0.97
rs11022105	11	Intron	.	T>C	0.395	0.318	1.46(1.08-1.99)	**0.02**	1.54(1.03-2.30)	**0.04**	1.83(0.95-3.52)	0.07
rs6485351	11	Intron	.	A>G	0.222	0.211	1.06(0.76-1.47)	0.74	1.10(0.73-1.65)	0.66	0.98(0.42-2.29)	0.96
rs7116879	11	Intron	.	T>C	0.220	0.202	1.15(0.81-1.63)	0.44	1.23(0.82-1.84)	0.32	0.88(0.32-2.44)	0.81
rs988666	11	Intron	.	C>T	0.401	0.387	1.03(0.77-1.39)	0.83	0.90(0.59-1.35)	0.60	1.41(0.78-2.53)	0.25
rs2403557	11	Intron	.	C>T	0.265	0.257	1.06(0.77-1.44)	0.74	1.07(0.72-1.59)	0.75	1.08(0.52-2.28)	0.83
rs6485345	11	Intron	.	G>A	0.255	0.214	1.28(0.92-1.79)	0.15	1.39(0.93-2.07)	0.11	1.19(0.50-2.83)	0.69
rs16910295	11	Intron	.	C>T	0.144	0.145	0.99(0.66-1.49)	0.97	0.97(0.62-1.51)	0.89	1.41(0.24-8.24)	0.70
rs1038908	11	Intron	.	C>T	0.131	0.107	1.30(0.83-2.06)	0.26	1.42(0.89-2.29)	0.14	.	.
rs12295349	11	Intron	.	T>C	0.182	0.211	0.82(0.58-1.16)	0.26	0.88(0.58-1.33)	0.54	0.41(0.15-1.14)	0.09
rs12288230	11	Intron	.	C>T	0.178	0.205	0.82(0.58-1.17)	0.28	0.89(0.59-1.34)	0.57	0.41(0.15-1.14)	0.09

rs16910294	11	Intron	.	A>G	0.164	0.145	1.14(0.77-1.70)	0.51	1.06(0.69-1.64)	0.79	4.73(0.57-39.47)	0.15
rs7113678	11	Intron	.	C>T	0.105	0.147	0.68(0.44-1.04)	0.07	0.65(0.41-1.03)	0.07	0.65(0.12-3.47)	0.62
rs12224675	11	Intron	.	C>T	0.164	0.142	1.20(0.82-1.76)	0.36	1.24(0.80-1.94)	0.33	1.18(0.36-3.90)	0.78
rs7109243	11	Intron	.	T>C	0.469	0.457	1.04(0.79-1.38)	0.77	1.06(0.69-1.63)	0.80	1.06(0.66-1.71)	0.81
rs4757595	11	Intron	.	A>G	0.086	0.078	1.07(0.64-1.81)	0.79	1.12(0.66-1.91)	0.68	.	.
rs11022104	11	Intron	.	A>C	0.254	0.266	0.93(0.68-1.28)	0.65	0.95(0.64-1.41)	0.79	0.80(0.37-1.72)	0.56
rs12225759	11	Intron	.	A>G	0.461	0.465	0.97(0.73-1.29)	0.82	1.22(0.79-1.89)	0.38	0.71(0.44-1.16)	0.17
rs10765917	11	Intron	.	T>C	0.378	0.393	0.93(0.69-1.25)	0.63	0.94(0.62-1.41)	0.75	0.87(0.49-1.53)	0.62
rs6416121	11	Intron	.	T>A	0.083	0.069	1.15(0.67-1.98)	0.61	1.21(0.69-2.12)	0.50	.	.
rs7478946	11	Intron	.	T>C	0.454	0.460	0.99(0.75-1.32)	0.97	1.23(0.80-1.90)	0.35	0.75(0.46-1.23)	0.25
rs4757519	11	Intron	.	G>A	0.331	0.384	0.80(0.61-1.06)	0.12	0.74(0.49-1.10)	0.14	0.76(0.44-1.30)	0.31
rs7395522	11	Intron	.	C>T	0.193	0.211	0.92(0.66-1.29)	0.63	0.91(0.60-1.37)	0.65	0.89(0.35-2.21)	0.79
rs16910272	11	Intron	.	G>A	0.189	0.159	1.18(0.83-1.69)	0.35	1.28(0.83-1.97)	0.27	1.04(0.40-2.70)	0.93
rs3750936	11	Intron	.	T>C	0.279	0.240	1.24(0.89-1.72)	0.20	1.35(0.91-2.01)	0.13	1.05(0.45-2.43)	0.91
rs4586138	11	Intron	.	T>C	0.136	0.078	1.75(1.09-2.80)	**0.02**	1.89(1.12-3.17)	**0.02**	1.85(0.34-9.97)	0.47
rs11022099	11	Intron	.	A>G	0.211	0.174	1.30(0.90-1.86)	0.16	1.48(0.97-2.25)	0.07	0.76(0.27-2.13)	0.60
rs6485328	11	Intron	.	G>C	0.248	0.295	0.82(0.60-1.11)	0.19	0.75(0.50-1.11)	0.15	0.85(0.42-1.74)	0.66
rs7396140	11	Intron	.	A>G	0.461	0.477	0.98(0.75-1.27)	0.86	0.91(0.59-1.40)	0.67	1.03(0.66-1.63)	0.89
rs1552796	11	Intron	.	C>T	0.322	0.373	0.81(0.61-1.07)	0.14	0.76(0.51-1.13)	0.17	0.74(0.42-1.30)	0.30
rs7480815	11	Intron	.	C>T	0.389	0.434	0.83(0.63-1.09)	0.18	0.78(0.52-1.18)	0.25	0.77(0.47-1.26)	0.30
rs11022098	11	Intron	.	C>T	0.104	0.142	0.68(0.43-1.05)	0.08	0.73(0.46-1.18)	0.20	.	.
rs1994843	11	Intron	.	C>T	0.072	0.072	0.94(0.54-1.62)	0.81	0.98(0.56-1.71)	0.93	.	.
rs2087882	11	Intron	.	G>A	0.287	0.358	0.73(0.55-0.97)	**0.03**	0.67(0.45-0.99)	**0.04**	0.64(0.36-1.17)	0.15
rs2291599	11	Intron	.	C>T	0.237	0.292	0.79(0.58-1.07)	0.13	0.72(0.49-1.08)	0.11	0.79(0.38-1.63)	0.52
rs7396187	11	Intron	.	G>C	0.241	0.292	0.80(0.59-1.09)	0.16	0.72(0.49-1.08)	0.11	0.86(0.42-1.76)	0.69
rs1472190	11	Intron	.	C>T	0.290	0.361	0.74(0.56-0.98)	**0.04**	0.69(0.47-1.03)	0.07	0.61(0.34-1.10)	0.10
rs7480026	11	Intron	.	G>A	0.086	0.081	1.06(0.64-1.77)	0.81	1.07(0.63-1.83)	0.80	0.91(0.06-14.97)	0.95
rs7480000	11	Intron	.	C>G	0.391	0.370	1.09(0.83-1.45)	0.54	1.22(0.81-1.82)	0.34	0.97(0.57-1.68)	0.92
rs11022095	11	Intron	.	A>G	0.339	0.329	1.04(0.78-1.41)	0.77	1.12(0.75-1.66)	0.59	0.92(0.49-1.73)	0.80

rs1472189	11	3'flanking	.	C>T	0.143	0.191	0.71(0.49-1.02)	0.07	0.76(0.50-1.17)	0.21	0.24(0.07-0.82)	0.02
DKK3_B1_ht1	11	.	.	.	0.426	0.413	1.10(0.82-1.49)	0.52	1.16(0.76-1.78)	0.49	1.09(0.62-1.89)	0.77
DKK3_B1_ht2	11	.	.	.	0.114	0.098	1.32(0.83-2.11)	0.25	1.26(0.77-2.05)	0.36	.	.
DKK3_B1_ht3	11	.	.	.	0.077	0.072	1.09(0.64-1.88)	0.75	1.15(0.66-2.02)	0.62	.	.
DKK3_B1_ht4	11	.	.	.	0.063	0.052	1.27(0.69-2.34)	0.45	1.24(0.66-2.32)	0.51	.	.
DKK3_B2_ht1	11	.	.	.	0.458	0.514	0.81(0.61-1.08)	0.15	0.79(0.50-1.25)	0.31	0.73(0.45-1.17)	0.19
DKK3_B2_ht2	11	.	.	.	0.204	0.165	1.28(0.89-1.85)	0.19	1.38(0.90-2.09)	0.14	1.02(0.33-3.18)	0.98
DKK3_B2_ht3	11	.	.	.	0.167	0.113	1.59(1.05-2.41)	**0.03**	1.63(1.03-2.59)	**0.04**	2.44(0.50-11.93)	0.27
DKK3_B2_ht4	11	.	.	.	0.110	0.107	1.02(0.65-1.61)	0.94	1.02(0.63-1.65)	0.95	1.09(0.11-10.79)	0.94
DKK3_B3_ht1	11	.	.	.	0.259	0.243	1.09(0.80-1.48)	0.60	1.12(0.75-1.66)	0.59	1.10(0.54-2.27)	0.79
DKK3_B3_ht2	11	.	.	.	0.178	0.205	0.82(0.58-1.17)	0.28	0.89(0.59-1.34)	0.57	0.41(0.15-1.14)	0.09
DKK3_B3_ht3	11	.	.	.	0.158	0.142	1.15(0.78-1.68)	0.48	1.18(0.75-1.84)	0.47	1.17(0.36-3.87)	0.79
DKK3_B3_ht4	11	.	.	.	0.134	0.139	0.96(0.63-1.47)	0.86	0.91(0.58-1.43)	0.69	2.81(0.29-27.01)	0.37
DKK3_B3_ht5	11	.	.	.	0.118	0.101	1.22(0.76-1.94)	0.41	1.33(0.82-2.16)	0.25	.	.
DKK3_B3_ht6	11	.	.	.	0.092	0.142	0.62(0.40-0.96)	**0.03**	0.58(0.36-0.94)	**0.03**	0.65(0.12-3.47)	0.62
DKK3_B4_ht1	11	.	.	.	0.373	0.387	0.94(0.70-1.25)	0.66	0.97(0.65-1.46)	0.88	0.83(0.47-1.46)	0.51
DKK3_B4_ht2	11	.	.	.	0.369	0.341	1.15(0.86-1.52)	0.35	1.41(0.95-2.10)	0.09	0.85(0.48-1.50)	0.57
DKK3_B4_ht3	11	.	.	.	0.167	0.191	0.86(0.60-1.23)	0.40	0.89(0.58-1.35)	0.58	0.55(0.19-1.59)	0.27
DKK3_B4_ht4	11	.	.	.	0.081	0.066	1.19(0.69-2.05)	0.54	1.25(0.71-2.21)	0.44	.	.
DKK3_B5_ht1	11	.	.	.	0.165	0.188	0.90(0.64-1.29)	0.57	0.89(0.58-1.35)	0.57	0.88(0.33-2.34)	0.79
DKK3_B5_ht2	11	.	.	.	0.162	0.150	1.12(0.76-1.65)	0.56	1.16(0.75-1.79)	0.52	1.03(0.29-3.70)	0.97
DKK3_B5_ht3	11	.	.	.	0.129	0.150	0.83(0.57-1.21)	0.34	0.81(0.52-1.28)	0.37	0.72(0.24-2.16)	0.55
DKK3_B5_ht4	11	.	.	.	0.077	0.127	0.55(0.34-0.88)	**0.01**	0.57(0.35-0.95)	**0.03**	.	.
DKK3_B5_ht5	11	.	.	.	0.119	0.069	1.67(1.02-2.73)	**0.04**	1.80(1.04-3.12)	**0.03**	1.85(0.34-9.97)	0.47
DKK3_B5_ht6	11	.	.	.	0.050	0.075	0.66(0.37-1.17)	0.16	0.64(0.35-1.17)	0.15	0.71(0.04-13.18)	0.82
DKK3_B5_ht7	11	.	.	.	0.063	0.055	1.10(0.59-2.02)	0.77	1.10(0.59-2.02)	0.77	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown.Significant associations are shown in boldface (P-value≤ 0.05).

MAF: minor allele frequency, OR: odd ratio, CI: confidence interval

Table 3 - Logistic analysis of DKK3 polymorphisms according to PSA criteria.

	Minor Allele Frequency			Co-dominant		Dominant		Recessive	
SNP ID	PSA≥10 (n=113)	4≤PSA<10 (n=98)	PSA<4 (n=62)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs16910308	0.243	0.222	0.105	1.77(1.17-2.67)	**0.007**	2.27(1.41-3.63)	**0.0007**	0.75(0.22-2.55)	0.64
rs7116879	0.257	0.237	0.121	1.71(1.14-2.56)	**0.009**	2.21(1.39-3.52)	**0.0008**	0.78(0.24-2.49)	0.67
rs988666	0.442	0.367	0.379	1.29(0.93-1.78)	0.13	1.17(0.74-1.85)	0.51	1.92(1.02-3.60)	**0.04**
rs16910295	0.173	0.134	0.105	1.64(1.02-2.63)	**0.04**	1.52(0.91-2.52)	0.11	.	.
DKK3_B2_ht2	0.243	0.219	0.105	1.77(1.17-2.67)	**0.007**	2.27(1.42-3.64)	**0.0007**	0.75(0.22-2.55)	0.64
DKK3_B3_ht5	0.080	0.143	0.145	0.59(0.35-0.99)	**0.05**	0.59(0.35-0.99)	**0.05**	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown. OR: odd ratio, CI: confidence interval * Statistically significant at $P \leq 0.05$

Supplementary Table 2 - Logistic analysis of DKK3 polymorphisms according to PSA criteria.

	Minor Allele Frequency			Co-dominant		Dominant		Recessive	
SNP ID	PSA≥10 (n=113)	4≤PSA<10 (n=98)	PSA<4 (n=62)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs11022110	0.097	0.102	0.089	1.05(0.62-1.79)	0.86	1.14(0.64-2.01)	0.65	0.26(0.02-4.04)	0.33
rs1493208	0.394	0.459	0.371	1.01(0.72-1.43)	0.94	1.17(0.73-1.89)	0.51	0.81(0.43-1.52)	0.50
rs751580	0.115	0.158	0.129	0.89(0.56-1.41)	0.62	0.85(0.51-1.41)	0.52	1.27(0.24-6.67)	0.78
rs16910327	0.146	0.143	0.113	1.18(0.73-1.89)	0.50	1.14(0.69-1.89)	0.60	2.71(0.23-31.39)	0.43
rs3750940	0.155	0.168	0.097	1.25(0.79-1.97)	0.35	1.31(0.80-2.15)	0.29	0.98(0.15-6.19)	0.98
rs11544817	0.119	0.143	0.129	0.95(0.60-1.49)	0.81	0.88(0.52-1.47)	0.62	1.70(0.36-8.02)	0.50
rs2896594	0.155	0.158	0.089	1.32(0.83-2.11)	0.24	1.41(0.85-2.33)	0.19	1.03(0.16-6.56)	0.97
rs7104941	0.142	0.141	0.073	1.39(0.85-2.25)	0.19	1.58(0.93-2.70)	0.09	0.74(0.12-4.62)	0.75
rs3750938	0.292	0.361	0.282	0.97(0.69-1.36)	0.85	1.12(0.72-1.75)	0.62	0.62(0.29-1.33)	0.21
rs7107048	0.111	0.102	0.089	1.13(0.69-1.85)	0.63	1.24(0.70-2.20)	0.47	0.76(0.17-3.42)	0.72
rs7478978	0.124	0.102	0.097	1.25(0.75-2.07)	0.40	1.36(0.78-2.35)	0.28	0.41(0.05-3.44)	0.41
rs12421658	0.137	0.168	0.218	0.70(0.46-1.06)	0.10	0.75(0.46-1.21)	0.24	0.27(0.07-1.02)	**0.05**
rs16910308	0.243	0.222	0.105	1.77(1.17-2.67)	**0.007**	2.27(1.41-3.63)	**0.0007**	0.75(0.22-2.55)	0.64
rs11022105	0.398	0.418	0.347	1.16(0.83-1.63)	0.38	1.23(0.77-1.96)	0.38	1.19(0.62-2.27)	0.61
rs6485351	0.186	0.230	0.274	0.73(0.51-1.06)	0.10	0.75(0.47-1.18)	0.21	0.45(0.17-1.19)	0.11
rs7116879	0.257	0.237	0.121	1.71(1.14-2.56)	**0.009**	2.21(1.39-3.52)	**0.0008**	0.78(0.24-2.49)	0.67
rs988666	0.442	0.367	0.379	1.29(0.93-1.78)	0.13	1.17(0.74-1.85)	0.51	1.92(1.02-3.60)	**0.04**
rs2403557	0.286	0.253	0.242	1.19(0.84-1.69)	0.33	1.23(0.79-1.93)	0.36	1.31(0.57-3.03)	0.53
rs6485345	0.292	0.211	0.250	1.26(0.87-1.81)	0.22	1.24(0.79-1.93)	0.35	1.81(0.68-4.78)	0.24

rs16910295	0.173	0.134	0.105	1.64(1.02-2.63)	**0.04**	1.52(0.91-2.52)	0.11	.	.
rs1038908	0.097	0.158	0.145	0.70(0.42-1.16)	0.17	0.70(0.42-1.16)	0.17	.	.
rs12295349	0.177	0.194	0.169	1.03(0.68-1.57)	0.88	1.15(0.72-1.83)	0.57	0.55(0.14-2.23)	0.40
rs12288230	0.177	0.189	0.161	1.07(0.71-1.63)	0.74	1.21(0.75-1.93)	0.44	0.55(0.14-2.23)	0.40
rs16910294	0.190	0.143	0.145	1.39(0.91-2.14)	0.13	1.43(0.88-2.34)	0.15	1.79(0.45-7.05)	0.41
rs7113678	0.102	0.107	0.105	0.90(0.54-1.50)	0.68	0.84(0.48-1.46)	0.53	2.31(0.20-26.72)	0.50
rs12224675	0.183	0.128	0.185	1.02(0.67-1.56)	0.92	1.03(0.63-1.68)	0.91	0.99(0.26-3.74)	0.99
rs7109243	0.438	0.474	0.508	0.82(0.60-1.13)	0.22	0.72(0.44-1.18)	0.20	0.83(0.49-1.40)	0.48
rs4757595	0.058	0.102	0.113	0.58(0.32-1.04)	0.07	0.58(0.32-1.04)	0.07	.	.
rs11022104	0.230	0.281	0.250	0.93(0.65-1.34)	0.69	1.07(0.69-1.68)	0.76	0.53(0.21-1.32)	0.17
rs12225759	0.442	0.459	0.492	0.85(0.60-1.19)	0.33	0.76(0.46-1.26)	0.28	0.88(0.50-1.56)	0.67
rs10765917	0.385	0.357	0.393	0.98(0.70-1.36)	0.90	0.90(0.57-1.42)	0.65	1.15(0.60-2.23)	0.68
rs6416121	0.058	0.102	0.097	0.66(0.37-1.20)	0.17	0.66(0.37-1.20)	0.17	.	.
rs7478946	0.469	0.454	0.435	1.17(0.83-1.63)	0.37	1.07(0.65-1.77)	0.79	1.45(0.81-2.60)	0.22
rs4757519	0.336	0.311	0.363	0.94(0.69-1.28)	0.69	0.83(0.53-1.30)	0.42	1.11(0.58-2.11)	0.75
rs7395522	0.177	0.235	0.153	1.05(0.71-1.55)	0.80	1.13(0.71-1.81)	0.60	0.81(0.28-2.39)	0.71
rs16910272	0.230	0.148	0.177	1.34(0.90-1.99)	0.15	1.43(0.89-2.30)	0.14	1.40(0.46-4.22)	0.55
rs3750936	0.257	0.296	0.290	0.87(0.60-1.25)	0.44	0.89(0.57-1.39)	0.61	0.65(0.26-1.65)	0.36
rs4586138	0.155	0.102	0.153	1.08(0.69-1.70)	0.73	1.02(0.61-1.69)	0.95	2.29(0.44-12.06)	0.33
rs11022099	0.212	0.209	0.208	1.04(0.70-1.55)	0.86	1.08(0.69-1.71)	0.74	0.81(0.24-2.78)	0.74
rs6485328	0.248	0.281	0.194	1.15(0.81-1.63)	0.43	1.32(0.84-2.08)	0.22	0.90(0.39-2.06)	0.80
rs7396140	0.447	0.490	0.435	1.03(0.77-1.38)	0.84	1.09(0.68-1.75)	0.71	0.99(0.59-1.66)	0.97

rs1552796	0.332	0.296	0.355	0.98(0.71-1.34)	0.88	0.85(0.54-1.32)	0.47	1.30(0.67-2.54)	0.44
rs7480815	0.385	0.388	0.410	0.93(0.69-1.26)	0.64	0.79(0.50-1.25)	0.32	1.12(0.63-1.99)	0.71
rs11022098	0.106	0.094	0.113	0.89(0.51-1.55)	0.67	0.89(0.51-1.55)	0.67	.	.
rs1994843	0.053	0.102	0.056	0.77(0.41-1.45)	0.42	0.77(0.41-1.45)	0.42	.	.
rs2087882	0.301	0.247	0.336	0.96(0.69-1.33)	0.80	0.87(0.56-1.36)	0.54	1.17(0.57-2.41)	0.67
rs2291599	0.243	0.270	0.169	1.25(0.88-1.79)	0.22	1.53(0.97-2.41)	0.07	0.86(0.36-2.05)	0.74
rs7396187	0.243	0.276	0.177	1.21(0.85-1.71)	0.29	1.53(0.97-2.41)	0.07	0.75(0.33-1.71)	0.49
rs1472190	0.301	0.255	0.339	0.96(0.69-1.33)	0.78	0.85(0.55-1.33)	0.48	1.22(0.59-2.51)	0.59
rs7480026	0.066	0.122	0.065	0.84(0.48-1.49)	0.55	0.85(0.47-1.52)	0.57	0.55(0.02-20.49)	0.75
rs7480000	0.407	0.398	0.344	1.17(0.85-1.61)	0.33	1.24(0.79-1.96)	0.36	1.22(0.66-2.25)	0.52
rs11022095	0.350	0.320	0.344	1.04(0.74-1.45)	0.84	1.06(0.68-1.65)	0.81	1.01(0.50-2.05)	0.98
rs1472189	0.150	0.115	0.169	0.96(0.60-1.51)	0.85	0.89(0.54-1.47)	0.66	1.96(0.28-13.53)	0.50
DKK3_B1_ht1	0.420	0.469	0.371	1.11(0.80-1.55)	0.54	1.23(0.76-1.98)	0.40	1.03(0.56-1.90)	0.92
DKK3_B1_ht2	0.102	0.122	0.121	0.87(0.53-1.44)	0.59	0.91(0.53-1.55)	0.72	0.43(0.05-3.68)	0.44
DKK3_B1_ht3	0.075	0.087	0.065	1.02(0.55-1.88)	0.95	1.02(0.55-1.88)	0.95	.	.
DKK3_B1_ht4	0.035	0.077	0.089	0.52(0.27-1.00)	**0.05**	0.54(0.27-1.06)	0.07	.	.
DKK3_B2_ht1	0.447	0.439	0.516	0.82(0.59-1.14)	0.24	0.73(0.44-1.21)	0.22	0.83(0.47-1.46)	0.51
DKK3_B2_ht2	0.243	0.219	0.105	1.77(1.17-2.67)	**0.007**	2.27(1.42-3.64)	**0.0007**	0.75(0.22-2.55)	0.64
DKK3_B2_ht3	0.137	0.168	0.218	0.70(0.46-1.06)	0.10	0.75(0.46-1.21)	0.24	0.27(0.07-1.02)	**0.05**
DKK3_B2_ht4	0.124	0.102	0.097	1.25(0.75-2.07)	0.40	1.36(0.78-2.35)	0.28	0.41(0.05-3.44)	0.41
DKK3_B3_ht1	0.274	0.265	0.234	1.13(0.81-1.59)	0.46	1.13(0.72-1.76)	0.60	1.35(0.62-2.94)	0.45
DKK3_B3_ht2	0.177	0.189	0.161	1.07(0.71-1.63)	0.74	1.21(0.75-1.93)	0.44	0.55(0.14-2.23)	0.40

DKK3_B3_ht3	0.173	0.122	0.185	0.96(0.63-1.47)	0.84	0.94(0.58-1.55)	0.82	0.99(0.26-3.71)	0.98
DKK3_B3_ht4	0.159	0.122	0.105	1.54(0.96-2.50)	0.08	1.41(0.84-2.37)	0.19	.	.
DKK3_B3_ht5	0.080	0.143	0.145	0.59(0.35-0.99)	**0.05**	0.59(0.35-0.99)	**0.05**	.	.
DKK3_B3_ht6	0.084	0.092	0.105	0.76(0.44-1.29)	0.31	0.67(0.37-1.21)	0.19	2.31(0.20-26.72)	0.50
DKK3_B4_ht1	0.381	0.357	0.379	1.00(0.72-1.40)	0.99	0.97(0.61-1.52)	0.88	1.09(0.56-2.12)	0.80
DKK3_B4_ht2	0.389	0.362	0.355	1.11(0.80-1.54)	0.54	1.13(0.72-1.79)	0.59	1.17(0.61-2.26)	0.64
DKK3_B4_ht3	0.164	0.179	0.153	1.06(0.69-1.61)	0.80	1.18(0.73-1.92)	0.49	0.55(0.14-2.23)	0.40
DKK3_B4_ht4	0.053	0.102	0.097	0.63(0.34-1.14)	0.13	0.63(0.34-1.14)	0.13	.	.
DKK3_B5_ht1	0.164	0.199	0.113	1.21(0.80-1.83)	0.36	1.36(0.83-2.21)	0.22	0.92(0.28-2.98)	0.88
DKK3_B5_ht2	0.168	0.158	0.153	1.13(0.74-1.73)	0.58	1.18(0.73-1.92)	0.50	0.90(0.22-3.61)	0.88
DKK3_B5_ht3	0.137	0.117	0.145	1.01(0.66-1.56)	0.96	0.96(0.57-1.62)	0.88	1.29(0.37-4.52)	0.69
DKK3_B5_ht4	0.080	0.061	0.097	0.83(0.45-1.53)	0.54	0.83(0.45-1.53)	0.54	.	.
DKK3_B5_ht5	0.137	0.092	0.129	1.10(0.69-1.76)	0.69	1.03(0.60-1.77)	0.91	2.29(0.44-12.06)	0.33
DKK3_B5_ht6	0.053	0.051	0.040	1.19(0.58-2.44)	0.63	1.24(0.58-2.65)	0.58	0.84(0.02-31.30)	0.92
DKK3_B5_ht7	0.049	0.087	0.048	0.83(0.42-1.61)	0.57	0.83(0.42-1.61)	0.57	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown.
Significant associations are shown in bold face (P-value≤0.05).

Table 4 - Logistic analysis of DKK3 polymorphisms according to clinical stage criteria.

SNP ID	Minor Allele Frequency			Co-dominant		Dominant		Recessive	
	Metastatic (n=8)	Locally advanced (n=10)	Localized (n=252)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs2403557	0.500	0.350	0.254	2.16(1.07-4.33)	**0.03**	3.53(1.21-10.26)	**0.02**	1.69(0.36-7.95)	0.50
rs12295349	0.250	0.300	0.175	1.98(0.88-4.48)	0.10	2.70(1.02-7.15)	**0.05**	.	.
rs12288230	0.250	0.300	0.171	2.02(0.90-4.56)	0.09	2.78(1.05-7.36)	**0.04**	.	.
rs4586138	0.438	0.100	0.129	2.25(1.03-4.92)	**0.04**	2.00(0.74-5.38)	0.17	8.71(1.60-47.51)	**0.01**
rs7480000	0.750	0.500	0.378	2.72(1.33-5.56)	**0.006**	4.82(1.08-21.53)	**0.04**	3.26(1.17-9.11)	**0.02**
DKK3_B3_ht2	0.250	0.300	0.171	2.02(0.90-4.56)	0.09	2.78(1.05-7.36)	**0.04**	.	.
DKK3_B5_ht5	0.438	0.100	0.111	2.53(1.17-5.47)	**0.02**	2.43(0.90-6.56)	0.08	8.71(1.60-47.51)	**0.01**

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown. OR: odd ratio, CI: confidence interval * Statistically significant at $P \leq 0.05$

Supplementary Table 3 - Logistic analysis of DKK3 polymorphisms according to clinical stage criteria.

	Minor Allele Frequency			Co-dominant		Dominant		Recessive	
SNP ID	Metastatic (n=8)	Locally advanced (n=10)	Localized (n=252)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs11022110	0.188	0.000	0.097	0.90(0.26-3.05)	0.86	0.92(0.26-3.28)	0.90	.	.
rs1493208	0.375	0.600	0.405	1.58(0.76-3.28)	0.22	2.61(0.73-9.33)	0.14	1.24(0.33-4.59)	0.75
rs751580	0.063	0.250	0.133	1.35(0.53-3.46)	0.53	1.55(0.55-4.35)	0.41	.	.
rs16910327	0.063	0.250	0.133	1.29(0.50-3.32)	0.60	1.11(0.38-3.26)	0.85	4.70(0.37-59.48)	0.23
rs3750940	0.125	0.100	0.145	0.73(0.25-2.14)	0.56	0.76(0.24-2.39)	0.64	.	.
rs11544817	0.063	0.250	0.129	1.38(0.55-3.45)	0.49	1.67(0.59-4.69)	0.34	.	.
rs2896594	0.125	0.100	0.139	0.75(0.25-2.22)	0.60	0.79(0.25-2.48)	0.68	.	.
rs7104941	0.063	0.100	0.126	0.60(0.17-2.06)	0.42	0.61(0.17-2.20)	0.45	.	.
rs3750938	0.313	0.400	0.311	1.30(0.62-2.71)	0.48	1.39(0.52-3.70)	0.51	1.41(0.30-6.67)	0.66
rs7107048	0.000	0.050	0.103	0.27(0.04-1.96)	0.19	0.25(0.03-1.99)	0.19	.	.
rs7478978	0.000	0.050	0.113	0.21(0.03-1.59)	0.13	0.20(0.03-1.58)	0.13	.	.
rs12421658	0.125	0.250	0.167	1.26(0.53-2.99)	0.60	1.56(0.57-4.21)	0.39	.	.
rs16910308	0.188	0.250	0.203	1.13(0.49-2.60)	0.78	1.08(0.40-2.88)	0.88	1.63(0.18-14.46)	0.66
rs11022105	0.313	0.500	0.394	1.14(0.56-2.36)	0.71	0.87(0.32-2.35)	0.79	1.90(0.58-6.29)	0.29
rs6485351	0.125	0.400	0.220	1.39(0.66-2.96)	0.39	1.70(0.64-4.49)	0.29	0.99(0.12-8.31)	1.00
rs7116879	0.188	0.250	0.219	1.03(0.44-2.41)	0.94	0.97(0.36-2.59)	0.95	1.54(0.18-13.52)	0.70
rs988666	0.563	0.400	0.397	1.38(0.70-2.74)	0.35	2.00(0.64-6.28)	0.23	1.10(0.31-3.91)	0.88
rs2403557	0.500	0.350	0.254	2.16(1.07-4.33)	**0.03**	3.53(1.21-10.26)	**0.02**	1.69(0.36-7.95)	0.50
rs6485345	0.438	0.150	0.253	1.18(0.54-2.59)	0.67	1.25(0.48-3.26)	0.65	1.13(0.14-9.04)	0.91
rs16910295	0.063	0.250	0.143	1.21(0.47-3.12)	0.69	1.35(0.48-3.80)	0.57	.	.
rs1038908	0.063	0.050	0.135	0.38(0.08-1.72)	0.21	0.38(0.08-1.72)	0.21	.	.
rs12295349	0.250	0.300	0.175	1.98(0.88-4.48)	0.10	2.70(1.02-7.15)	**0.05**	.	.
rs12288230	0.250	0.300	0.171	2.02(0.90-4.56)	0.09	2.78(1.05-7.36)	**0.04**	.	.
rs16910294	0.063	0.250	0.165	1.02(0.42-2.52)	0.96	1.20(0.43-3.37)	0.72	.	.
rs7113678	0.000	0.150	0.105	0.67(0.20-2.29)	0.53	0.71(0.19-2.60)	0.60	.	.
rs12224675	0.500	0.050	0.159	1.69(0.77-3.73)	0.19	1.92(0.73-5.08)	0.19	1.80(0.22-15.05)	0.59
rs7109243	0.813	0.400	0.460	1.70(0.85-3.37)	0.13	2.13(0.59-7.68)	0.25	1.94(0.70-5.37)	0.20
rs4757595	0.000	0.000	0.091	.	.	.	.	.	.

rs11022104	0.188	0.300	0.252	1.05(0.47-2.35)	0.91	1.32(0.51-3.46)	0.57	.	.
rs12225759	0.188	0.450	0.470	0.50(0.23-1.07)	0.07	0.39(0.15-1.02)	0.06	0.52(0.11-2.37)	0.39
rs10765917	0.188	0.450	0.382	0.74(0.36-1.55)	0.43	0.68(0.26-1.79)	0.43	0.70(0.15-3.30)	0.65
rs6416121	0.000	0.000	0.087	.	.	.	.	.	.
rs7478946	0.563	0.550	0.446	1.78(0.86-3.70)	0.12	3.64(0.80-16.54)	0.10	1.45(0.45-4.67)	0.53
rs4757519	0.313	0.350	0.329	0.99(0.50-1.95)	0.97	0.88(0.34-2.29)	0.78	1.24(0.34-4.54)	0.75
rs7395522	0.188	0.300	0.190	1.47(0.67-3.22)	0.34	2.05(0.78-5.40)	0.15	.	.
rs16910272	0.500	0.100	0.185	1.64(0.78-3.45)	0.19	1.59(0.60-4.21)	0.35	3.09(0.65-14.68)	0.16
rs3750936	0.000	0.250	0.288	0.37(0.14-1.01)	**0.05**	0.37(0.13-1.07)	0.07	.	.
rs4586138	0.438	0.100	0.129	2.25(1.03-4.92)	**0.04**	2.00(0.74-5.38)	0.17	8.71(1.60-47.51)	**0.01**
rs11022099	0.000	0.250	0.216	0.56(0.20-1.53)	0.26	0.58(0.20-1.68)	0.31	.	.
rs6485328	0.250	0.300	0.248	1.16(0.56-2.42)	0.69	1.37(0.52-3.58)	0.52	0.75(0.10-5.73)	0.78
rs7396140	0.250	0.550	0.466	0.86(0.45-1.64)	0.65	0.76(0.28-2.03)	0.58	0.89(0.28-2.82)	0.84
rs1552796	0.313	0.350	0.319	1.06(0.53-2.13)	0.87	0.92(0.35-2.39)	0.86	1.52(0.41-5.66)	0.53
rs7480815	0.313	0.350	0.390	0.79(0.39-1.58)	0.50	0.62(0.24-1.63)	0.33	0.99(0.27-3.59)	0.98
rs11022098	0.063	0.150	0.100	1.00(0.31-3.22)	1.00	1.00(0.31-3.22)	1.00	.	.
rs1994843	0.000	0.000	0.075	.	.	.	.	.	.
rs2087882	0.250	0.200	0.292	0.71(0.32-1.55)	0.39	0.69(0.26-1.83)	0.45	0.51(0.07-3.91)	0.51
rs2291599	0.313	0.300	0.234	1.47(0.71-3.02)	0.30	1.90(0.72-5.01)	0.19	0.91(0.12-6.95)	0.92
rs7396187	0.250	0.300	0.240	1.23(0.59-2.56)	0.58	1.48(0.57-3.87)	0.42	0.80(0.11-6.14)	0.83
rs1472190	0.250	0.200	0.296	0.71(0.32-1.55)	0.39	0.68(0.25-1.81)	0.44	0.52(0.07-4.03)	0.53
rs7480026	0.000	0.000	0.091	.	.	.	.	.	.
rs7480000	0.750	0.500	0.378	2.72(1.33-5.56)	**0.006**	4.82(1.08-21.53)	**0.04**	3.26(1.17-9.11)	**0.02**
rs11022095	0.125	0.450	0.340	0.81(0.38-1.71)	0.58	0.89(0.34-2.33)	0.81	0.45(0.06-3.56)	0.45
rs1472189	0.125	0.100	0.144	0.72(0.24-2.17)	0.56	0.51(0.14-1.84)	0.31	6.07(0.64-57.40)	0.12
DKK3_B1_ht1	0.438	0.650	0.417	1.90(0.91-3.95)	0.09	2.38(0.67-8.49)	0.18	2.23(0.73-6.75)	0.16
DKK3_B1_ht2	0.063	0.250	0.111	1.66(0.63-4.38)	0.30	1.86(0.66-5.26)	0.24	.	.
DKK3_B1_ht3	0.000	0.100	0.077	0.66(0.14-3.03)	0.59	0.66(0.14-3.03)	0.59	.	.
DKK3_B1_ht4	0.125	0.000	0.063	0.96(0.22-4.19)	0.95	0.97(0.22-4.37)	0.97	.	.
DKK3_B2_ht1	0.688	0.300	0.458	1.05(0.51-2.14)	0.90	0.95(0.32-2.81)	0.93	1.21(0.38-3.84)	0.75
DKK3_B2_ht2	0.188	0.250	0.202	1.13(0.49-2.61)	0.78	1.08(0.40-2.90)	0.88	1.63(0.18-14.49)	0.66
DKK3_B2_ht3	0.125	0.250	0.167	1.26(0.53-2.99)	0.60	1.56(0.57-4.21)	0.39	.	.
DKK3_B2_ht4	0.000	0.050	0.113	0.21(0.03-1.59)	0.13	0.20(0.03-1.58)	0.13	.	.
DKK3_B3_ht1	0.188	0.200	0.264	0.72(0.32-1.63)	0.43	0.51(0.18-1.47)	0.21	1.36(0.28-6.49)	0.70

DKK3_B3_ht2	0.250	0.300	0.171	2.02(0.90-4.56)	0.09	2.78(1.05-7.36)	**0.04**	.	.
DKK3_B3_ht3	0.438	0.050	0.155	1.47(0.65-3.31)	0.35	1.56(0.58-4.20)	0.38	1.81(0.22-15.12)	0.58
DKK3_B3_ht4	0.000	0.250	0.135	1.03(0.38-2.81)	0.96	1.12(0.38-3.32)	0.84	.	.
DKK3_B3_ht5	0.063	0.050	0.121	0.43(0.10-1.95)	0.27	0.43(0.10-1.95)	0.27	.	.
DKK3_B3_ht6	0.000	0.150	0.091	0.76(0.22-2.57)	0.66	0.81(0.22-3.03)	0.76	.	.
DKK3_B4_ht1	0.188	0.450	0.377	0.77(0.37-1.60)	0.48	0.71(0.27-1.86)	0.48	0.73(0.16-3.44)	0.69
DKK3_B4_ht2	0.625	0.250	0.367	1.28(0.63-2.60)	0.50	1.03(0.38-2.76)	0.96	2.17(0.66-7.07)	0.20
DKK3_B4_ht3	0.188	0.300	0.161	1.83(0.81-4.16)	0.15	2.44(0.93-6.43)	0.07	.	.
DKK3_B4_ht4	0.000	0.000	0.085	.	.	.	.	.	.
DKK3_B5_ht1	0.125	0.300	0.163	1.50(0.66-3.41)	0.34	1.98(0.74-5.25)	0.17	.	.
DKK3_B5_ht2	0.000	0.250	0.165	0.82(0.30-2.19)	0.69	0.89(0.30-2.62)	0.83	.	.
DKK3_B5_ht3	0.125	0.000	0.135	0.41(0.10-1.69)	0.22	0.41(0.09-1.80)	0.24	.	.
DKK3_B5_ht4	0.000	0.100	0.077	0.59(0.13-2.75)	0.50	0.59(0.13-2.75)	0.50	.	.
DKK3_B5_ht5	0.438	0.100	0.111	2.53(1.17-5.47)	**0.02**	2.43(0.90-6.56)	0.08	8.71(1.60-47.51)	**0.01**
DKK3_B5_ht6	0.063	0.000	0.052	0.54(0.07-3.98)	0.54	0.54(0.07-4.11)	0.55	.	.
DKK3_B5_ht7	0.000	0.000	0.065	.	.	.	.	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown.
Significant associations are shown in bold face (P-value≤0.05).

Table 5 - Logistic analysis of DKK3 polymorphisms according to pathological stage criteria.

SNP ID	Minor Allele Frequency		Co-dominant		Dominant		Recessive	
	Locally advanced (n=100)	Localized (n=152)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs751580	0.180	0.107	1.90(1.11-3.23)	**0.02***	2.08(1.16-3.72)	**0.01***	1.63(0.23-11.79)	0.63
rs11544817	0.170	0.107	1.72(1.02-2.92)	**0.04***	1.82(1.01-3.29)	**0.05***	2.37(0.39-14.51)	0.35
rs4757519	0.418	0.290	1.64(1.13-2.38)	**0.009***	1.85(1.09-3.14)	**0.02***	2.13(1.02-4.44)	**0.04***
rs1552796	0.397	0.290	1.54(1.06-2.23)	**0.02***	1.83(1.08-3.09)	**0.02***	1.67(0.79-3.54)	0.18
rs11022098	0.144	0.084	1.88(1.01-3.52)	**0.05***	1.88(1.01-3.52)	**0.05***	.	.
rs1994843	0.041	0.097	0.36(0.16-0.82)	**0.02***	0.36(0.16-0.82)	**0.02***	.	.
rs2087882	0.356	0.253	1.54(1.05-2.26)	**0.03***	1.74(1.03-2.92)	**0.04***	1.87(0.82-4.26)	0.13
rs1472190	0.351	0.263	1.46(1.00-2.14)	**0.05***	1.55(0.92-2.59)	0.10	1.93(0.85-4.37)	0.12
rs11022095	0.387	0.304	1.44(0.98-2.12)	0.06	1.70(1.00-2.88)	**0.05***	1.40(0.63-3.11)	0.40
rs1472189	0.216	0.101	2.66(1.54-4.59)	**0.0005***	2.61(1.47-4.62)	**0.001***	.	.
DKK3_B1_ht2	0.155	0.087	2.00(1.12-3.58)	**0.02***	1.93(1.04-3.55)	**0.04***	.	.
DKK3_B5_ht7	0.036	0.090	0.33(0.14-0.80)	**0.01***	0.33(0.14-0.80)	**0.01***	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown. **OR:** odd ratio, **CI:** confidence interval * Statistically significant at $P \leq 0.05$

Supplementary Table 4 - Logistic analysis of DKK3 polymorphisms according to pathological stage criteria.

	Minor Allele Frequency		Co-dominant		Dominant		Recessive	
SNP ID	Locally advanced (n=97)	Localized (n=150)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs11022110	0.082	0.107	0.76(0.40-1.45)	0.41	0.80(0.41-1.57)	0.52	.	.
rs1493208	0.428	0.393	1.20(0.81-1.78)	0.37	1.34(0.77-2.34)	0.30	1.12(0.53-2.35)	0.77
rs751580	0.180	0.107	1.90(1.11-3.23)	**0.02**	2.08(1.16-3.72)	**0.01**	1.63(0.23-11.79)	0.63
rs16910327	0.103	0.143	0.68(0.38-1.21)	0.19	0.59(0.32-1.11)	0.10	2.67(0.24-30.24)	0.43
rs3750940	0.124	0.150	0.79(0.46-1.36)	0.39	0.80(0.44-1.44)	0.45	0.47(0.05-4.64)	0.52
rs11544817	0.170	0.107	1.72(1.02-2.92)	**0.04**	1.82(1.01-3.29)	**0.05**	2.37(0.39-14.51)	0.35
rs2896594	0.124	0.137	0.89(0.51-1.53)	0.66	0.91(0.50-1.66)	0.77	0.48(0.05-4.80)	0.54
rs7104941	0.113	0.122	0.92(0.53-1.62)	0.78	1.05(0.57-1.95)	0.88	.	.
rs3750938	0.325	0.292	1.19(0.80-1.77)	0.39	1.20(0.72-2.00)	0.50	1.40(0.58-3.42)	0.46
rs7107048	0.098	0.103	0.97(0.54-1.71)	0.90	1.07(0.55-2.08)	0.84	0.36(0.04-3.30)	0.37
rs7478978	0.103	0.110	0.95(0.52-1.73)	0.86	1.03(0.54-1.94)	0.93	.	.
rs12421658	0.165	0.173	0.96(0.59-1.55)	0.86	0.88(0.50-1.54)	0.65	1.60(0.39-6.59)	0.51
rs16910308	0.175	0.211	0.77(0.48-1.24)	0.28	0.78(0.46-1.35)	0.38	0.47(0.09-2.37)	0.36
rs11022105	0.366	0.409	0.81(0.55-1.21)	0.31	0.74(0.43-1.27)	0.28	0.84(0.38-1.84)	0.65
rs6485351	0.237	0.227	1.08(0.71-1.64)	0.73	1.11(0.66-1.88)	0.69	1.02(0.35-2.98)	0.97
rs7116879	0.186	0.232	0.74(0.46-1.17)	0.20	0.75(0.44-1.28)	0.29	0.42(0.08-2.06)	0.28
rs988666	0.433	0.373	1.27(0.88-1.84)	0.20	1.22(0.72-2.09)	0.46	1.67(0.84-3.32)	0.14
rs2403557	0.250	0.252	1.00(0.66-1.53)	1.00	0.98(0.58-1.65)	0.94	1.08(0.37-3.16)	0.89
rs6485345	0.268	0.245	1.11(0.72-1.69)	0.64	0.97(0.58-1.63)	0.90	2.22(0.74-6.63)	0.15
rs16910295	0.129	0.154	0.83(0.48-1.44)	0.51	0.82(0.46-1.47)	0.50	0.81(0.07-9.11)	0.86
rs1038908	0.134	0.130	1.14(0.63-2.06)	0.67	1.14(0.63-2.06)	0.67	.	.
rs12295349	0.170	0.170	1.03(0.63-1.69)	0.90	1.14(0.66-1.98)	0.64	0.34(0.04-2.98)	0.33
rs12288230	0.170	0.167	1.06(0.64-1.73)	0.83	1.18(0.68-2.04)	0.57	0.34(0.04-2.98)	0.33
rs16910294	0.134	0.187	0.70(0.42-1.16)	0.16	0.66(0.37-1.18)	0.16	0.60(0.11-3.17)	0.55
rs7113678	0.134	0.093	1.46(0.81-2.63)	0.21	1.51(0.81-2.81)	0.19	1.24(0.08-20.34)	0.88
rs12224675	0.139	0.164	0.77(0.46-1.30)	0.33	0.82(0.46-1.46)	0.50	0.27(0.03-2.40)	0.24
rs7109243	0.448	0.450	1.00(0.69-1.45)	0.99	1.10(0.63-1.93)	0.74	0.89(0.47-1.71)	0.73
rs4757595	0.067	0.103	0.62(0.31-1.27)	0.19	0.62(0.31-1.27)	0.19	.	.
rs11022104	0.216	0.260	0.83(0.54-1.28)	0.39	0.90(0.53-1.53)	0.70	0.40(0.11-1.49)	0.17
rs12225759	0.469	0.467	1.02(0.68-1.52)	0.93	0.96(0.53-1.74)	0.89	1.11(0.57-2.16)	0.76
rs10765917	0.407	0.362	1.21(0.82-1.78)	0.34	1.22(0.71-2.08)	0.47	1.39(0.66-2.94)	0.39
rs6416121	0.062	0.103	0.57(0.28-1.18)	0.13	0.57(0.28-1.18)	0.13	.	.
rs7478946	0.469	0.440	1.18(0.80-1.74)	0.41	1.68(0.92-3.07)	0.09	0.82(0.41-1.63)	0.57
rs4757519	0.418	0.290	1.64(1.13-2.38)	**0.009**	1.85(1.09-3.14)	**0.02**	2.13(1.02-4.44)	0.04
rs7395522	0.149	0.207	0.71(0.44-1.15)	0.16	0.79(0.45-1.38)	0.40	0.16(0.02-1.26)	0.08
rs16910272	0.165	0.200	0.77(0.49-1.23)	0.28	0.77(0.44-1.35)	0.36	0.53(0.14-2.06)	0.36
rs3750936	0.263	0.293	0.87(0.57-1.34)	0.54	0.93(0.55-1.55)	0.77	0.57(0.18-1.85)	0.35

rs4586138	0.113	0.147	0.72(0.42-1.25)	0.25	0.74(0.40-1.35)	0.32	0.34(0.04-3.13)	0.34
rs11022099	0.214	0.201	1.13(0.71-1.79)	0.62	1.17(0.69-2.00)	0.56	0.96(0.22-4.12)	0.96
rs6485328	0.206	0.263	0.75(0.49-1.14)	0.18	0.72(0.42-1.22)	0.22	0.61(0.21-1.78)	0.36
rs7396140	0.428	0.467	0.91(0.64-1.29)	0.58	0.79(0.46-1.36)	0.39	1.00(0.54-1.85)	1.00
rs1552796	0.397	0.290	1.54(1.06-2.23)	**0.02**	1.83(1.08-3.09)	**0.02**	1.67(0.79-3.54)	0.18
rs7480815	0.438	0.383	1.20(0.84-1.71)	0.32	1.21(0.71-2.08)	0.48	1.38(0.72-2.65)	0.33
rs11022098	0.144	0.084	1.88(1.01-3.52)	**0.05**	1.88(1.01-3.52)	**0.05**	.	.
rs1994843	0.041	0.097	0.36(0.16-0.82)	**0.02**	0.36(0.16-0.82)	**0.02**	.	.
rs2087882	0.356	0.253	1.54(1.05-2.26)	**0.03**	1.74(1.03-2.92)	**0.04**	1.87(0.82-4.26)	0.13
rs2291599	0.206	0.247	0.82(0.54-1.25)	0.36	0.81(0.48-1.37)	0.42	0.68(0.23-2.01)	0.48
rs7396187	0.211	0.247	0.85(0.56-1.29)	0.43	0.81(0.48-1.37)	0.43	0.82(0.29-2.27)	0.70
rs1472190	0.351	0.263	1.46(1.00-2.14)	0.05	1.55(0.92-2.59)	0.10	1.93(0.85-4.37)	0.12
rs7480026	0.062	0.107	0.53(0.26-1.08)	0.08	0.54(0.26-1.11)	0.09	.	.
rs7480000	0.340	0.413	0.73(0.51-1.07)	0.10	0.67(0.40-1.14)	0.14	0.65(0.31-1.35)	0.25
rs11022095	0.387	0.304	1.44(0.98-2.12)	0.06	1.70(1.00-2.88)	**0.05**	1.40(0.63-3.11)	0.40
rs1472189	0.216	0.101	2.66(1.54-4.59)	**0.0005**	2.61(1.47-4.62)	**0.001**	.	.
DKK3_B1_ht1	0.438	0.403	1.20(0.81-1.76)	0.36	1.30(0.74-2.27)	0.36	1.19(0.59-2.42)	0.62
DKK3_B1_ht2	0.155	0.087	2.00(1.12-3.58)	**0.02**	1.93(1.04-3.55)	**0.04**	.	.
DKK3_B1_ht3	0.067	0.073	0.90(0.43-1.89)	0.78	0.90(0.43-1.89)	0.78	.	.
DKK3_B1_ht4	0.041	0.073	0.57(0.25-1.32)	0.19	0.58(0.24-1.37)	0.21	.	.
DKK3_B2_ht1	0.474	0.450	1.10(0.75-1.62)	0.62	1.17(0.65-2.10)	0.60	1.09(0.56-2.09)	0.80
DKK3_B2_ht2	0.175	0.210	0.78(0.48-1.25)	0.30	0.79(0.46-1.36)	0.40	0.47(0.09-2.38)	0.36
DKK3_B2_ht3	0.165	0.173	0.96(0.59-1.55)	0.86	0.88(0.50-1.54)	0.65	1.60(0.39-6.59)	0.51
DKK3_B2_ht4	0.103	0.110	0.95(0.52-1.73)	0.86	1.03(0.54-1.94)	0.93	.	.
DKK3_B3_ht1	0.289	0.263	1.12(0.76-1.65)	0.57	1.22(0.73-2.04)	0.45	0.99(0.41-2.39)	0.98
DKK3_B3_ht2	0.170	0.167	1.06(0.64-1.73)	0.83	1.18(0.68-2.04)	0.57	0.34(0.04-2.98)	0.33
DKK3_B3_ht3	0.134	0.157	0.78(0.46-1.32)	0.35	0.83(0.46-1.49)	0.53	0.28(0.03-2.42)	0.24
DKK3_B3_ht4	0.119	0.143	0.83(0.47-1.45)	0.51	0.82(0.45-1.49)	0.50	0.82(0.07-9.16)	0.87
DKK3_B3_ht5	0.124	0.113	1.21(0.66-2.22)	0.55	1.21(0.66-2.22)	0.55	.	.
DKK3_B3_ht6	0.119	0.080	1.46(0.78-2.71)	0.24	1.52(0.78-2.93)	0.22	1.24(0.08-20.34)	0.88
DKK3_B4_ht1	0.397	0.363	1.15(0.78-1.69)	0.47	1.11(0.65-1.88)	0.71	1.40(0.66-2.97)	0.38
DKK3_B4_ht2	0.376	0.377	0.97(0.66-1.42)	0.87	0.88(0.52-1.49)	0.63	1.14(0.54-2.41)	0.72
DKK3_B4_ht3	0.155	0.157	1.03(0.62-1.70)	0.91	1.14(0.65-2.02)	0.64	0.34(0.04-2.98)	0.33
DKK3_B4_ht4	0.057	0.103	0.52(0.25-1.10)	0.09	0.52(0.25-1.10)	0.09	.	.
DKK3_B5_ht1	0.134	0.180	0.75(0.45-1.23)	0.25	0.81(0.45-1.43)	0.46	0.21(0.03-1.74)	0.15
DKK3_B5_ht2	0.144	0.173	0.83(0.50-1.38)	0.48	0.89(0.50-1.56)	0.67	0.31(0.04-2.67)	0.28
DKK3_B5_ht3	0.124	0.133	0.92(0.55-1.54)	0.74	0.95(0.52-1.75)	0.87	0.61(0.12-3.22)	0.56
DKK3_B5_ht4	0.108	0.063	1.81(0.91-3.60)	0.09	1.81(0.91-3.60)	0.09	.	.
DKK3_B5_ht5	0.093	0.137	0.64(0.36-1.13)	0.12	0.63(0.33-1.19)	0.16	0.34(0.04-3.13)	0.34
DKK3_B5_ht6	0.052	0.053	0.96(0.43-2.15)	0.92	0.84(0.36-2.00)	0.70	.	.
DKK3_B5_ht7	0.036	0.090	0.33(0.14-0.80)	**0.01**	0.33(0.14-0.80)	**0.01**	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown.
Significant associations are shown in bold face (P-value≤0.05).

One SNP (rs1994843) and one haplotype (Block5_ht7) exhibited a protective effect from pathological cancer stage in both co-dominant and dominant model (OR 0.36, p=0.02; OR 0.33, p=0.01). Among 9 SNPS, 2 SNPs (rs4757519, rs1472189) exhibited a markedly significant association with greater aggressiveness.

The association between DKK3 polymorphisms and Gleason score in prostate cancer group

When we stratified the patients according to Gleason score, we observed that the association of prostate cancer risk with one SNP (rs7478946) and one haplotype (Block4_ht2) was strongest among patients with high compared to low grade Gleason scores (OR 2.32, p=0.01 in the dominant model; OR 2.23, p=0.05 in the recessive model, respectively; Table-6, Supplementary Table-5).

DISCUSSION

SNPs are the most common polymorphisms in the genomes of many species. The definition of a SNP is a variation of the DNA sequence at a frequency larger than 1% of the allele of a population (5). In this study, we examined whether genetic variations in the DKK3 gene alter the risk of developing prostate cancer. A total of 53 SNPs located in the DKK3 gene were genotyped in 272 patients with prostate cancer and 173 control subjects with BPH. We found that three SNPs and two haplotypes were significantly associated with prostate cancer risk ($p<0.05$). Also, we found that two SNPs were markedly significantly associated with prostate cancer aggressiveness ($P<0.001$). These findings suggest that DKK3 gene polymorphisms may alter susceptibility to prostate cancer and could thus possibly be used as biomarkers for the disease and predictors for aggressivenessin patient with prostate cancer.

The Dickkopf (DKK) family consists of four genes (DKK1-4) and a DKK3-related gene (20). DKK3 is the most divergent member of the DKK family by DNA sequence, function, and evolution. Unlike the other DKK members, DKK3 does not modulate Wnt signaling (11) and shows no affinity to the Wnt co-receptor LRP5/6 and Kremen (21). Human DKK3 was proposed to function as a tumor suppressor, since its expression is down-regulated in many types of cancer cells (11). DKK3 down-regulation has been reported in endometrial cancer (22), lung cancer (23), gastrointestinal cancer (24), breast cancer (25), prostate cancer (26, 27), and renal carcinomas (28). In support of the hypothesis that DKK3 functions in prostate as a tumor suppressor, overexpression of DKK3 suppresses cell growth and the invasive capacity of prostate cancer cell lines (26, 27). Zenzmaier et al. reported that in normal prostate tissue the secreted glycoprotein DKK3 is expressed in the epithelial compartment but expression is lost in BPH and prostate cancer (29). DKK3 promotes fibroblast proliferation and myofibroblast differentiation and represents a potential therapeutic target for stromal remodeling in BPH and prostate cancer (30).

To our knowledge, one epidemiological investigation between DKK3 and prostate cancer have been reported. Zenzmaier et al. reported that DKK3 levels in seminal plasma were significantly elevated in biopsy-confirmed prostate cancer patients, because loss of expression seems to be counterbalanced by upregulation of DKK3 expression (31). In this study, we epidemiologically investigated whether SNPs of the DKK3 gene were related to the risk and aggressiveness of prostate cancer for the first time.

We note that this study has several limitations. Our sample size was relatively small for a case-control association study; it is limited in subgroup analysis. Therefore, the study requires further confirmation in much larger cohorts. However this study included a unique racial population, and the prostate cancer cohorts had a similar clinical characters of a previous Korean study (13) and the control group also had little selection bias due to their exclusion by biopsy. Although control group underwent prostate biopsy which reveals negative malignancy, all the men in the BPH group are potentially at risk for development of prostate cancer and may have latent prostate cancer at time of designation as controls, leading to disease misclassification. In addition we could not evaluate the effect of treatment related to BPH or prostate cancer such as medication or surgery in individuals. For many gene-exposure studies, a key limitation is the quality of the exposure information. Few studies have the ability to examine interactions between pesticide exposure and genetic risk factors

Table 6 - Logistic analysis of DKK3 polymorphisms according to Gleason score criteria.

	Minor Allele Frequency			Co-dominant		Dominant		Recessive	
SNP ID	High grade (n=39)	Intermediate (n=202)	Low grade (n=29)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs7478946	0.458	0.379	0.454	1.37(0.90-2.07)	0.14	2.32(1.22-4.40)	0.01	0.85(0.41-1.74)	0.65
DKK3_B4_ht2	0.410	0.364	0.345	1.17(0.78-1.75)	0.46	0.90(0.52-1.58)	0.72	2.23(1.01-4.91)	0.05

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown. **OR:** odd ratio, **CI:** confidence interval * Statistically significant at $P \leq 0.05$

Supplementary Table 5 - Logistic analysis of DKK3 polymorphisms according to Gleason score criteria.

	Minor Allele Frequency			Co-dominant		Dominant		Recessive	
SNP ID	High grade (n=39)	Intermediate (n=202)	Low grade (n=29)	OR(95%CI)	P-value	OR(95%CI)	P-value	OR(95%CI)	P-value
rs11022110	0.111	0.052	0.098	0.99(0.52-1.91)	0.98	1.10(0.55-2.20)	0.78	0.13(0.01-2.14)	0.15
rs1493208	0.401	0.500	0.411	0.80(0.53-1.22)	0.30	0.98(0.55-1.76)	0.94	0.47(0.21-1.03)	0.06
rs751580	0.136	0.121	0.135	1.13(0.64-1.98)	0.67	1.07(0.57-1.99)	0.84	2.32(0.34-15.84)	0.39
rs16910327	0.121	0.224	0.135	0.70(0.39-1.25)	0.22	0.68(0.36-1.27)	0.22	0.72(0.05-9.91)	0.81
rs3750940	0.144	0.207	0.144	0.60(0.34-1.07)	0.08	0.61(0.33-1.13)	0.12	0.27(0.03-2.24)	0.23
rs11544817	0.131	0.121	0.131	1.14(0.65-1.99)	0.64	0.99(0.52-1.88)	0.98	3.85(0.73-20.42)	0.11
rs2896594	0.139	0.190	0.139	0.65(0.37-1.15)	0.14	0.66(0.35-1.23)	0.19	0.29(0.04-2.34)	0.24
rs7104941	0.130	0.121	0.125	0.89(0.50-1.60)	0.70	0.87(0.46-1.66)	0.67	0.95(0.10-9.10)	0.96
rs3750938	0.333	0.276	0.316	0.93(0.61-1.41)	0.72	0.97(0.56-1.68)	0.92	0.75(0.29-1.93)	0.54
rs7107048	0.099	0.103	0.102	1.06(0.59-1.92)	0.84	1.02(0.50-2.05)	0.97	1.53(0.26-9.08)	0.64
rs7478978	0.094	0.207	0.111	0.68(0.36-1.26)	0.22	0.58(0.30-1.14)	0.12	2.68(0.26-27.99)	0.41
rs12421658	0.168	0.155	0.169	1.14(0.68-1.90)	0.62	1.04(0.57-1.87)	0.91	2.54(0.57-11.28)	0.22
rs16910308	0.209	0.172	0.204	1.12(0.69-1.82)	0.66	1.12(0.64-1.96)	0.70	1.30(0.29-5.79)	0.73
rs11022105	0.405	0.328	0.396	1.21(0.80-1.82)	0.37	1.15(0.65-2.04)	0.64	1.53(0.70-3.35)	0.28
rs6485351	0.228	0.155	0.224	1.40(0.89-2.21)	0.15	1.31(0.75-2.30)	0.35	2.74(0.91-8.27)	0.07
rs7116879	0.226	0.172	0.219	1.16(0.72-1.87)	0.55	1.17(0.67-2.04)	0.58	1.30(0.32-5.39)	0.72
rs988666	0.371	0.534	0.402	0.91(0.61-1.35)	0.63	0.85(0.48-1.50)	0.58	0.93(0.44-1.96)	0.85
rs2403557	0.241	0.310	0.265	1.23(0.80-1.89)	0.35	1.08(0.63-1.88)	0.78	2.44(0.92-6.44)	0.07
rs6485345	0.259	0.190	0.255	1.30(0.83-2.04)	0.25	1.24(0.72-2.15)	0.44	2.12(0.70-6.45)	0.19
rs16910295	0.142	0.155	0.145	1.05(0.60-1.84)	0.87	1.08(0.58-1.98)	0.82	0.82(0.09-7.83)	0.86

rs1038908	0.139	0.155	0.131	0.64(0.34-1.21)	0.17	0.64(0.34-1.21)	0.17	.	.
rs12295349	0.176	0.190	0.181	1.11(0.67-1.86)	0.68	1.25(0.70-2.21)	0.46	0.51(0.10-2.74)	0.43
rs12288230	0.173	0.172	0.178	1.19(0.71-1.98)	0.51	1.35(0.76-2.41)	0.31	0.51(0.10-2.74)	0.43
rs16910294	0.166	0.155	0.165	1.07(0.64-1.79)	0.79	1.14(0.63-2.07)	0.66	0.80(0.16-4.02)	0.79
rs7113678	0.104	0.052	0.104	1.61(0.87-3.00)	0.13	1.64(0.83-3.25)	0.15	2.58(0.25-26.98)	0.43
rs12224675	0.147	0.224	0.164	0.93(0.55-1.57)	0.79	1.03(0.57-1.88)	0.92	0.39(0.08-1.87)	0.24
rs7109243	0.455	0.552	0.469	0.86(0.58-1.26)	0.43	0.77(0.42-1.40)	0.39	0.88(0.46-1.69)	0.70
rs4757595	0.082	0.121	0.087	0.86(0.42-1.77)	0.68	0.86(0.42-1.77)	0.68	.	.
rs11022104	0.250	0.293	0.254	0.91(0.58-1.43)	0.69	0.95(0.55-1.65)	0.86	0.70(0.23-2.14)	0.53
rs12225759	0.465	0.483	0.463	0.88(0.58-1.33)	0.53	0.87(0.47-1.63)	0.67	0.81(0.40-1.64)	0.55
rs10765917	0.389	0.357	0.379	0.93(0.62-1.39)	0.71	0.85(0.48-1.50)	0.58	1.02(0.46-2.28)	0.96
rs6416121	0.077	0.121	0.083	0.87(0.42-1.81)	0.71	0.87(0.42-1.81)	0.71	.	.
rs7478946	0.458	0.379	0.454	1.37(0.90-2.07)	0.14	2.32(1.22-4.40)	**0.01**	0.85(0.41-1.74)	0.65
rs4757519	0.351	0.241	0.331	1.06(0.72-1.56)	0.77	1.24(0.71-2.14)	0.45	0.82(0.37-1.81)	0.62
rs7395522	0.196	0.155	0.193	1.22(0.76-1.96)	0.42	1.35(0.76-2.41)	0.30	0.94(0.25-3.54)	0.92
rs16910272	0.181	0.190	0.189	1.14(0.71-1.84)	0.59	1.09(0.61-1.94)	0.77	1.73(0.49-6.17)	0.40
rs3750936	0.270	0.362	0.280	0.79(0.50-1.25)	0.31	0.85(0.49-1.46)	0.55	0.46(0.15-1.43)	0.18
rs4586138	0.116	0.190	0.137	1.16(0.67-2.00)	0.60	1.13(0.61-2.12)	0.70	1.75(0.30-10.15)	0.53
rs11022099	0.206	0.268	0.213	0.83(0.51-1.36)	0.46	0.93(0.53-1.63)	0.80	0.31(0.07-1.30)	0.11
rs6485328	0.257	0.190	0.246	1.10(0.71-1.69)	0.68	1.29(0.74-2.25)	0.36	0.69(0.24-1.97)	0.49
rs7396140	0.460	0.500	0.461	0.91(0.63-1.31)	0.61	1.15(0.64-2.06)	0.65	0.63(0.33-1.21)	0.17
rs1552796	0.342	0.241	0.322	1.04(0.71-1.55)	0.83	1.16(0.67-2.01)	0.59	0.86(0.38-1.97)	0.72
rs7480815	0.408	0.310	0.388	1.03(0.70-1.50)	0.89	1.18(0.68-2.07)	0.56	0.84(0.41-1.72)	0.63
rs11022098	0.105	0.069	0.103	1.26(0.64-2.48)	0.51	1.26(0.64-2.48)	0.51	.	.
rs1994843	0.072	0.069	0.070	0.92(0.42-2.02)	0.84	0.92(0.42-2.02)	0.84	.	.
rs2087882	0.316	0.196	0.289	0.97(0.65-1.46)	0.89	1.17(0.68-2.03)	0.57	0.59(0.24-1.44)	0.24
rs2291599	0.240	0.190	0.235	1.18(0.76-1.82)	0.47	1.42(0.81-2.48)	0.22	0.70(0.23-2.11)	0.53
rs7396187	0.248	0.190	0.239	1.11(0.72-1.72)	0.62	1.32(0.76-2.30)	0.33	0.72(0.25-2.04)	0.53
rs1472190	0.317	0.224	0.293	0.93(0.62-1.40)	0.73	1.06(0.62-1.83)	0.83	0.61(0.25-1.50)	0.28
rs7480026	0.087	0.086	0.085	0.92(0.45-1.86)	0.81	0.92(0.44-1.91)	0.82	0.74(0.01-67.85)	0.90
rs7480000	0.376	0.393	0.390	1.20(0.81-1.79)	0.36	1.14(0.64-2.01)	0.66	1.57(0.75-3.31)	0.23
rs11022095	0.331	0.357	0.340	1.05(0.70-1.59)	0.80	0.84(0.48-1.46)	0.54	1.92(0.83-4.46)	0.13
rs1472189	0.155	0.052	0.144	1.50(0.86-2.63)	0.15	1.66(0.90-3.06)	0.11	0.87(0.09-8.24)	0.90
DKK3_B1_ht1	0.423	0.411	0.534	0.81(0.53-1.21)	0.30	0.82(0.46-1.48)	0.52	0.66(0.31-1.41)	0.28
DKK3_B1_ht2	0.128	0.116	0.086	1.28(0.70-2.34)	0.43	1.20(0.62-2.31)	0.59	4.24(0.40-44.36)	0.23
DKK3_B1_ht3	0.051	0.079	0.086	0.73(0.34-1.56)	0.41	0.73(0.34-1.56)	0.41	.	.
DKK3_B1_ht4	0.013	0.072	0.069	0.56(0.25-1.24)	0.15	0.62(0.27-1.43)	0.26	.	.

DKK3_B2_ht1	0.410	0.463	0.466	0.83(0.55-1.25)	0.38	0.67(0.37-1.24)	0.20	0.98(0.48-1.97)	0.95
DKK3_B2_ht2	0.205	0.208	0.172	1.12(0.69-1.82)	0.66	1.12(0.64-1.96)	0.71	1.31(0.29-5.81)	0.73
DKK3_B2_ht3	0.179	0.168	0.155	1.14(0.68-1.90)	0.62	1.04(0.57-1.87)	0.91	2.54(0.57-11.28)	0.22
DKK3_B2_ht4	0.128	0.094	0.207	0.68(0.36-1.26)	0.22	0.58(0.30-1.14)	0.12	2.68(0.26-27.99)	0.41
DKK3_B3_ht1	0.244	0.265	0.241	1.00(0.66-1.52)	1.00	0.80(0.46-1.39)	0.43	1.90(0.75-4.80)	0.18
DKK3_B3_ht2	0.205	0.173	0.172	1.19(0.71-1.98)	0.51	1.35(0.76-2.41)	0.31	0.51(0.10-2.74)	0.43
DKK3_B3_ht3	0.179	0.146	0.207	0.87(0.52-1.48)	0.61	0.95(0.52-1.74)	0.86	0.39(0.08-1.86)	0.24
DKK3_B3_ht4	0.115	0.136	0.155	0.85(0.48-1.51)	0.58	0.83(0.45-1.57)	0.57	0.82(0.09-7.86)	0.86
DKK3_B3_ht5	0.064	0.124	0.155	0.57(0.30-1.10)	0.09	0.57(0.30-1.10)	0.09	.	.
DKK3_B3_ht6	0.115	0.092	0.052	1.40(0.73-2.69)	0.31	1.39(0.67-2.86)	0.37	2.58(0.25-26.98)	0.43
DKK3_B4_ht1	0.346	0.381	0.362	0.93(0.62-1.39)	0.71	0.84(0.48-1.47)	0.55	1.05(0.47-2.36)	0.90
DKK3_B4_ht2	0.410	0.364	0.345	1.17(0.78-1.75)	0.46	0.90(0.52-1.58)	0.72	2.23(1.01-4.91)	**0.05**
DKK3_B4_ht3	0.154	0.168	0.172	0.96(0.57-1.61)	0.86	1.03(0.57-1.85)	0.93	0.51(0.10-2.74)	0.43
DKK3_B4_ht4	0.090	0.074	0.121	0.87(0.42-1.84)	0.72	0.87(0.42-1.84)	0.72	.	.
DKK3_B5_ht1	0.179	0.168	0.121	1.28(0.78-2.11)	0.33	1.42(0.78-2.58)	0.25	1.00(0.23-4.32)	1.00
DKK3_B5_ht2	0.167	0.153	0.224	0.84(0.50-1.43)	0.53	0.89(0.49-1.62)	0.70	0.45(0.08-2.39)	0.35
DKK3_B5_ht3	0.051	0.144	0.138	0.65(0.38-1.12)	0.12	0.54(0.28-1.05)	0.07	0.90(0.18-4.53)	0.90
DKK3_B5_ht4	0.090	0.079	0.052	1.28(0.60-2.71)	0.52	1.28(0.60-2.71)	0.52	.	.
DKK3_B5_ht5	0.179	0.106	0.138	1.29(0.73-2.26)	0.39	1.30(0.67-2.50)	0.44	1.75(0.30-10.15)	0.53
DKK3_B5_ht6	0.026	0.054	0.034	0.84(0.34-2.03)	0.69	0.81(0.32-2.09)	0.67	1.04(0.01-96.11)	0.99
DKK3_B5_ht7	0.064	0.062	0.052	1.11(0.49-2.54)	0.81	1.11(0.49-2.54)	0.81	.	.

Minor allele frequencies and P-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age as covariate are shown.
Significant associations are shown in bold face (P-value≤0.05).

for prostate cancer, thus replication of these findings may be difficult. Despite these limitations, the present study provides the first evidence of an important and novel association between DKK3 polymorphisms and the risk for prostate cancer, so will be a basis for future study.

CONCLUSIONS

We have observed several positive interactions between DKK3 gene polymorphisms and the risk of prostate cancer in a Korean population for the first time. These findings could be helpful to diagnose and predict the prognosis of prostate cancer. We anticipate that patients' genomic data could be used in clinical practice to investigate associations between the risk of prostate cancer and polymorphisms in the DKK3 gene in the near future.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Soon Chul Myung, MD, PhD
Department of Urology
Chung-Ang University, College of Medicine
221 Heukseok-dong, Dongjak-ku
Seoul 156-756, Korea
E-mail: uromyung@cau.ac.kr

ORIGINAL ARTICLE

Vol. 41 (5): 898-905, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0036

Concurrent Down-Regulation of PTEN and NKX3.1 Expression in Iranian Patients with Prostate Cancer

Vahideh Nodouzi [1], Mohammadreza Nowroozi [2], Mehrdad Hashemi [3], Gholareza Javadi [1], Reza Mahdian [4]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran; [2]Urooncology Research Center, Tehran University of Medical Sciences, Tehran, Iran; [3]Department of Genetics Tehran Medical Branch, Islamic Azad University, Tehran, Iran; [4]Department of Molecular Medicine, Pasteur Institute of Iran, Biotechnology Research Center, Tehran, Iran

ABSTRACT

NKX3.1 and PTEN genes are involved in the development and progression of prostate cancer (PCa). Here, in line with other studies that correlated the expression of these two genes, we aimed at evaluating the expression pattern of these genes in clinical PCa samples. Collectively, 81 tissue samples including 45 human PCa and 36 benign prostatic hyperplasia (BPH) specimens were included in the study. The tissue samples were subjected to RNA extraction and subsequently to cDNA synthesis according to the kit manufacturer's protocol. Quantitative Real-Time PCR assay was performed for each sample in triplicate reactions. REST and SPSS software were used to statistically analyze PTEN and NKX3.1 gene expression data.

Expression level of both NKX3.1 and PTEN genes was down-regulated in PCa samples compared to BPH samples. The relative expression ratio of PTEN and NKX3.1 was decreased to 0.155 and 0.003, respectively (P=0.000). The results of Chi-Square analysis revealed a significant correlation between the expression of these genes in both BPH and cancer groups (P=0.004 and 0.001, respectively).

According to previous studies and our data, we concluded that the association between the down-regulation of PTEN and NKX3.1 genes contributed to the prostate tumorigenesis. This might highlight the interaction between the proteins encoded by these genes. Furthermore, this finding might be exploited for the development of innovative diagnostic and therapeutic approaches in PCa.

ARTICLE INFO

Key words:
Prostatic Neoplasms; NKX3-1 protein, human [Supplementary Concept]; PTEN Phosphohydrolase; Real-Time Polymerase Chain Reaction; Biological Markers

Int Braz J Urol. 2015; 41: 898-905

Submitted for publication:
January 25, 2014

Accepted after revision:
May 22, 2014

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer worldwide. It is the second most common type of cancer in men and causes the death of approximately 250,000 men annually (1). PCa is a heterogeneous disease with variable clinical behavior. This heterogeneity increases significantly with progression from benign to malignant form (2). Since there are no effective therapeutic options for advanced PCa, the identification of high risk individuals and early detection of the tumor when it is still confined to the prostate tissue are highly desired.

Although different grades of PCa (including prostatic intraepithelial neoplasia, invasive adenocarcinoma and metastatic forms) have been well defined histologically (3), molecular mechanisms involved in the progression of the disease have not been fully described yet. Recently, developments

in molecular genetics techniques have led to the identification of more than 200 genes related to PCa. These genes are predominantly expressed in PCa epithelial cells and affect the initiation and progression of PCa.

NKX3.1 is an androgen-regulated homeodomain gene, whose expression is restricted to the prostate epithelium (4). As a prostate-specific transcription factor with relative molecular mass of 26 kDa, NKX3.1 is necessary for normal development and function of the prostate (5). NKX3.1 gene is affected by the loss of heterozygosity in 60-80% of prostate carcinomas (6), whereas no point mutations were observed in its coding sequence (7). The loss of a single allele of the gene may be sufficient to promote prostate carcinogenesis in humans, confirming haploinsufficiency for this phenotype (8). So far, several mechanisms have been proposed for the loss of NKX3.1 expression in human PCa, including both transcriptional and post transcriptional modifications as well as epigenetic regulation and protein degradation (9).

PTEN was first identified as a tumor suppressor gene in 1997 (10, 11). PTEN gene is located on chromosome 10q23 and encodes an amino acid sequence with relative molecular mass of 47 kDa (10). It is mostly expressed in brain, colon, breast as well as gastric and prostate epithelial cells. After P53, PTEN is the second most mutated tumor suppressor gene. It is frequently inactivated as a result of loss of heterozygosity in up to 70% of primary PCa cases (11, 12).

PTEN contributes as a hub protein in cellular pathways, such as angiogenesis, apoptosis, cell cycle and cell migration. Moreover, it is frequently inactivated in somatic cancers such as PCa (12). Homology of tyrosine phosphatase domain of PTEN to tensin protein suggests that PTEN may suppress tumor cell growth. This activity is accomplished by antagonizing protein tyrosine kinases. Hypothetically, PTEN can regulate tumor cell invasion and metastasis by arrested angiogenesis, which is required for cancer growth and metastasis. This effect is mediated by blocking the transcription of VEGF gene (13). All these effects are likely mediated via PIP3 hydrolysis by PTEN (14).

Some studies have indicated that loss of PTEN function correlates with the decreased expression of NKX3.1 and PCa progression in both mice and humans (15-17). A pioneering study showed that PTEN controls the activity of NKX3.1 through the regulation of its expression (16). The exogenous up-regulation of NKX3.1 obviously blocked the proliferative and anti-apoptotic effects of PTEN loss in PCa cells. Furthermore, the mice compound heterozygous for NKX3.1 and PTEN gene deletion showed fast progression to invasive and androgen independent disease (17).

In this study, we aimed to evaluate the changes in the pattern of NKX3.1 and PTEN gene expression and their contribution in the prostate tumorigenesis in Iranian PCa patients.

MATERIALS AND METHODS

Sample collection

Prostate tissue samples, including both tumor and benign prostatic hyperplasia (BPH) samples were selected from patients who were referred to the urology department at Uro-oncology Research Center (UORC) at Clinical Research Development Center (Tehran, Iran) from June 2011 to February 2013. A written informed consent was obtained from each participating patient. The study was also approved by the Ethics and Clinical Research Committee of Pasteur Institute of Iran (Tehran, Iran). Relevant clinical and pathological data were collected for the patients. All patients were new cases with no medical history of surgery or chemotherapy. The median age of the patients was 63 years, ranging from 47 to 75. The patients were examined by an expert urologist and evaluated according to standard imaging procedures and laboratory analyses for PCa. The studied samples consisted of 81 tissue samples including 45 PCa and 36 BPH specimens. The median age of the patients was 68 years, ranging from 49 to 83 years. The mean plasma PSA level was 17.82±2.13ng/mL and 7.71±1. 80ng/mL (Mean±SEM) in cancer and BPH groups, respectively. The Gleason score for the PCa tissues varied from 4 to 10. Tissue samples were obtained via open radical prostatectomy or needle biopsy. Each tissue sample was obtained in two replicates; one replicate was examined by a pathologist for the detection of malignant changes and the evaluation of tumor grade as Gleason score. The other replicate was ins-

tantly immersed in RNAlater solution (Qiagen, Germany) and kept at room temperature for 24 hours and subsequently stored at -80°C. Then, the tissue samples were transferred into liquid nitrogen containers for long term storage. Pathological examinations confirmed either the presence of cancerous cells or BPH. Only tissue sections containing at least 80% tumor cells were included in the study.

RNA extraction and cDNA synthesis

Total RNA was extracted from 80-100mg of tissue samples using TRI reagent (RiboPure™ Kit, Ambion, USA) according to the manufacturer's instructions. The concentration and quality of the purified RNA were determined by spectrophotometry (IMPLEN, Germany). High quality RNA samples (A260/280>1.8) were used as templates for cDNA synthesis. Selected RNA samples were kept at -80°C for subsequent cDNA synthesis. RNA samples (up to 1µg) were converted to cDNA using Quantitect® Reverse Transcription Kit (Qiagen, Germany) according to the kit instructions. The cDNA integrity was verified by validation experiments using GAPDH specific primers. The cDNA samples with satisfactory quality were diluted in a ratio of 1:10 and stored at -20°C until use for further analysis.

Primer design

NKX3.1 and PTEN genes were considered as target genes and PSA (prostate-specific antigen) and GAPDH as reference genes. Primers design was performed by Primer Express V.3.0 (Applied Biosystems, USA) software and verified by Gene runner V.3.05 and Allele ID V. 6 (Biosoft Int.) software. Moreover, BLAST analysis was used to test the specificity of the primers for their targets (http://www.ncbi.nlm.nih.gov/blast). To further improve the accuracy of the quantitation of mRNA expression, the target fragments spanning distinct exon-exon boundaries of the mRNA transcripts were chosen. Primer sequences were synthesized at Bioneer Corporation (South Korea). The sequence of the primers are shown in Table-1.

Quantitative Real-Time PCR

SYBR Green Master Mix was used to perform a 40-cycle PCR reaction using SDS v.1.0.1 software (ABI System 7300, Applied Biosystems, USA) in MicroAmp Optical 96-well plates. Each PCR amplification reaction (20µL) contained 10µL Power SYBR Green PCR Master Mix (2x), 1µL from each forward and reverse primers, 3µL of first-strand cDNA and 5µL D.D.W. Also, non-template control (NTC) reactions were included in all experiments. The thermal-cycling conditions were as follows: stage 1, 1 cycle for 10 min at 95°C as first denaturation and Hot-start enzyme activation, followed by stage 2, 40 cycles at 95°C for 15 s and at 60°C for 1 min as annealing and extension stages. This stage or process was followed by a dissociation stage (95°C for 15s, 60°C for 30 s and 95°C for 15s) to verify the specificity of the PCR products. Analysis of the results confirmed the specific amplification of the interest gene fragments. Each amplicon was identified by its specific melting curve Tm. Additionally, electrophoresis of the PCR products on 2% agarose gel (V: 80, time 45 min) revealed a single sharp band with expected length of 143, 161, 112 and 140 related to NKX3.1, PTEN, GAPDH and PSA, respectively.

Quantitative data analysis of Real-time PCR

Serial dilutions were prepared as ten-fold dilutions of the target nucleic acid to draw a standard curve by plotting related Ct values against log cDNA concentrations. To analyze the amount of change in gene expression, we used comparative threshold cycle (Ct) and to calculate the ΔΔCt values, mean threshold cycle (mCt) was obtained from triplicate series of amplifications during the exponential phase. Then, mCt value of reference gene (PSA or GAPDH) was subtracted from mCt value of the target gene (NKX3.1 or PTEN) to obtain ΔCt. Subsequently, ΔΔCt values were calculated from corresponding ΔCt values separately for each sample, using the following formula: ΔΔCt=[mCt target--mCt reference] (cancer) - [mCt target - mCt reference] (BPH). Finally, using the ratio formula (ratio = $2^{-\Delta\Delta Ct}$), up or Down-Regulation of target gene was achieved in proportionate to the reference gene.

RESULTS

Melting curve analysis and gel electrophoresis

Based on the temperature and dF/dT derivation, the melting curve was drawn. This cur-

Table 1 - Forward and reverse primers for NKX3.1, PTEN, GAPDH and PSA genes.

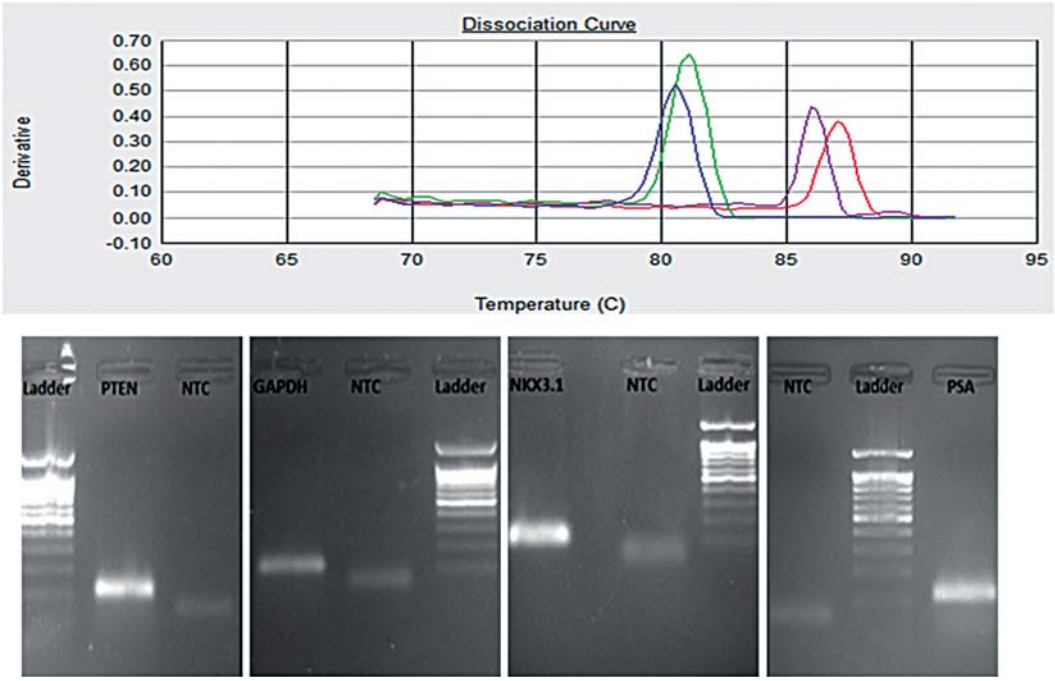
Gene	Forward primer	Reverse primer	Amplicon (bp)
NKX3.1 3-1 (NM_006167.3)	CCAGAGCCAGAGCCAGAGG	TCCAACAGATAAGACCCCAAGTG	143
PTEN (NM_000314.4)	CACACGACGGGAAGACAAGTTC	CCTCTGGTCCTGGTATGAAGAATG	161
GAPDH NM_006167.3	ACACCCACTCCTCCACCTTTG	TCCACCACCCTGTTGCTGTAG	112
PSA (NM_001648.2)	TCTGCGGCGGTGTTCTGG	GCTGTGGCTGACCTGAAATACC	140

ve was made to differentiate between the primer dimmers or non-specific products with the desired amplicons. The melting peaks for PTEN, GAPDH, NKX3.1 and PSA genes have been drawn at 81, 81.5, 85.5 and 87°C, respectively. Moreover, gel electrophoresis of PCR products showed the specific amplification of the genes of interest (Figure-1).

Data analysis

Comparison between mean gene expression levels in the study groups (PCa Vs BPH) and graph preparation were performed using Relative Expression Software Tool software (REST© 2009, Qiagen, Germany) (Figure-2). REST© software compares two or more treatment groups or conditions with data points (CT) in sample or control group for multiple reference and target genes. As expected from previous studies (15, 16), the expression level of NKX3.1 gene was significantly different between cancer and BPH group (P=0.000). In fact, the relative expression of NKX3.1 gene was drastically decreased in the PCa patient samples

Figure 1 - Dissociation curves of PTEN, GAPDH, NKX3.1 and PSA genes drawn through Real-time PCR assay experiments. The corresponding PCR bands revealed by agarose gel electrophoresis are also shown (from left to right).



compared to BPH samples (relative ratio=0.003, P=0.000). In addition, similar result was achieved for PTEN gene (relative ratio =0.155, P=0.000) as its relative expression was declined in comparison with BPH samples (Table-2). Interestingly, our data suggested the complete loss of NKX3.1 expression in high-grade and metastatic PCa samples. Also, the consistent alteration in the expression of NKX3.1 and PTEN genes was associated with prostate tumorigenesis. Chi-square analysis (SPSS software, V 16.0) was performed to evaluate the correlation between the expression level of these two genes in the study group. P values<0.05 were considered statistically significant. It showed a significant relation in both BPH (P=0.001) and cancer groups (P=0.004).

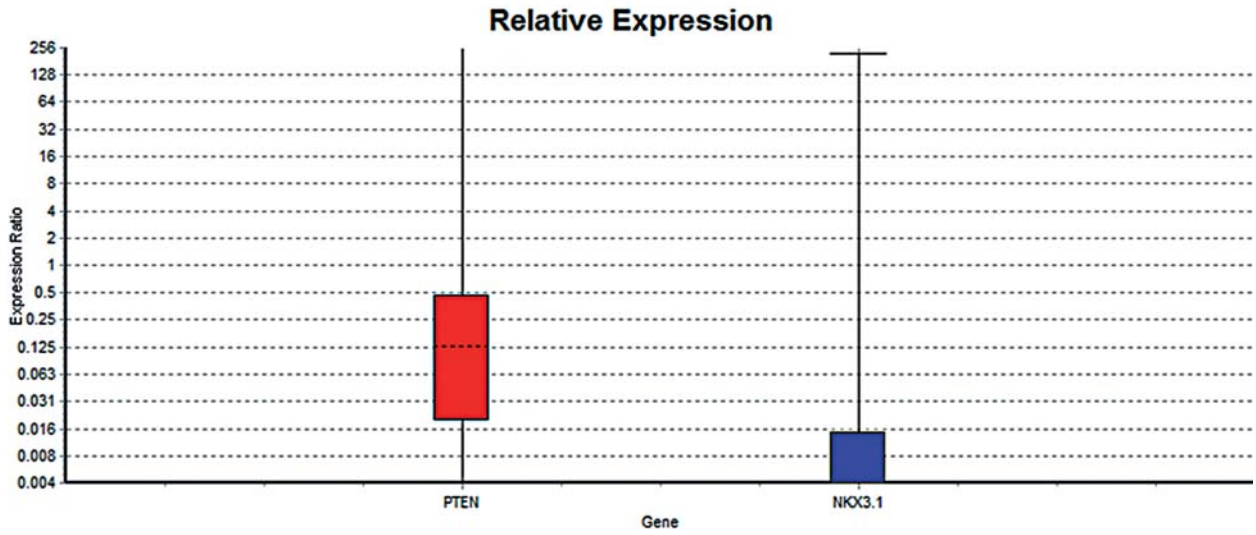
DISCUSSION

There is a general consensus that PCa is the second most common cancer worldwide, causing over 250,000 death in men each year (1). Owing to the fact that there are no effective therapeutic options for advanced PCa, early detection of cancer patients and identification of high risk cases is highly desired. Currently, both PCa diagnosis and management are mainly dependent on serum levels of PSA. In spite of the fact that PSA testing has

Table 2 - The results of the gene expression analysis by REST software. PTEN is down-regulated in sample group (in comparison to control group) by a mean factor of 0.155 (Std. Error range is 0.033-0.745). PTEN in sample group is different to control group. P (H1) = 0.000. NKX3.1 is down-regulated in sample group (in comparison to control group) by a mean factor of 0.003 (Std. Error range is 0.000-0.026). NKX3.1 sample group is different from control group. P (H1) = 0.000.

	Type	Reaction efficiency	Expression	Std. Error	95% C.I.	P (H1)	Result
PSA, GAPDH	REF	1.0	1.000				
PTEN	TRG	1.0	0.155	0.033-0.745	0.033-0.745	0.000	DOWN
NKX3.1	TRG	1.0	0.003	0.000-0.026	0.000-0.296	0.000	DOWN

Figure 2 - The results of the gene expression analysis obtained by REST software. Box-plots represent the interquartile range or the middle 50% of observations.



been used for a long time as a diagnostic aid for PCa detection, it has certain limitations (18, 19). The lack of specificity has been one of these major hurdles resulting in high rate of negative biopsies. Therefore, further investigation is needed to establish more specific and sensitive markers of PCa (18, 20). The identification of PCa specific genes can effectively pave the way for improving the accuracy of PCa diagnostic tests. Achievement of this goal requires the study of gene expression patterns in different stages of PCa from BPH to metastatic PCa (21). More recently, breakthroughs in molecular genetics techniques have contributed to the identification of genes, which are expressed in PCa epithelial cells and related to PCa growth (22).

Alteration in the expression of PTEN has been strongly implicated in PCa development, since mutations in its gene are found in a large proportion of both primary and metastatic PCa (30% and 63%, respectively) (19). Moreover, PTEN mutations are among the most frequent genetic alterations in human PCa (23). Our findings provide new insights into the PTEN role as a biomarker in prostate tumorigenesis. This gene can be included in gene signatures for early diagnosis of PCa in combination to other biomarkers (20).

NKX3.1 is expressed exclusively in prostatic cells and considered as a differentiation-related gene (4). Murine NKX3.1 is the earliest known marker of prostate epithelium during embryogenesis, that subsequently is expressed in all stages of prostate differentiation (24, 25). NKX3.1 protects differentiated prostate epithelium from oxidative DNA damage and inhibits the AKT phosphorylation/activation via an androgen receptor-dependent mechanism (26, 27). Previous studies have shown that NKX3.1 expression is down-regulated during early stages of prostate tumorigenesis (19, 21 28). Because of its chromosomal localization to a PCa hot spot (minimal region of 8p21), several studies have proposed that NKX3.1 is a prostate-specific tumor suppressor gene in which loss of a single allele may predispose to prostate carcinogenesis (4, 6, 8). Interestingly, complete loss of NKX3.1 expression in high-grade tumor samples indicates that it could precisely predict PCa. Despite strong correlation between loss of NKX3.1 expression and PCa initiation and progression, the involved mechanisms are still remained to be described. So far, several mechanisms which contribute to the loss of NKX3.1 in PCa have been proposed, including allelic loss, post-transcriptional control and epigenetic mechanisms (9, 19). However, Lind et al. study on the methylation status of NKX3.1 promoter showed that the promoter was unmethylated (29). In 2010, Jong et al. suggested that NKX3.1 down-regulation is not caused by promoter hypermethylation. They also proposed that other epigenetic mechanisms such as structure modulation of chromatin or histone modifications might be involved (29). In another study, Kunderfranco et al. (30) examined the expression of transcription factor ERG (ETS related gene), NKX3.1 and androgen receptor using immune histochemistry. Surprisingly, they observed that NKX3.1 is directly controlled by ERG in prostate tumors. In a study conducted by Lei et al., it has been suggested that PTEN largely modulates the NKX3.1 function through regulating of its expression (16). They demonstrated that PTEN loss causes reduced NKX3.1 expression in both murine and human PCa. Interestingly, the NKX3.1 restoration can alleviate the adverse phenotype related to PTEN loss. In Pten null prostate epithelium, the gene restoration to wild type resulted in reduced cell proliferation and a rise in cell apoptosis (15). The assessment of molecular changes caused by homozygous PTEN deletion clearly identified important human related PCa genes such as NKX3.1 gene (17). These findings emphasize the cooperative effects of PTEN as a tumor suppressor gene and prostate-specific expressed NKX3.1 in PCa development (15-17).

CONCLUSIONS

In the present study, we evaluated the expression of NKX3.1 and PTEN genes in clinical prostate samples to verify the correlation between the expression level of these two genes in human BPH and PCa. Our data confirmed the results from the previous studies regarding a concurrent decrease in both NKX3.1 and PTEN expression in prostate tumors. Moreover, statistical data analysis indicated a significant correlation between the expression level of the two genes in both BPH and

PCa samples. This study further confirmed that these two genes may be considered as potential biomarkers in PCa besides other prostate biomarkers (2, 4).

However, we have not studied the expression of the proteins encoded by these genes. Therefore, it is highly favorable for future studies to evaluate the expression of both genes at protein level. To our knowledge, this is the first study to assess the simultaneous changes in the expression of PTEN and NKX3.1 in clinical PCa samples. According to our results, PTEN loss not only contributes to the reduced expression of NKX3.1 but also to prostate tumorogenesis. On the other hand, the link between the expression levels of PTEN and NKX3.1 genes could be implemented for the design of novel therapeutics for human PCa.

ACKNOWLEDGEMENT

We would like to thank Mr. J. Alizadeh, Dr. B. Yusefi, Dr. M. Asgari, and Dr. M. Rezaei for their kind cooperation in this study.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Vahideh Nodouzi, MD, PhD
Department of Biology
Science and Research Branch,
Islamic Azad University, Tehran, Iran
E-mail: vahidehnorouzi82@gmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 906-910, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0419

The efficacy of duration of prophylactic antibiotics in transrectal ultrasound guided prostate biopsy

Volkan Bulut [1], Ali Feyzullah Şahin [2], Yavuz Balaban [3], Muammer Altok [4], Rauf Taner Divrik [2], Ferruh Zorlu [5]

[1] Department of Urology, Akyazi Public Hospital, Sakarya, Turkey; [2] Department of Urology, Şifa University Medicine School, İzmir, Turkey; [3] Department of Urology, Kahta Public Hospital, Adiyaman, Turkey; [4] Department of Urology, Suleyman Demirel University Medicine School, Isparta, Turkey; [5] Department of Urology, Tepecik Training Hospital, İzmir, Turkey

ABSTRACT

Introduction: We aimed to evaluate the efficacy of the duration of prophylactic antibiotic administration in patients undergoing transrectal ultrasound (TRUS) guided biopsy.
Material and Methods: A total of 367 patients undergoing a prostate biopsy between September 2007 and June 2009 was reviewed retrospectively and divided into 2 groups according to prophilaxy: oral ciprofloxacin (750 mg every 12 hours) for 3 or more days in Group-1 and single day in Group-2. Demographic characteristics of patients, symptoms, PSA values, IPSS scores, prostate sizes, pathologic results and complications were compared between the groups.
Results: The mean age of all patients was 63.92 years and the mean PSA was 13.61ng/dL. The pre-biopsy mean IPSS score was 12.47 and mean prostate volume 52.53 mL. For 78.2% of patients the current biopsy was their first biopsy. Cancer detection rate was 24.2%. Fever was observed in 3 (1.2%) patients in Group-1 and 5 (4.0%) patients in Group-2. Local infections occurred in 2 (0.8%) and 1 (0.8%) patients respectively in Groups 1 and 2. Acute prostatitis was observed in only 1 (0.8%) patient in Group-2. None of the patients developed septicemia or other serious infection. There was no statistically significant difference in terms of fever, local infections (epididimitis, orchitis, etc.) and acute prostatitis.
Conclusions: In a selected patient population single dose prophylaxis with ciprofloxacin can be safely administered compared to other regimens of 3 or more days. Increasing the duration of antibiotic prophylaxis does not decrease infectious complications.

ARTICLE INFO

Key words:
Antibiotic Prophylaxis; Prostate; Biopsy; Ultrasound, High-Intensity Focused, Transrectal

Int Braz J Urol. 2015; 41: 906-10

Submitted for publication:
August 24, 2014

Accepted after revision:
January 28, 2015

INTRODUCTION

The necessity of antibiotic prophylaxis before biopsy is well established by randomized, controlled trials in literature. Despite the existing consensus, the type, dosage and duration of treatment have not become clear (1). It is known that antibiotic prophylaxis before transrectal prostate biopsy reduces infectious complications (2). Up to date both oral and intravenous administrations of several different prophylactic regimens have been investigated and different recommendations on drug selection have been made. One of the frequently used regimens is oral administration of a flouroquinolone 30-60 min before biopsy which is continued for 2-3 days after biopsy. Although

minimal infectious complications occur with the existing prophylaxis protocols the 0.1-0.5% rate of bacteremia or sepsis is still an important problem (3). In this study the efficacy of the duration of prophylactic antibiotic administration in patients undergoing transrectal ultrasound (TRUS) guided biopsy was evaluated.

MATERIALS AND METHODS

The study was initiated after the approval of local ethics committee. The data of patients undergoing a prostate biopsy between September 2007 and June 2009 with a suspicion of prostate cancer were retrospectively reviewed. A total of 367 patients with complete records were included. Patients with a positive urine culture before the biopsy, a history of urologic intervention in the last 3 months, immunodeficiency state and catheterized patients were excluded. Patients were divided into 2 groups according to the duration of antibiotic prophylaxis they received. Oral prophylactic antibiotics were administered to all patients before biopsy. Patients receiving oral ciprofloxacin (750 mg every 12 hours) for 3 or more days were assigned to Group-1 and for single day to Group-2.

In our clinic prostate biopsy is offered to patients who have positive findings in digital rectal examination and/or a total PSA level >2.5ng/mL. Routinely 10-12 cores biopsies are taken. Additional cores are also included in case of any suspicious area. All biopsies are performed by using 7.5 MHz transrectal probe and its fitting attachment of Sonoscape SSI-2000 BW system (Sonoscape. co. Ltd.) ultrasound device. The anticoagulation treatment is discontinued 1 week before the biopsy. Bowel preparation, dietary modifications and local area preparation are not performed routinely. The first dosage of the prophylactic antibiotic was administered orally 30-60 min before the procedure. The second dose was suggested to be taken 12 hours after biopsy.

Standard patient forms including biopsy related complications were used in biopsy unit of our clinic. Demographic characteristics of patients, symptoms, PSA values, IPSS scores, prostate sizes, pathologic results and complications occuring after biopsy were recorded in those forms. Haematuria was defined as persisting bleeding for more than 24 hours after biopsy. Fever was defined as the presence of any temperature higher than 38°C in the first week after biopsy without any local infection findings and with negative urine culture. Acute prostatitis was defined as fever higher than 39°C dysuria, frequency, perineal pain or discomfort with positive urine culture.

Acquired data were transfered to a computer environment and statistical analysis was performed by software. The results were expressed as mean ± standard deviation. Categorical and continous variables were analysed with Chi-square and Student T tests respectively. A p value of <0.05 was considered statistically significant.

RESULTS

Of 367 patients 243 were classified as Group-1 and the rest 124 were grouped as Group-2. The mean age of all patients was 63.92 years and the mean PSA was 13.61ng/dL. The pre-biopsy mean IPSS score was 12.47 and mean prostate volume 52.53 mL. For 78.2% of patients the current biopsy was their first biopsy. Cancer detection rate was 24.2%. These characteristics for each group are shown in Table-1. There was no significant difference between groups in terms of age, first biopsy rate, PSA values, IPSS, prostate volume and cancer detection rates. In terms of complications, hematuria was recorded in 25 (10.3%) patients in Group-1 and 27 (21.8%) patients in Group-2 (p=0.003). Fever was observed in 3 (1.2%) patients in Group-1 and 5 (4.0%) patients in Group-2. While comparing local infection (epididimitis, orchitis, etc.) rates in Groups 1 and 2, local infections occurred in 2 (0.8%) and 1 (0.8%) patients respectively in Groups 1 and 2. Acute prostatitis was not observed in any patients in Group-1 and was observed in only 1 (0.8%) patient in Group-2. None of the patients developed septicemia or other serious infection. Only in one patient hospitalization due to acute prostatitis was required. When the two groups were evaluated according to their infectious complications there was no statistically significant difference in terms of fever, local infections (epididimitis, orchitis, etc.) and acute prostatitis. The complication rates in the two groups are shown in Table-2.

Table 1 - Patient characteristics in the two groups

	Total	Group-1	Group-2	P value
Number of patients	367	243	124	-
Age (range)	63.92 ± 7.18 (48-83)	63.67 ± 7.06 (46-79)	64.41 ± 7.42 (48-85)	0.353
First biopsy rate (%)	287 (78.2)	190 (78.2)	97 (78.2)	0.553
PSA (ng/dL) (range)	13.61 ± 20.04 (2.77-130)	13.15 ± 19.84 (2.77-121)	14.51 ± 20.47 (3.04-130)	0.537
IPSS	12.47 ± 7.82	12.74 ± 7.76	11.94 ± 7.95	0.377
Prostate volume (mL) (range)	52.53 ± 32.30 (11.25-191.89)	51.68 ± 32.31 (11.25-173.56)	54.04 ± 32.35 (18.76-191.89)	0.515
Cancer detection rate (%)	88 (24.2)	56 (23.2)	32 (26.2)	0.838

Mean ± SD

Table 2 - Complication rates among groups.

	Total	Group-1	Group-2	P value
Number of patients	367	243	124	-
Hematuria (%)	52 (14.2)	25 (10.3)	27 (21.8)	0.003
Fever (%)	8 (2.2)	3 (1.2)	5 (4.0)	0.090
Local infections (%) (epididimitis, orchitis, etc)	3 (0.8)	2 (0.8)	1 (0.8)	0.735
Prostatitis (%)	1 (0.3)	-	1 (0.8)	0.338

DISCUSSION

Transrectal biopsy is frequently performed nowadays and it is regarded as a relatively safe procedure in general. But, owing to its traumatic nature this procedure may cause hemorrhagic (hematuria or rectal bleeding) and infectious complications. Infectious complications may be in the form of asymptomatic bacteriuria as well as it can present as urinary infections, fever or sepsis. It can even cause death following septicemia (4). American Urology Association recommends single dose antibiotic prophylaxis before prostate biopsy (5). Currently prophylaxis with quinolones is still recommended despite increased rate of infectious complications (5).

In a study conducted in 144 hospitals in England and Ireland (6) the use of 13 antibiotics in 48 different regimens was reported. They suggested ciprofloxacin or norfloxacin regimens as inexpensive and efficient. An evaluation of 88 different centres in USA has revealed that 83% of patients received antibiotic prophylaxis before biopsy and in 81% of these cases quinolones were used (7). One of the most frequently used protocols is to administer a quinolone orally 30-60 min before the biopsy and continue for 2-3 days after the biopsy. In spite of this it was reported that no hospital admissions due to febrile urinary tract infection were required with the use of a single dose of ciprofloxacin (8).

In a randomized controlled trial, Aron et al. (9) divided 231 patients into 3 groups: Group-1 (n=75) received placebo twice daily for 3 days, Group-2 (n=79) received single doses of ciprofloxacin 500 mg and tinidazole 600mg and Group-3 (n=77) received the same treatment as Group-2 for 3 days. The complications were classified as infectious or noninfectious. Noninfectious complications were

defined as lower urinary tract symptoms, rectal bleeding for more than 12 hours, hematuria for more than 12 hours and perineal pain. Infectious complications included fever (body temperature>38°C), urinary tract infection, prostatitis, fever+urinary tract infection and prostatitis+fever+urinary tract infection. When complications rates were compared between the two groups, infectious complications were significantly higher in the placebo group compared to single dose and 3-day treatment groups. The groups were similar in terms of fever and bacteremia while there was a significant difference in terms of urinary tract infections. At the end of the study hematuria was present in 8 of 75 (10.7%), 13 of 79 (16.5%) and, 12 of 77 (15.6%) patients in Groups 1, 2 and, 3 respectively. Our results showed a significant difference between the groups in terms of hematuria while Aron et al. did not find such a difference. It may be a result of the fact that mean number of cores were not the same in both groups. Patients with elevated PSA (>20ng/dL) were biopsied with fewer cores. By using single dose prophilaxy safely during the period more cores were taken routinely (upgraded 12 from 10). Aron et al. detected fever in 5, 2 and 2 of patients in Groups 1, 2 and 3 respectively. In our study we detected fever in 3 and 5 of the patients in Groups 1 and 2 respectively. In both of these studies there was not a statistically significant difference in terms of fever detection. When these two studies are evaluated in terms of prostatitis, while Aron et al. reported prostatitis in 6, 8 and 3 of patients in each of the three groups we have found prostatitis in only one patient in Group-2. A statistically significant difference in prostatitis rates between groups were reported in both studies. Aron et al. concluded that antibiotic prophylaxis was mandatory in prostate biopsy and prophylaxis for 3 days was superior to single dose prophylaxis.

Also Cam et al. (10) divided 400 patients into three groups in a randomized study to show that a single dose of antibiotic is adequately effective in prophylaxis. Patients were administered 1 gr of intramuscular ceftriaxone single dose, oral ciprofloxacin 500 mg twice daily for 3 days and oral ciprofloxacin 500 mg single dose in Groups 1, 2 and 3 respectively. Their results, similar to our study showed no significant difference in terms of infectious complications between the 3 groups.

Shigemura et al. (11) evaluated a total of 236 patients in two groups in a randomized study to prove the efficacy of single day prophylaxis for prostate biopsy. One hundred twenty four patients in Group-1 received 600 mg levofloxacin once daily and 112 patients in Group-2 received levofloxacin 300 mg daily for 3 days. They observed febrile infectious complications in 2 patients in each group. Their results showed no significant difference in terms of febrile complications between the groups, similar to our study.

Briffaux et al. (12) treated 139 patients with 2 tablets of ciprofloxacin 500mg 2 hours before the biopsy and 149 patients with the same dose of ciprofloxacin for 3 days, to prove the efficacy of single dose in prophylaxis. They observed asymptomatic bacteriuria in 6 patients and prostatitis in 1 patient in both groups. In line with our results there was no signifant difference in the post-biopsy symptom scores at both groups. The groups were similar in terms of complication rates.

Sabbagh et al. (13) administered a fluoroquinolone for durations of 1 and 3 days as a prophylaxis before prostate biopsy in a randomized study. They did not find significant difference in complication rates.

In a study of Aslan et al. (14) the febrile and non febrile urinary infection rates were reported to be similar with treatment regimens of single dose levifloxacin and single dose fosfomycin+500 mg of ciprofloxacin twice daily for 5 days. But they observed higher rates of fluorochinolone resistant and extended spectrum beta lactamase (ESBL) resistant E.Coli in urine cultures of those patients receiving ciprofloxacin.

Taylor et al. (15) obtained urine and rectal swab cultures of patients before biopsy in his prospective study. In 19% of 865 patients ciprofloxacin resistant gram negative coliform bacteria was detected. In 90.6% of these the bacteria were E.Coli. The high risk factors for developing resistance were a history of using ciprofloxacin in the last 3 months and surgery relating to cardiac valve diseae. Infectious complications were detected in 3.6% (n=31) of patients. In 48% of these ciprofloxacin resistant organisms were detected in rectal swabs. Among pa-

tients who had positive rectal swab cultures for ciprofloxacin resistant bacteria 9% developed infectious complications. Despite a positive rectal swab culture for ciprofloxacin resistant E.Coli, infectious complications did not occur in those patients receiving ciprofloxacin prophylaxis. Since rates of infectious complications were similar in single dose and longer prophylaxis with ciprofloxacin, the most appropriate prophylactic regimen was suggested as single dose applications despite increasing rates of resistance.

The major limitations of the current study are its retrospective design and absence of data regarding antibiotic usage in the last 3 months. More Urologists are performing prostate biopsy in their daily practice. Antibiotic resistance and infectious complications are increasing throughout the World and especially in our country. We tried to reflect the results of our daily practice to navigate clinicians more antibiotic is not resulting low infectious complications.

CONCLUSIONS

In a selected patient population single dose prophylaxis with ciprofloxacin can be safely administered compared to other regimens of 3 days or more duration. Increasing the duration of antibiotic prophylaxis does not decrease infectious complications. Nevertheless it should be kept in mind that there is always some risk of infectious complications. Single dose prophylaxis is easy to administer and reduces costs.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Ali Feyzullah Şahin, MD
Assistant Professor in Urology
Department of Urology
Şifa University Medicine School, İzmir, Turkey
Sanayi cad. No:7 35050 Bornova, İzmir, Turkey
E-mail: ali.sahin@sifa.edu.tr

ORIGINAL ARTICLE

Vol. 41 (5): 911-919, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0371

Effect of utilization of veno-venous bypass vs. cardiopulmonary bypass on complications for high level inferior vena cava tumor thrombectomy and concomitant radical nephrectomy

Ross M. Simon [1], Timothy Kim [2], Patrick Espiritu [2], Tony Kurian [1], Wade J. Sexton [2], Julio M. Pow-Sang [2], Einar Sverrisson [2], Philippe E. Spiess [2]

[1] University of South Florida, Department of Urology, Tampa, FL, USA and [2] Department of Genitourinary Oncology, Moffitt Cancer Center, Tampa, FL, USA

ABSTRACT

Purpose: To determine if patients with renal cell carcinoma (RCC) with levels III and IV tumor thrombi are receive any reduction in complication rate utilizing veno-venous bypass (VVB) over cardiopulmonary bypass (CPB) for high level (III/IV) inferior vena cava (IVC) tumor thrombectomy and concomitant radical nephrectomy.

Materials and Methods: From May 1990 to August 2011, we reviewed 21 patients that had been treated for RCC with radical nephrectomy and concomitant IVC thrombectomy employing either CPB (n =16) or VVB (n=5). We retrospectively reviewed our study population for complication rates and perioperative characteristics.

Results: Our results are reported using the validated Dindo-Clavien Classification system comparing the VVB and CPB cohorts. No significant difference was noted in minor complication rate (60.0% versus 68.7%, P=1.0), major complication rate (40.0% versus 31.3%, P=1.0), or overall complication rate (60.0% versus 62.5%, P=1.0) comparing VVB versus CPB. We also demonstrated a trend towards decreased time on bypass (P=0.09) in the VVB cohort.

Conclusion: The use of VVB over CPB provides no decrease in minor, major, or overall complication rate. The use of VVB however, can be employed on an individualized basis with final decision on vascular bypass selection left to the discretion of the surgeon based on specifics of the individual case.

ARTICLE INFO

Key words:
Venae Cavae; Nephrectomy; Neoplasms

Int Braz J Urol. 2015; 41: 911-9

Submitted for publication:
July 24, 2014

Accepted after revision:
December 18, 2014

INTRODUCTION

With the increasing use of cross sectional imaging over recent years, the incidence of renal cell carcinoma (RCC) has increased at an average of 2.5% yearly (1). Although this has led to earlier detection of RCC, 5-10% of patients continue to present with tumor thrombi formation in the inferior vena cava (IVC) at the time of diagnosis. The presence of thrombi itself portends a poorer prognosis; however it has been demonstrated that thrombi level I-IV have equivalent 5-year cancer specific survival of 32%-68% after surgical intervention (1-5). Even though surgery is warranted in most patients regardless of the level of thrombus, an increased perioperative complication rate has been observed proportional to the proximal extent of the tumor thrombus. This increase in complica-

tion rate is in part caused by the frequent need for vascular bypass to successfully resect bulky level III and IV IVC tumor thrombi (1-3, 5-9).

In an effort to reduce perioperative complications, liver transplantation techniques that do not utilize vascular bypass have been successfully applied for certain level III thrombi. However, there still exists a population of patients that cannot tolerate the decrease in cardiac return after cross clamping of the IVC, and require vascular bypass for successful resection (3, 10-13). Although it is widely accepted that resection of most level IV thrombi must be accomplished with cardiopulmonary bypass (CPB), there is controversy over which type of bypass should be used for level III thrombi and selected level IV thrombi (1, 6-8, 14, 15). In these patients the use of veno-venous bypass (VVB) has been utilized in effort to reduce the risk of perioperative coagulopathy and neurologic and systemic complications associated with CPB (4, 10-11). Most prior studies examining the use of VVB have been descriptive in nature except for a prior peer reviewed study that demonstrated decreased operative time and bypass time when compared to CPB (16). The aim of the present study was to validate this prior study's results at our own institution while taking an in-depth look at perioperative complications among RCC patients with level III or IV IVC tumor thrombi submitted to such surgery on either VVB or CPB.

MATERIALS AND METHODS

A retrospective study protocol was approved by our institutional review board prior to identifying patients at our tertiary care referral center from May 1990 to August 2011 with RCC and level III-IV IVC thrombi who underwent a radical nephrectomy and IVC thrombectomy on either VVB or CPB. Prior to their operation, a complete metastatic evaluation was conducted which included history, physical examination, and serological studies that included serum creatinine, complete blood count, calcium assessment, and liver function studies. Patients were also screened with chest x-ray or non-contrast computed tomography (CT) or magnetic resonance imaging (MRI), both with intravenous contrast when no contraindication was present (i.e. allergy to contrast or renal insufficiency). This was performed to assess the presence of metastases as well as differentiate the level of the tumor thrombus and carefully clinically stage patients using the American Join Committee on Cancer (AJCC, 2010) classification (17, 18). Additional tests such as bone scintigraphy were performed at the discretion of the referring urologist based on the patient's clinical presentation. The level of tumor thrombus was determined using the Mayo Classification Scale of IVC tumor thrombi (1, 18, 19).

A retrospective chart review was performed for demographics, estimated blood loss (EBL), transfusion of packed red blood cells (PRBC), bypass pump time, operative time, anesthesia time, length of hospital stay and overall survival. Complications were also retrospectively assigned utilizing the Dindo-Clavien classification system (20). All patients with IVC tumor thrombi who did not undergo vascular bypass (n=103) were excluded from the study.

Our surgical technique for resection of level III and IV IVC tumor thrombi has been previously described in the literature and is individualized based on the clinical characteristics of the patient and at the discretion of the multidisciplinary surgical team comprised of a cardiothoracic, hepatobiliary, and/or vascular surgeon (8). Most patients with Level IV thrombi underwent CPB with the exception of those patients in which the tumor thrombi could be manually migrated caudally. In patients with level III tumor thrombi we rely primarily on VVB when vascular bypass is necessary. However, in instances where level III tumor thrombi cannot be adequately controlled at the level of the suprahepatic IVC we typically utilize CPB. In either case the decision to undergo bypass is determined by our multidisciplinary team based in part on the height of the thrombus, magnitude of IVC involvement, bulk of the tumor thrombi, and the anticipated ability of the patient to tolerate cross-clamping of the IVC. After surgical resection, patients were followed routinely every 3-6 months with history and physical examination, serological testing, and radiographic imaging of the chest (chest x-ray, non-contrast CT) and abdomen (CT or MRI with intravenous contrast provided there were no contraindications).

Statistical analysis

Estimated blood loss, intra-operative PRBC transfusions, post-operative PRBC transfusions, time on bypass, operative time, anesthesia time, length of hospital stay, overall survival (OS), disease specific survival (DSS) and complication rates were compared between the CPB and VVB groups. Comparisons between groups were made using Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables. The Kaplan-Meier method was used to estimate overall and disease-specific survival from the time of surgery, with comparisons made using the log-rank test. Two patients that died in the peri-operative period were omitted from the survival analysis as they died prematurely on the study. As such an intention to treat analysis was not performed. All p-values reported are two-tailed with statistical significance set when $p<0.05$. Statistical analyses were conducted using SPSS 21 (IBM Software division, Somers, NY, USA).

RESULTS

Our patient population consisted of 21 patients that had been treated by nephrectomy and concomitant IVC thrombectomy for RCC utilizing either CPB (n=16) or VVB (n=5). Of this group, 17 patients were classified as having level IV thrombi (81%) and 4 were classified as having level III thrombi (19%). The median age of the population was 64 years (43-84). Patients undergoing surgical resection had an overall good performance status, with 20 of 21(95.0%) patients having an Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 0 or 1. The clinical and pathological characteristics of the two patient subsets (VVB and CPB) are summarized in Table-1.

The type of bypass utilized was not predictive of overall, minor, or major complication rate. These complication rates were determined using the validated Dindo-Clavien classification system. The overall complication rate was 60.0% in the VVB group versus 62.5% in the CPB group (P=1.0, Table-2). Additionally the minor complication rate (Clavien I and II) was 60.0% versus 68.7% (P=1.0) and the major complication rate (Clavien IIIa-V) was 40.0% versus 31.3% (P=1.0) in the VVB versus the CPB group. Notably, two perioperative mortalities occurred in the CPB group (on postoperative days 2 and 13). The death at postoperative 2 day occurred from renal insufficiency and haemothorax formation. The death at postoperative day 13 occurred from sepsis caused by an enterococcus infection. Additionally, the need for post-operative blood transfusions occurred in 0 of the VVB group and 43.8% of the CPB group (P=0.12).

Overall, we did not discover any statistical difference in the perioperative characteristics between VVB versus CPB when analyzing median EBL (2300 mL versus 3250 mL, P=0.35), intraoperative pRBC's transfused (6 units versus 8 units, P=0.66), operative time (362 minutes versus 403 minutes, P=0.28), anesthesia time (407 minutes vs. 473 minutes, P=0.18), and length of hospital stay (8 days versus 11 days, P=0.21). There was a trend however, towards decreased total time on vascular bypass in patients undergoing VVB (29 minutes versus 60 minutes, P=0.09). These results are shown in Table-3.

The median post-operative follow-up for the entire population was 11.93 months (IQR: 5.59-29.92 months). The median OS for the entire population was relatively low at 16.1 months (IQR: 6.3-32.5 months) with a comparable median estimated DSS for the entire population of 20.6 months (IQR: 6.3-84.8 months). Utilization of one form of bypass over the other did not predict OS or DSS. Median OS in the VVB group was 20.6 months versus 10.16 months (IQR: 5.6-84.8) in the CPB group (P=0.80) with 2-year OS rates of 50% (VVB) and 40% (CPB). The overall DSS for the VVB versus the CPB group was 20.6 months (IQR: 6.3-29.9 months) versus 10.2 months (IQR 5.6-84.8 months, P=0.60) with 2-year DSS rates of 50% (VVB) and (50%).

DISCUSSION

In our current study we attempted to determine if any benefit exists in utilizing VVB over CPB in patients undergoing IVC tumor thrombectomy with concomitant radical nephrectomy for RCC. We assessed our surgical experience in conducting high level IVC tumor thrombi (level III

Table 1 - Patient Clinical and Pathological Characteristics.

Feature	VVB (n=5)	CPB (n=16)
Tumor Thrombus Level		
Level III	3(60.0)	1(6.25)
Level IV	2(40.0)	15(93.75)
Age at Surgery		
Median (Range)	45(43-83)	65(53-84)
Gender M/F	3/2	8/8
Extent of Disease at Time of Surgery		
N+	2(40.0)	5(31.25)
M+	0	5(31.25)
Histologic Subtype		
Clear Cell	4(80.0)	7(43.75)
Papillary	0	6(37.50)
Chromophobe	0	0
Not Specified	1(20.0)	3(18.75)
Nuclear Grade		
1	0	0
2	1(20.0)	2(12.50)
3	3(60.0)	4(25.00)
4	1(20.0)	5(31.25)
Not Otherwise Specified	0	5(31.25)
ECOG		
0	3(60.0)	9(56.25)
1	2(40.0)	6(37.50)
2	0	1(6.25)
BMI		
Median (Range)	29.3 (20.9-35.9)	27.6 (19.5-42.3)

CPB = Cardiopulmonary Bypass; **VVB =**Veno-Venous Bypass; **BMI =** Body Mass Index, RCC = Renal Cell Carcinoma; **M =** male; **F =** Female; **ECOG =** Eastern Cooperative Oncology Group Status
(Data in parenthesis are percentages)

and IV) using either VVB or CPB techniques. We have shown that both approaches can be successfully performed safely acknowledging a high peri-operative complication rate in such challenging surgical procedures for locally advanced disease.

Traditionally the use of CPB was utilized in almost all cases of level III and IV tumor thrombi. Due to the known complications of renal and hepatic failure, neurologic dysfunction, postoperative sepsis, and systemic coagulopathy associated with CPB, alternative techniques have been attempted to reduce these complications (1, 16, 21, 22). Some level III thrombi can be successfully managed utilizing orthotopic liver transplant techniques that involves cross clamping of the IVC. This technique was reported by Cianco et al., and reduces the inherent risk associated with vascular bypass. The decrease in cardiac return after IVC cross clamping however is sometimes not tolerable in a select group of patients (Supp. Figure-1).

Table 2 - Overall Complication Rate By Clavien Classification.

Complication By Clavien Classification	VVB(n=5)	CPB(n=16)
Atrial Fibrillation II	0	3(18.75)
Cephalic Vein Thrombus II	0	1(6.25)
Chylous Fistula II	0	1(6.25)
Deep Vein Thrombosis II	1(20.0)	0
Volume Overload II	0	1(6.25)
Pneumothorax IIIa	0	1(6.25)
Cardiac Tamponade IIIb	0	1(6.25)
Myocardial Infarction IV	1(20.0)	0
Pulmonary Embolus IV	1(20.0)	0
Mortality V	0	2(12.5)

Data in parenthesis are percentages. (P=1.0)

Table 3 - Perioperative characteristics.

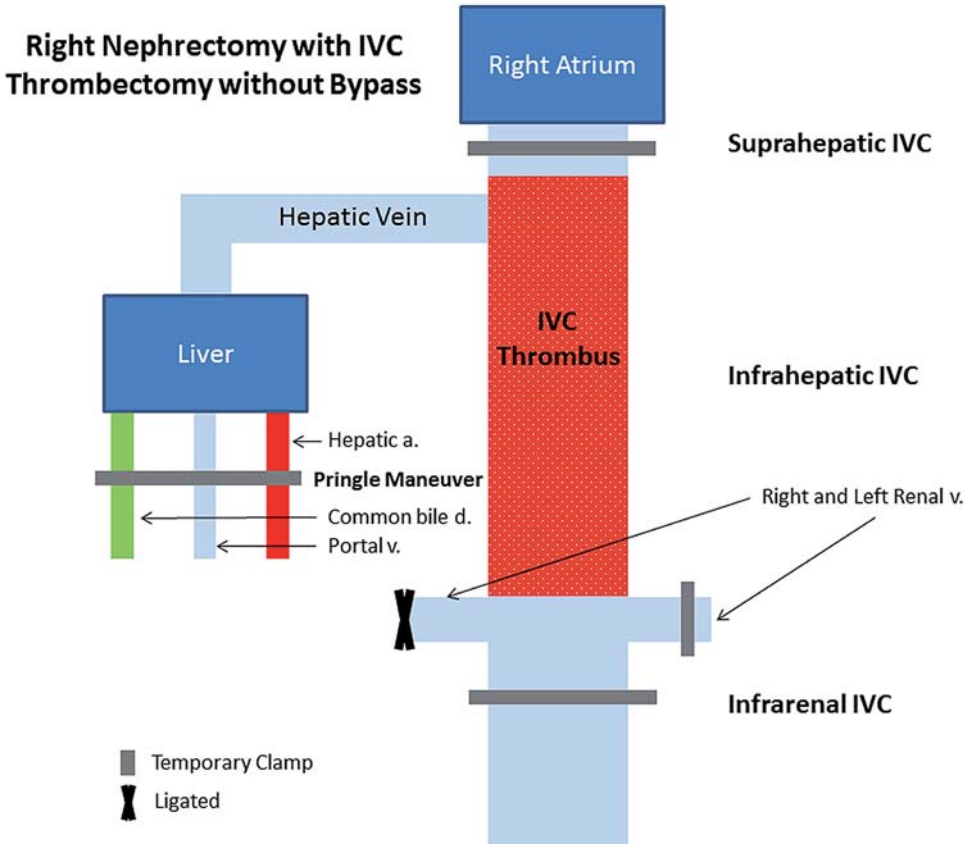
Features	VVB(n=5)	CPB(n=16)	P Value
Estimated Blood Loss (mL)	2300(1300-5200)	3250(900-9000)	0.35
Intra-operative pRBC's (units)	6(4-12)	8(1-38)	0.66
Bypass Time (min)	34 (20-50)	64 (16-138)	0.09
Operative Time (min)	362 (288-478)	403 (248-865)	0.28
Anesthesia Time (min)	407 (300-541)	473 (384-955)	0.18
Length of Hospital Stay (min)	8 (5-10)	11 (2-20)	0.21

pRBC-packed red blood cells
Data is reported as medians with range demonstrated in parentheses.

As such, the use of bypass is clearly beneficial and encouraged (Supp. Figure-2). As excessive post-operative bleeding can occur in up to 11% of patients after undergoing CPB however, VVB has been used to possibly reduce the risk of post-toperative coagulopathy (24). Initially utilized for liver transplantation, VVB has the advantage that it does not require systemic anti-coagulation, as the cannulas are pre-coated with heparin (16, 22, 23, 25).

The use of VVB in IVC thrombectomy has been described extensively in the literature (10, 11, 16, 26-29). However, only one prior retrospective study conducted by Granberg et al. has compared VVB versus CPB bypass in the setting of RCC and IVC tumor thrombi (16). This study demonstrated patients undergoing VVB (n=13) had significantly shorter bypass, operative, and anesthesia times than did patients treated with CPB (n=28). The study also demonstrated trends towards decreased intraoperative blood loss, reduced transfusion requirements, and a shorter length of hospitalization with VVB. In our current study, we sought to perform an extensive analysis of complications while comparing similar peri-operative characteristics to the previous study. In our study we did

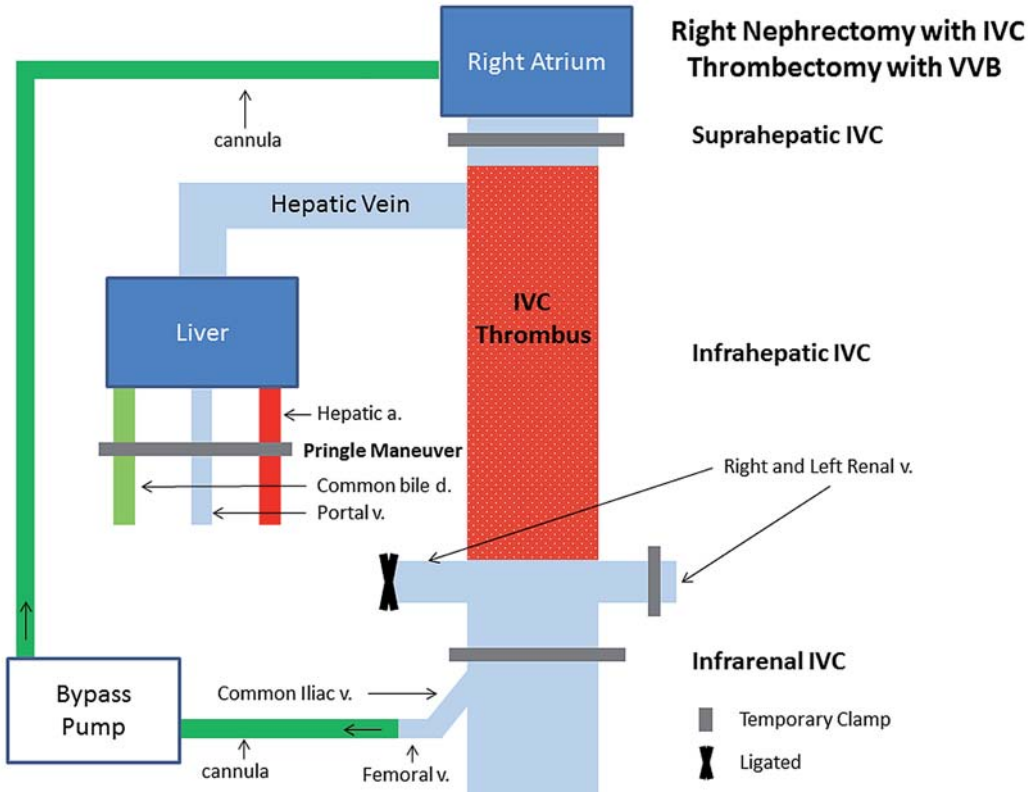
Supplementary Figure 1 - Vascular control during right radical nephrectomy with inferior vena cava (IVC) thrombectomy without bypass utilizing the orthotopic liver transplant technique. Temporary clamps are placed on the hepatic hilum (hepatic artery, portal vein, and common bile duct) via the Pringle maneuver, suprahepatic IVC, infrarenal IVC, and left renal vein. If no collateral circulation exists between the suprahepatic IVC and the right atrium, decreased cardiac preload can lead to hypotension.



not discover trends towards decreased intraoperative blood loss, reduced transfusion requirements, and shorter length of hospital stay with the use of VVB. This could in part be from the limited power of our study or perhaps a selection bias, as any patient undergoing IVC thrombectomy for RCC is subject to substantial blood loss, leading to increased transfusion requirements, and possibly an increased hospital stay. Our study also differed from the previous, (16) as we only demonstrated a trend in decreased time on bypass and showed no statistical difference in operative time and anesthesia time. It could be assumed that since VVB only requires percutaneous access and not direct access to the vasculature like CPB, there would be a decrease in operative and anesthesia time. However since at our institution vascular bypass is reserved for cases that require extensive mobilization and resection of the tumor thrombi, increased operative time, anesthesia time, and time on bypass would be increased in all cases (27).

Prior studies demonstrated comparable survival rates of patients with level III and IV tumor thrombi after surgical resection (5). As evidenced by our current study as well the study conducted by Granberg et al., utilizing VVB versus CPB provides no increase in OS or DSS with one form of bypass versus another. This is conceivable as both modalities allow for adequate resection of tumor thrombi and both involve a substantial and comparable insult to the cardiovascular system. As such, it is quite feasible that if the patient successfully recovers from the perioperative period, there will be no differences in intermediate long-term survival.

Supplementary Figure 2 - Vascular control during right radical nephrectomy with inferior vena cava (IVC) thrombectomy utilizing veno-venous bypass (VVB). Similar to the orthotopic liver transplant technique, temporary clamps are placed on the hepatic hilum (hepatic artery, portal vein, and common bile duct) via the Pringle maneuver, suprahepatic IVC, infrarenal IVC, and left renal vein. Cardiac preload is restored by the bypass of the portal and venous circulation via cannulation (direction of flow depicted by arrows) of the femoral vein returning blood flow to the right atrium.



We discovered no difference in minor, major, or overall complication rate when comparing VVB versus CPB utilizing the Dindo-Clavien Classification system. Significant major complication rates (Dindo-Clavien IIIa-V) were evident in both the VVB (40.0%) and the CPB (31.3%) group. Notably there were two post-operative deaths (Clavien V) as well as one pneumothorax (Clavien IIIa) and one case of cardiac tamponade (Clavien IIIb) in the CPB cohort. Similarly, one intra-operative myocardial infarction (Clavien IVa) and one post-operative pulmonary embolus (Clavien IVa) occurred in the VVB group. Additionally no difference in minor complications between VVB and CPB were observed. As there is no statistical difference in minor, major, or overall complication rates between the VVB and CPB groups, our study demonstrates that both modalities are associated with significant complications in the perioperative period. Although the use of VVB eliminates the use of systemic anticoagulation, this is only one variable that contributes to post-operative coagulopathy. Consumptive coagulopathy, which is caused by introducing red blood cells to foreign surfaces such as connecter tubing used in both VVB and CPB, increases the expression of tissue factor, which in turn initiates the coagulation cascade leading to consumption of coagulation factors and platelets (23, 24). As substantial post-operative complications can occur when using either VVB or CPB to successfully resect IVC tumor thrombi, knowledge of these complications is paramount for surgical planning and post-operative management.

We acknowledge that our study is limited by its relatively small sample size as well as our single-institution retrospective design. We also acknowledge an inherent selection bias in our study as we utilize a multidisciplinary decision making process and not specific criteria to determine which patients undergo VVB versus CPB. However, in light of this, our study does not necessarily support the use of VVB over CPB in the setting of IVC thrombectomy. As both methods have similar survival and complication rates, the select use of VVB could be employed on high level thrombi (III/IV) on an individualized basis.

CONCLUSIONS

It has been speculated that the use of VVB could potentially mitigate complications associated with CPB in patients with tumor thrombi undergoing IVC thrombectomy and radical nephrectomy for RCC. However, our study demonstrated that no decrease in complication rate exists with VVB, and that both modalities come with considerable complications that must acknowledged for surgical planning as well as patient education. Although there is no clear-cut benefit to VVB, we discovered a trend of decreased time on bypass, which would possibly be significant in a larger multi-center study. We suggest that CPB is still a valid method for assisting in resection of level III and IV tumor thrombi in patients with RCC, however the use VVB could also be considered on an individualized basis at the discretion of the multi-disciplinary surgical team.

SUPPORT/FINANCIAL DISCLOSURES

Patrick Espiritu, MD - Employee (Moffitt Cancer Center)

Wade J. Sexton, MD - Employee (Moffitt Cancer Center)

Julio M. Pow-Sang, MD - Employee (Moffitt Cancer Center)

Philippe E. Spiess, MD - Employee (Moffitt Cancer Center)

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Correspondence address:
Philippe E. Spiess, MD
Associate Member
Department of Genitourinary Oncology
Moffitt Cancer Center
12902 Magnolia Dr.
Tampa, FL 33612, USA
Fax: + 1 813 745-8494
E-mail: philippe.spiess@moffitt.org

ORIGINAL ARTICLE

Vol. 41 (5): 920-926, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0402

Impact of retrograde flexible ureteroscopy and intracorporeal lithotripsy on kidney functional outcomes

Nicolas Hoarau [1], Francois Martin [1], Souhil Lebdai [1], Denis Chautard [1], Thibaut Culty [1], Abdel Rahmene Azzouzi [1], Pierre Bigot [1]

1 Département d'urologie, Hôpital universitaire d'Angers, Angers, France

ABSTRACT

Objective: The aim of the study was to evaluate renal function and to identify factors associated with renal function deterioration after retrograde intrarenal surgery (RIRS) for kidney stones.
Materials and Methods: We retrospectively analyzed patients with renal stones treated by RIRS between January 2010 and June 2013 at a single institute. We used the National Kidney Foundation classification of chronic kidney disease (CKD) to classify Glomerular Filtration Rate (GFR) in 5 groups. The baseline creatinine level was systematically pre-operatively and post-operatively evaluated. All patients had a creatinine blood measurement in June 2013. A change toward a less or a more favorable GFR group following RIRS was considered significant.
Results: We included 163 patients. There were 86 males (52.8%) and 77 females (47.3%) with a mean age of 52.8±17 years. After a mean follow-up of 15.5±11.5 months, median GFR was not significantly changed from 84.3±26.2 to 84.9±24.5 mL/min (p=0.675). Significant renal function deterioration occurred in 8 cases (4.9%) and significant renal function amelioration occurred in 23 cases (14.1%). In univariate analysis, multiple procedures (p=0.023; HR: 5.4) and preoperative CKD (p=0.011; HR: 6.8) were associated with decreased renal function. In multivariate analysis these factors did not remain as predictive factors.
Conclusion: Stone management with RIRS seems to have favorable outcomes on kidney function; however, special attention should be given to patients with multiple procedures and preoperative chronic kidney disease.

ARTICLE INFO

Key words:
kidney stones, holmium laser lithotripsy, renal function, retrograde intrarenal surgery, urolithiasis

Int Braz J Urol. 2015; 41: 920-6

Submitted for publication:
August 08, 2014

Accepted after revision:
December 04, 2014

INTRODUCTION

Retrograde intrarenal surgery (RIRS), combined with Holmium laser lithotripsy is widely applied for the management of intra-renal stones. For large or multiple stones, RIRS may represent an alternative therapy to percutaneous nephrolithotomy (PNL) even if multiple procedures are often required (1, 2). It is accepted that stone removal can improve renal function; however, a stone-removing procedure may negatively impact the kidney parenchyma (3). Recently, El-Tabey et al. reported that Percutaneous Nephrolithotomy (PNL) for calculi in solitary kidneys provided significant improvement in renal function at long-term follow-up (4).

To date, no study has evaluated the impact of RIRS on renal function. Nevertheless, in case of RIRS, the flexible ureteroscope is introduced into the upper urinary tract collecting system

under water pressure. Irrigation during endoscopy cool the tip of energy-delivering devices and helps to maintain a clear visual field by displacing blood, stone fragments, and cellular debris. However, this leads to prolong renal calices distension. Moreover, delivering laser energy next to or directly onto renal tissue may cause damage to the renal papillae. Thus, one could advocate neither prolonging nor multiplying the surgical procedure to avoid renal function deterioration and to decrease post-operative complications. The type and the time of post-operative ureteral catheterization could also protect from renal dysfunction.

The aim of this study was to evaluate renal function and identify factors associated with renal function deterioration or amelioration after RIRS.

MATERIALS AND METHODS

Patients

After approval from an institutional review board, we retrospectively analyzed 163 patients undergoing 205 RIRS for intra-renal stones between January 2010 and June 2013 in an academic department of Urology. All patients with a unique radio-opaque stone were previously treated by at least one procedure of Shock Wave Lithotripsy (SWL) without result. Patients were pre and post-operatively evaluated by computed tomography. All procedures were conducted under sterile urine and verified by a urine culture seven days before the RIRS. Confirmed urinary infection or bacteriurias were systematically treated during at least five days with adapted antibiotherapy. We recorded patient age, gender, body mass index, number of stones, diameter of the largest stone, cumulative stone diameter, and stone composition. The perioperative parameters analyzed were the use of pre-operative stenting, the mean operative time, the use of a UAS (Ureteral Access Sheath), the presence of a post-operative ureteral stent or a JJ-stent, the number of procedures and the mean follow-up. The stone free (SF) status was defined when no residual fragments (<2mm) were seen on a non-contrast enhanced tomography performed 1 to 2 months after the last retrograde flexible ureteroscopy session. We performed another RIRS in case of significant persistent stone (>4mm) on the post-operative tomography. RIRS were performed between 2 and 3 months after the previous procedure.

Surgical procedure

The procedures were performed by 10 different surgeons from the same institution. All procedures were done under general anesthesia in a standard flexible ureteroscopy installation. A 0.035 inch polytetrafluoroethylene coated wire was placed in the upper urinary tract under visual and fluoroscopic control through a rigid cystoscope. A safety wire was routinely used. The use of a UAS (Ureteral Access Sheath with AQ, Cook Medical, Spencer, USA) was done under the assessment of the surgeon, as well as its insertion method. When no UAS was used, the flexible ureteroscope (Flex-X2 TM, Karl Storz endocopy, Tuttlingen, Germany) was inserted in a monorail way over the second wire. Normal saline irrigation was performed at a pressure of 60 cm H_2O through the same channel used for working instruments. If necessary a transient water pressure was carried out using a hand pump. A holmium--YAG laser was used at an energy varying between 0.6 to 0.8 Joules, and at a frequency of 8 to 10 Hertz. A 270µm or 400µm laser fibre was used for delivering laser energy. At the end of the procedure a JJ stent or a ureteral stent was inserted under the assessment of the surgeon.

Renal function evaluation

The evaluation of the Glomerular Filtration Rate (GFR) was derived from the Modification of Diet in Renal Disease (MDRD) study group equation (5). The baseline creatinine level was systematically pre-operatively and post-operatively (day one) evaluated. All patients had a creatinine blood measurement in June 2013.

We used the National Kidney Foundation classification of chronic kidney disease (CKD) which classifies estimated GFR in the following ranges: at least 60, 45 to 59 (stage 3a), 30 to 44 (stage 3b), 15 to 29 (stage 4), and less than 15 mL per minute per 1.73m^2 (stage 5) (6). A change toward a less or a more favorable GFR group following surgery was considered significant.

Statistical analysis

Pair t-test was used for comparisons of GFR before and after RIRS. Independent-sample t-test and chi-square tests were used for comparisons of means and proportions, respectively. Univariate and multivariate regression models were used to assess the influence of different variables on renal function outcomes. Only factors that were significant in univariate analysis were considered for multivariate analysis. All tests were done using SPSS® version 10.

RESULTS

Patient and stone characteristics

We included 163 patients. There were 86 males (52.8%) and 77 females (47.3%) with a mean age of 52.8±17 years. The mean BMI was 26.2±5.9 cm/kg. Patients presented a GFR greater than 60 mL/min/1.73m^2 in 128 (78.5%) cases. Patients presented 3a, 3b and 4 preoperative CKD stage in 27 (16.6%), 7 (4.3%) and 1 (0.6%) cases, respectively. The mean GFR was 84.30±26.2 mL/min/1.73m^2.

Multiple stones were present in 73 patients (44.7%). The mean diameter of the largest stone was 12.9±5.7mm, and the cumulative stone diameter was 15±8.6mm. Stone composition was calcium oxalate monohydrate, calcium oxalate dehydrate, uric acid, carbapatite, and unknown in 56 (34%), 8 (5%), 13 (8%), 27 (16.6%) and 86 (53%) cases, respectively. A preoperative stent was inserted in 46 (63.8%) cases. The mean operative time was 96.4±40.78 minutes. A ureteral access sheath was used in 144 (88.3%) procedures and a postoperative ureteral JJ stent was left in 115 (70.6%) cases. Multiple procedures were performed in 29 (17.8%) patients 24 (14.7%) patients had two procedures and 5 (3%) patients had 3 procedures). At the end of the follow-up, 121 (74.2%) patients were stone free.

Perioperative complications occurred in 16 (9.8%) patients (5 pyelonephritis, 3 macroscopic hematuria, 6 pains with the necessity of grade II analgesia, and 1 urinoma). Patient and stone characteristics are reported in the Table-1.

Table 1 - Characteristics of patients and Calculi.

	N=163 patients
Sex, male (n,%)	86 (54.6 %)
Age (y)	52.8±17
BMI (cm/kg^2)	26.2±5.9
Multiple stones (n,%)	73 (44.7%)
Diameter of the largest stone	12.9±5.7
Cumulative stone diameter (mm)	15±8.6
Stone composition	
Calcium oxalate monohydrate (n,%)	56 (34%)
Calcium oxalate dehydrate (n,%)	8 (4.9%)
Uric acid (n,%)	13 (8%)
Carbapatite (n,%)	27 (16.6%)
Unclear (n,%)	59 (36.1%)
Preoperative stenting (n,%)	46 (63.8%)
Mean operation time (min)	96.4±40.78
Ureteral Access Sheath (n, %)	144 (88.3%)
Postoperative ureteral JJ stent	115 (70.6%)
Multiple procedures (n, %)	29 (17.8%)
Stone free rate (n,%)	121 (74.2%)
Follow-up (months)	15.5±11.5

Analysis of the immediate post-operative renal function

The median GFR after procedure was 89.45 mL/min/1,73m^2. An acute kidney injury, defined as any GFR less than 15 mL/minute/1.73m^2, occurred in one (0.06%) patient and no patients needed dialysis. Significant renal function change occurred in 18 cases (11%) with 13 (7.9%) amelioration and 5 (3%) deterioration.

Analysis of long-term renal function

After a mean follow-up of 15.5±11.5 months, 14 (8.6%), 8 (4.9%) and 0 (0%) patients presented 3a, 3b and 4 CKD stages, respectively. The median GFR after procedure was 84.9±24.4 mL/min/1.73m^2. There was no significant difference with the preoperative median GFR (p=0.675). A

change towards a worse or a better CKD group was observed in 8 (4.9%) and 23 (14.1%) cases, respectively (Table-2).

Analysis of predicting factors of renal function changes

The univariate Cox regression analysis showed only two significant factors for renal deterioration: presence of multiple procedures and pre-existing chronic kidney disease (p=0.023; HR=5.4 and p=0.011; HR=6.8 respectively). In multivariate analysis these factors did not remain as predictive (p=0.156; HR=3.12 and p=0.054; HR=4.7) (Table-3). None of the analysed factors were predictive of renal function amelioration (Table-4).

DISCUSSION

In this study, we intended to evaluate the incidence of CKD after RIRS for stone management. A key finding of this study is that RIRS seemed to have a small impact on kidney function and was associated with 14.1% of long-term improvement.

Ureteroscopy is recommended by the European Association of Urology guidelines as a first-line treatment for proximal ureteral stones greater than 1 cm, but they do not recommend this procedure as a first-line therapy for intra-renal stones (7). This is essentially due to the efficiency and the non-invasive nature of SWL. Moreover, complications of RIRS are often arguments for its detractors. Nonetheless, if infection or ureteral injuries have been widely reported and studied, the renal functional outcomes after RIRS are unknown (8-10).

As we know, the use of pressurized water during RIRS leads to a dilatation of renal cavities. However, the consequences of these high intrarenal pressures have not been studied. What we do know is that excessive renal pressure is the main factor of kidney destruction during acute obstructions. Tubular function is threatened by acute excessive urinary pressure. The renal tubular cells stretched by the hydrostatic pressure leads to a tubular interstitial inflammation with macrophages proliferation and myoblasts accumulation. Alteration of the tubular cells associated with macrophages and myoblasts infiltration lead to the production of cytokines and growth factors which are responsible for renal tubular cell apoptosis. This results in chronic obstructive nephropathy with tubular atrophy and loss of nephrons which are replaced by interstitial fibrosis (11-13).

Concerning evaluation of the GFR, we focused on pre-operative, early post-operative and long-term blood creatinine level measurement. Even if the mean GFR before and after RIRS was not significantly different, we found a trend towards GFR improvement. This can be explained by numerous factors. The true prevalence of obstructive nephropathy is unknown but it

Table 2 - Analysis of renal function after ureteroscopy and intracorporeal lithotripsy for intrarenal stones.

	Preoperative	Postoperative	p
CKD group, n (%)			
GFR>60 mL/min/1.73m^2	128 (78.5%)	141 (86.5%)	0.003
45≤GFR<59 mL/min/1.73m^2	27 (16.6%)	14 (8.6%)	0.018
30≤GFR<45 mL/min/1.73m^2	7 (4.3%)	8 (4.9%)	0.529
15≤GFR<30 mL/min/1.73m^2	1 (0.6%)	0 (0%)	0.319
GFR<15 mL/min/1.73m^2	0 (0%)	0 (0%)	1
Change to a worse CKD group		8 (4.9%)	
Change to a better CKD group		23 (14.1%)	
Mean GFR (mL/min/1.73m^2)	84.30±26.2	84.90±24.4	0.675

Table 3 - Univariable and multivariable Cox regression analysis of predicting factors for renal function deterioration in 163 patients treated by retrograde flexible ureteroscopy and intracorporeal lithotripsy for intrarenal stones.

	Univariable		Multivariable	
	p value	HR (CI)	p value	HR (IC)
Female gender (versus male)	0.109	0.26 (0.05-1.3)	-	-
Age (continuous)	0.069	1.043 (0.99-1.09)	-	-
Multiple stones (vs unique stone)	0.3	3.1 (0.36-25.6)	-	-
Cumulative stone diameter (continuous)	0.587	0.96 (0.819-1.120)	-	-
Stone free (vs stone persistence)	0.088	0.26 (0.06-1.22)	-	-
Multiple procedures	0.023	5.4 (1.2 - 23.1)	0.156	3.12 (0.65-15)
Preoperative stenting (vs. no stent)	0.117	5.4 (0.65-45.5)	-	-
Preoperative CKD group >1	0.011	6.8 (1.6-30.4)	0.054	4.7 (0.97-23.4)
Operation time (continuous)	0.522	1.005 (0.98-1.02)	-	-
Postoperative double pigtail stent (vs ureteral stent)	0.773	1.2 (0.2-7.3)	-	-
Postoperative pyelonephritis	0.416	2.5 (0.27-23.3)		

HR=Hazard ratio; **CI=**Confidence interval

Table 4 - Univariable Cox regression analysis of predicting factors for renal function amelioration in 163 patients treated by retrograde flexible ureteroscopy and intracorporeal lithotripsy for intrarenal stones.

	Univariable	
	p value	HR (CI)
Female gender (versus male)	0.251	1.7 (0.7- 4.1)
Age (continuous)	0.051	1.028 (0.99- 1.056)
Multiple stones (vs unique stone)	0.546	0.75 (0.29-1.9)
Cumulative stone diameter (continuous)	0.445	0.97 (0.9-1.05)
Stone free (vs stone persistence)	0.121	2.1 (0.82-5.35)
Multiple procedures	0.546	0.75 (0.295 - 1.9)
Preoperative stenting (vs. no stent)	0.765	0.872 (0.35-2.1)
Operation time (continuous)	0.311	1.006 (0.99-1.02)
Postoperative double pigtail stent (vs ureteral stent)	0.087	3. (0.85-10.6)

HR=Hazard ratio; **CI=**Confidence interval

is known that nephrolithiasis duration, subsequent urinary tract infections, and size of stones are factors that influence renal function (14-16). As a consequence, removing these factors may reasonably influence GFR positively.

In our study, multiple procedures appear as predicting factors of renal function loss in univariate analysis but did not stay significant in multivariate analysis. This could be explained by the effect of RIRS on the kidney but also by the

fact that patients with multiple procedures often have larger stones and more advanced nephrolithiasis disease. Interestingly, operative time was not a predictive factor of renal function loss. In this context, kidney function did not seem to be a good argument for limiting the RIRS procedure time. Moreover, if a UAS is known to decrease intra-renal pressure, we did not find any influence on post-operative renal functional outcomes (17). The univariate cox regression analysis also showed that pre-existing renal dysfunction was another factor correlated with renal function. This result is not surprising considering that renal insufficiency is known as a risk factor for intra and post-operative complications in general surgery and, more particularly, in renal surgery (18). Only a 63 years old man with previous stage 3b CKD (37 mL/min) had an acute renal failure (13 mL/min) after the second procedure (120 min) for multiple renal stones (cumulative stone diameter of 15mm). He did not need hemodyalisis. Three months after RIRS and with hyperhydratation, his renal function was 28 mL/min.

Considering the superior number of patients changing to a better CKD group, we researched predictive factors of renal function improvement but no significant result clearly appears. However, our result confirmed that removal of a stone improves postoperative renal function (3).

To our knowledge, there is no study assessing the renal functional outcomes after flexible ureteroscopy. Nevertheless, this point is essential in the therapeutic decision for the management of kidney stone diseases. PNL is considered as a more invasive procedure and is indicated for larger stone diameters (more than 2 cm). For these larger stones, if RIRS is performed, multiple procedures might be required, whereas usually only a single session is required for PNL. Moreover, PNL is known to have a good functional outcome even on solitary kidneys (3, 19). A kidney functional approach in stone management is primordial. Indeed, nephrolithiasis is considered as a chronic disease, and patients often undergo multiple procedures. Thus, the management of nephrolithiasis must take into consideration the kidney functional outcomes especially in fragile patients. According to our data, we believe that the improvements of laser and flexible ureteroscope technologies raise the possibility of mini-invasive approaches.

There are several limitations to this study. First, this is a retrospective review from a single institution. The results are based on a relatively small sample size and we could not confirm that multiple procedures and preoperative kidney disease were prognostic factors of kidney function deterioration in multivariate analysis. Moreover, there might have been a confusing factor due to the contralateral compensation of the non-treated kidney for all patients with two kidneys. However, a solitary kidney model could have been more informative but it is rare and represents a very different clinical situation. Moreover, kidney function deterioration could also be linked to urolithiasis disease and it is difficult to know the part played by either natural history of the disease or RIRS. Larger cohorts and longer periods of follow-up will be necessary to consolidate these data and to confirm the identified predictive factors.

CONCLUSIONS

After RIRS, 8 (4.9%) patients had a decrease and 23 (14.1%) patients had an improvement of their renal function. Stone management with RIRS seems to have favorable outcomes on kidney function; however, special attention should be given to patients with multiple procedures and preoperative chronic kidney disease.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Pierre Bigot, MD
Département d'urologie,
Hôpital universitaire d'Angers, Angers, France
5 rue Larrey
49000 Angers, France
Fax: +02 41 355323
E-mail: pibigot@chu-angers.fr

ORIGINAL ARTICLE

Vol. 41 (5): 927-934, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0024

Mini incision open pyeloplasty – Improvement in patient outcome

Vishwajeet Singh [1], Manish Garg [1], Pradeep Sharma [1], Rahul Janak Sinha [1], Manoj Kumar [1]

[1] *Department of Urology, King George Medical University, Chhatrapati Shahuji Maharaj Medical University), Lucknow, India*

ABSTRACT

Purpose: To assess the subjective and objective outcomes of mini-incision dismembered Anderson-Hynes pyeloplasty in the treatment of primary ureteropelvic junction obstruction (UPJO).

Materials and Methods: Between January 2008 to January 2013, Anderson-Hynes pyeloplasty was performed in 71 patients diagnosed with primary UPJO. Small subcostal muscle splitting incision was used in all cases. Sixteen patients with renal calculi underwent concomitant pyelolithotomy. Subjective outcome was assessed using visual pain analogue score (VAS). For objective assessment, the improvement in differential renal function (DRF) and radio-tracer wash out time (T1/2) on Tc-99m DTPA scan and decrease in hydronephrosis (HDN) on renal ultrasound (USG) and urography (IVU) were assessed.

Results: Mean incision length was 5.2 cm. The average operating time and postoperative hospital stay was 63 (52-124) minutes and 2.5 (2-6) days respectively. Concomitant renal calculi were successfully removed in all the patients. Overall complication rates were 8.4% and overall success rate was 98.6% at median follow-up of 16 months. There was significant improvement in pain score (p=0.0001) and significant decrease in HDN after the procedure. While preoperative mean T1/2 was 26.7±6.4 minutes, postoperative half-time decreased to 7.8±4.2 minutes at 6 months and to 6.7±3.3 minutes at 1 year. Mean pre-operative DRF was 26.45% and it was 31.38% and 33.19% at 6 months and 1 year respectively.

Conclusions: Mini-incision pyeloplasty is a safe and effective technique with combined advantage of high success rates of standard open pyeloplasty with decreased morbidity of laparoscopic approach. Excellent functional and objective outcomes can be achieved without extra technical difficulty.

ARTICLE INFO

Key words:
Laparoscopy; Minimally Invasive Surgical Procedures; Postoperative Period

Int Braz J Urol. 2015; 41: 927-34

Submitted for publication:
January 16, 2014

Accepted after revision:
March 21, 2014

INTRODUCTION

In the current era, the treatment options of ureteropelvic junction obstruction (UPJO) vary from standard open pyeloplasty to various minimally invasive approaches. Traditionally, open pyeloplasty is considered as the reference standard for UPJO with success rates of 90% to 100%, against which all other treatment options are usually compared (1, 2). In standard open pyeloplasty large muscle cutting incision (about 20 to 24 cm) is generally used, which is the major cause of postoperative morbidity and pain (2, 3).

With the advancement of technology, various endoscopic and laparoscopic procedures were proposed for the treatment of UPJO. The

laparoscopic techniques are increasingly employed for urological diseases and can be done via both transperitoneal or retroperitoneal approaches (4, 5) while procedures of antegrade and retrograde endopyelotomies, although minimally invasive, tend to have lower success rates with significant risk of bleeding (6). Though laparoscopic pyeloplasty is gaining popularity in urological practice because of less morbidity as compared to open standard pyeloplasty, laparoscopy is technically more difficult and challenging especially suturing. The laparoscopic pyeloplasty is also time consuming especially in inexperienced hands. Longer and steep learning curve is the main limiting factor of the procedure. In addition, laparoscopy requires specialized and expensive equipment thus increasing the overall cost of procedure. The technique is even more difficult while employing the retroperitoneoscopic laparoscopic approach (7, 8).

There are few reports which used small mini-incision for urological procedures for ureterolithotomy, nephrectomy or pyeloplasty which is defined as less than 10 cm in adults in different series (9-12). We contend that by refining the technique of open pyeloplasty to a much smaller incision, morbidity of the standard open technique can be minimized without compromising the success rates. Due to sheer advantages of mini-incision pyeloplasty (MIP), we decided to describe our experience and results of MIP in the present series.

MATERIALS AND METHODS

A total of 71 consecutive patients were enrolled in this study from January 2008 to January 2013. The institutional ethical approval was obtained and it was in accordance with the declaration of Helsinki. All patients with BMI < 30kg/m^2 with primary UPJO were included in the study.

The patients with renal function of less than 15%, uncorrected coagulopathy, vertebro--spinal deformity, and the presence of cardiopulmonary or respiratory compromise were excluded from study. Apart from the clinical history, physical examination and blood investigations, imaging studies including ultrasonography, contrast enhanced computerized tomography (CECT KUB) or intravenous pyelography (IVP) were done. Diuretic Tc-99m DTPA renal scan was done in all cases to assess the drainage pattern of the kidney, radio-tracer wash out time and differential renal function (DRF). The chest X-ray P.A view, electrocardiogram and pulmonary function test were performed to assess fitness for anesthesia. Urinary tract infection was treated preoperatively considering antibiotic sensitivity. Informed written consent was obtained prior to surgical intervention. Sixteen patients were found to have associated renal pelvic or calyceal calculi at diagnosis and two patients had horse-shoe kidney with unilateral UPJO.

Open technique

All operations were done by a single urologist. For open pyeloplasty lateral decubitus position was used and lumber subcostal muscle splitting incision was made with incision length of less than 8 cm (Figures 1 and 2). The abdominal muscles were separated and the peritoneum was pushed back. Gerota's fascia was opened and pelvis approached by identifying ureter over psoas muscle and by dissecting the ureter proximally. All dissections were performed with help of long retractors and instruments. The redundant part of pelvis was excised. After adequate spatulation of ureter, the anastomosis was performed by 4-0/5-0 vicryl continuous suture. Anderson Hynes dismembered pyeloplasty was done and antegrade DJ stent was placed in all cases (Figure-3). We found the advantage of direct access to the UPJ with good exposure of pelvis and renal vessels with this extraperitoneal approach without the need of extending the incision.

Postoperative follow-up and care

The patient was kept on intravenous fluid till recovery of bowel sounds. Intravenous broad spectrum antibiotic (ceftriaxone) and tramadol on patient demand were administered. Subjective outcome was assessed using visual pain analog scoring (VAS) which was done one day before surgery, on first and second post-operative days and 6 months after surgery in follow-up period. When the drainage tube output was less than 30 mL in 24 hours it was removed in all the patients. The subjects were discharged following Foley catheter and drainage tube removal. The DJ stent

Figure 1 - Subcostal muscle splitting incision (mean length 5.2 cm).

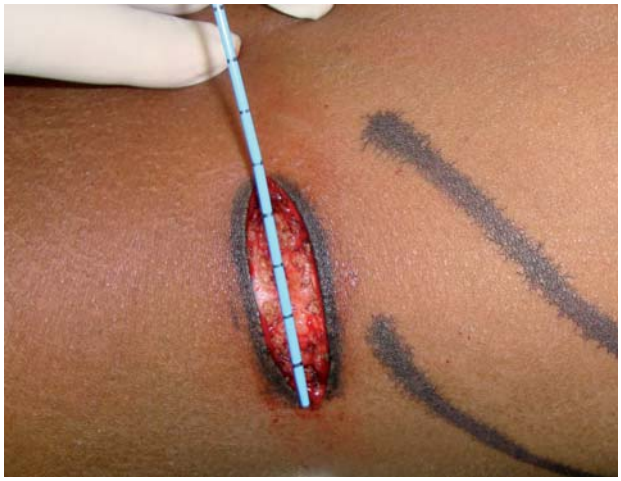
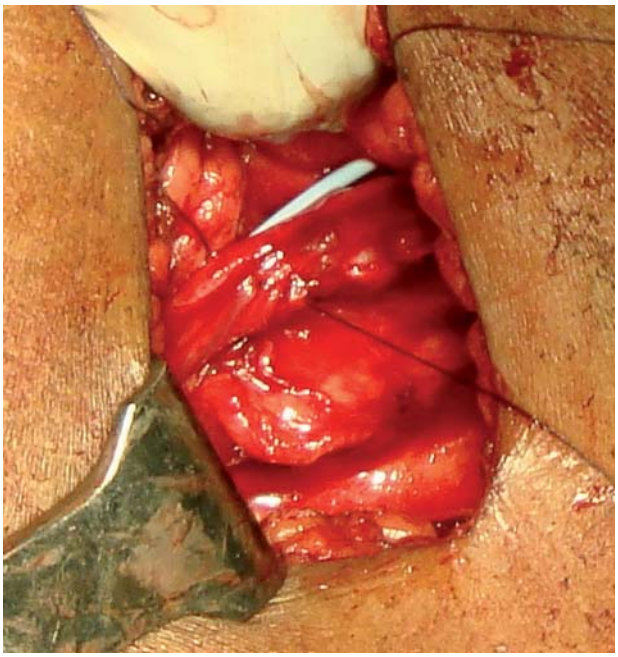
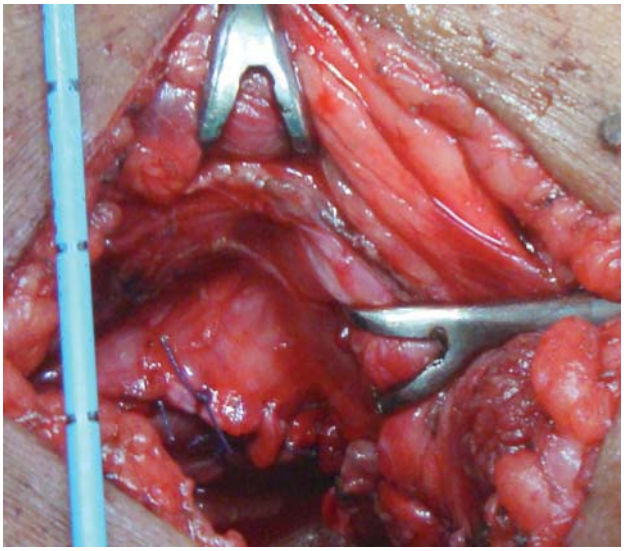


Figure 2 - Antegrade DJ stent placement.



was removed after 6 weeks of operation under IV sedation and antibiotic use. Objective parameters were evaluated by an IVP or USG performed at 3 months of follow-up. Subsequent follow-up of patients were done at 6 months and then annually. At each visit, apart from history and clinical examination, serum creatinine and USG were obtained. DTPA scan was done at 6 months and repeated at 1 year of follow-up and yearly afterwards.

Figure 3 - Water-tight anastomosis.



Statistical analysis

The analysis was carried out by using SPSS version 16.0. The results are presented in mean ± standard deviation and percentages. The continuous variables were compared by unpaired t-test and changes in variables from pre-operative to post operatives were compared by paired t-test. The p-value <0.05 was considered as significant.

RESULTS

The baseline demographic characteristics of patients are shown in Table-1. A total of 71 patients were included. Among them, 17 patients were in pediatric age group (less than 18 years) and the remaining 54 were more than 18 years old. All patients underwent Anderson Hynes dismembered pyeloplasty through muscle-splitting mini--incision approach. Mean incision length was 5.2 cm. The mean operating time was 63 (52-124) minutes and the mean blood loss was 41.23 (27-78) mL (Table-2). None of the patients required blood transfusion. In 27 (38%) patients, anterior crossing vessels at UPJ were encountered and transposed during UPJ reconstruction. Complications were recorded and graded using Dindo-modified Clavien classification of surgical complications

Table 1 - Baseline demographic characteristics of patients.

Total No of patients	71
Mean (range) (years)	23(07-42)
No. M/F	44/27
Mean BMI (kg/m2)	23.6 (18-27)
Mean (range) S. Creatnine	1.1 (0.3-1.5)
No. Rt/Lt side of involvement	23/48
Symptoms	
Lumbar pain	59
Abdominal mass	10
Asymptomatic	2
No. of pts with associated stones	16
Horse-shoe kidney with UPJO	2
No. of pts with	
pre-op PCN	4
No. of pts with co-morbidity	
DM	4
HT	6

Table 2 - Peri-operative and postoperative parameters.

Mean (range) operative time (mints)	63 (52-124)
Mean±SD VAS pre-operatively	2.6±0.46
Incision length (cm)	5.2 (3.8-7.4)
Mean±SD VAS on day 1	3.8±0.73
Mean±SD VAS on day 2	1.2±0.26
Mean±SD VAS post- operatively at 6 months after surgery	0.27±0.43
No. of pts with anterior crossing vessels	27
Mean (range) blood loss (mL)	41.23(27-78)
Mean (range) days of drain removal	2.1 (2 to 5)
Mean(range) hosp stay (days)	2.5 (2-6)
Median Follow-up (months)	16(9-22)
Overall complications (%)	5(8.4%)
Overall Success rate (%)	98.6

(Table-3). The overall complication rate was 8.4%. No major complications occurred during or after the surgery. DJ stent was found to be blocked in one patient with persistent pain and fever with excessive drain output. The patient was managed by replacement of double J catheter. Three patients developed post-operative febrile urinary tract infection, managed by culture specific antibiotics. No patient developed hernia at incision site or other wound related complications. Mean visual pain analog score (VAS) pre--operatively was 2.6±0.46; it was 3.8±0.73 and 1.2±0.26 on the first and second post- operative day respectively and 0.27±0.43 at 6 months after surgery. The mean hospital stay in this study was 2.5 (2-6) days (Table-2). The double-J stent was removed 6 weeks postoperatively under local anesthesia and sedation.

The overall success rate was 98.6%. In 16 cases, concomitant renal stones were successfully removed and no recurrence of renal calculi was noted till last follow-up. The median follow-up period was 16 months (range 9-22 months). All the patients were symptom free during the follow--up period. Comparison of pre and post-operative parameters are shown in Table-4. Most patients had grade III or IV hydronephrosis on ultrasound and severe to moderate hydronephrosis on IVP before surgical intervention. Post-operative renal ultrasound or IVP demonstrated decrease or complete resolution of hydronephrosis in almost all patients. The follow-up DPTA scan was suggestive of significant improvement in drainage in comparison to previous scans. While preoperative mean T1/2 was 26.7±6.4 minutes, postoperative half-time decreased to 7.8±4.2 minutes at 6 months and to 6.7±3.3 mints at 1-year after surgery. Mean pre--operative DRF on DTPA scan was 26.45±10.32% and it was 31.38±8.74% and 33.19±06.13% at 6 months and 1 year respectively.

DISCUSSION

Open pyeloplasty was originally described by Foley in 1937 and modified by Anderson and Hynes and since then, the open pyeloplasty is considered the gold standard treatment for UPJO with success rates of more than 90% (13).

A significant post-operative pain and a long recovery time with incision site scar are the major disadvantages of the open pyeloplasty. Many minimally invasive approaches were introduced to minimize the drawbacks of open pyeloplasty which include endopyelotomies either by antegrade or retrograde route, acucise endopyelotomy, balloon dilation, laparoscopic pyeloplasty or more recently robotic pyeloplasties (14). By using endoscopy and laparoscopy, the morbidity of surgery can be

Table 3 - Post operative complications by Dindo-modified Clavien Classification of Surgical Complications.

Clavien Grading	Complication	No. of pts in open group
I	Wound infection	1
II	Febrile UTI	3
IIIa	Displaced stent in post op period	1
IIIb	Re- surgery	1

Table 4 - Comparison of pre and post operative parameters.

Subjective outcome	Pre-operatively	On post op day 1	6 months after surgery	P value blw pre-op and at 6 months
Pain Score (VAS)	2.6±0.46	3.8±0.73	0.27±0.43	0.0001
Objective outcome	**Pre-operatively**	**At 6 months of follow-up**	**At 1 year follow-up**	**P value blw pre-op and at 1 year**
Mean DRF (%)	26.45±10.32	31.38±8.74	33.19±06.13	0.0001
T1/2 (min)	26.7±6.4	7.8±4.2	6.7±3.3	0.0001

HDN Grades on Renal USG	Pre-operatively (no. of pts)	At 6 month follow up (no. of pts)	At 1 year follow up(no. of pts)
Grade IV	59	10	3
Grade III	10	14	2
Grade II	2	17	9
Grade I	--	27	16
No HDN	--	3	41

IVP Grading of HDN	Pre-operatively (no. of pts)	At 6 month follow up (no. of pts)	At 1 year follow-up (no. of pts)
Severe	58	11	2
Moderate	13	17	5
Mild	--	39	13
NO HDN	--	4	51

minimized especially in ablative and reconstructive urologic procedures. However, with the exception of laparoscopic and robotic pyeloplasties, other minimally invasive surgeries present lower success rates (61% to 89%) with significant risk of bleeding compared with open pyeloplasty (15).

Laparoscopic pyeloplasty as a treatment option for the UPJO combines the advantage of an open reconstruction under direct magnified vision with the low morbidity of an endoscopic approach (16, 17). Schuessler et al. described the first transperitoneal access in 1993 (18); the retroperitoneoscopic approach to pyeloplasty was first reported by Janetschek et al. in 1996 (19). Laparoscopic pyeloplasty is reported in several series but transperitoneal route was more commonly employed in those studies (20). Open pyeloplasty is performed by lumbar posterior approach rather than the transperitoneal (a more anatomical direct approach), with better exposition of the renal pelvis (19). In our opinion, the use of laparoscopic techniques should not involve a change in the surgical approach. Also, the steep learning curve has hindered laparoscopic pyeloplasty acceptance into mainstream urological practice (21).

There are few non randomized studies comparing conventional open pyeloplasty with laparoscopic pyeloplasty (22, 23) which seem to favour the latter. However, this comparison studies involved the standard open technique, not the 'mini' approach (incision length <8 cm), which results in far less morbidity than the conventional operation. In a study, Chacko et al. concluded that minimal invasive open pyeloplasty is a safe and effective treatment of choice for UPJO in pediatric age group with success rates reaching 95% after surgery (24). Minimally invasive surgery could be performed with a small incision without excessive post-operative analgesic requirement. Sutherland et al. achieved a success rate of 95% in children <1 year and 96% in children >1 year in a study of 234 pediatric pyeloplasties (25). The present cohort study using mini incision open pyeloplasty technique compares favorably with previously reported literature in children and even in adults.

Minimally invasive open pyeloplasty has the distinctive advantages over endourological procedures in terms of overall success rates and in the management of associated crossing vessels. Another issue is that endourological procedures are compromised by varied surgical anatomy of PUJ (high ureteric insertion or huge dilatation). These are also associated with a higher risk of peri-operative hemorrhage with blood transfusions rate of 3-11% (6, 8, 13). In comparison to open or laparoscopic pyeloplasty, success rates of these minimally invasive endoscopic surgeries (Acucise cutting balloon, antegrade percutaneous endopyelotomy, and retrograde endopyelotomy) are approximately 10% to 25% lower and hemorrhagic complications are more frequent (26, 27).

Soulie et al. reported that shorter anterior incision (mean 5 cm) with muscle splitting reduces the risk of chronic pain and wound herniation (28). The results of this study revealed no significant difference between the two techniques (LP and OP with minimal incision) in terms of morbidity, functional results, post-operative pain and return to normal activity. Laparoscopic pyeloplasty was described as a difficult procedure requiring careful ureteral dissection and intra-corporeal suturing.

In a recent meta-Analysis done by Stefanie A et al., re-intervention (RI) rate and re-do--pyeloplasty rate were twice more frequent in laparoscopy group (LP) in comparison to open pyeloplasty (29). The reason for higher RI in LP group was absence of tactile sensation and more tissue trauma at site of anastomosis.

In the present study, we used relatively shorter muscle-splitting incision, thus, avoiding injury to subcostal neurovascular bundle which lies between internal oblique and transversus abdominis muscles to reduce postoperative pain and other complications. As patients of different age groups were included in the present study (both children and adults), mean length of 5.2 cm incision was used (3.8-7.4 cm). While Klingler and Zhang et al. used 23.8±9.1 cm and 21 cm incision respectively in their comparison study of open versus laparoscopic pyeloplasty with consequent abdominal wall herniations and thromboembolism due to long incision and subsequent prolonged hospital stay (2, 3), no such complications occurred in the present study.

Mean operating time was 63 (52-124) minutes in the present series. The mini-incision tech-

nique has the definite advantage of reduced overall duration of surgery in comparison to most of published series of laparoscopic pyeloplasty and thus, less anesthesia related complications (30). Mean blood loss in this study was 41.23 (27-78) mL, which is comparable to other laparoscopic and open studies (30).

In the present series, complications were recorded and graded according to Dindo-modified Clavien classification of surgical complications. Overall complication rate was 8.4% which is comparable to those reported in laparoscopic and open literature (30). There was no major complication in our series and most of them were managed conservatively.

In our study, the success rate was 98.6% which is comparable to standard open and laparoscopic pyeloplasty. Most of the patients in present series had BMI less than 25 (mean 23.6); minimal incision approach may not be successful in more obese patients. High success rates are the sheer advantage of mini-incision pyeloplasty over other less invasive techniques. The results of present study clearly indicate that reconstructive urological procedures can effectively be done by mini-incision approach with minimal morbidity and comparable success rates.

Because of smaller operative space, laparoscopic pyeloplasty was found to be technically challenging in children in comparison to adults. So, pediatric population seems to be specially benefitted by mini-incision approach as equal cosmesis can be achieved, avoiding technical difficulty of laparoscopic approach (22). We also observed that mini-incision approach was also feasible in the anomalous kidneys. In the present study, 2 of the 71 patients had a horseshoe kidney with UPJO. In another patient, UPJO was present in solitary functioning kidney. Pyeloplasty was performed in those patients uneventfully.

In the present study, mean visual pain analog score (VAS) pre-operatively was 2.6±0.46. VAS was 0.27±0.43 when measured at 6 months of follow-up period, showing significant decrease of subjective symptoms. The lower pain score and the decreased consumption of postoperative analgesics allow early ambulation and resumption of oral intake.

There was significant improvement in the objective parameters after the surgery in the follow up period. There was either decrease or complete resolution of hydronephrosis on renal ultrasound or IVP which further decreased on serial follow--up scans. There was significant improvement in drainage pattern on follow-up DTPA renal scans in comparison of preoperative scans in the majority of patients. Mean radiotracer wash out time was more than 20 minutes in the majority of the patients pre-operatively. Significant decrease in half time was observed post-operatively at 6 month renal scan and reached a non-obstructed level. Mean differential renal function on follow up DTPA scan showed improvement in differential function in comparison to previous scan.

Thus, mini incision pyeloplasty can easily be adapted by surgeons who are experienced in standard open pyeloplasty. This technique has a shorter hospital stay, early convalescence and better cosmesis. It is cost-effective and is an ideal substitute for the centres where laparoscopy is still evolving.

CONCLUSIONS

Mini-incision pyeloplasty is a safe and effective technique of open pyeloplasty. It has high success rates similar to open standard pyeloplasty with lower morbidity than laparoscopic approach. Excellent functional and objective outcomes can be achieved if performed by an experienced surgeon.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Manish Garg, MD
Senior Resident
Department of Urology,
King George Medical University, Lucknow, India
Telephone: + 91 760 7333618
E-mail: dr_manugarg@yahoo.co.in

ORIGINAL ARTICLE

Vol. 41 (5): 935-944, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0547

In vitro studies reveal antiurolithic effect of *Terminalia arjuna* using quantitative morphological information from computerized microscopy

A. Mittal [1], S. Tandon [2], S.K. Singla [3], C. Tandon [4]

[1] Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan 173234, Himachal Pradesh, India; 2 Amity Institute of Molecular Medicine and Stem Cell Research (AIMMSCR), Amity University Uttar Pradesh,Noida; [3] Department of Biochemistry, Panjab University, Chandigarh – 160014, India and [4] Amity Institute of Biotechnology, Amity University Uttar Pradesh, Sector – 125, Noida, U.P., 201313 India

ABSTRACT

Purpose: For most cases, urolithiasis is a condition where excessive oxalate is present in the urine. Many reports have documented free radical generation followed by hyperoxaluria as a consequence of which calcium oxalate (CaOx) deposition occurs in the kidney tissue. The present study is aimed to exam the antilithiatic potency of the aqueous extract (AE) of *Terminalia arjuna* (*T. arjuna*).

Materials and Methods: The antilithiatic activity of Terminalia arjuna was investigated in vitro nucleation, aggregation and growth of the CaOx crystals as well as the morphology of CaOx crystals using the inbuilt software 'Image-Pro Plus 7.0' of Olympus upright microscope (BX53). Antioxidant activity of AE of Terminalia arjuna bark was also determined in vitro.

Results: Terminalia arjuna extract exhibited a concentration dependent inhibition of nucleation and aggregation of CaOx crystals. The AE of Terminalia arjuna bark also inhibited the growth of CaOx crystals. At the same time, the AE also modified the morphology of CaOx crystals from hexagonal to spherical shape with increasing concentrations of AE and reduced the dimensions such as area, perimeter, length and width of CaOx crystals in a dose dependent manner. Also, the Terminalia arjuna AE scavenged the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radicals with an IC_{50} at 13.1µg/mL.

Conclusions: The study suggests that Terminalia arjuna bark has the potential to scavenge DPPH radicals and inhibit CaOx crystallization in vitro. In the light of these studies, Terminalia arjuna can be regarded as a promising candidate from natural plant sources of antilithiatic and antioxidant activity with high value.

ARTICLE INFO

Key words:
Kidney; Calculi; Calcium Oxalate; Phytotherapy; Terminalia

Int Braz J Urol. 2015; 41: 935-44

Submitted for publication:
October 29, 2014

Accepted after revision:
February 16, 2015

INTRODUCTION

Urinary stones affect a large proportion of the population. Approximately 85% of urinary stones are calcium stones, which consist of oxalate and phosphate, either alone or in combination (1). The mechanisms involved in the formation of urinary stones are not fully understood but it is generally agreed that urinary lithiasis is a multifaceted process involving events leading to crystal nucleation, aggregation and growth of insoluble particles (2). Crystal growth and agglomeration

may be due to supersaturation with respect to stone forming constituents or the presence of various inhibitory or stimulatory biomolecules or even pH (3). Urine is always supersaturated with common stone forming minerals, however, the crystallization inhibiting capacity of urine does not allow urolithiasis to happen in most of the individuals, whereas this natural inhibition is impaired in stone formers (4).

CaOx stones are mostly found in two different varieties, CaOx monohydrate (COM) or Whewellite, and CaOx dihydrate (COD) or Weddellite. COM, the thermodynamically most stable form, is observed more frequently in clinical stones than COD and has a greater affinity for renal tubular cells, thus responsible for the formation of stones in the kidney (5). CaOx monohydrate (COM) has been found to initiate mineralization followed by the deposition of CaOx dehydrate (COD) on it (6).

Development of modern techniques such as extracorporeal shock wave lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL), or ureteroscopy (URS) have revolutionized surgical management of kidney stones in recent years but do not give satisfactory results as these techniques do not prevent the likelihood of new stone formation (7). Many medicinal plants have been used since ages to treat urinary stones though the rationale behind their use is not well established through systematic and pharmacological studies, except for some composite herbal drugs and plants (8).

In recent years, numerous studies describing the therapeutic properties of extracts from different parts of various medicinal plants have been developed. Indeed, the use of such extracts as complementary and alternative medicine has lately increased, and also serves as an interesting source of drug candidates for the pharmaceutical industrial research (9). *Terminalia arjuna*, belonging to the family Combretaceae, holds a reputed position in Ayurvedic system of medicine since ancient times (10). Experimental and clinical studies revealed the beneficial effects of this plant against all sorts of conditions of cardiac failure (11), dropsy, anti-infective (12), anti-asthmatic and for the treatment of rheumatoid arthritis and is traditionally used to prevent the formation of kidney stone.

The present study aims to exam the antilithiatic potency of the AE of *Terminalia arjuna* bark on crystallization of CaOx in vitro and antioxidant activity of the same.

MATERIALS AND METHODS

Plant

The dried bark of was purchased from Natural Remedies Pvt. Ltd., Bangalore, India. A collection of voucher specimen is available at the company.

Preparation of the AE of *Terminalia arjuna*

The dried fine powdered *Terminalia arjuna* bark was soaked in distilled water for 24 hours at 4°C. The extract was then filtered through muslin cloth followed by centrifugation at 10,000 rpm for 20 mins at 4°C and the filtrate was lyophilized to obtain the dried powder referred to as AE of *Terminalia arjuna* bark. The dried AE was stored in labeled sterile bottles and kept at -20°C. The various concentrations of the plant sample tested for their inhibitory potency were: 10µg/mL, 25µg/mL, 50µg/mL, 100µg/mL, 200µg/mL, 500µg/mL and 1000µg/mL which were prepared at the time of experiment.

DPPH radical scavenging Assay

The effect of AE of *Terminalia arjuna* bark on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi (13). A solution of 0.135 mM DPPH in methanol was prepared and 2.0mL of this solution was mixed with 2.0mL of extract in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517nm. Ascorbic acid was used as reference. The IC_{50} value was defined as the concentration (µg/mL) of extracts that scavenges the DPPH radicals by 50%. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%)=((Acontrol-Asample)/Acontrol) × 100 where Acontrol is the absorbance of DPPH radical plus methanol, Asample is the absorbance of DPPH radical plus sample extract/standard. Methanol was used as a blank.

CaOx crystallization assay

Stock solutions of 10.0mM calcium chloride ($CaCl_2$) and 1.0mM sodium oxalate ($Na_2C_2O_4$), containing 200mM sodium chloride (NaCl) and 10mM sodium acetate, were adjusted to pH 5.7. All chemicals were of the highest purity grade available. Before being used in crystallization experiments, solutions were filtered through Millex-GV membranes with a pore diameter of 0.22µm and warmed up to 37°C in a water bath. For crystallization experiments, 1.5mL of the $CaCl_2$ solution was transferred into a 10mm light path quartz cuvette and an additional 1.5mL of the $Na_2C_2O_4$ solution was then added to final assay concentrations of 5.0mM for calcium and 0.5mM for oxalate, respectively. In the cuvette, the final solutions were stirred continuously and maintained at 37°C (14, 15).

Control experiment was performed with calcium/oxalate concentration ratio, i.e., 5.0/0.5mM/mM. After addition of the oxalate--containing solution, automated time-course measurements of optical density at 620nm (OD_{620}) were performed, i.e., OD_{620} was recorded after every 60 seconds over 40 minutes. Experiments with added sample (100µL), where rates of nucleation and aggregation were considerably lower, had to be extended to 60 minutes. All crystallization experiments were performed at least in triplicate. Percentage inhibition produced by the AE was calculated as (1-(Tsi/Tsc)) X100, where Tsc was the turbidity slope of the control and Tsi the turbidity slope in the presence of the inhibitor.

CaOx crystal growth assay

Inhibitory activity against CaOx crystal growth was measured using the seeded solution--depletion as say described previously by Nakagawa and colleagues (16). Briefly, an aqueous solution of 10mM Tris-HCl containing 90mM NaCl was adjusted to pH 7.2 with 4N HC1. Stone slurry (1.5mg/mL) was prepared in 50mM sodium acetate buffer (pH 5.7). CaOx monohy drate crystal seed (from FTIR identified clinical kidney stones) was added to a solution containing 1mM $CaCl_2$ and 1mM $Na_2C_2O_4$. The reaction of $CaCl_2$ and $Na_2C_2O_4$ with crystal seed led to deposition of CaOx (CaC_2O_4) on the crystal surfaces, thereby decreasing free oxalate that is detectable by spectrophotometry at λ214nm. When AE is added into this solution, depletion of free oxalate ions will decrease if the test sample inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 60 seconds incubation for 20 minutes, with or without test sample. The relative inhibitory activity was calculated as follows: % Relative inhibitory activity=((C-S)/C)x100, where C is the rate of reduction of free oxalate without any test sample and S is the rate of reduction of free oxalate with a test sample.

Image Analysis of Crystal Morphology

The stock solutions of 12.75mM $CaCl_2$ and 2.25 mM $Na_2C_2O_4$ were used to observe the size and morphology of the crystals and to verify the effect of incubation with the test material on CaOx crystal formation. 50µL of $CaCl_2$ solution was added to wells in a 96-well plate. To each of the wells, 50µL of test sample and $Na_2C_2O_4$ solution were added to obtain final concentrations of 4.25mM calcium and 0.75mM oxalate (17, 18). Each concentration of AE was prepared in triplicate. The plates were then incubated at 37°C for 45 minutes. Crystal morphology was examined in five randomly selected fields at 100x magnification under an upright microscope (Olympus Corporation, Japan). Images were captured from different fields. The measurement parameters in terms of area, perimeter, length and width of CaOx crystals in the absence and presence of various concentrations of *Terminalia arjuna* AE were measured using the inbuilt software 'Image-Pro Plus 7.0' to show the efficacy of AE of *Terminalia arjuna*. Cystone was used as a positive control.

Statistical analysis

Data were expressed as mean values of three independent experiments (each in triplicate) and analyzed by ANOVA ($p<0.05$) to estimate the differences between values of extracts tested using the software GraphPad Prism version 6.01.

RESULTS

DPPH radical scavenging Assay

The antioxidant activity of AE of *Terminalia arjuna* was determined by measuring the DPPH

radical scavenging activity. The AE of *Terminalia arjuna* bark displayed the DPPH radical scavenging activity. The ability to scavenge the DPPH radical increased with increasing concentrations of the extract in a dose-dependent manner as shown in Figure-1. The percentage of DPPH radical inhibition ranged from 25.82% at 5µg/mL to 93.87% at 50µg/mL. The AE caused scavenging of DPPH radical with IC_{50} value of 13.11µg/mL. The chemical, ascorbic acid was used as a standard and similarly inhibited DPPH with IC_{50} value of 5.84µg/mL.

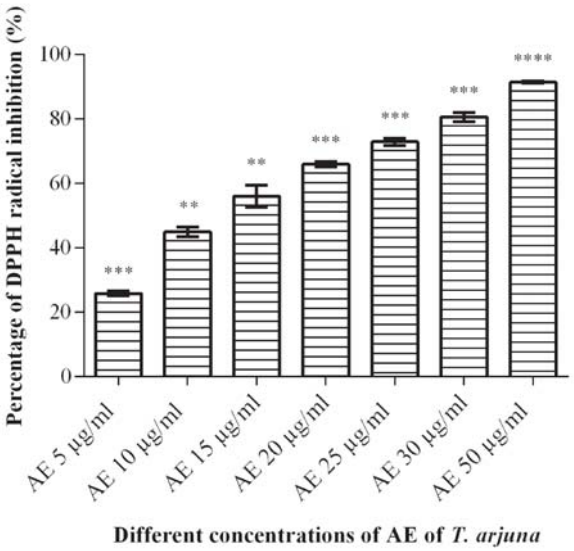
CaOx crystallization assay

At final concentrations of 5.0mmol/L calcium and 0.5mmoL/L oxalate, the CaOx crystallization inhibitory activity of *Terminalia arjuna* AE increased with increasing concentrations of the extract in a dose-dependent manner from 10µg/mL to 1000µg/mL as shown in Figure-2. The cystone drug at a dosage of 1000µg/mL was used as a positive control.

For CaOx crystal nucleation, the percentage inhibition shown by the *Terminalia arjuna* bark AE at 10µg/mL, 25µg/mL and 50µg/mL were found to be 20.8±3.6%, 27.3±2.8% and 31.1±2.5% respectively. As the concentration of AE was increased to 100µg/mL, the percentage inhibition increased to 42.63±1.4%. The inhibition was almost constant in the range of 45-50% at 200µg/mL and 500µg/mL and increased upto 61.35±0.6% at 1000µg/mL when compared to the control (with no plant extract). Addition of 1000µg/mL cystone resulted in a nucleation percentage inhibition of 57.53±3.5%.

For CaOx crystal aggregation, the percentage inhibition shown by the *Terminalia arjuna* bark AE at 10µg/mL was found to be 31.05±5.3 % which remained almost constant at 25µg/mL and 50µg/mL. As the concentration of AE was increased to 100µg/mL, 200µg/mL and 500µg/mL, the percentage inhibition increased to 34.47±2.7%, 39.9±2.3% and 44.6±2.4% respectively. The percentage inhibition increased to 49.8±2.4% at 1000µg/mL when compared to the control (with no plant extract). Addition of 1000µg/mL cystone resulted in an aggregation percentage inhibition of 69.7±4.2%.

Figure 1 - Effect of aqueous extract of Terminalia arjuna on DPPH radicals inhibition. Data are mean±S.D of three independent observations.



**p<0.005, ***p<0.0005, ****p<0.0001

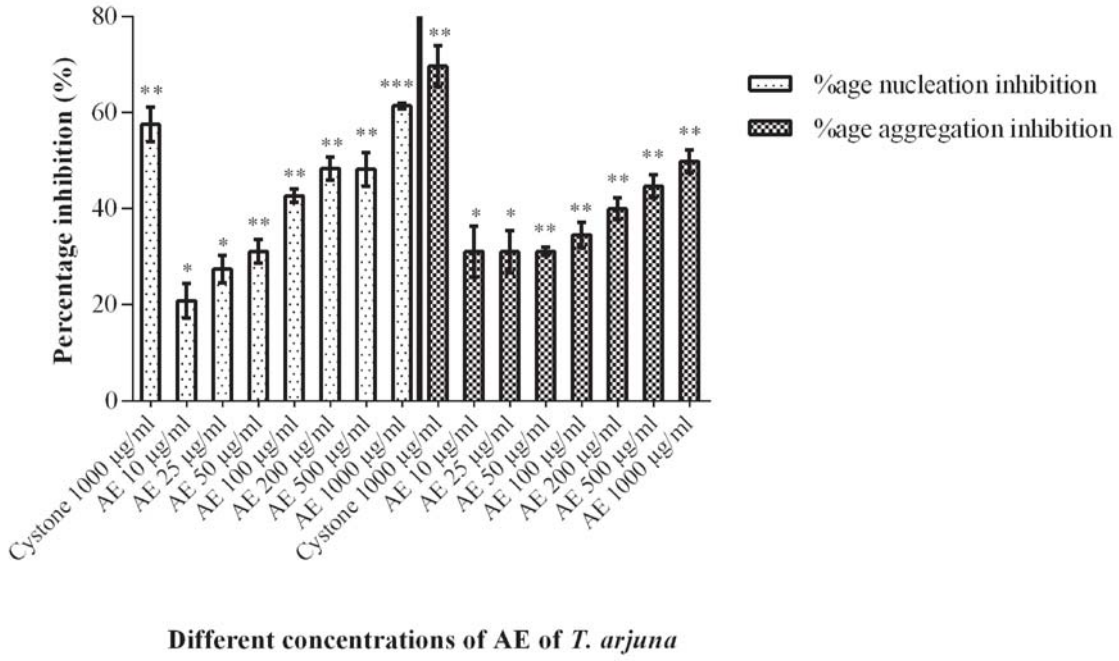
CaOx crystal growth assay

The AE of *Terminalia arjuna* bark showed the inhibitory effect on the growth of CaOx crystals as shown in Figure-3. When compared to the control (with no plant extract), the percentage inhibition at 10µg/mL was found to be 13.8±1.9%, which increased to 30.4±0.4% and 34.34±4.5% at 25µg/mL and 50µg/mL AE respectively. As the concentration of AE was increased to 100µg/mL, the percentage inhibition decreased to 24.97±4.6%. The percentage inhibition was significantly increased to 41.82±2.03% and 96.4±1.4% at 200µg/mL and 500µg/mL respectively and again reduced to 86.89±1.9% at 1000µg/mL. The cystone drug at a dosage of 1000µg/mL was used as a positive control. Addition of 1000µg/mL cystone resulted in a growth percentage inhibition of 90.67±5.9%.

Image Analysis of CaOx Crystal Morphology

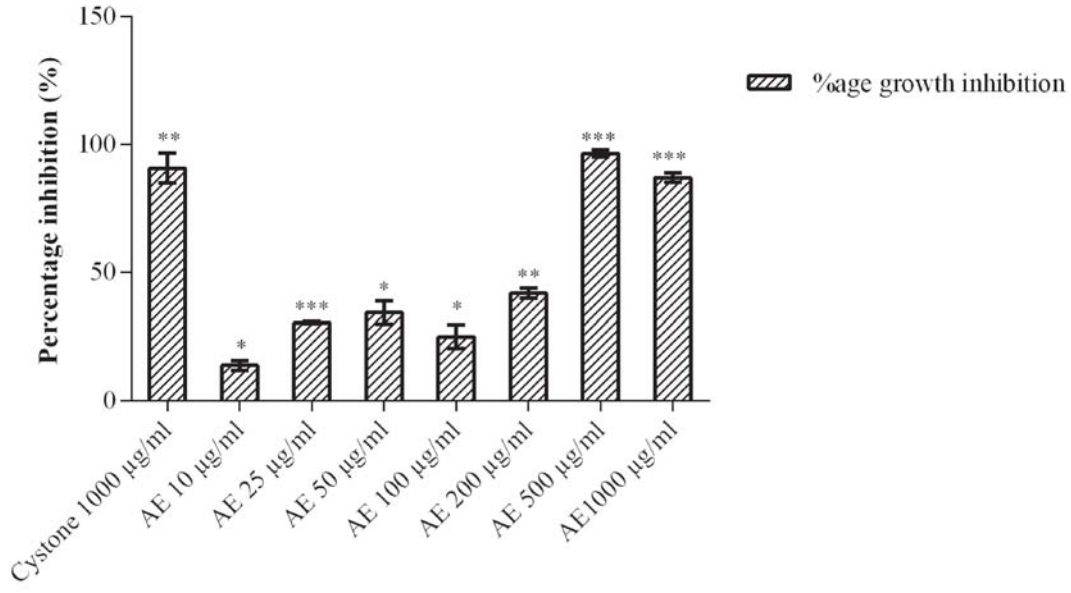
The incubation of metastable solutions of 4.25mM calcium and 0.75mM oxalate resulted in the formation of CaOx crystals composed predominately of hexagonal CaOx monohydrate as

Figure 2 - Effect of aqueous extract of Terminalia arjuna on nucleation and aggregation of calcium oxalate crystals. Data are mean±S.D of three independent observations.



*p<0.05, **p<0.005, ***p<0.0005

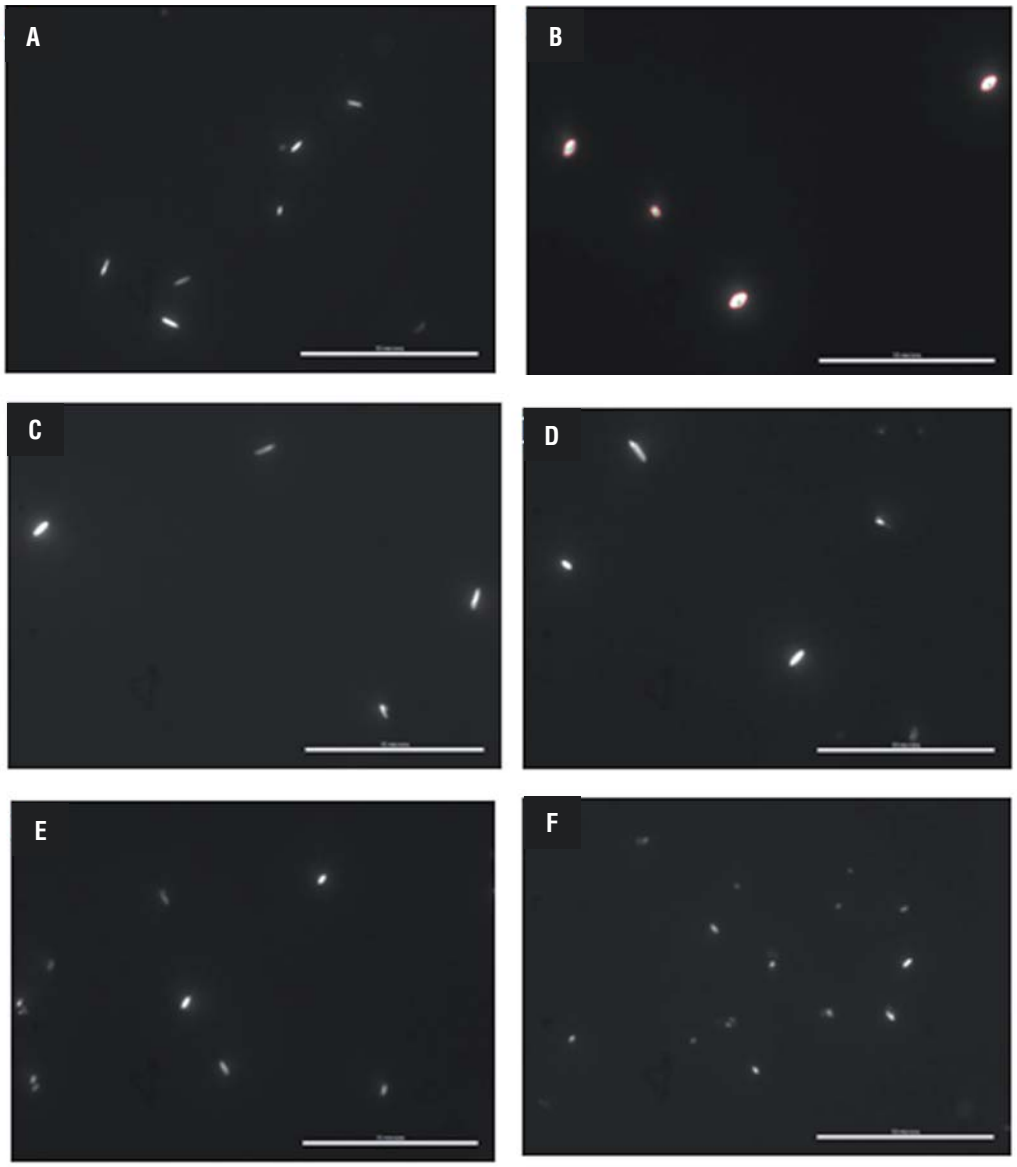
Figure 3 - Effect of aqueous extract of Terminalia arjuna on growth of calcium oxalate crystals. Data are mean±S.D of three independent observations.

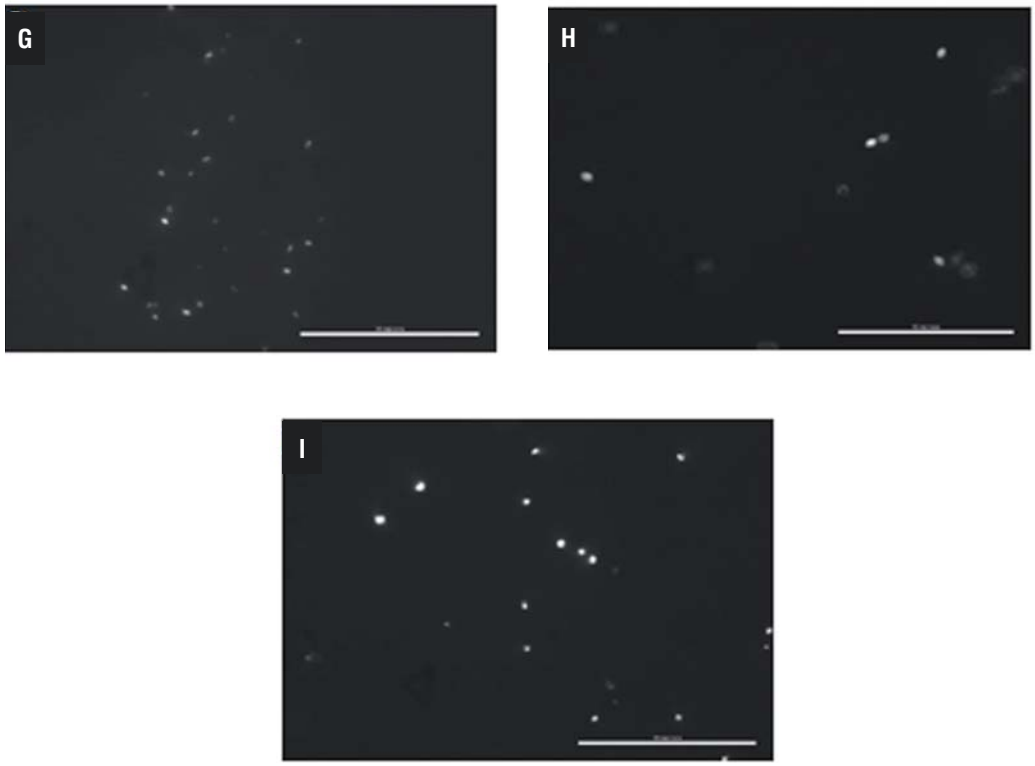


*p<0.05, **p<0.005, ***p<0.0005

shown in Figure-4A. Microphotography studies showed that the AE of *Terminalia arjuna* bark resulted in the formation of rounded CaOx crystals. When compared to control (with no plant sample), the AE of *Terminalia arjuna* bark modified the morphology of CaOx crystals from hexagonal to spherical shape with increasing concentrations of AE as shown in Figure 4A-I and reduced the dimensions such as area, perimeter, length and width of CaOx crystals in a dose dependent manner as shown in Table-1. The CaOx crystal area, perimeter, length and width was reduced from 0.3µm^2, 2.31µm, 0.85µm and 0.54µm to 0.06µm^2, 0.85µm, 0.31µm and 0.23µm by the addition of 1000µg/mL of AE respectively. The cystone drug at a dosage of 1000µg/mL was

Figure 4 - The calcium oxalate crystals, observed under upright microscope (100x), formed in the metastable solution of calcium oxalate in the absence (A) and the presence of (B) cystone (1000µg/mL) and Terminalia arjuna bark aqueous extract, (C-I) 10, 25, 50, 100, 200, 500 and 1000µg/mL.





used as a positive control. Addition of 1000µg/mL cystone decreased the area, perimeter, length and width of CaOx crystals to 0.23µm^2, 1.73µm, 0.64µm and 0.41µm respectively.

DISCUSSION

Hyperoxaluria is a major risk factor for CaOx nephrolithiasis, which in turn is associated with renal injury. High level of oxalate causes a variety of changes in the renal epithelial cells, such as an increase in free radical production and a decrease in antioxidant status, followed by cell injury and cell death. These changes are significant predisposing factors for the facilitation of crystal adherence and retention (19). Oxalate induced toxicity and free radical production are attenuated in vivo (20) and in vitro (21) by antioxidants.

A dramatic advancement in using phytotherapy for urolithiasis treatments has been observed in the recent years and many investigators have proposed scientific studies on its efficacy. Many medicinal plants have been used since ages to treat urinary stones though the rationale behind their use is not well established. One such unexplored plant is *Terminalia arjuna*, commonly called as 'arjuna' or 'arjun'. The plant is said to be a divine medicine in Vedas and has a special mention in Charaka Samhita, though not much scientific study has been done to explore the antiurolithiatic potency of *Terminalia arjuna*, which has been established through ethnobotanical studies (10). In view of its medicinal use, *Terminalia arjuna* bark extract was studied to evaluate its antiurolithiatic potential using different in vitro models.

Terminalia arjuna bark AE possess very high antioxidant activity due to the presence of Terpenoids. It has the ability to scavenge the free radicals with an IC_{50} of 13.11µg/mL. Thus, pretreatment with antioxidants can block oxalate induced increases in ceramide (22). Antioxidant treatments also block oxalate-induced cell death (21), suggesting a role for oxidant stress in these responses.

Table 1 - Effect of Terminalia arjuna aqueous extract on the morphology and dimension of calcium oxalate crystals in vitro at concentrations of 10, 25, 50, 100, 200, 500 and 1000µg/mL as compared to control having Millipore water instead of aqueous extract. Data are mean±S.D of three independent observations.

	Control	Cystone 1000µg/mL	AE 10µg/mL	AE 25µg/mL	AE 50µg/mL	AE 100µg/mL	AE 200µg/mL	AE 500µg/mL	AE 1000µg/mL
Area (μm^2)	0.30±0.05	0.23±0.14	0.25±0.03	0.24±0.06	0.12±0.02	0.09±0.03	0.06±0.07	0.06±0.002	0.06±0.001
Perimeter (µm)	2.31±0.21	1.73±0.72	2.04±0.26	1.92±0.24	1.25±0.2	1.02±0.19	0.84±0.07	0.92±0.11	0.85±0.007
Length (µm)	0.85±0.0	0.64±0.29	0.88±0.13	0.84±0.11	0.51±0.07	0.41±0.07	0.32±0.007	0.35±0.026	0.31±0.01
Width (µm)	0.54±0.07	0.41±0.15	0.35±0.02	0.32±0.05	0.26±0.05	0.23±0.04	0.20±0.006	0.22±0.017	0.23±0.02
Shape	Hexagonal	Hexagonal with rounded edges	Hexagonal	Hexagonal with rounded edges	Hexagonal with rounded edges	Hexagonal with rounded edges	Hexagonal with rounded edges	Hexagonal with rounded edges	Spherical

The effect of *Terminalia arjuna* bark extract on CaOx crystallization kinetics was studied by the time course measurement of turbidity. In this study, AE inhibited the CaC_2O_4 crystal aggregation in a concentration-dependent manner, similar to cystone drug, a well-known drug formulated by the Himalaya to cure kidney stones and widely clinically used for the management of urolithiasis.

In the microscopic study, AE modified CaOx monohydrate crystal morphology. A similar change in the morphology of CaOx monohydrate crystals has been previously reported with citrate and Mg^{2+} (23). Microphotography studies verified that AE of *Terminalia arjuna* resulted in the formation of round CaOx crystals. COM and COD are the major forms found in most urinary calculi. AE of *Terminalia arjuna* inhibited the growth of COM crystals, prevented the aggregation of COM crystals, and induced the formation of spherical COM crystals. These spherical COM crystals are thermodynamically less stable phase and have weaker affinity for cell membranes than hexagonal COM crystals. Both *Terminalia arjuna* AE (1mg/mL) and cystone (1mg/mL) resulted in the shape changes of CaOx crystals, as shown in Figures 4B and 4I; a more rounded polygonal crystals shape. This shape may prevent the formation of kidney stones, because crystals with this shape are more easily excreted in the urine compared with the COM.

Formation of crystals along urinary tract, driven by urinary supersaturation, is a primary requisition for the subsequent stone formation (24), although crystal formation does not necessarily lead to stone formation. Researchers have identified crystal retention as a critical step for the formation of clinically symptomatic stone from a free particle. Various physiological inhibitors of urolithiasis found in urine including citrate have been shown to decrease the saturation of CaC_2O_4 and inhibit crystal nucleation, growth and aggregation, while reduced crystallization inhibiting capacity of urine can play a role in stone formation (3). Interference with crystal growth and aggregation therefore seems a possible therapeutic strategy for the prevention of recurrent stone disease.

Terminalia arjuna bark extract is previously reported to inhibit CaOx crystal precipitation and growth (25). These results were verified in previous studies and also showed the change in the shape CaOx crystals in the presence of *Terminalia arjuna* bark AE. Recently, several plants including Herniaria hirsuta (26), Tribulus terrestris (5), Terminalia chebula (27) and Bergenia ligulata (17), are being explored for their antiurolithiatic

property on the basis of their usage in the traditional medicine. An extract of H. hirsuta increased the CaOx crystal number but decreased their size. It also promoted the formation of CaOx dihydrate crystals, despite the presence of CaOx monohydrate particles (26). B. ligulata is a widely used plant in South Asia, mainly India and Pakistan, as a traditional medicine for the treatment of urolithiasis. The crude aqueous-methanolic extract of B. ligulata rhizome (BLR) was studied using in vitro and in vivo methods and the extract showed the antiurolithic activity through CaOx crystal inhibition, diuretic, hypermagneseuric and antioxidant effects (17). Very recently, in our lab, antilithiatic potency of Dolichos biflorus (28) and Trachyspermum ammi (29) has been evaluated in vitro and in vivo. Antilithiatic proteins were identified and characterized from these plants adding a new vista to study therapeutic proteins from plants.

CONCLUSION

This study demonstrated that AE of *Terminalia arjuna* possess a high antioxidant activity and an ability to inhibit the CaOx crystallization in vitro. In addition, this extract changed the morphology and reduced the dimensions of hexagonal COM crystals to spherical COM crystals. This shape may prevent the formation of kidney stones. In the light of these studies, *Terminalia arjuna* can be regarded as a promising candidate from natural plant sources of antilithiatic and antioxidant activity with high value.

ABBREVIATIONS

CaOx = Calcium oxalate
AE = Aqueous extract
T. arjuna = *Terminalia arjuna*
COM = Calcium oxalate monohydrate
COD = Calcium oxalate dihydrate
ESWL = Extra corporeal shock wave lithotripsy
PCNL = Percutaneous nephrolithotomy
URS = Ureteroscopy
DPPH = 2, 2-diphenyl-1-picrylhydrazyl

CONFLICT OF INTEREST

None declared.

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Correspondence address:
C. Tandon, PhD
Director,
Amity Institute of Biotechnology
Amity University Uttar Pradesh,
Sector – 125, Noida – 201313 (U.P.), India
Fax: +91 120 439-2947
E-mail: ctandon@amity.edu

ORIGINAL ARTICLE

Vol. 41 (5): 945-952, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0471

The efficacy of peritubal analgesic infiltration in postoperative pain following percutaneous nephrolithotomy – A prospective randomized controlled study

Bannakij Lojanapiwat [1], Tanarit Chureemas [2], Pruit Kittirattarakarn [2]

[1] Division of Urology - Surgery 110 intravaroros Muang Chiangmai, Chiang Mai, Thailand; [2] Faculty of medicine - Surgery, Chiang Mai, Thailand

ABSTRACT

Objective: To study the efficacy of peritubal infiltration in postoperative pain following percutaneous nephrolithotomy in general PCNL patients and PCNL patients with supracostal renal access.
Patients and Methods: A total of 105 PCNL patients were randomized into two groups, 53 patients receiving peritubal analgesic infiltration (study group) and 52 patients as the control group. Of these patients, supracostal access was performed in 22 patients of study group and 23 patients of control group. The study group received peritubal injection with 10mL of bupivacain. Postoperative pain as the primary outcome was assessed by using visual analogue scale at 1, 4, 12, 24 and 48 hours postoperatively. The secondary outcomes were the total postoperative morphine usage in 24 hours and time of the first analgesic demand.
Results: The average VAS pain at 1 and 4 hours after the operation in the study group were significant lower in the control group (P≤0.001 and 0.026). Doses of morphine usage for controlling postoperative pain and the first analgesic demand were significantly lower and longer in study group. Among patients submitted to supracostal access, the average VAS pain at 1 hour after operation in the study group was lower (P=0.018). Doses of morphine usage for controlling postoperative pain also was lower in the study group (P=0.012).
Conclusion: The peritubal local anesthetic infiltration is effective in alleviating immediate postoperative pain after percutaneous nephrolithotomy even with supracostal access.

ARTICLE INFO

Key words:
Nephrostomy, Percutaneous; Postoperative Period

Int Braz J Urol. 2015; 41: 945-52

Submitted for publication:
September 20, 2014

Accepted after revision:
December 15, 2014

INTRODUCTION

Traditionally, opioid analgesics such as meperidine and morphine are used in postoperative pain management. High doses of these drugs lead to higher rates of side effects including postoperative nausea and vomiting, drowsiness, respiratory depression, ileus, urinary retention and constipation (1, 3-6). Several techniques have been used to overcome these problems such as multimodal analgesic regimens, PCNL with small nephrostomy tube, tubeless PCNL, mini-PCNL, local analgesic infiltration and renal capsule analgesic infiltration (1, 2, 7-9). Another modality is peritubal local anesthetic infiltration which was developed under the rationale to relief the pain that might be originated in renal capsule after PCNL surgery (1, 10-12).

We studied the efficacy of peritubal infiltration of 0.25% bupivacaine in postoperative

pain following percutaneous nephrolithotomy with percutaneous nephrostomy tube. We also studied the efficacy of this technique in patients with supracostal renal access.

PATIENTS AND METHODS

Patients

A total of 105 patients who underwent single tract PCNL with postoperative nephrostomy tube placement were recruited. The patients were randomized into two groups: 53 patients received peritubal analgesic infiltration (study group) and 52 patients were included in the control group. Twenty-two patients of study group and 23 patients of control group received supracostal access. Exclusion criteria included patients with a history of local analgesic allergy, patients who underwent a second nephroscopy, patients who required more than one puncture, and patients who had excessive intra-operative bleeding.

Methods

After general anesthesia was administered, an open-end 6 F ureteral catheter was placed transurethrally into the ureter in supine position. Under fluoroscopic guidance in prone position, contrast media was injected via ureteral catheter. Renal access was created by the biplane technique of standard PCNL. For the supracostal access the needle puncture was performed through the diaphragm and retroperitoneum in full inspiration, whereas the needle was passed through the kidney during deep inspiration. After the tip of the needle was located in the collecting system, working and safety guide wires were inserted followed by tract dilatation with telescopic metal dilators sizes 8F to 30F with 30F Amplatz sheath. Stone was disintegrated with ultrasonic and/or pneumatic lithotripsy. The nephrostomy tube size 20F was routinely inserted in all cases.

In the patients of the study group, the 23-gauge, 90mm spinal needle was inserted up to the renal capsule under fluoroscopic guidance along the nephrostomy tube at 6 and 12 o'clock positions (cranial and caudal); then 0.25% bupivacaine was infiltrated into the nephrostomy tract, including renal capsule, muscle, subcutaneous tissue and skin, 10mL in each position (Figure-1). The control group did not receive any infiltration. Chest X-ray (CXR) and complete blood count were performed to evaluate blood loss and pulmonary complications.

Demographic and clinical characteristics of the patients were recorded at the time of enrollment. Postoperative pain as the primary outcome was assessed by an independent observer blinded to the infiltration using a 0-10 point visual analogue scale for pain (VAS pain) where 0 on the scale meant no pain and 10 meant very severe pain. VAS pain was recorded at 1, 4, 12, 24 and 48 hours postoperatively. The secondary outcomes were the total postoperative morphine usage in 24 hours, time of the first analgesic demand and adverse effects.

Statistical analysis was performed using SPSS® version 13. Continuous variables were compared using t-test for two independent samples. Categorical variables were compared using Chi-square analysis. P-value<0.05 was considered to be statistically significant.

All patients provided written informed consent. The ethical approval was obtained from the Institutional Review Board for human research project of Faculty of Medicine, Chiang Mai University.

RESULTS

Profiles of patients were not clinically significant different between the two groups (Table-1).

Figure 1 - Intraoperative fluoroscopic view of peritubal injection.

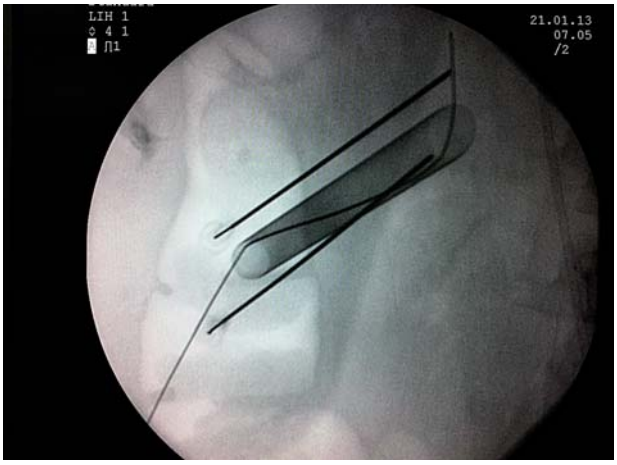


Table 1 - Profiles of patients (Total patients).

	Group I (Study group)	Group II (Control group)	P-value
Patients	53	52	
Gender (M:F)	35:18	36:16	0.83
Age (years)	56.64±11.34	53.84±10.65	0.19
BMI	22.54±3.46	23.81±3.97	0.085
Stone size (cm)	4.00±1.83	4.05±1.88	0.845
ASA status (N, %)			
ASA 1	20 (37.74)	18 (34.62)	0.94
ASA 2	30 (56.60)	31 (59.62)	
ASA 3	3 (5.66)	3 (5.77)	
Previous surgery (N, %)	10(19.87)	10 (19.23)	1.00
Access site Upper pole (N)			
Supracostal	22	23	0.847
Subcostal	19	22	
Middle	4	3	
Lower	8	4	

Postoperative pain as the primary outcome evaluated by VAS is shown in Figure-2. The average VAS pain at 1 and 4 hours after the operation in the study group was 4.64±2.73 and 3.41±2.28 compared with 7.11±2.33 and 4.40±2.21 in the control group (P≤0.001 and 0.026), respectively. The postoperative VAS pain at 12, 24 and 48 hours were not significant different between both groups (Figure-3). Doses of morphine usage for controlling postoperative pain was 4.43±2.78mg in study group and 7.52±5.12mg in control group (P=0.002, Table-2). The first analgesic demand

Figure 2 - Visual Analog Score at postoperative times of 1, 4, 12, 24, 48 hours (total patients).

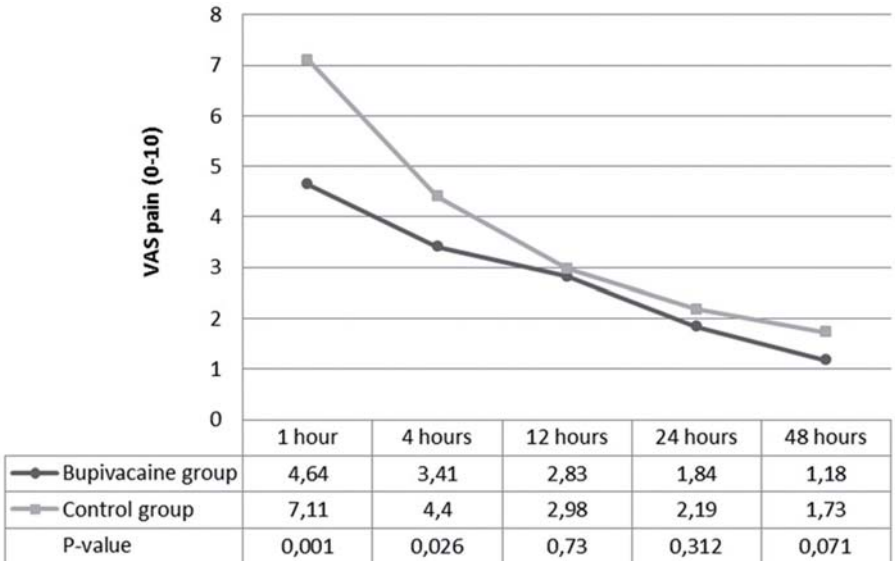
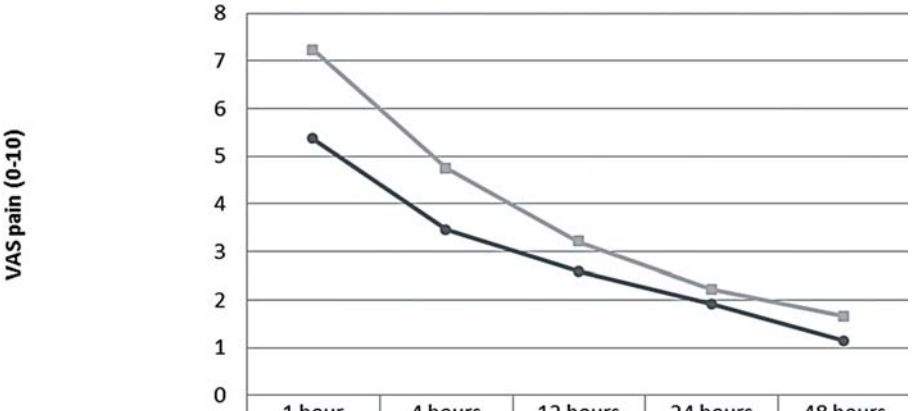


Figure 3 - Visual Analog Score at postoperative times of 1, 4, 12, 24, 48 hours (supracostal patients).



	1 hour	4 hours	12 hours	24 hours	48 hours
Bupivacaine group	5,36	3,45	2,59	1,91	1,14
Control group	7,22	4,74	3,21	2,22	1,65
P-value	0,018	0,103	0,357	0,53	0,199

Table 2 - Clinical outcomes and complications (Total patients).

	Group I (Study group)	Group II (Control group)	P-Value
Stone Free (%)	38 (72%)	36 (69%)	0.84
Stone fragment≤4mm (%)	9 (17%)	10 (21%)	
Operative time (min)	90.09±28.05	86.15±27.45	0.46
Pulmonary complication	0	0	1.00
Morphine usage (mg)	4.43±2.78	7.52±5.12	0.002
First analgesic demand (min)	97.00±87.74	55.10±60.50	0.007
Side effect (N, %)			
Nausea/vomiting	7 (13%)	13 (25%)	0.22

was longer in study group compared with the control group (97.00±87.74 min VS 55.10±60.50mg, P=0.007) (Table-2).

Supracostal access was performed in 55 patients, 22 patients in study group and 23 patients in control group. Profiles of patients are shown in Table-3. The average VAS pain at 1 hour after operation in the study group was 5.36±2.87 compared with 7.22±2.15 in the control group (P=0.018). The postoperative VAS pain at 4, 12, 24 and 48 hours were not significantly different between both groups. Doses of morphine usage for controlling postoperative pain was 4.92±2.96mg in study group and 8.81±6.36mg in control group (P=0.012, Table-4). The first analgesic demand was longer in study group compared with the control group, but was not significantly different (97.69±94.29min and 61.91±67.48min, P=0.165) (Table-4).

DISCUSSION

Postoperative pain is an important issue following the surgery. This affects the postope-

Table 3 - Profiles of patients (supracostal access patients).

	Group I (Study group)	Group II (Control group)	P-value
Patients	22	23	
Gender (M:F)	17:5	17:6	1.00
Age (years)	54.18±9.33	53.69±9.32	0.86
BMI	23.50±2.75	24.89±3.60	0.16
Stone size (cm)	4.43±2.29	4.37±1.87	0.93
ASA status (N, %)			
ASA 1	9 (40.9)	6 (26.09)	0.62
ASA 2	11 (50.00)	14 (60.87)	
ASA 3	2 (5.66)	3 (13.04)	
Previous surgery			
(N, %)	6 (27.27)	5 (21.74)	0.74

Table 4 - Clinical outcomes and complications (supracostal access patients).

	Group I (Study group)	Group II (Control group)	P-Value
Stone Free (%)	17 (78%)	18(79%)	0.87
Stone fragmen≤t4mm (%)	2 (10%)	3(13%)	
Operative time (min)	92.50±33.54	92.61±30.37	0.99
Pulmonary complication	0	0	1.00
Morphine usage (mg)	4.92±2.96	8.81±6.36	0.012
First time of analgesic demand (min)	97.69±94.29	61.91±30.37	0.165
Side effect (N, %)			
Nausea/vomiting	2 (9.1%)	8 (34.79%)	0.03

rative quality of life especially in recovery period with patient's anxiety and several negative aspects such as delayed mobilization, increased postoperative complications and prolonged hospitalization (3-6). Recently, several techniques have been developed for improvement of postoperative pain management due to better understanding of acute pain physiology, development of new analgesic agents, better analgesia delivery procedures and better local anesthetic infiltration techniques (10-15). Gender also affects the level of postoperative pain. Women have more pain sensitivity and therefore most women need more analgesic consumption than men (16, 17).

Percutaneous nephrolithotomy (PCNL) is accepted to be the minimally invasive procedure for large renal and ureteral calculi with less morbidity and mortality compared to open surgery. Even in minimally invasive nature, PCNL still causes significant postoperative pain especially in standard PCNL with nephrostomy tube. The purpose of nephrostomy tube placement following PCNL is for the tamponade of the bleeding along the tract, adequate drainage and maintenance of

tract for a second nephroscope (8, 9). A significant number of PCNL patients have been distressed from postoperative pain mostly due to the presence of nephrostomy tube. Various techniques were reported to minimize postoperative pain following PCNL such as small bored nephrostomy tube, tubeless PCNL, lignocaine infiltration at renal capsule and peritubal infiltration (7-12).

Patients with small bored nephrostomy tube have less postoperative pain score and less narcotic requirement (2, 7). Tubeless PCNL is recommended in uncomplicated cases without increasing complication (8, 9). Techniques of small bored nephrostomy and tubeless PCNL have been shown to have the advantage of less postoperative pain, but these techniques are not recommended in patients with significant bleeding, significant extravasation and second nephroscope required (tubeless PCNL). As standard technique of PCNL, the placement of large nephrostomy tube follows completion of the procedure is recommended for general cases. Postoperative pain usually is caused by the dilatation of renal capsule and parenchyma of access tract with local inflammation reaction along the nephrostomy tube (10-12). Pain following PCNL that involved nephrostomy tube might originate from renal capsule, muscle, subcutaneous tissue and skin. Renal capsule and parenchyma are richly innervated of pain-conductive neurons; the pain is therefore not only at the skin (11).

Opioid analgesics are traditionally used for controlling postoperative pain, but these drugs usually have side effects. The usage of multimodal or a combination of lower doses of opioid analgesics with non-opioid analgesics could avoid these side effects. Several studies demonstrated the efficacy of acetaminophen with and without opioid in management of postoperative pain (3-6, 18, 19). Maghsoudi et al. reported the positive effect of intravenous paracetamol as part of multimodal analgesia regimen for postoperative pain management following PCNL. Fifty patients who received 1gram intravenous paracetamol had significantly less visual analog score at 6 and 24 hours postoperative period compared with patients that received placebo. The meperidine consumption was also lower in paracetamol group (54.40mg VS 77.60mg, $P<0.001$) (18).

The benefit of local anesthesia was demonstrated in previous studies of general surgery, gynecology and anesthesia such as cesarean sections, hysterectomy, thyroid surgery, mastectomy, total-hip arthroplasty and cervical spine surgery, where marcaine was used as anesthesia agent (20-23). From the previous studies, the maximal benefit of marcaine infiltration will be met if the infiltration is performed before the incision. Haleblian et al. studied the effect of local anesthetic (Marcaine®) infiltration at the incision wound (subcutaneous) of PCNL with 10 Fr. nephrostomy tube in 10 patients compared with 12 patients with saline infiltration. It was observed no significant differences between both groups in the aspect of pain scores and postoperative narcotic use. The sample size of the study was small and difficult to interpret, and marcaine was infiltrated subcutaneously, which was not adequate for the local pain control following this operation (11).

Jonnavithola et al. studied the randomized control of peritubal infiltration of bupivacaine of renal capsule and demonstrated the effectiveness of this technique. The technique consisted of the use of a 23 gauge spinal needle (10cm in length) along nephrostomy tube at 6 and 12 o'clock and each infiltrated 10mL of 0.25% bupivacaine. The pain free period and mean total consumption of tramadol following operation of controlled group and blocked group were 4.6 ± 5.4 hours and 105 ± 85 mg and 14.7 ± 9.6 hours and 31 ± 44 mg, respectively. The mean AUC-UAS was 39.2 hours in control group and 18.9 hours in infiltration group (12).

Ugras et al. demonstrated the positive effect on postoperative pain and ventilatory function following ropivacaine infiltration of skin, nephrostomy tract and renal puncture site in combination with parenteral analgesia (metamizol). The aim of the study was to evaluate visual analog score (VAS), peak expiratory flow rate (PEF) and blood gas analysis. The time of first analgesic demand, total analgesic need and VAS at 6 hours were significantly lower, and PEF at 2 and 6 hours were significantly higher in patients with combinded ropivacaine infiltration and parenteral analgesic. Combination treatment for postoperative pain control lead to better pain management, which resulted in better patient's ventilation (1).

Parikh et al. reported a prospective randomized study of the efficacy of 0.25% bupivacaine peritubal infiltration in 60 PCNL patients; 30 patients were included in the treated group (0.25% bupivacaine infiltration) and 30 in the controlled group (normal saline infiltration). Exclusive criteria of the study were multiple punctures, supracostal puncture, stone size larger than 2.5cm, duration of procedure more than 3 hours and excessive intraoperative bleeding. Visual analogue scale (VAS) and dynamic visual analogue scale (DVAS) were lower in bupivacaine injected patients in early and late postoperative times. Mean of first tramadol demand was significantly shorter in normal saline infiltration patients (1.96hours VS 4.4hours). Total tramadol consumption was higher in normal saline patients (276.8mg VS 119.3mg) (10).

Our patient recruitment criteria included all single tract PCNL patients with postoperative nephrostomy tube placement including supracostal puncture without considering operative time and stone size, which is different from previous studies. Our study confirms the benefit and safety of peritubal analgesic infiltration in controlling postoperative pain (lower VAS number), lower use of morphine and longer time of first analgesic requirement. These results were also observed in the subgroup analysis of supracostal access, which should have more pain after this operation.

CONCLUSIONS

Peritubal local anesthetic infiltration with 0.25% bupivacaine resulted in beneficial effects in alleviating immediate postoperative pain after percutaneous nephrolithotomy even with supracostal access. This effect resulted in lower early postoperative pain (lower VAS score), lower number of morphine usage and longer time of first analgesic requirement.

ACKNOWLEDGEMENTS

We thank Faculty of Medicine, Chiang Mai University for granting this study and Miss Wilaiwan Chongruksut for statistical analysis.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Bannakij Lojanapiwat, MD
Division of Urology - Surgery
110 intravaroros Muang Chiangmai
Chiang Mai, 50200, Thailand
Telephone: +66 818 824-085
E-mail: dr.bannakij@gmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 953-958, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0586

Percutaneous puncture of renal calyxes guided by a novel device coupled with ultrasound

Chen Jen Chan [1], Victor Srougi [1], Fabio Yoshiaki Tanno [1], Ricardo Duarte Jordão [1], Miguel Srougi [1]

[1] Seção de Endorologia e Videolaparoscopia, Divisão de Urologia, Hospital das Clínicas, Universidade de São Paulo, Escola de Medicina, São Paulo, Brasil

ABSTRACT

Purpose: To evaluate the efficiency of a novel device coupled with ultrassound for renal percutaneous puncture.
Materials and Methods: After establishing hydronephrosis, ten pigs had three calyxes of each kidney punctured by the same urology resident, with and without the new device ("Punctiometer"). Time for procedure completion, number of attempts to reach the calyx, puncture precision and puncture complications were recorded in both groups and compared.
Results: Puncture success on the first attempt was achieved in 25 punctures (83%) with the Punctiometer and in 13 punctures (43%) without the Punctiometer (p=0.011). The mean time required to perform three punctures in each kidney was 14.5 minutes with the Punctiometer and 22.4 minutes without the Punctiometer (p=0.025). The only complications noted were renal hematomas. In the Punctiometer group, all kidneys had small hematomas. In the no Punctiometer group 80% had small hematomas, 10% had a medium hematoma and 10% had a big hematoma. There was no difference in complications between both groups.
Conclusions: The Punctiometer is an effective device to increase the likelihood of an accurate renal calyx puncture during PCNL, with a shorter time required to perform the procedure.

ARTICLE INFO

Key words:
Punctiometer, percutaneous access, kidney punction, nephrolitotripsy, ultrassound

Int Braz J Urol. 2015; 41: 953-8

Submitted for publication:
November 13, 2014

Accepted after revision:
February 10, 2015

INTRODUCTION

Minimally invasive surgical techniques have gained increasing prominence because of their advantages of shortening the convalescence period and producing less pain during postoperative recovery. In accordance with this trend, percutaneous techniques have been developed to treat urinary tract stones that previously required open surgery with long and painful incisions.

During percutaneous nephrolithotomy (PCNL) (1-3), the urinary tract is accessed by a renal puncture, followed by dilation and the use of optical instruments to find and remove the stones. Percutaneous access to the excretory pathway is an essential step, but it is not always easily achieved, and more than one attempt is frequently necessary. Furthermore, the procedure is often performed using fluoroscopic guidance, which exposes the surgeon and patient to radiation (4). The procedure can also be performed using ultrasound (5, 6), with the advantage of avoiding radiation but the disadvantage of requiring the presence of an ultrasound expert.

To facilitate renal calyx puncture, we developed an apparatus called the "Punctiometer"

for use with ultrasound guidance. This device can be extremely helpful, especially in the presence of ureteral obstruction or when it is not possible to position a ureteral catheter to inject the excretory pathway with contrast before conventional fluoroscopic puncture. The purpose of the current study is to evaluate the efficacy of the Punctiometer device in aiding percutaneous puncture of renal calyxes in a pig model.

MATERIALS AND METHODS

This experimental study was approved by the Ethics Committee in Research of the University of São Paulo School of Medicine (Protocol 432/11). It was performed in the CEPEC-Center of Training and Research in Surgery "Vicky Safra" of Faculty of Medicine University of São Paulo (FMUSP) from December 2012 to January 2013. Ten 30-kg Duroque pigs of the MC60 lineage were used. The animals were anesthetized with 100mg ketamine, 400mg xylazine, 15mg midazolam, 250mg thiopental, 4mg pancuronium, and 0.05mg fentanyl; intubated and mechanically ventilated with 100% O2; and placed in the lateral position.

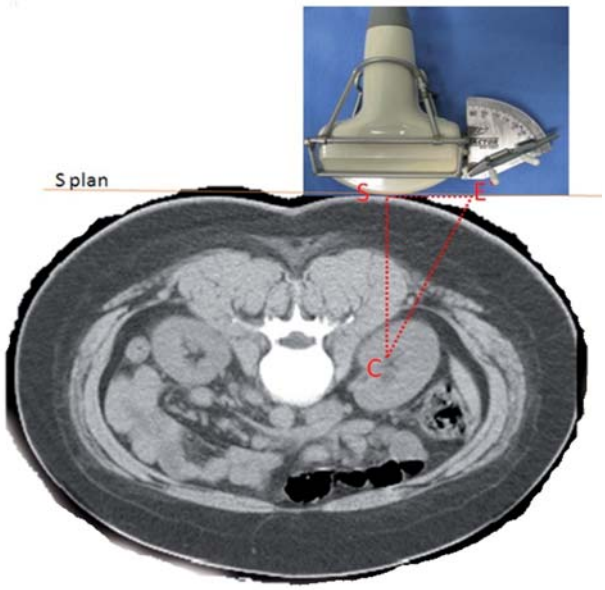
All kidneys were punctured using ultrasound guidance, after blinded randomization to either the Punctiometer or no Punctiometer Groups. Laparoscopic access was used to ligate the ureters bilaterally to promote mild dilation of the excretory pathways. The ligation was executed with a cotton thread and endocorporeal knot in the proximal portion of the ureter, ensuring total luminal occlusion. Approximately 20 minutes after, the animals were then positioned in the prone position for the renal punctures, which were performed by a senior urology resident with previous training in percutaneous surgery. A 2-0 nylon thread was used as a guidewire to identify the exact puncture location in the calyx. Three calyxes of each kidney were punctured (superior, middle, and inferior). Bilateral nephrectomy was subsequently performed to verify the position of the guidewire. If the nylon guidewire was lost, the puncture was excluded from the final evaluation. At the end of the procedure, the pigs were sacrificed with 20mL potassium chloride and discarded in accordance with the local law ("RSS-Resíduos Sólidos de Serviços de Saúde (Solid residuals of Health Services)"-São Paulo (7).

The final evaluation involved determination of the following: number of attempts to reach each calyx, precision index score, total time to perform the procedure for each kidney, and occurrence of complications. The precision index score was rated on a 0 to 2 point scale: 2 points, reached the collecting system in the target calyx; 1 point, reached the renal pelvis; or 0 points, did not reach the collecting system. Complications were rated on a 1 to 4-point scale: 1 point, small hematoma; 2 points, moderate hematoma; 3 points, big hematoma; or 4 points, huge hematoma. The definition of the hematoma grade was subjective and evaluated by the surgeon who performed the puncture.

Punctiometer

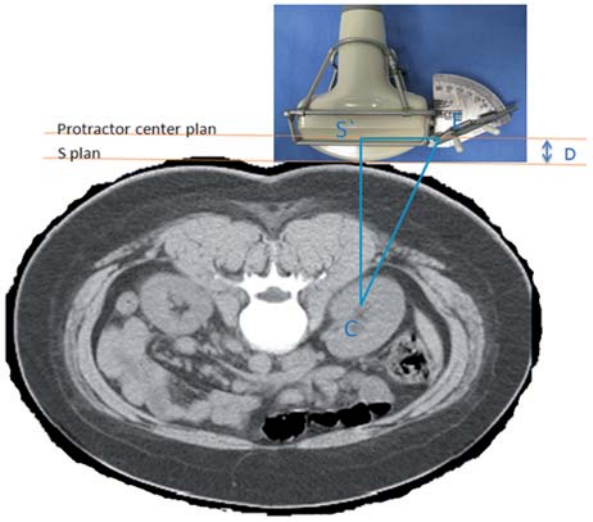
Use of the Punctiometer involves the general principle of first identifying a triangle (Figure-1) formed by the target renal calyx (C), the "apex" of the ultrasound transducer at a right angle with the calyx (S), and the orthogonal projection of the protractor with the skin (E´). Using a triangular Figure, it is possible to calculate the hypotenuse when it is known the length of the

Figure 1 - Vertices of the initial right triangle: the target calyx (C); the "apex" of ultrasound transducer in contact with the skin, perpendicular to the aimed calyx (S); and the orthogonal projection of the center of the protractor in the skin plane (E´).



opposite and adjacent sides. From the triangle in Figure-1, we derive the triangle in Figure-2, which is the triangle actually used for the puncture. One of the sides of this second triangle is the distance between the center of the protractor (E) and the point of the orthogonal projection of the target calyx to the surface of the protractor (S´E). This distance is fixed in the apparatus. The other side of the triangle is the distance between the calyx and the skin where there is contact with the apex of the ultrasound transducer. This corresponds to the distance measured by ultrasound, added to the distance (D) from the ultrasound apex to the center of the protractor (S`C).

Figure 2 - Formation of the right triangle. S`C: Distance from the target calyx (C) to its orthogonal projection on the plane of the center of the protractor (S`). S`E: Distance between point S` and the center of the protractor (E). CE: Distance from the target calyx (C) to the center of the protractor (E). D: Distance between the planes.



Statistical analyses

Descriptive analyses of the quantitative data with a normal distribution included determination of the means, with their respective standard deviations (±SD). Qualitative data were presented as frequencies and percentages. We used Student´s t-tests to compare quantitative variables. Qualitative variables were evaluated by likelihood ratio tests for comparing proportions. For all inferential analyses, we considered the probability of a type I error (α level) of 0.05 to be statistically significant. Statistical analyses were performed using SPSS software, version 21 (SPSS 21.0 for Windows) (8).

RESULTS

After randomization, 30 calyxes were successfully punctured in each Group (10 pigs), with the number of attempts ranging from 1 to 5. The average diameter of the calyxes measured by ultrasound was 9.1mm (7-17mm) in the Punctiometer Group and 10.5mm (5-17mm) in the no Punctiometer Group.

Puncture success on the first attempt was achieved in 25 punctures (83%) with the Punctiometer and in 13 punctures (43%) without the Punctiometer (p=0.011). The guidewire was lost in 1 calyx in the Punctiometer Group and in 5 calyxes in the no Punctiometer Group; these punctures were not included in the final analyses. A precision index score of 2 was noted in 21 of the 29 remaining punctures (72%) in the Punctiometer Group and in 13 of the 25 remaining punctures (52%) in the no Punctiometer Group (p=0.028). The mean time required to perform three punctures in each kidney was 14.5 minutes with the Punctiometer and 22.4 minutes without the Punctiometer (p=0.025).

There were no lesions produced in other organs. The only complications noted were renal hematomas of an intensity of 1 to 3 points. In the Punctiometer Group, all kidneys had small hematomas (designated as 1 point). In the no Punctiometer Group, 8 kidneys (80%) had small hematomas (1 point), 1 (10%) had a medium hematoma (2 points), and 1 (10%) had a big hematoma (3 points). The percentage of 1-point hematomas did not differ significantly between the two groups (p=0.224). Results are summarized in Table-1 and Figure-3

DISCUSSION

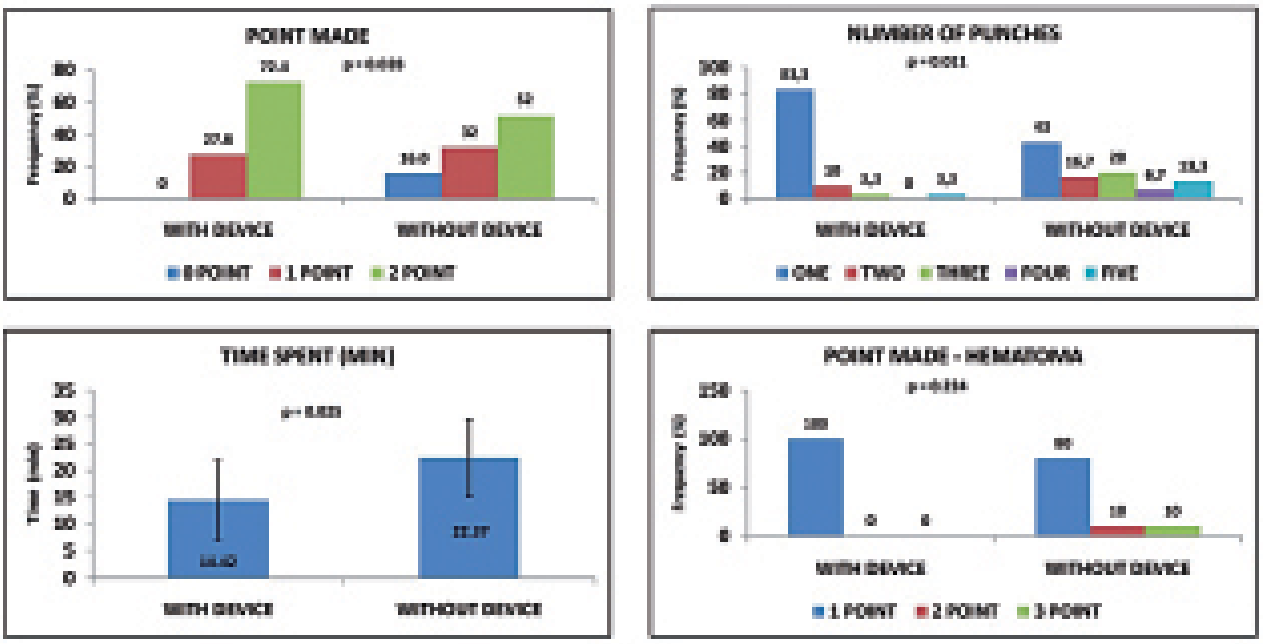
Percutaneous access to the excretory system is a relatively easy procedure when the system is clearly dilated. However, it may be challenging when there is minimal or no dilation, as is fre-

Table 1 - Results of the puncture procedures in the Punctiometer and no Punctiometer groups.

	Punctiometer		p-value
	With	Without	
Calyxes with a successful 1st puncture	25 (83%)	13 (43%)	0.011
Calyxes with a precision index rating of 2	21 (72%)	13 (52%)	0.028
Time for three punctures in each kidney (min)	14.42±7.39	22.37±7.21	0.025
Kidneys with 1-point complications	10 (100%)	8 (80%)	0.224

Data are mean±standard deviation or number (percentage)

Figure 3 - Comparison of frequencies and means between the Punctiometer and no Punctiometer groups: A) Precision index; B) number of puncture attempts required; C) mean time required to perform three punctures in each kidney; D) grade of complications (which were all hematomas).

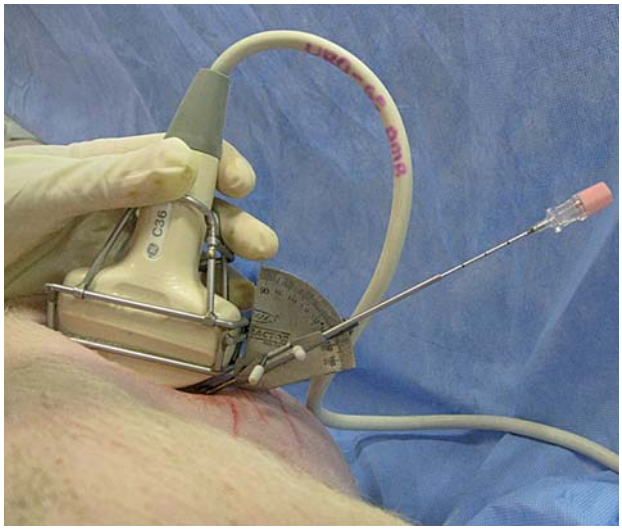


quently seen in patients with renal stones. The use of ultrasound to guide renal puncture may be valuable in this situation. In 1974, Pedersen and colleagues (9), were the first to describe the use of ultrasound during percutaneous nephrostomy, obtaining a success rate of 75%. Other studies addressing access to the excretory system have been published, most of which have reported on the accuracy of the technique used (10) without mentioning the number of attempts necessary for a successful puncture. Agostini et al. (11) reported that 9.2% of patients required more than one puncture attempt, and Krombach et al. (12) noted that use of a magnetic field-based navigation device to guide the puncture in a porcine model failed in 2 of 12 kidneys.

In our study, we successfully reached the excretory system in all cases, whether or not we used the Punctiometer (Figure-4).. This high success rate may be due to the relatively short dis-

tance between the skin and renal calyxes in the pig, compared to humans. Most punctures were successful on the first attempt, but some required up to 5 attempts, which was inferior to the success rate reported by Agostini et al. (11). The discrepancy may be explained by the presence of urinary tract obstruction in the Agostini series, whereas in our study, the mean diameter of the calyxes was 9 to 10mm. Our findings regarding the need for more than one puncture attempt with the Punctiometer are comparable to those reported by Krombach and colleagues (12) using the ultrasound and magnetic field-based navigation device.

Figure 4 - Ilustration of the Punctiometer in use.



Regarding the precision index, we could find no previously published similar study with which to compare our results. Punctures were more precise in the Punctiometer Group than in the no Punctiometer Group. The difference was statistically significant, thereby suggesting that the device was effective in improving the likelihood of success of the puncture procedure.

Previously, Agostini et al. (11) reported that the time to place a percutaneous nephrostomy catheter was 7 to 15 minutes. Karim and colleagues (6) reported a longer time, 39 (25-55) minutes, to perform the same procedure. In our study, the time required to perform three punctures in each kidney was 14.4 minutes with the Punctiometer and 22.4 minutes without the Punctiometer, representing mean times of 4.8 minutes and 7.5 minutes per calyx in both Groups, respectively. Use of the Punctiometer was associated with significantly shorter renal puncture times. These results may be attributed to our use of only one guidewire in each calyx, without placing a nephrostomy catheter.

The most common complications of renal puncture accessed percutaneously are unintentional perforation of abdominal organs or pleura, and bleeding from renal or the great vessels. Although the risk of complications is small (6, 13, 14), the risk is higher when there is minimal or no dilation of the excretory system. In the present series, we observed no organ or pleura perforation and no bleeding arising from the great vessels; our complications were limited to renal bleeding. The difference in rate of 1 point hematomas between the Punctiometer and no Punctiometer Groups was not significant and the overall complication score was low in both Groups. However, it is possible that the short time between the punctures and nephrectomy may have limited the size of the observed hematomas.

CONCLUSIONS

The renal Punctiometer is an effective device to increase the likelihood of an accurate renal calyx puncture during PCNL, with improved precision and a shorter time required to perform the procedure.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Chen J. Chan, MD
Departamento de Urologia,
Hospital das Clínicas de São Paulo
Av Enéas de Carvalho Aguiar, 255
São Paulo, SP, 05403-000, Brasil
Telephone: +55 11 2661-8080
E-mail: vsrougi@uol.com.br

ORIGINAL ARTICLE

Vol. 41 (5): 959-966, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0170

The vascular and neurogenic factors associated with erectile dysfunction in patients after pelvic fractures

Yong Guan [1], Sun Wendong [2], Shengtian Zhao [2], Tongyan Liu [3], Yuqiang Liu [2], Xiulin Zhang [2], Mingzhen Yuan [2]

[1] ShanDong University, Jinan, China; [2] Department of Urology, the Second Hospital of ShanDong University, Jinan, China; [3] The Second Hospital of ShanDong University, Jinan, China

ABSTRACT

Erectile dysfunction (ED) is a common complication of pelvic fractures. To identify the vascular and neurogenic factors associated with ED, 120 patients admitted with ED after traumatic pelvic fracture between January 2009 and June 2013 were enrolled in this study. All patients answered the International Index of Erectile Function (IIEF-5) questionnaire. Nocturnal penile tumescence (NPT) testing confirmed the occurrence of ED in 96 (80%) patients on whom penile duplex ultrasound and neurophysiological testing were further performed. Of these ED patients 29 (30%) were demonstrated only with vascular abnormality, 41 (42.7%) were detected only with neural abnormality, 26 (27.1%) revealed mixed abnormalities. Of the 55 patients (29+26) with vascular problems, 7 patients (12.7%) with abnormal arterial response to intracavernous injection of Bimix (15mg papaverine and 1mg phentolamine), 31 (56.4%) with corporal veno-occlusive dysfunction and 17 (30.9%) had both problems. Of the 67 (41+26) patients with abnormal neurophysiological outcomes, 51 (76.1%) with abnormal bulbocavernosus reflex (BCR), 20 (29.9%) with pathological pudendal nerve evoked potentials (PDEPs) and 25 (37.3%) with abnormal posterior tibial somatosensory nerve evoked potentials (PTSSEPs). Our observation indicated that neurogenic factors are important for the generation of ED in patients with pelvic fracture; venous impotence is more common than arteriogenic ED.

ARTICLE INFO

Key words:
Erectile Dysfunction; Urethra; Pelvis; Penis

Int Braz J Urol. 2015; 41: 959-66

Submitted for publication:
April 07, 2014

Accepted after revision:
October 23, 2014

INTRODUCTION

Erectile dysfunction (ED) is defined as the inability to achieve or maintain an erection adequate for sexual satisfaction (1). It has been reported that 3% of cases of ED may result from pelvic fractures or perineal blunt trauma (2). The incidence of ED ranges from 20% to 84% in patients with urethral injury secondary to perineal trauma or pelvic fractures (3). ED caused by pelvic fractures, especially associated with urethral injuries, is more common than previously described (2). It is assumed that ED caused by such reasons is due to lesions of the cavernous nerves or branches of the internal pudendal arteries that pass in close proximity to the pelvic bones and posterior urethra. The intimate relationship of the soft tissues and the bony pelvic ring result in a high risk of concomitant local injury associated with fractures of the pelvis (4). Even without severe urological injury, damage to the delicate vascular and nervous tissues supplying the genitalia can result in

sexual dysfunction (3, 5). While knowledge from pelvic microscopic anatomy and erectile physiology provided insights for alternative pathways in the development of ED (2, 6, 7), few studies have been designed to analysis the exact pathophysiological factors in this type of ED patients (8, 9), particularly no study was performed to differentiate the neurogenic ED from vasculogenic ED and it was just assumed that patients with normal vascular response are neurogenic ED (3, 10); furthermore, the number of the patients observed in most of the reports are not big enough which for some extent may impact the interpretation of the results (3, 11). Therefore, the main purpose of our study was to evaluate the vascular and neurogenic factors associated with ED in a relatively large population of patients after pelvic fracture.

MATERIALS AND METHODS

General information of the patients

120 patients who were admitted to the Second Hospital of Shandong University between January 2009 and June 2013 for the complaint of ED were enrolled in this study. All patients had a history of pelvic fractures associated with urethral injuries and were submitted to urethral realignment by traction. According to patient history, they were free from ED before the injury. The age ranged from 21 to 48 years old (mean age 37.6±6.3). Imaging studies taken at admission (e.g., pelvic radiographs, computed tomography scans) were used to classify the injury according to the modified Tile's classification and Denis' classification for sacral fractures (12-14). Based on the criteria we classified these patients into type A (Stable, minimally displaced), type B (rotationally unstable, vertically stable) and type C (rotationally and vertically unstable).

Blood tests

To exclude hormonal factors related with ED the levels of testosterone (T), estradiol (E2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the peripheral blood plasma were measured by radioimmunoassay. Blood lipid, blood glucose level and blood pressure were also checked after admission.

IIEF-5 questionnaires

IIEF-5 questionnaires were answered by all patients. A modification of the method developed by Cappelleri was used for grading of ED into four categories: no ED (scores 26-30), mild ED (scores 17-25), moderate ED (scores 11-16) and severe ED (scores 6-10) (15).

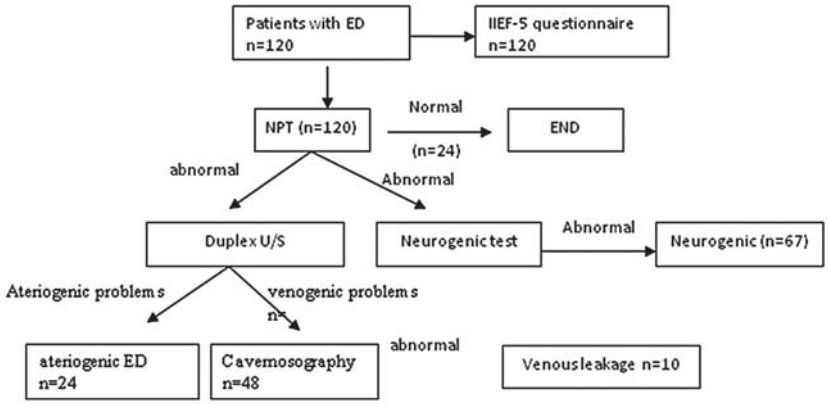
Nocturnal penile tumescence (NPT) test

NPT tests were performed using the RigiScan® device in all the patients. To ensure a restful night sleep the patient was asked to avoid napping, caffeine or alcohol intake and to evacuate the bladder prior to going to sleep. The data were collected each morning. The test was conducted over two consecutive nights in order to avoid the "first night effect". Normal nocturnal erectile function was defined as at least 3 tumescence periods lasting more than10 minutes with rigidity at the penile tip of at least 70% (3).

Duplex ultrasonography and cavernosography

Patients with abnormal NPT outcome were submitted to duplex ultrasonography test (GE LOGIQ9, America) (Figure-1). Patients received a single intracavernous injection of Bimix (15mg papaverine and 1 mg phentolamine). The erectile response was evaluated for tumescence and rigidity by palpation of the penis. The erectile status after intracavernous injection was also compared with that demonstrated at home before trauma in order to obtain the best quality erection (16). The penis was scanned by a ventral approach at the base with the probe held transversally or in an oblique-longitudinal position. Peak systolic velocity (PSV), end diastolic velocity (EDV) and resistance index (RI) within the cavernosal arteries were measured. Patients with PSV greater than 35cm/sec were considered with a normal arterial response, while less than 25cm/sec signified severe arterial insufficiency. Corporal veno-occlusive dysfunction was defined as EDV >5cm/sec and RI <0.85 (3, 17, 18). If venogenic ED was highly suspected, cavernosography was performed by intracavernous injection of contrast following administration of vasodilators (15mg papaverine and 1 mg phentolamine).

Figure 1 - Flow diagram for evaluation of ED patients. All patients answered IIEF-5 questionnaire and were submitted to NPT. Duplex ultrasonography and neurophysiological tests were performed if problems were detected with NPT. Cavernosography was undertaken to determine if there was venous leakage in patients suspected with venogenic ED.



Neurophysiological tests

Posterior tibial somatosensory nerve evoked potentials (PTSSEPs), pudendal nerve evoked potentials (PDEPs) and the bulbocavernosus reflex (BCR) test were conducted for all the ED patients confirmed by NPT test. PTSSEPs and PDEPs were performed according to the International Federation of Clinical Neurophysiology (IFCN) standards (19). The latency of cortical P40>45ms or left–right difference >2.5ms were considered abnormal in PTSSEPs test. P40 latency>44.1 ms was considered pathological in PDEPs test (20). BCR test was performed by applying electrical pulses on the penis and the responses were recorded from both bulbocavernosus muscles with concentric needle electrodes (20 21). Abnormal results include absent responses, response latency >37ms and interside differences >1.5ms.

RESULTS

All of the patients had normal levels of blood hormones including testosterone, estradiol, LH and FSH. Blood lipid, blood glucose and blood pressure level of these patients were also normal. According to the modified Tile's classification and Denis' classification for sacral fractures, 72 patients (60.8% of 120) were classified as type A fracture, 35 patients (29%) were type B, and 12 patients (10%) were type C (Table-1).

All of the patients answered the IIEF-5 questionnaire and 115 (95.8%) were considered with ED based on the Cappelleri's grading criteria (15); of them 16 patients were mild ED, 34 patients were mild to moderate ED, 47 patients were moderated ED and 18 patients had severe ED (Table-2).

NPT tests showed normal nocturnal erections in 24 (20.0%) patients, abnormal nocturnal erections in 96 patients (80.0%) in whom further Duplex ultrasonography, cavernosography and neurophysiologic testing revealed organic lesions (Figure 1 and Table-3). Penile duplex Doppler ultrasonography detected abnormal responses to intracavernous injection of Bimix in 55 patients (45.8% of 120) (Table-3), of them 29 patients only with abnormal vascular response, 26 with abnormal vascular as well as neurogenic problems, 7 patients (12.7%) with abnormal arterial response, 31 (56.4%) with corporal veno-occlusive dysfunction and 17 (30.9%) had both problems (Table-4, Figure-2). Of the 31 patients with corporal veno--occlusive dysfunction, there were 5 patients with penile venous leakage revealed via cavernosography (Figure-3). Abnormal neurophysiologic outcomes were seen in 67 patients, of them 41 patients were only with abnormal neurophysiological ou-

Table 1 - Patients distribution based on the type of pelvic fracture.

	Type A	Type B	Type C
Number of patients.	73	35	12
%	60.8% (73 of 120)	29.2% (35 of 120)	10.0% (12 of 120)

Table 2 - Grading and distribution of ED patients based on IIEF-5 scores.

ED Status	Theoretical EF Domain Scores*	The Number of Patients (%)
Without ED	(26–30)	5 (4.2%)
With ED	(≤25)	115 (95.8%)
Mild ED	(22–25)	16 (13.3%)
Mild to moderate ED	(17–21)	34 (28.3%)
Moderate ED	(11–16)	47 (39.2%)
Severe ED	(6–10)	18 (15.0%)

*Criteria from Cappelleri et al. [13].

Table 3 - Distribution of ED patients based on etiology.

Origin	NPT	Vasculogenic ED			Neurogenic ED	Vasculogenic and Neurogenic
		Arteriogenic	Venogenic	Mixed		
Number	96	3	16	10	41	26

One of the neurophysiologic tests (BCR, PDEP or PTSSEPs) was abnormal indicating the patients had neurogenic ED.

Table 4 - Vasculogenic ED patient distribution.

	Arteriogenic	Venogenic	Mixed
Number of patient.	7	31	17
%	12.7% (7 of 55)	56.4% (31of 55)	30.9% (17 of 55)

These 55 vasculogenic ED patients include 29 only with vasculogenic and 26 with both neurogenic and vasculogenic origin.

tcome and 26 with both abnormal neurophysiologic outcome and vascular problem. Pathological PDEP responses were seen in 20 patients (29.9% of 67), abnormal latencies of PTSSEPs were observed in 25 patients (37.3%), problematic BCR were found in 51 patients (76.1%) (Table-5); of them 9 patients (17.6%) with bilaterally abnormal, 37 (72.5%) with unilaterally abnormal and 5 (9.8%) with no response (Table-6).

DISCUSSION

Pelvic fractures, particularly those associated with posterior urethral injury often cause

Figure 2 - Abnormal arterial or venous responses to intracavernous injection of Bimix in ED patients. Patients received a single intracavernous injection of Bimix (15 mg papaverine and 1 mg phentolamine) then ultrasonography was performed. (a) peak systolic velocity (PSV)>35cm/sec and the resistance index (RI)=1 in a patient indicating a normal arterial and venous response. (b) In one ED patient PSV<35cm/sec with the end-diastolic velocities (EDV)<5cm/sec suggesting abnormal arterial response. (c) in another ED patient PSV>35cm/sec with EDV>5cm/sec indicating veno-occlusive dysfunction. (d) PSV<35cm/sec with EDV>5cm/sec suggesting both arterial and venous abnormal responses.

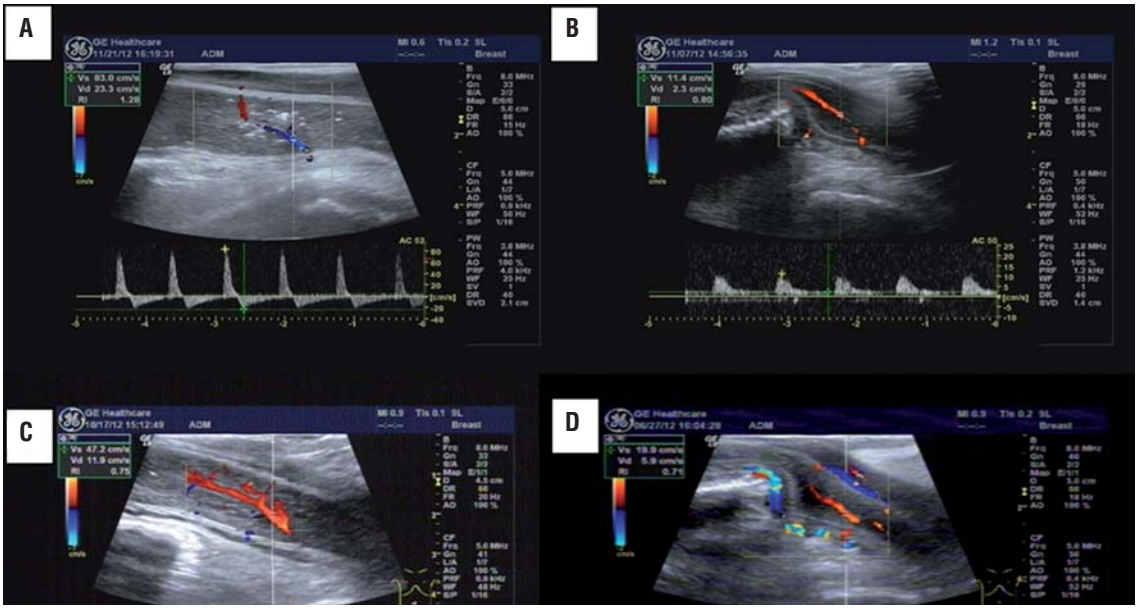
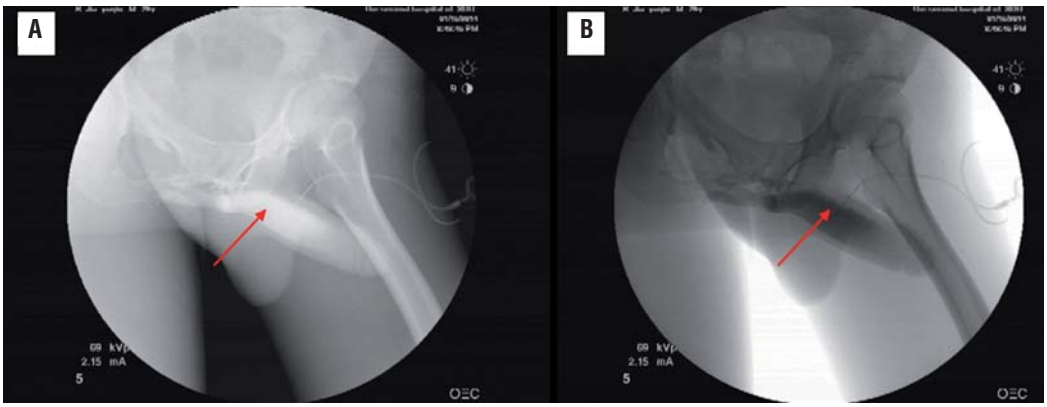


Figure 3 - Venous leakage revealed by cavernosography in venogenic ED patients. Contrast was injected intracavernously following administration of 15 mg papaverine and 1 mg phentolamine. Solid arrow indicates leakage from the vein. (a) the positive film (b) the negative film.



ED. It had been reported ED occurred in 20-84% of cases when pelvic fractures were associated with urethral injury (3, 6, 10). In our study all of the patients had a history of pelvic fractures associated with urethral injuries; NPT test demonstrated 80% of those patients had organic ED. The criterion used in our study for NTP testing was the same as Shenfeld's report (3) and the incidence of ED (80%) was closer to 72% reported by them and was also similar to 84% reported by Flynn et al. (22). Our observation that neurogenic ED accounted for most of the ED patients is consistent with

Table 5 - Neurogenic ED patient distribution.

	BCR	PDEP	PTSSEPs
Number of patients.	51	20	25
%	76.1% (51 of 67)	29.9% (20 of 67)	37.3% (25 of 67)

These 67 patients included 41 only with neurogenic and 26 with both neurogenic and vasculogenic origin. One patient may have two or three abnormal outcomes.

Table 6 - ED Patients with abnormal BCR outcomes.

	Bilateral	Unilateral	No response
Number of patients.	9	37	5
%	17.6% (9 of 51)	72.5% (37 of 51)	9.8% (5 of 51)

other reports indicating neurogenic factor is the principal etiology of organic ED associated with urethral injury (3, 10).

The IIEF was used as a self-administered questionnaire and proved an adequate tool in bringing forward the latent expectations of the patients; it might be used at the time of rehabilitation to identify those patients who would need further evaluation and treatment (23). The limitation of self-administered questionnaires is that they do not distinguish an etiologic basis for ED. In our study IIEF scores were used to assess the severity of ED and 95.8% of patients were considered with ED, compared with the 80% ED patients detected with NPT test indicating some of the ED patients might had a psychogenic origin.

Erections are initiated by a combination of psychic and physical stimuli, and erectile function is controlled by parasympathetic fibers originating from S2 to S4. These fibers travel through the pelvic nerve and the pelvic plexus to the cavernous nerve, which enters the corpora cavernosa. As these fibers pass through the pelvis, the nerves run in close proximity to the prostate and rectum, which makes them prone to injury during surgical procedures as well as after pelvic trauma. It has been speculated that the etiology of ED after pelvic fracture is neurovascular injury. Most previous studies that address the etiology of ED with pelvic fracture did not differentiate neurogenic from the vascular factors (3, 10, 24), they just indirectly concluded that most of the ED patients were neurogenic, since most of the ED patients had a normal arterial response to intracavernous injection of either Trimix (3) or Bimix (10). To our knowledge our study is the first report that electrophysiological testing was performed to identify the neurological pathologies related with ED after pelvic fracture. We found that 69.7% of ED patients had abnormal electrophysiological outcomes and were diagnosed as neurogenic ED. As mentioned above, penile erection is primarily an autonomic nerve function (cavernous nerve), but currently there are no sensitive and direct neurophysiologic tools to test on it. Electrophysiological tests including BCR, PTSSEPs and PDEPs were used to diagnose neurogenic ED in our study based on the following considerations: (1) while these tests mainly evaluate somatic nerve functions, there is increasing evidence in literature that the autonomic and somatic functions are anatomically and physiologically connected in the pudendal nerve (25, 26), therefore, abnormal outcomes of these tests particularly BCR and PDEPs can indirectly reflect the insufficiency of cavernous nerve mediated erection; (2) they are widely used for assessing neurological alterations related with ED in literature reports (20, 21, 27), for example, neurophysiological testing including BCR, PTSSEPs, PDEPs etc was used to detect peripheral neuropathy in ED patients (20). BCR was performed to predict the response of ED patients following radical prostatectomy to sildenafil citrate (28), BCR testing was used to detect neurogenic impotence in patients

after radical prostatectomy (3, 29) no other neurological pathologies that are closely related with ED such as diabetic neuropathy, hypertension, hypercholesterolemia etc were detected in all the patients enrolled in our study, therefore, abnormal neurophysiologic findings must be pelvic fracture related. The detected rate of abnormal BCR (76.1%) was much higher than abnormal PTSSEPs (37.3%) and PDEPs (29.9%) among neurogenic ED which is expected, since the afferent and efferent nerve of BCR test are pudendal nerves.

Duplex ultrasonography is the most reliable and less invasive diagnostic modality for assessing ED. The most important parameters are the peak systolic velocity (PSV) and end-diastolic velocities (EDV) measured in the central penile arteries. PSV equal 35cm/sec or greater indicates normal arterial response to adequate pharmacological stimulation, whereas PSV below 25cm/sec indicate arterial insufficiency; intermediate values are not specific (30). The EDV and the corresponding semi-quantitative measurement of the RI may be informative about penile veno-occlusion. An EDV >5cm/sec combined with a normal arterial response is accepted as the measurement at which a venous leak is present (31). In our study, Bimix of 15mg papaverine and 1mg phentolamine was injected intracavernously to relax the vasculature as other reports (10), and the same evaluation criterion as above mentioned was applied; we found that ED caused by pelvic fracture had a vasculogenic etiology in 45.8% (55 of 120) of patients, of them 12.7% were subclassified as arteriogenic, 56.4% venogenic and 30.9% arteriovenogenic. Penile venous leakage occurred in 5 patients (Figure-3).

There are some limitations in our study. First, since invasive arteriography could not be accepted by most patients in our department, we could not determine whether the patients who showed a normal penile vascular response in ultrasound suffered extensive arterial lesions in the pudendal axis. Second, the three electrophysiological tests used in our study only accessed the large fiber functions (mainly A_{β}or A_{δ}); a careful study of neurogenic etiology in ED must necessarily assess the small fiber (c-fiber) pathways which might be detected with heat stimuli or capsaicin. These points might be further resolved in our future studies.

CONCLUSIONS

ED is very common in patients with pelvic fractures associated with urethral injury. Neurovacular injuries contribute to the occurrence of ED. The neurogenic factor is the main etiology in relation to the vascular factor. Venous impotence is more common than arteriogenic ED. Most of the neurogenic ED patients had abnormal BCR outcomes. Our findings provide a detailed profile for the etiology of ED in patients after pelvic fracture.

ACKNOWLEDGEMENTS

This work was supported by the Second Hospital of Shandong University, P.R.China.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Mingzhen Yuan, Pro.
Department of urology
The second hospital,Shandong University
beiyuan street No.274
Jjinan, 250033, China
E-mail: yuanmingzhen2005@126.com

ORIGINAL ARTICLE

Vol. 41 (5): 967-974, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0386

Safety and efficacy of low intensity shockwave (LISW) treatment in patients with erectile dysfunction

A. Ruffo [1], M. Capece [1], D. Prezioso [1], G. Romeo [1], E. Illiano [1], L. Romis [2], G. Di Lauro [2], F. Iacono [1]

[1] Department of Urology, Federico II University, Naples, Italy; [2] Department of Urology, Hospital Santa Maria delle Grazie, Naples, Italy

ABSTRACT

The primary goal in the management strategy of a patient with ED would be to determine its etiology and cure it when possible, and not just to treat the symptoms alone. One of the new therapeutic strategies is the use of low intensity extracorporeal shockwave (LISW) therapy. The mechanism of shockwave therapy is not completely clear. It is suggested that LISW induces neovascularization and improvement of cavernosal arterial flow which can lead to an improvement of erectile function by releasing NO, VEGF and PCNA.

Materials and Methods: 31 patients between February and June 2013 with mild to severe ED and non-Phosphodiesterase 5 inhibitors responders were enrolled. Patients underwent four weekly treatment sessions. During each session 3600 shocks at 0.09mJ/mm^2 were given, 900 shocks at each anatomical area (right and left corpus cavernosum, right and left crus). Improvement of the erectile function was evaluated using the International Index of Erectile Function (IIEF-EF), the Sexual Encounter Profile (SEP) diaries (SEP-Questions 2 and 3) and Global Assessment Questions (GAQ-Q1 and GAQ--Q2).

Results: At 3-month follow-up IIEF-EF scores improved from 16.54±6.35 at baseline to 21.03±6.38. Patients answering 'yes' to the SEP-Q2 elevated from 61% to 89% and from 32% to 62% in the SEP-Q3. A statistically significant improvement was reported to the Global Assessment Questions (GAQ-Q1 and GAQ-Q2).

Conclusion: In conclusion, we can affirm that LISW is a confirmed therapeutic approach to erectile dysfunction that definitely needs more long-term trials to be clarified and further verified.

ARTICLE INFO

Key words:
Erectile Dysfunction; Therapeutics; Lithotripsy

Int Braz J Urol. 2015; 41: 967-74

Submitted for publication:
October 30, 2014

Accepted after revision:
April 25, 2015

INTRODUCTION

Erectile dysfunction (ED) is the main complaint in male sexual medicine and is defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance. Although ED is a benign disorder, it may affect physical and psychosocial health and may have a significant impact on the quality of life (QoL) of patients and their partners (1).

ED seems to affect 52% of 40-70-year-old men (2). Advances in basic and clinical research on ED during the past 15 years have led to the development of a variety of new treatment options, including pharmacological agents for intracavernous, intraurethral, and oral use and the use of vacuum erection devices (1).

Oral therapies have changed the diagnostic and therapeutic approach to ED becoming a major tool in treating ED. In fact, phosphodiesterase-5

inhibitors (PDE5-i) in the late 1990s and early 2000s completely revolutionized the field of sexual medicine becoming the most popular treatment and the first-line monotherapy for ED (3).

Unfortunately, they are limited for being used before the sexual act and do not modify the physiologic mechanism of penile erection (4).

After the initial enthusiasm of the use of the PDEi, the psychological impact–artificiality of erections and planning for sexual intercourse as well as a not proven curative effect (5) have slightly limited the use of these drugs, leaving the field open to the development of new therapies to treat or maybe cure patients with ED. Furthermore, the frequently reported side-effects of PDE5i, such as headache, dyspepsia, muscular pains, and hot flushes can affect a normal sexual intercourse (6).

The primary goal in the management strategy of a patient with ED would be to determine its etiology and cure when possible, and not just the treatment of symptoms. One of the new therapeutic strategies is the use of low intensity extracorporeal shockwave (LISW) therapy.

Shockwaves (SWs) are longitudinal acoustic waves that travel in the speed of water in ultrasound through body tissue and that carry energy (7). SWs have been widely used in urology to treat urinary stone disease (8), and less often in Peyronie's disease (9) or chronic pelvic pain syndrome (CPPS) in males (10).

The mechanism of action of low-intensity shock waves (LISW) is still not very clear. Many authors suggested that LISW improves erectile function increasing cavernous blood flow and inducing a neovascularization (11). Neovascularization is promoted by the expression of angiogenesis-related growth factors, such as endothelial nitric oxide synthase (NOS), vascular endothelial growth factor (VEGF), and endothelial cell proliferation factors, e.g., proliferating cell nuclear antigen (PCNA) (12).

The aim of our study is to evaluate the improvement of erectile function after therapy with LISW in men affected by mild to moderate ED.

MATERIALS AND METHODS

Study population

31 patients between February and June 2013 with mild to severe ED, and non-Phosphodiesterase 5 inhibitors responders were assessed for this study. Only 2 (6.4%) underwent treatment with PDE5-i in the last four weeks before starting the treatment (Table-1). They all signed an informed consent.

Inclusion criteria were: good general health, ED for at least six months, IIEF-EF between 7 to 24 (=mild to moderate).

Exclusion criteria included: neurological pathology, past radical prostatectomy or extensive pelvic surgery, recovering from cancer during the last year, any unstable medical, psychiatric disorder, spinal cord injury, penile anatomical abnormalities, clinically significant chronic hematological disease, anti-androgens or radiotherapy treatment of the pelvic region.

The medical and psychosexual history of all patients were evaluated at baseline to detect comorbidities. Table-2 summarizes the patients' organic co--morbidities: cardiovascular diseases in 7 pts (22%), hypertension in 18 pts (58%), diabetes in 12 pts (38%) and abnormal total serum cholesterol in 13 pts (41%).

Table 1 - The pretreatment characteristics of population.

Variable	Patients	P value
Age (years)		0.39
Mean±SD	59.93±12.16	
N.of subjects analysed	31	
Time suffering from ED (yrs)		0.50
Mean±SD	3.66±4.57	
N.of subjects analysed	31	
Treatment with PDE5-I in the last 4 weeks (%)	6.45	0.12
Proportion	2/31	

Table 2 - Analysis of self-reported measures at baseline , 1-month and 3-month follow up by treatment cohort.

Variable	Baseline	Follow-up 1 month	p value	Follow-up 3 months	P value
IIEF – EF	16.54±6.35	21.13±6.31	P=0.0075	21.03±6.38	p=0.0096
SEP-Q_2 (%)	61 (yes)	86 (yes)	P=0.0292	89 (yes)	P=0.0112
	38 (no)	13 (no)		10 (no)	
		2 drop-out			
SEP-Q_3 (%)	32 (yes)	58 (yes)	P=0.0402	62 (yes)	P=0.0207
	67 (no)	41 (no)		37 (no)	
		2 drop-out			

(IIEF-EF): International Index of Erectile Function; **(SEP-Q2):** Sexual Encounter Profile-Q2; **(SEP-Q3):** Sexual Encounter Profile-Q3

Study design

This is a pilot clinical study evaluating safety and efficacy of LISW treatment (performed with Renova ®) on symptomatic ED patients versus baseline.

Study schedule

a) screening

Patients were visited (visit 1) and those who were using PDE5-i had to go to a flush-out period of three weeks before starting the treatment. Furthermore, they committed to refrain from usage of PDE5-i during the duration of the treatment session.

b) Treatment

Patients underwent four weekly treatment sessions. During each session 3600 shocks at 0.09 mJ/mm2 were given. Shocks were applied at the penis shaft at right corpus cavernosum and left corpus cavernosum, right crus and left crus, 900 shocks at each area.

The treatment areas were the same for every session, so that at the end of the full treatment (four sessions) each area received 3600 shocks at an average 0.09mj/mm. We used this protocol under the guidance of Direx Group LTD.

LISW utilize low energy-0.09mJ/mm^{2-} -equivalent to 10% of the energy used by conventional kidney stone lithotripters in the treatment of urinary tract stones. This device generates a low intensity shockwave focused along a line of 70mm and hence is able to apply shockwaves to the corpora cavernosa and crura effectively.

For the past 3 years, a similar LISW technique has been used in different sites using the same level of energy density to treat ED (13). Shockwaves are created by a special generator and are focused using a specially designed shockwave applicator apparatus. The shockwaves are delivered through the applicator covering the entire corpora cavernosa of the penis.

The treatment does not inflict pain and does not require any anesthesia or sedation.

Each session lasts approximately 30 minutes.

c) Primary efficacy objective

To evaluate the increase of number of points in the International Index of Erectile Function (IIEF-EF) questionnaire from baseline (visit 1) to 1 and 3 months after treatment regarding the severity of the symptoms according to minimal clinically important differences in the erectile function domain of the IIEF scale (14). The IIEF-EF was chosen as primary clinical efficacy assessment tool in this study. It has been reported to be brief and reliable, psychometrically sound, and easy to administer in both research and clinical settings. It is available (and cross-culturally validated) in 10 languages and demonstrates adequate sensitivity and specificity for detecting treatment-related changes in erectile function (15).

d) Secondary efficacy objective

To study the clinical efficacy of LISW in terms of improvement in sexual activity leading to optimal penetration at 1 and 3 months post--treatment by using the Sexual Encounter Profile

(SEP) diaries (SEP-Questions 2 and 3). Patients recorded efficacy information after each sexual encounter by answering the two yes/no questions of the test: SEP Question 2:''Were you able to insert your penis into your partner's vagina?'' and SEP Question 3:''Did your erection last long enough for you to have successful intercourse?''.

In addition, patients underwent further evaluation with the Global Assessment Question (GAQ) by answering the two yes/no questions of the test: (GAQ-Q1) ''Over the past four weeks has the treatment you have been taking improved your erectile function?'' and (GAQ-Q2) ''If yes, has the treatment improved your ability to engage in sexual activity over the past four weeks''.

Statistical analysis

Statistical analysis was performed by the program Statistical Package for Social Sciences for Windows, version 11.5.1 (SPSS Inc., Chicago, IL, USA), using X^2 test and T-student for categorical data comparisons.

RESULTS

All patients had mild to severe ED at least six months, were non PDE-5i responders, with a mean age of 59.93±12.16 years. Median follow-up was of 3 months (range 2-5 months). Global patient perceptions after treatment with LISW significantly improved. Indeed IIEF--EF score showed significant improvement (baseline 16.54±6.35 vs 21.13±6.31 after 1 month P=0.0075; baseline 16.54±6.35 vs 21.03±6.38 after 3 months p=0.0096) (Table-2; Figure 1-3). About 86% (P=0.0292) and 89% (P=0.0112) of patients answered with a positive answer to SEP Q2 question (''Were you able to insert your penis into your partner's vagina?'') 1 month and 3 months after treatment, respectively, versus 61% positive answers pre-treatment (Table-2). SEP Q3 question (''Did your erection last long enough for you to have successful intercourse?'') was answered positively by 58% (P=0.0402) 1 month after LISW treatment and 62% (P=0.0207) after 3 months. After 1 month of treatment there were two drop-outs (Table-2). Table-3 shows patients' satisfactions of treatment with GAQ-Q1 ("Over the past four weeks has the treatment you have been taking improved your erectile function?'') and GAQ-Q2 questions ("If yes, has the treatment improved your ability to engage in sexual activity over the past four weeks''). Regarding the individual answers for the GAQ questions, we noticed that 89% and 62% of patients at 1 and 3 months respectively answered "Yes" to the GAQ-Q1 while in the same period 79% and 76% of patients answered "Yes" to the

Figure 1 - IIEF-EF score at baseline and after 1 month follow-up

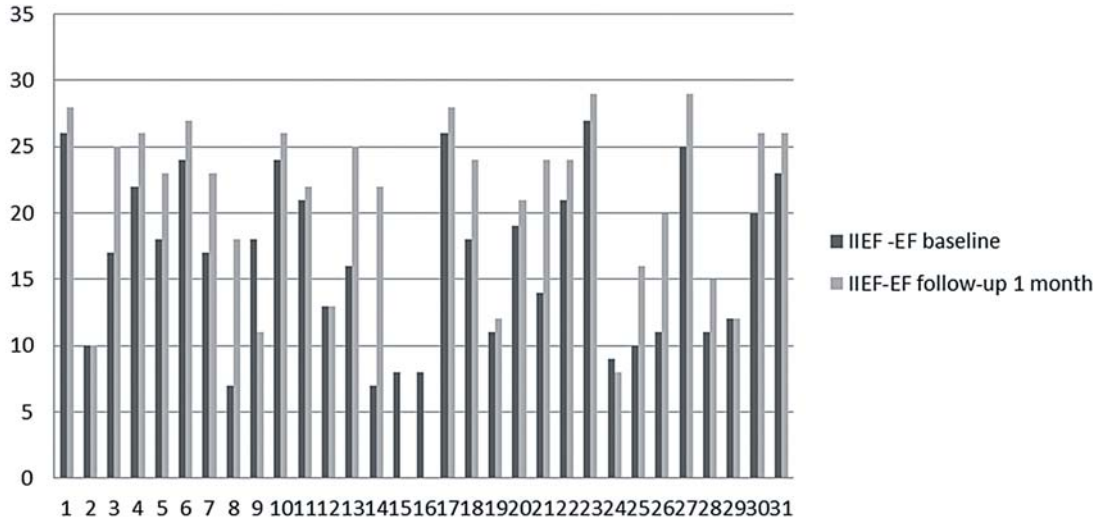


Figure 2 - IIEF-5 score at baseline and after 3 month follow-up

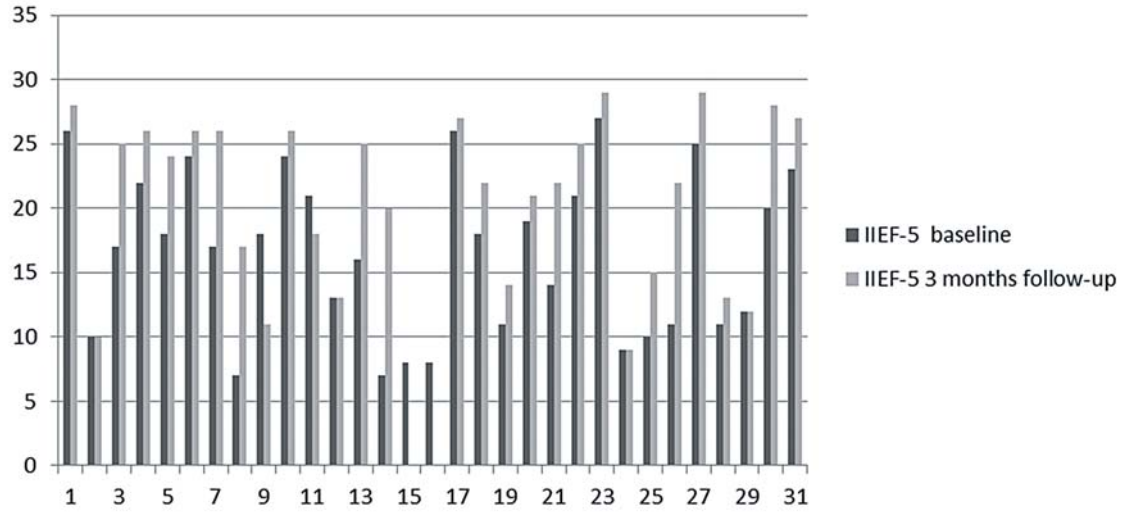
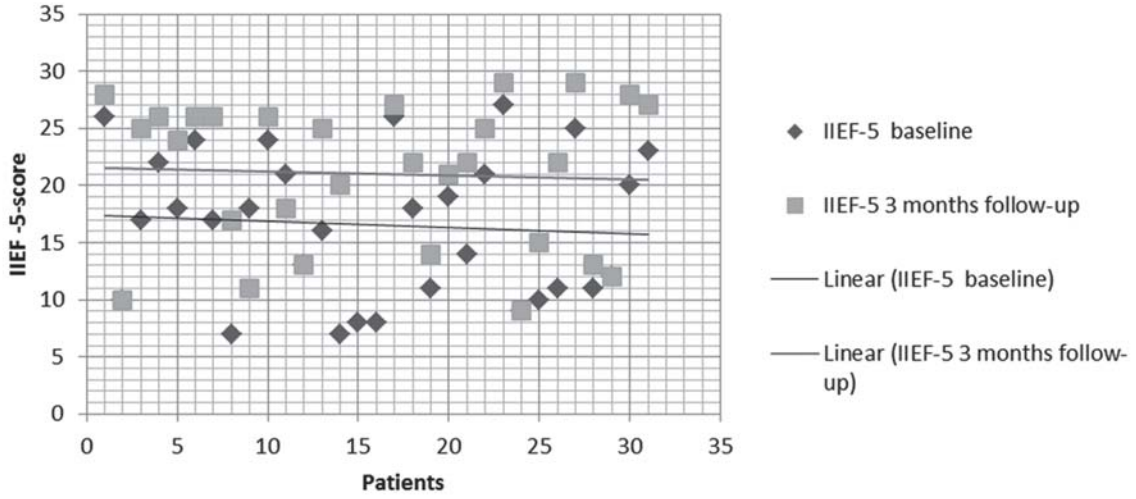


Figure 3 - Dispersion date IIEF score baseline and 3 month follow-up



GAQ-Q2 demonstrating success with the treatment (Table-3).

No adverse events were reported during and following treatment.

DISCUSSION

According to others author's data LISW appears to be significantly effective for increasing erectile function thanks to the improvement in penile hemodynamics (13, 11). By releasing neo--angiogenic factors and subsequent neovascularization of the treated tissue, LISW therapy leads to tissue regeneration (16). In fact, it has been shown that this low intensity energy acts on vascularization inducing a non-enzymatic production of physiologic amounts of nitric oxide (17). Nitric oxide (NO), the smallest known signaling molecule, is produced by three isoforms of NO synthase (NOS; EC 1.14.13.39). Neuronal NOS (nNOS, NOS I) is

Table 3 - Analysis of self-reported measures at 1-month and 3-month follow up by treatment cohort.

Variable	Follow-up 1 Month	Follow-up 3 Month	P value
GAQ-Q_1 (%)	89 (yes)	62 (yes)	P=0.141
	10 (no)	38 (no)	
	2 droup-out	2 droup-out	
GAQ-Q_2 (%)	79 (yes)	76 (yes)	P=0.7259
	20 (no)	24 (no)	
	2 droup-out	2 droup-out	

(GAQ-Q1): Global Assessment Question- Q1; **(GAQ-Q2):** Global Assessment Question- Q2

constitutively expressed in central and peripheral neurons and in some other cell types. Its functions include synaptic plasticity in the central nervous system (CNS), central regulation of blood pressure, smooth muscle relaxation, and vasodilatation via peripheral nitrergic nerves. Nitrergic nerves are of particular importance in the relaxation of corpus cavernosum and penile erection (18). In corpus cavernosum nNos-derived NO activates guanylyl cyclase which synthesizes cyclic GMP (cGMP) from GTP which in turn is the basis for the pro--erectile function of PDE5 inhibitors (19).

The most important isoform is eNOS, which keeps blood vessels dilated, controls blood pressure, and has numerous other vasoprotective and anti-atherosclerotic effects inhibiting DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells as well as smooth muscle cell migration. eNOS is mostly expressed in endothelial cells and synthesizes NO in a pulsatile manner (20).

eNOS appears to be a homeostatic regulator of numerous essential cardiovascular functions: in fact, eNOS-derived NO causes vasodilation in all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells that regulates the activity of calcium channels as well as intracellular contractile proteins that affect the relaxation of corpus cavernosum smooth muscle (21). Qiu et al. reported that LISW can partially ameliorate Diabetes Mellitus (DM)-associated ED in rat model by promoting regeneration of nNOS-positive nerves, endothelium, and smooth muscle in the penis. These beneficial effects appear to be mediated by recruitment of endogenous mesenchymal stem cells (MSCs) (22). Wang and colleagues discovered that LISW stimulates the expression of angiogenesis-related growth factors, such as endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF), and endothelial cell proliferation factors, such as proliferating cell nuclear antigen (PCNA).

The eNOS and VEGF began to rise in as early as one week and remained high for 8 weeks, then declined to baseline in 12 weeks; whereas the increase of PCNA and neo-vessels began in 1 week and persisted for 12 weeks and longer (12).

The effect of LISW on intracellular VEGF levels in human umbilical vein endothelial cells (HUVECs) has also been reported by Nishida et al. (23), who found that LISW significantly increased the expression of VEGF mRNA and its receptor, Flt-1. Their studies on the effects of LISW on a porcine model of chronic myocardial ischemia also showed that VEGF expression was significantly upregulated in the ischemic myocardial cells after treatment inducing neovascularization and improving myocardial perfusion (24).

Furthermore, it has been proved that SW therapy improved symptoms and myocardial perfusion in patients with severe coronary artery disease without any complications or adverse effects (24-26).

Regarding erectile dysfunction, Vardi et al. have been the first ones to believe in the use of

LISW to improve male sexual function (27). In the first randomized, double-blind, sham-controlled study, they demonstrated a positive short-term clinical and physiological effect on the erectile function of men who respond to oral PDE5Is (28). In another trial they reported an improvement in penile hemodynamics and endothelial function, as well as IIEF-EF domain score in severe ED patients who were poor responders to PDE5Is.

In this paper we demonstrated the efficacy of LISW in the medical management of ED. Our data show a statistically significant improvement of IIEF-EF score (5 points) and an increase of SEP and GAQ scores after treatment.

Limitations of this study are the lack of a sham controlled arm and the relatively low number of participants.

CONCLUSIONS

LISW has a well-documented positive clinical and physiological effect on erectile function. The preliminary data at 1 and 3 months follow-up are very encouraging and indicate a therapeutic success of this second generation technology for treating ED with linear low-intensity shockwaves. We also noticed that this treatment is feasible and easy to administer and with no side effects reported. Clearly, we cannot assure the long-term efficacy of LISW, so further studies are needed in order to strengthen these results and to assess whether is possible to repeat cyclically the treatment.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Antonio Ruffo, MD
Department of Urology
University of Naples Federico II, Naples, Italy
Via S.Pansini 5
CAP 80100, Naples, Italy
Telephone: + 39 081 746-2607
Email: antonio.ruffo7@gmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 975-981, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0544

Clinical profile of 93 cases of 46, XY disorders of sexual development in a referral center

Bianca Costa Mota [1], Luciana Mattos Barros Oliveira [2], Renata Lago [3], Paula Brito [2], Ana Karina Canguçú-Campinho [1], Ubirajara Barroso [4], Maria Betânia Pereira Toralles [5]

[1] Universidade Federal da Bahia - Hospital Universitário Professor Edgard Santos, Laboratório de Pesquisa em Infectologia (LAPI), Salvador, BA, Brasil; [2] Universidade Federal da Bahia, Salvador, BA, Brasil; [3] Departamento de Pediatria, Universidade Federal da Bahia, Salvador, BA, Brasil; [4] Departamento de Urologia, Universidade Federal da Bahia, Salvador, BA, Brasil; [5] Departamento de Genética, Universidade Federal da Bahia, Salvador, BA, Brasil

ABSTRACT

The term DSD refers to disorders that affect the normal process of sexual development causing disagreement between chromosomal, gonadal and phenotypic sex, and this study aimed to describe the clinical profile of a group with DSD 46, XY joined on DSD Clinic of Hospital of Salvador, Bahia Clinics. It was a retrospective study of medical records of survey data of 93 patients with DSD 46, XY. Among the patients studied 50.5% had no defined etiology and 20.4% had androgen insensitivity syndrome (AIS), 63.4% had been initially recorded in males, 31 (33.3%) in females, being that in two it was necessary to reassignment. All patients with complete AIS pure gonadal dysgenesis and had female genitalia. Others have been diagnosed with genital ambiguity or severe hypospadias and cryptorchidism. The gonads were palpable at the first consultation in 75.3% of patients. It is important to establish an active surveillance program for these patients. The first assessment took place before the age of ten in more than 50% of cases, which shows that much needs to be done for medical education and community about the DSD. Because the phenotypic variability of sexual development disorders was noted that the clinical profile of patients studied ranged between different etiologies, including hindering the diagnostic conclusion of these individuals.

ARTICLE INFO

Key words:
Epidemiology; Endocrinology; Pediatrics; Cryptorchidism

Int Braz J Urol. 2015; 41: 975-81

Submitted for publication:
October 29, 2014

Accepted after revision:
December 15, 2014

INTRODUCTION

Individuals with 46, XY karyotype and disagreement between external genitalia and gonadal sex are classified as individuals with 46, XY disorders of sexual development (DSD). Most of these patients have a autosomal recessive pattern of inheritance linked to X chromosome (1, 2).

Patients with 46, XY DSD have lower virilization of genitals compared to normal 46, XY individuals. Etiology may be associated to hypoplasia of Leydig cells, enzyme disturbances of testosterone synthesis, deficit of 5-alfa-reductase enzyme (DEF5α), testicular regression syndrome, gonadal disgenesia (GD), anorquia, androgen insensitivity syndrome (AIS) or ovotesticular DSD (3).

At birth, patients with 46, XY DSD show an array of external genitalia patterns, from a male looking phallus to an almost normal female genitalia with slight increase of clitoris. Testes may be abdominal or at inguinal region. The development of genitals will depend on the capacity of testos-

terone synthesis of testicles, of the transformation of testosterone in dehydrotestosterone (DHT) by 5-alpha-reductase enzyme or of the presence of receptors sensitive to testosterone. The diagnosis of patients with 46, XY DSD is mainly clinical and laboratorial (4) and the treatment requires a multidisciplinary approach in order to determine the social sex. Besides, these individuals may be referred to certain surgical procedures and hormonal treatment (3, 5, 6).

Initial clinical approach must evaluate the physical characteristics of the individual genitalia. In the presence of ambiguous genitalia (single urethral orifice at the basis of the phallus, non-palpable gonads or presence of gonads at the inguinal region) DSD must be suspected and the urologist must perform a karyotype exam to determine genotypic sex, as well refer the patient to endocrinologists and psychologists (4).

Most published literature regarding DSD is related to patients with XX, DDS rather than XY, DDS. This is a very interesting group of patients, since the presence of chromosome Y and testicles lead to several phenotypes, and adaptation to gender designation (male or female) and or sexual satisfaction may be troublesome. There is a trend that these patients, due to the presence of Y chromosome and probable cerebral masculinization, be characterized as males. However, patients with CAIS (complete androgen insensivity syndrome) and pure gonadal disgenesia (GD) present a better adaptation to female sex (2). The objective of the present paper is to describe the epidemiological and clinical profile of patients with DSD 46, XY syndrome followed in a tertiary referral center ambulatory.

MATERIALS AND METHODS

This is a retrospective study (approved by the ethical committee, protocol 024/2007) that included the review of the charts of all patients with diagnosis of DSD 46, XY followed in the ambulatory of a tertiary center of disorders of sexual development (state of Bahia, Brazil). Data collection was completed from March to September 2013.

Etiologic diagnosis was based upon clinical and laboratory tests: hormonal profiles, cytogenetic analysis, clinical evaluation of genitalia and pathological exam.

That ambulatory attends referred patients with signs of DSD. At present, it attends 341 patients. Epidemiological data included: birth date, age at first consultation, similar cases among relatives, living at the capital or inner region of Bahia State, sex creation, change of civil registration, age at last consultation, reason of referral and loss of ambulatory follow-up. Abandonment of ambulatory follow-up was considered for those that had not returned for consultation for more than five years.

Clinical aspects included: a description of genitalia according to the size of the phallus, number of orifices, localization of gonads, labioscrotal status and established etiological diagnosis; it was used an Investigative Protocol of 46, XY DSD proposed by the institution. Data were distributed and categorized for each patient in an Excel spread sheet. Target population included 110 elected individuals and the descriptive statistical data were calculated (average, median, interquartile interval for age at diagnosis) and two stratified analyses were proposed. Inferential statistics were not calculated (hypothesis test and confidence interval) since this was a study that covered the whole population.

RESULTS

Among 93 patients with syndromic diagnosis of DSD 46, XY, 47 (50.5%) had no defined etiologic diagnosis; 19 (20.4%) had AIS (androgen insensitivity syndrome) and 10 PAIS (partial androgen insensitivity syndrome), 16 (17.2%) presented DEF5α, 7 (7.5%) GD (4 pure and 3 mix) and 4 (4.3%) had ovotesticular DSD. 52 of all patients (55.9%) lived outside our city.

Among those 93 patients, 59 (63.4%) had been initially registered as males, 31 (333%) females and there were 3 newborns (3.2%) without civil registration. Initial designation of female sex was observed in 9 (100%) patients with CAIS, 4 without diagnosis, 4(100%) in patients with pure GD, 1 (33.3%) with mix GD, 11 (68.7%) with DEF5α, 1 (25%) with ovotesticular DSD and 1 (10%) with PAIS. In two of these patients it was necessary

redesignation of sex. One patient without defined diagnosis was initially considered female and after orthophallusplasty and orchipexy at 13 years old had his civil registration altered to male. Another patient with DEF5α, initially registered as a girl, was submitted at 21 years of age to a masculinizing genitoplasty and had also his civil registration altered to male.

Median age of patients at first consultation was 1 year and 10 months (1.8 years), varying from 4 days to 28 years of age, with interquartile intervals of 7.3 years; 56 patients were referred to ambulatory (60.2%) after the first year of age. The diagnosis of patients with pure GD and CAIS was established in a median age of 7 years and 3 months (varying from 6 months to 27 years). Among those with pure GD 3 (75%) attended the first consultation with more than 14 years old and those 9 patients with CAIS, 2 (22.2%) had more than 12 years old. Among other etiologies, median age was 1 year and 4 months (varying from 11 days old to 29 years), among individuals without defined diagnosis 8 (17%) was more than 12 years at first consultation, among those with DEF5α 1 (20%) was more than 15 years old, with PAIS 1 (10%) was 11 years old and all those with mix GD and ovotesticular DSD were less than 10 years old.

Family history of the occurrence of the same type of DSD was positive in 14 patients (15.05%): 5 (35.7%) had CAIS, 1 (7, 1%) had PAIS, 2 (14.3%) DEF5α, 1 (7.1%) mix GD and 5 (35.7%) had no defined diagnosis. Consanguinity was found in 8 patients: 3 (37.5%) with pure GD, 2 (25%) with DEF5α, 1 (12.5%) with PAIS. Two (25%) had no definitive etiology.

In relation to the aspect of genitals, all patients with CAIS and pure GD had female genitalia. All other were diagnosed with ambiguous genitalia or severe hypospadias and criptorquidism. In patients with mix GD, the median of the size of the phallus at diagnosis was 5cm (varying from 2 to 5.5 cm). In 2 patients, this information was not recorded. Single genital orifice was found in 65 patients (69.9%): 5 were considered boys, 12 as girls and 3 had no information. There were 26 patients with two genital orifices (27.9%) (19 considered boys and 7 girls, and 2 had no information).

Gonads were palpated at first consultation in 70 patients (75.3%). Among these, 32 were unilateral at the inguinal region and 6 were bilateral, at the inguinal region and at the scrotum. All others were in other regions, such as only in the scrotum, abdominal region or the information was not available. Table-1 shows the distribution of palpable gonads among groups with DSD 46, XY.

A total of 35 patients (35.5%) were followed-up. Graph-1 presents the frequency of abandonment of patients of the ambulatory according to etiological diagnosis.

DISCUSSION

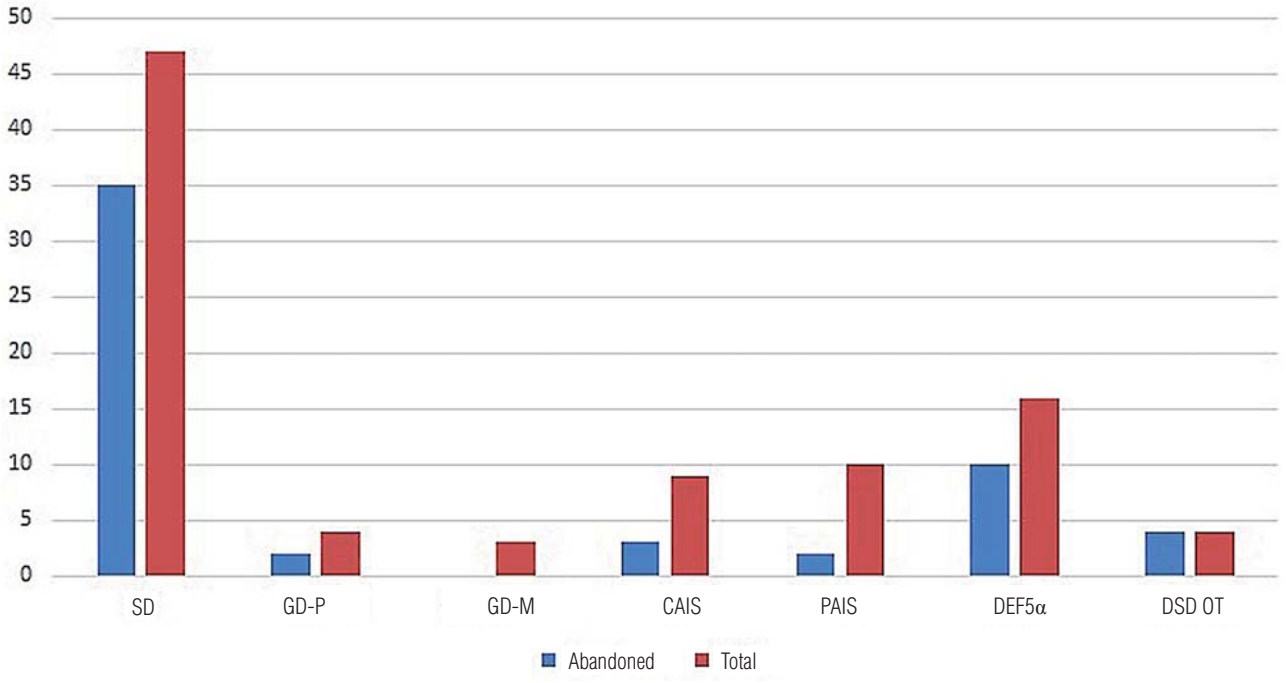
Among all our patients with defined diagnosis, 44% presented AIS, and this was the most

Table 1 - Palpaple gonads in different groups of patients with 46, XY DSD.

Etiology	Palpaple gonads			Not informed	Total
	Bilateral	Unilater	No	-	
Without diagnosis	26	09	12	-	47
DEF 5α	10	03	03	-	16
CAIS	05	02	02	-	09
PAIS	07	02	01	-	10
GD	-	-	04	-	04
Mix GD	01	01	01	-	03
Ovotesticular DSD	-	03	-	01	04

GD = Gonadal disgenesia; **CAIS =** Complete androgen insensitivity syndrome; **PAIS =** Partial androgen insensitivity syndrome; **DEF5α =** Deficiency of 5α reductase; Ovotesticular DSD-ovotesticular disturbance of sexual develpment.

Figure 1 - Distribution of patients by diagnosis DSD 46, XY HUPES.



SD=No Diagnosis; **GD-P=**Gonadal dysgenesis pure; **GD-M=**Gonadal dysgenesis mixed; **CAIS=**Complete androgen insensitivity syndrome; **PAIS=**Partial androgen insensitivity syndrome; **DEF5α** =efficiency 5α–redutase; **DSD OT=**Ovotesticular disorder of sex development

frequent diagnosis. Abdullah et al. (7) analyzed 45 cases of 46, XY DSD and 48% had AIS, showing that this condition is the commonest among individuals with 46, XY DSD. Laino et al. (8) studied 46 individuals with 46, XY DSD and 29 (63%) had deficiency of synthesis or androgenic action, 15 (51.7%) showed mutation of the gene of androgenic receptor, suggesting that most had AIS. In the present study, 50% had no defined diagnosis. Other authors agree that many patients with 46, XY DSD are not diagnosed (7, 9, 10). Even with the more available molecular tests that aid the diagnosis of these patients, not always they reach a diagnosis, and, besides very expensive, are not easily available in clinical practice (11, 12).

59 patients (63.4%) were registered as males, 31 (33.3%) were raised or registered as females, two had their civil registration altered to male. Andrade et al. (13) described the clinical profile of 62 patients with DSD, and 36 of them were registered as males, and among the 28 patients with X, Y karyotype, only 2 had a female civil registration. Brandão (14) studied 50 patients with DSD 46, XY and 66% were defined as males. Such studies demonstrate that most patients with DSD 46, XY are initially registered as males. All patients with CAIS in our study were registered as females according to their phenotypes and this was confirmed in other researches (10, 15-20).

DEF5α type 2 is a condition with a wide variety of phenotypes, from typical female external genitalia (pseudovaginal hypospadias perineal-scrotal) to a typical male phenotype, without any sign of this condition (11, 21-23). When the patient is considered and designed as female, during puberty it can occur virilization and it may be necessary to redefine the sex as male (24-26). Dessouky et al. study (27) presented 186 cases of DSD 46, XY, and 31 had changed their gender to male and this fact was more frequent among those with DEF5α (52%). Cassia et al. (28) studied 96 cases of DSD 46, XY patients and showed that

20% changed their gender to male. In the present study, among the 16 patients with DEF5α, 11 were registered as females and one of them altered his registration to male. Due to the higher possibility of incongruity of gender definition among individuals with DEF5α, it is more often recommended to designate these individuals as males. Other studies must be carried out in order to confirm that hypothesis in patients with minimal virilization.

Individuals with 46, XY karyotype, disagreement between external genitalia, gonadal and chromosomal sex are classified as carriers of DSD 46, XY syndrome. In those cases, it is necessary an early recognition of the disease, referral to specialists, laboratory tests and surgeries to optimize long term results (2). Treatment of DSD 46, XY individuals requires a multidisciplinary approach and they must be followed along with their families to verify their satisfaction with the gender they are been raised (5, 6). However, although we could count on professionals such as geneticists, urologists, endocrinologists and psychologists, the collected data demonstrated the difficulty to stablish a diagnosis and to follow-up those patients. Although many of them are born with genital abnormalities, only 42% are referred to consultation with few months of life. Abdullah et al. (11) showed that more than 60% of patients with DSD 46, XY are referred to clinical consultation up to one year of age. On the other hand, patients with GD were those who attended the ambulatory more lately in life. Gomes (29) studied 41 female patients with GD 46, XY and all searched medical attention in the second and third decades of life, with primary amenorrhea. Among patients with CAIS, 7 attended firstly the ambulatory with 0 to 9 years old, and 2 at 12 years of age. Late referral usually refers to patients with female phenotypic sex with symptoms of primary amenorrhea at puberty (30, 31). The most precocious diagnosis of CAIS usually occurs due to identification of a testicle during hernia repair surgery in a patient with a phenotypic female genitalia or through palpation of testicles at inguinal region. On the other hand, patients with pure GD are usually diagnosed due to primary amenorrhea or inguinal hernia repair surgery. In our series, several patients with ambiguous genitalia looked for specialized consultation only at puberty. Precocious diagnosis, right after birth, is fundamental, in order to provide medical and psychological counseling to the patient and relatives, to reduce suffering.

Familial history of similar cases were observed in 15.1% of our patients, more frequent in AIS cases (6 patients-42.8%) (1 PAIS and 5 CAIS). Dessouky (27) revised 317 charts of patients with DSD and showed that 210 had DSD 46, XY and among these 36 (17, 1%) had familial history, and higher incidence among patients with PAIS (47.2%) and DEF5α (27.7%). In a study of 33 cases of androgenic insensitivity syndrome, familiar history was positive in 70% of cases (17), this high prevalence of familiar history among patients with AIS may be due to the small number of studied patients in the casuistic and the single nature of the etiology. We suggest that this frequency may vary according to different etiologies but is higher in individuals with AIS.

In the present work, 10% of parents presented consanguinity. The incidence of consanguinity seem higher in countries that allow for endogamy, also, consanguinity is more prevalent in individuals with DSD 46, XY (7, 32). We could not find any paper that described consanguinity rate among patients with 46, XY DSD.

The limitations of the present study include: the retrospective characteristic and the high rate of abandonment of follow-up, and this may imply that the data here presented is related to a selected group of patients.

CONCLUSION

The disturbances of sexual development 46 XY are rare, and the most frequent etiology in the present study was AIS. The high rate of abandonment of ambulatory follow-up indicates the need for an active search of these patients. First evaluation occurred prior to 10 years of age in more than 50% of cases that warrants for education of medical and lay communities about DSDs. The main reason of referral was genital ambiguity. The clinical profile of patients varied according to etiology, with several phenotypes that

made diagnosis difficult. It is recommended to use complementary tools such as molecular biology to evaluate and follow-up patients with 46, XY DSD.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Bianca Costa Mota, MD
Universidade Federal da Bahia
Hospital Universitário Professor Edgard Santos,
Laboratório de Pesquisa em Infectologia (LAPI),
Rua Augusto Viana, snº
Salvador, BA, 40110-060, Brasil
Telefone: +55 71 8100-6036
E-mail: biancamota_biomed@hotmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 982-989, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0351

Are the urology operating room personnel aware about the ionizing radiation?

Adem Tok [1], Alparslan Akbas [2], Nimet Aytan [3], Tamer Aliskan [1], Izzet Cicekbilek [1], Mehmet Kaba [4], Abdulkadir Tepeler [3]

[1] Department of Urology, Faculty of Medicine, Bulent Ecevit University, Zonguldak, Turkey; [2] Department of Urology, Faculty of Medicine, Canakkale 18 Mart University, Canakkale, Turkey; [3] Department of Urology, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey; [4] Department of Urology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey

ABSTRACT

Purpose: We assessed and evaluated attitudes and knowledge regarding ionizing radiation of urology surgery room staff.
Materials and Methods: A questionnaire was sent by e-mail to urology surgery room personnel in Turkey, between June and August 2013. The questionnaire included demographic questions and questions regarding radiation exposure and protection.
Results: In total, 127 questionnaires were answered. Of them, 62 (48.8%) were nurses, 51 (40.2%) were other personnel, and 14 (11%) were radiological technicians. In total, 113 (89%) participants had some knowledge of radiation, but only 56 (44.1%) had received specific education or training regarding the harmful effects of radiation. In total, 92 (72.4%) participants indicated that they used a lead apron and a thyroid shield. In the subgroup that had received education about the harmful effects of radiation, the use ratio for all protective procedures was 21.4% (n=12); this ratio was only 2.8% (n=2) for those with no specific training; the difference was statistically significant (p=0.004). Regarding dosimeters, the use rates were 100% for radiology technicians, 46.8% for nurses, and 31.4% for other hospital personnel; these differences were statistically significant (p<0.001). No significant relationship between working period in the surgery room, number of daily fluoroscopy procedures, education, task, and use of radiation protection measures was found.
Conclusions: It is clear that operating room-allied health personnel exposed to radiation do not have sufficient knowledge of ionizing radiation and they do not take sufficient protective measures.

ARTICLE INFO

Key words:
Radiation Injuries; Minimally Invasive Surgical Procedures

Int Braz J Urol. 2015; 41: 982-9

Submitted for publication:
July 15, 2014

Accepted after revision:
January 26, 2015

INTRODUCTION

Recently, the use of open surgical interventions has decreased notably, due to technological developments, improvements, and an increase in the use of minimally invasive methods. Indeed, it has been reported at major medical centers that the ratio of open surgical interventions for urinary system stones is now as low as 1-4% (1). Shock wave lithotripsy, percutaneous nephrolithotomy, endoscopic ureter stone treatments, and retrograde intrarenal operations are frequently used minimally invasive treatment methods for ureter and kidney stones.

During these minimal invasive methods, imaging techniques (e.g. fluoroscopy, ultrasonography, computed tomography) are usually used as guidance. The most commonly used technique is fluoroscopy. However, a major disadvantage of fluoroscopy use is radiation exposure of the patient, surgeon, and other operating room personnel. As a result of this exposure to radiation, deterministic and stochastic effects (mutations and carcinogenesis) occur in the body (2). For this reason, the International Radiation Commission recommends not exceeding 20 mSv/year during a 5-year occupational period (3). The effects of ionizing radiation change depending on the radiation dose, duration, and whether and how much protection is used.

Preventive measures should be taken to protect against the effects of radiation. The most important include use of lead aprons, thyroid shields, and radiation-protective gloves and eyeglasses. Furthermore, it is important to use dosimeters to determine the cumulative amount of radiation exposure. With such information, it can be determined which subjects do not have enough protection against exposure to radiation (4). Attitudes and behavior of endourologists and other team members exposed to radiation during surgiry were evaluated by some investigators (4, 5).

Although operating room personnel are an important occupational group with increased risk of radiation exposure, there is no study specifically evaluating their awareness about this risk. In this study, we evaluated attitudes and behavior regarding ionizing radiation of urology operating room personnel.

MATERIALS AND METHODS

With the approval of the local ethics committee, a questionnaire survey was sent by e-mail to 183 operating room personnel (nurses, radiology technicians and other personnel) (age range 20-50 years) working in urology surgery rooms at various state hospitals, private hospitals, training research hospitals, and medical faculty hospitals between June and August 2013. The survey was prepared on the Google Docs™ website. Because of the characteristics of the software, the participants remained anonymous. Participants were informed that the results of the survey would be used for scientific purposes only and that their identities would not be determined or recorded.

The questionnaire included 13 questions. These concerned demographic informations, such as job, age, educational background, work duration in surgery rooms, and number of daily endourological cases and how many used fluoroscopy, whether a dosimeter was used, and, if so, whether periodic exposure measurements were made, whether the participant had an understanding of the harmful effects of radiation, whether they had received specific training about the harmful effects of radiation and about protective methods against radiation and which one(s) they used, and whether there was a radiation warning sign in areas where fluoroscopy was used.

The survey was self-administered and was not validated. After gathering the results of the survey, data were analyzed using the SPSS software (ver. 18.0).

RESULTS

In total, 127 (69.4%) participants completely answered and returned the questionnaire. Of the participants, 62 (48.8%) were nurses, 51 (40.2%) were other operating room personnel, and 14 (11%) were radiology technicians. The average age of responders was calculated as 32.0±5.9 (range 20-50) years. Regarding education, in order, 47 (37%) had a bachelor's degree, 30 (23.6%) had a 2-year associate degree, and 30 (23.6%) had completed high school. Other demographic information is provided in Table-1. Most of the participants were staff at university hospitals (31.5%) and training and research hospitals (40.9%). Most of the personnel indicated that they were involved in two (30.7%) or three (30.7%) endourological surgeries per day. In these cases, fluoroscopy use was typically once (34.6%) or twice (12.6%) per day. Regarding work experience in urology surgery rooms, 16 (12.6%) indicated that they had worked there less than 1 year, 70 (55.1%) between 1 and 5 years, 24 (18.9%) between 5 and 10 years, and 17 (13.4%) for more than 10 years. No significant relationship was found between period

Table 1 - Demographic characteristics of participants.

Questions	Answers	n	(%)
Job	Other personnel	51	(40.2%)
	Nurse	62	(48.8%)
	Radiological technician	14	(11%)
Mean age	32.01±5.9 years		
Education	Primary school	19	(15%)
	High school	30	(23.6%)
	2-years associate degree	30	(23.6%)
	Bachelor's degree	47	(37%)
	Postgraduate	1	(0.8%)
For how long (years) have you worked in a urology surgery room?	<1 year	16	(12.6%)
	1-5 years	70	(55.1%)
	6-10 years	24	(18.9%)
	>10 years	17	(13.4%)
Your corporation	Private Hospital	16	(12.6%)
	State Hospital	19	(15%)
	Training and research Hospital	52	(40.9%)
	University Hospital	40	(31.5%)

of working in the surgery room, number of daily fluoroscopy procedures, education, task, and use of protection from radiation.

For surgeries using fluoroscopic imaging, 59 (46.5%) participants indicated that they used dosimeters, and 54 indicated that they gathered monthly and yearly measurements. Although 50% of personnel working at university hospitals and 55.8% at training and research hospitals indicated that they used dosimeters, participants from private hospitals indicated that they did not (p=0.001).

The dosimeter usage rate was 100% for radiology technicians, 46.8% for nurses, and 31.4% for other operating room personnel; these differences were statistically significant (p<0.001). Of the participants, 113 (89%) reported having information about the harmful effects of radiation, but the number of participants who had been specifically educated about these effects was 56 (44.1%); the training rate was 100% for radiology technicians. Of the participants, 92 (72.4%) indicated that they used lead aprons along with thyroid shields. Protective measures are presented in Table-2. In the group who had received education about the harmful effects of radiation, the use ratio for all protective measures combined, that is, lead apron + thyroid shield + eyeglasses +l eaded gloves was 21.4% (n=12), compared to 2.8% (n=2) in the group without specific training; this difference was statistically significant (p=0.004). Of the 14 personnel who reported using all four protective measures, 11 were nurses.

Of the participants, 65 (51.2%) indicated that there was no warning sign in areas where radiation was used. Moreover, 25 (40.3%) participants who indicated that warning signs were present worked at training and research hospitals (p=0.005).

DISCUSSION

Ionizing radiation to which staff members are exposed during medical diagnostic interventions and treatments is a health issue, and the

Table 2 - Other questionnaire responses by participants.

Questions	Answers	n (%)	
What is the average number of endourology cases you attend daily?	1	7	(5.5%)
	2	39	(30.7%)
	3	39	(30.7%)
	4	18	(14.2%)
	>4	24	(18.9%)
Of the daily operations you attend, how many involve fluoroscopy?	1	44	(34.6%)
	2	40	(31.5%)
	3	27	(21.3%)
	4	9	(7.1%)
	>4	7	(5.5%)
Do you have dosimeter?	Yes	59	(46.5%)
	No	68	(53.5%)
Do you have monthly and yearly measurements from your dosimeter?	Yes	54	(42.5%)
	No	73	(57.5%)
Do you have an understanding of the harmful effects of radiation?	Yes	113	(89%)
	No	14	(11%)
Did you take specific training about the harmful effects of radiation?	Yes	56	(44.1%)
	No	71	(55.9%)
Which protective methods do you use against the effects of radiation?	Lead apron	13	(10.2%)
	Thyroid shield	4	(3.1%)
	Apron-Thyroid shield	92	(72.4%)
	Apron-Gloves	1	(0.8%)
	Apron-Thyroid shield-Gloves	1	(0.8%)
	Apron-Thyroid shield-Eyeglasses	2	(1.6%)
	Apron-Thyroid shield-Gloves-Eyeglasses	14	(11.0%)
Is there a radiation warning sign in the area(s) where fluoroscopy is used?	Yes	62	(48.8%)
	No	65	(51.2%)

harmful effects of radiation must be taken into consideration. Particularly, in recent years, because of the increasing number of endourological interventions, urologists have a key role in controlling exposure to ionizing radiation for themselves, other health personnel, and, indeed, their patients (6). The deterministic effects of ionizing radiation—that is, cell death and ultimately organ dysfunction in sufficiently large doses—are rarely encountered, even in those working with radiation. However, long-term exposure to low doses that do not cause immediate cell damage can modify cells

and result in stochastic effects (mutations and carcinogenesis). To minimize these effects, "ALARA" (as low as reasonably achievable) principles must be followed (7).

'It has been shown in many studies that most urologists are poorly aware of the radiation exposure to themselves, and their patients, and that insufficient precautions are commonly taken against radiation (4, 8, 9). Indeed, it has been reported that urologists and their assistants are exposed to considerably greater levels of radiation than are other operating room personnel (10). Although the dose of radiation exposure per case that personnel receive is lower than the level that surgeons receive, the cumulative level of radiation exposure may be higher for operating room personnel. Although statistically insignificant, nurses gave more importance to protective measures (11). This fact probably was due to nurses work closer to the radiation source than the radiology technicians and other personnel. In our study, although fluoroscopy was used commonly in urological procedures and the harmful effects of radiation are known generally by all personnel exposed, insufficient precautions were taken.

The use of appropriate protective equipment greatly reduces the harmful effects of ionizing radiation (6, 12). However, studies have shown that lead aprons, thyroid shields, leaded gloves, and eyeglasses are not commonly used in combination (4, 6). Also in this study, most of the participants who used protection used aprons and thyroid shields; the usage of gloves and eyeglasses was rare. The ratio of the combined use of apron + thyroid shield + eyeglasses + leaded gloves in personnel educated regarding radiation was 21.4%, compared to 2.8% in personnel without specific education (p=0.004). Training regarding the harmful effects of radiation can substantially increase the use of protective measures. Söylemez et al. reported that because most protective equipment was inappropriate ergonomically, it was little used (13).

According to Turkish Radiation Safety Regulations about radiation protection, doses cannot exceed the individual dose limits (14). Moreover, if the yearly dose could exceed 30% of the permitted level, personal dosimeters must be used. In this study, 46.5% of the participants (59 persons) used dosimeters; of them, 49 were from university and training and research hospitals. The less frequent use of dosimeters in state and private hospitals may be due to the less common performance of fluoroscopy or inadequate education about radiation and its affects in these institutions.

Although most of the participants (89%) in the survey reported some knowledge of the harmful effects of radiation, only 44.1% of them had received specific education regarding the harmful effects of radiation. Using inadequate protection against radiation is a result of inadequate education concerning the issue (4, 6). Radiation technicians attended endourological surgeries as part of their undergraduate and associate degree programs, with the expected results, presumably due to their education.

The use of appropriate warning signs in radiation areas is important for patients, relatives, and other health personnel working in these areas. Although warnings were displayed in many surgery rooms, inadequate care and insufficient preventative measures were being taken when entering such areas. Most of the participants indicated that they were involved in surgeries using fluoroscopy at least once or twice per day. However, despite the increase in the number of fluoroscopy procedures, there has been no corresponding change in the radiation protection methods used by surgery room personnel. Unwanted side effects of radiation exposure may be seen in surgery room personnel over the long term.

In the survey, fluoroscopy was performed frequently at university and training and research hospitals. This can be explained by the availability of appropriate equipment at these hospitals, as indicated in other studies (4). Although the physicians and their assistants involved differ among the surgical procedures performed in these centers, the allied health personnel tend to be identical, which causes them to be exposed to ionizing radiation often, if not continuously. However, because the radiation dose decreases with distance from the fluoroscopy source, this may be militate in favor of nurses and other personnel who are not directly involved in the surgery and so are more distant from the radiation.

The main limitation of this study is the small number of responders. Although only a small group of personnel working in different hospitals of Turkey were included in this study, we believe that further studies in larger populations in different age groups will provide more information about this specific topic. The other point is that the responders were young. This may be related to the fact that Internet usage is more common in young age population. Endourology procedures using fluoroscopy guidance have been more popular in recent years. Young urologists and operating room personnel are especially more involved in these procedures. Despite these limitations we believe that this study emphasize the importance of radiation protection for operation room personnel. To the best of our knowledge this is the first study evaluating knowledge and attitude of operation room personnel about radiation exposure in the literature.

CONCLUSIONS

The application of ALARA principles in areas where fluoroscopy is used is necessary and, indeed, essential for occupational health. However, surgery room personnel who are subjected to radiation exposure did not have sufficient information regarding ionizing radiation and did not take sufficient preventive measures. We consider that this was likely due to insufficient education. It is important that personnel who work with radiation in these departments receive training during their basic education or as a part of in-service training. Beyond this, the use of dosimeters and determination of exposure levels must be enforced by the authorities.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Adem Tok, MD
Department of Urology,
Faculty of Medicine, Bulent Ecevit University,
Kozlu, Zonguldak, Turkey
Telephone:+ 90 505 373-7138
E-mail: ademtok2003@yahoo.com

SURVEY
Survey regarding knowledge level of surgery room personnel about ionizing radiation

This form was prepared only to assess the level of knowledge of radiation of surgery room personnel in Turkey. The information obtained from this survey will not be used to criticise and/or accuse any individual or corporation of anything. The objective of this survey is to attract the attention of medical staff to an important issue, namely radiation exposure.

Job

- Other personnel ( )
- Nurse ( )
- Radiological technician ( )

Age ( )

Education

- Primary school ( )
- High school ( )
- 2-years associate degree ( )
- Bachelor's degree ( )
- Postgraduate ( )

For how long (years) have you worked in a urology surgery room?

- <1 year ( )
- 1-5 years ( )
- 6-10 years ( )
- >10 years ( )

Your corporation

- Private Hospital ( )
- State Hospital ( )
- Training and research hospitals ( )
- University Hospital ( )

What is the average number of endourology cases you attend daily?

- 1 ( )
- 2 ( )
- 3 ( )
- 4 ( )
- >4 ( )

Of the daily operations you attend, how many involve fluoroscopy?

1	( )
2	( )
3	( )
4	( )
>4	( )

Do you have dosimeter?

Yes	( )
No	( )

Do you have monthly and yearly measurements from your dosimeter?

Yes	( )
No	( )

Do you have an understanding of the harmful effects of radiation?

Yes	( )
No	( )

Did you take specific training about the harmful effects of radiation?

Yes	( )
No	( )

Which protective methods do you use against the effects of radiation?

Lead apron	( )
Thyroid shield	( )
Gloves	( )
Eyeglasses	( )

Is there a radiation warning sign in the area(s) where fluoroscopy is used?

Yes	( )
No	( )

ORIGINAL ARTICLE

Vol. 41 (5): 990-1001, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0422

Acellular human glans extracellular matrix as a scaffold for tissue engineering: in vitro cell support and biocompatibility

Fernanda M. Egydio [1], Luiz G. Freitas Filho [1], Kleber Sayeg [1], Marcus Laks [1], Andréia S. Oliveira [2], Fernando G. Almeida [3]

[1] Departamento de Cirurgia, Universidade Federal de São Paulo São Paulo, SP, Brasil; [2] Departamento de Nefrologia Universidade Federal de São Paulo São Paulo, SP, Brasil; [3] Deparamento de Urologia Feminina, Universidade Federal de São Paulo São Paulo, SP, Brasil

ABSTRACT

Objectives: Diseases of the genitourinary tract can lead to significant damage. Current reconstructive techniques are limited by tissue availability and compatibility. This study aims to assess if the decellularized human glans can be used as a biomaterial for penile reconstruction.
Materials and Methods: Samples of the glans matrices were descellularized. We evaluate the presence of collagen type I and III, and elastic fibers. Biocompatibility assays were performed to assess the cytotoxic and non-cytotoxic interactions between the acellular matrix and 3T3 cells. The matrices were seeded with mesenchymal stem cells and were assessed for viability and integration of these cells. Biomechanical tests in native tissue, descellularized matrix and seeded matrix were performed to characterize their biomechanical properties.
Results: The tissue architecture of the decellularized matrix of human glans was preserved as well as the maintenance of the biomechanical and biological properties. The analyzes of glans seeded with mesenchymal stem cells revealed the integration of these cells to the matrices, and its viability during two weeks "in vitro".
Conclusion: The decellularization process did not alter the biological and biomechanical characteristics of the human glans. When these matrices were seeded they were able to maintain the cells integrity and vitality.

ARTICLE INFO

Key words:
Extracellular Matrix; Materials Testing; Neoplasms

Int Braz J Urol. 2015; 41: 990-1001

Submitted for publication:
August 16, 2014

Accepted after revision:
May 24, 2015

INTRODUCTION

Congenital diseases, cancer, trauma, and other conditions can lead to significant glans damage requiring reconstruction. This is a major challenge because there are great limitations of available compatible grafts due to glans complex anatomical arrangement, and the psychological impact of its absence is usually very important. In addition, complications can occur when tissue reconstruction of glans is performed due to a transient ischemia caused by vascular impairment, which may even evolve to necrosis (1). In fact, all alternative surgical techniques described for tissue reconstruction result in an organ anatomically similar to a penis, but erectile function can almost never be restored, and the results, both functional and aesthetic, are thus considered inadequate (2-5).

This study aims to assess if the decellularized human glans can be used as a biomaterial for penile reconstruction, and whether the decellula-

rization process changes the biomechanical and biological characteristics of the matrices. Furthermore, we analyzed the integration of mesenchymal stem cells into decellularized matrices of human glans.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the local Research Ethics Committee (0878/09) and conducted in strict conformity with local institutional guidelines and with international standards for the manipulation and care of laboratory animals.

Obtaining the glans matrices

The matrices were obtained from the glans penis of human donor tissue for transplantation. Each fragment extended from the coronal region to the urethral meatus, maintaining the dorsal neuro-vascular bundle and was stored in a PBS solution (Phosphate-Buffered Saline) at 4°C, supplemented with penicillin 10,000 IU/L and streptomycin 50 mg/L.

Decellularization of human glans matrices

Dissection of the fragments exposed the deep dorsal vein of the penis, which was then isolated for insertion of a catheter through which 1200 mL of distilled water at 4°C was perfused. 500 mL of decellularization solution with 1% Triton X-100 and 0.1% ammonium hydroxide at 4°C was then perfused and the fragments were maintained in this solution for 7 days for complete removal of cellular components. The matrices were then perfused with 600 mL of distilled water and 300 mL of PBS and stored in glycerol 87% at 4°C.

Cell Culture of Mesenchymal stem cells

The mesenchymal stem cells were isolated from femurs and tibiae of male Wistar rats ranging in weight from 150-250 g. For the separation of the mononuclear fraction of bone marrow, the material was subjected to the Histopaque protocol (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 1800 rpm for 30 minutes, the mononuclear cells were selected from the interface established between the Histopaque and PBS, and were washed with sterile PBS. These cells were then cultured in 25 cm^2 polystyrene flasks with 5 mL of DMEM low glucose (Dulbecco's Modified Eagle's medium, DMEM Low Glucose - Sigma Chemical Company, St. Louis, USA), supplemented with 10% fetal bovine serum (FBS), and maintained at 37°C, in a humidified 5% (v/v) CO_2 in air atmosphere for 10 to 15 days. For each cell culture passage, the cells were subjected to trypsin-EDTA (Sigma Chemical Company, St. Louis, USA), seeded, and cultured until to obtain adequate cell number for the experiments.

Determination of cellularity

The decellularized glans samples were fixed in 10% buffered formalin, paraffin embedded, sectioned transversely (5µm), and stained with hematoxylin-eosin (H&E). Using a light microscope interfaced with a digital image analysis system, the samples were analyzed to confirm complete removal of the cellular components of the tissue. Transverse cross sections (10µm) of native tissue and decellularized glans matrix were blocked for 10 minutes with a solution containing PBS, 0.2% gelatin, and 0.1% saponin, incubated for 15 minutes with DAPI (4′,6-diamidine-2-phenyl indole dihydrochloride 0.1 mg/mL, Sigma), washed twice with PBS, and finally mounted in buffered glycerol with 0.1 M Tris/HCl, pH 8.8, and 0.01% p-fenilenodiamino. To detect the presence of intact cell nuclei, images were analyzed under a microscope SP5 Confocal Leica TS (200x), NA=0.7.

Histology evaluation

To characterize the preservation of biological properties of the decellularized glans matrices, some samples were fixed in 10% buffered formalin, paraffin embedded, sectioned transversely (5µm), and stained using the Verhoff-Von Gieson and Masson's Trichrome methods. Using a light microscope interfaced with a digital image analysis system, these samples were analyzed by assessing the preservation of collagen fibers by Masson Trichrome staining, and elastic fibers by Verhoff-Von Gieson staining, and comparing with the pattern found in the control group that corresponded to the glans tissue.

Cytotoxicity and biocompatibility evaluation

Cytotoxicity tests were performed using the direct contact method described by Ciapetti et al. (6) in order to detect potential lethal effects on the cells. A small amount of the decellularized glans samples were put directly on a monolayer of cultured cells covered with culture medium. To evaluate the biocompatibility of decellularized matrix glans, the following tests were performed: Metabolic Activity of Mitochondria (MTT), Lysosome Activity (Neutral Red) and quantification of genetic material (Violet Crystal). Previously the 96-well plates were seeded with a monolayer of mouse dermal fibroblasts 3T3, at a density of $1x10^3$ cells per well and incubated for 24 hours at 37°C, in a humidified 5% (v/v) CO_2 air atmosphere with DMEM supplemented with 1% FBS. The fragments of decellularized glans matrices were incubated in fibroblast cultures at 37°C, in a humidified 5% (v/v) CO_2, air atmosphere for 24, 48, and 72 hours. As a positive control for cytotoxic effects, dimethyl sulfoxide solution (DMSO) at 0.2% was used, and as a negative control, mouse fibroblast 3T3 cells without exposure to any external agent were used. All samples were tested in replicates of four wells (n=4), and the experiments were triplicated (n=12).

Seeding of mesenchymal stem cells on decellularized matrices

Fragments of the decellularized glans matrices measuring 5 x 5 x 2 mm were placed in each well of the 96-well plate. A cell suspension of mesenchymal stem cells containing $6.5x10^5$ cells/cm^2 was seeded under static conditions in each of the fragments. After 30 minutes, 500µL of DMEM supplemented with 10% FBS was added to each well. The culture medium was changed every 2-3 days to ensure sufficient nutrients for the cells. After 7, 14, and 28 days of the static seeding, histological and biomechanical evaluations of the matrices were performed.

In Vitro analysis of the decellularized glans seeded with mesenchymal stem cells

The seeded matrices after 7, 14, and 28 days cultured in vitro were fixed in 10% buffered formalin, paraffin embedded, sectioned transversely (5µm), and stained with H&E. Using a light microscope interfaced with a digital image analysis system, the samples were evaluated to check the integration in vitro of mesenchymal stem cells into the decellularized glans matrices. These same samples histologically processed were now sectioned transversely (10µm), blocked for 10 minutes with a solution containing PBS, 0.2% gelatin, and 0.1% saponin, incubated for 15 minutes with DAPI (4′,6-diamidine-2-phenyl indole dihydrochloride 0.1 mg/mL, Sigma), washed twice with PBS, and mounted in buffered glycerol with 0.1 M Tris, HCl pH 8.8 and 0.01% p-fenilenodiamino. The samples were analyzed to evaluate the integration of mesenchymal stem cells to the seeded glans matrices.

Biomechanical testing

Fragments measuring 15x5 mm were seeded with mesenchymal stem cells at a concentration of 6.5 x 10^5 cells/cm^2 and maintained in culture at 37°C, in a humidified 5% (v/v) CO_2 air atmosphere for 7, 14, and 28 days. Samples were then analyzed to evaluate the maintenance of biomechanical characteristics by seeded matrices. In biomechanical testing, specimens were tensioned longitudinally by a specific machine AME – 2KN (Oswaldo Filizola), pulling at both ends in opposite directions at a speed of 18 mm/min until breakage. The results were analyzed using software "DynaView Standard / Pro M". The maximum force and deformation to which each sample was subjected were measured at the rupture moment. As a positive control the native tissue of the glans was used, and as a negative control, the decellularized glans matrices were used. The elasticity modulus (E = the force required to deform a sample) is defined as the ratio of tension (T) and relative deformation (Rel.Def) in this tension.

E=T / Rel.Def

Tension (T) is the ratio between maximum force (F) and the area (SecA) of the sample. Sectional area is the product of width (W) and thickness (Tk). The relative deformation is the ratio of deformation (Df) and initial length (L0). The elasticity modulus can be rewritten as follows:

E = (F / W. Tk) / (Df / L0)

Statistical analysis

Statistical analyses are reported as mean value ± standard deviation. To evaluate the differences between groups, two-way analyses of variance (ANOVA) for repeated measures were performed, followed by post hoc Tukey tests. For the statistical analyses of biocompatibility testing, the following nomenclature was used: group effect and interaction time versus group. Group effect means the difference between the analyzed groups, and interaction time versus group means the variation of analyzed group over time. A p-value < 0.05 was defined as significant.

RESULTS

Histological evaluation

The standardized decellularization protocol used was effective in promoting the complete removal of cellular components of the tissue after 2 weeks (Figure-1).

Distribution of collagen and elastic fibers

No significant changes in the distribution of collagen were observed after the removal of the cells from native tissue. Elastic fibers were also preserved in decellularized tissue, with similar patterns to native tissue (Figure-2).

Cytotoxicity and biocompatibility

Mitochondrial metabolic activity

The metabolic activity of mitochondria of the 3T3 cells was quantified over time, which revealed a significant group effect (Figure-2; $F(5.52) = 267.9$; $p<0.0001$), showing that decellularized glans matrices group had similar patterns than 3T3 cells alone and was significantly different from DMSO group independent of the exposure time ($p<0.0001$ for both groups). There was an interaction time

Figure 1 – Photomicrograph of native tissue and decellularized matrix of human glans. A and C correspond to the native tissue; B and D to the decellularized matrix glans. A and B (100X) H&E and C and D (200X) DAPI. In B and D, complete decellularization of the tissue glans and the preservation of the architecture of the extracellular matrix of native tissue can be observed. SM - Smooth Muscle layer B - Blood vessels.

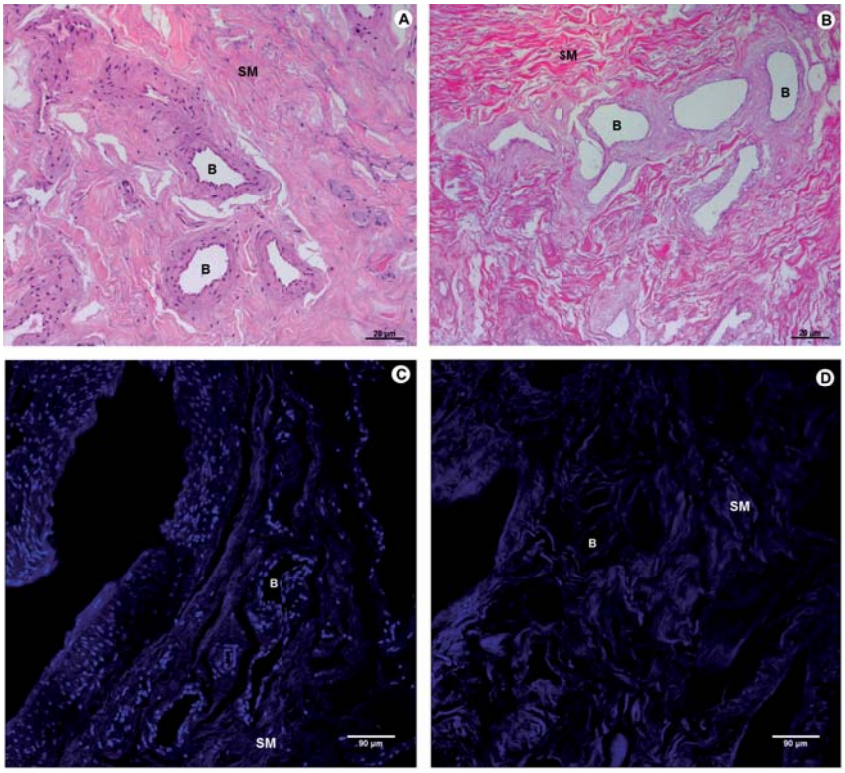
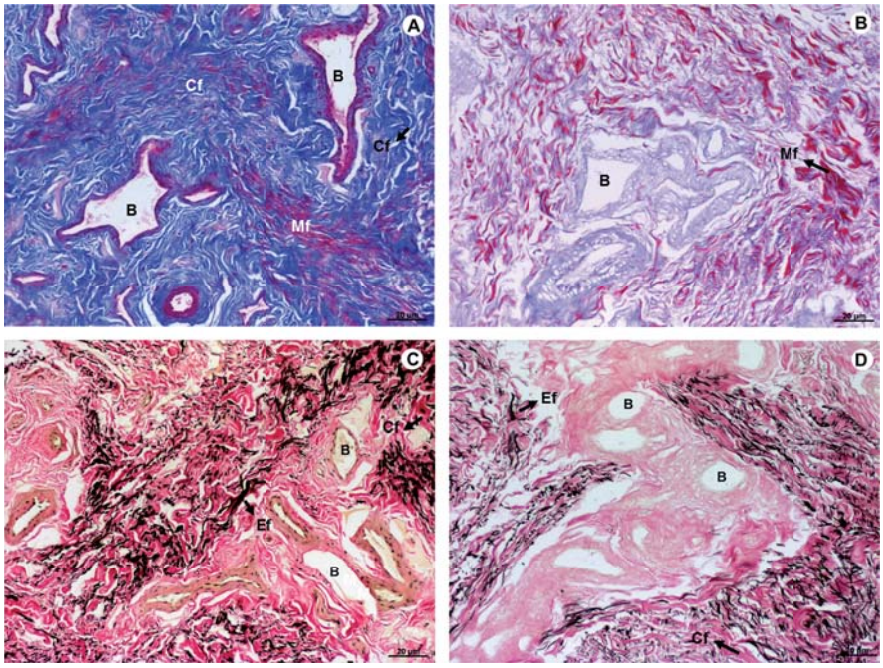


Figure 2 - Photomicrograph of native tissue (A and C), and decellularized matrix of human glans (B and D). Masson's Trichrome – A and B; Verhoff-Van Gieson – B and D revealed the preservation of collagen fibers (blue), architecture of muscle fibers (red), and elastic fibers (black), after the decellularization process. A - D (100X). Mf – Muscle fibers; Cf – Collagen fibers; Ef – Elastic fibers; B - Blood vessels.



versus group effect (Figure-3; F(8.86)=9.5; p<0.0001), revealing that the decellularized glans matrices differed statistically at 48 (0.353±0.069) and 72 hours (0.320±0.032) from the values obtained at 24 hours (0.207±0.026, p<0.0001). The 3T3 cell group at 48 (0.342±0.080) and 72 hours (0.322±0.038) was statistically different from the baseline time point at 24 hours (0.115±0.018, p<0.0001). At 72 hours the 0.2% DMSO group (0.067±0.024) was statistically different from the respective baseline time at 24 hours (0.056±0.010, p<0.028) (Figure-3). The results obtained through the quantification of mitochondrial metabolic activity indicate that 3T3 cells, noncytotoxic group, and the decellularized glans matrices showed a similar pattern of cytotoxicity.

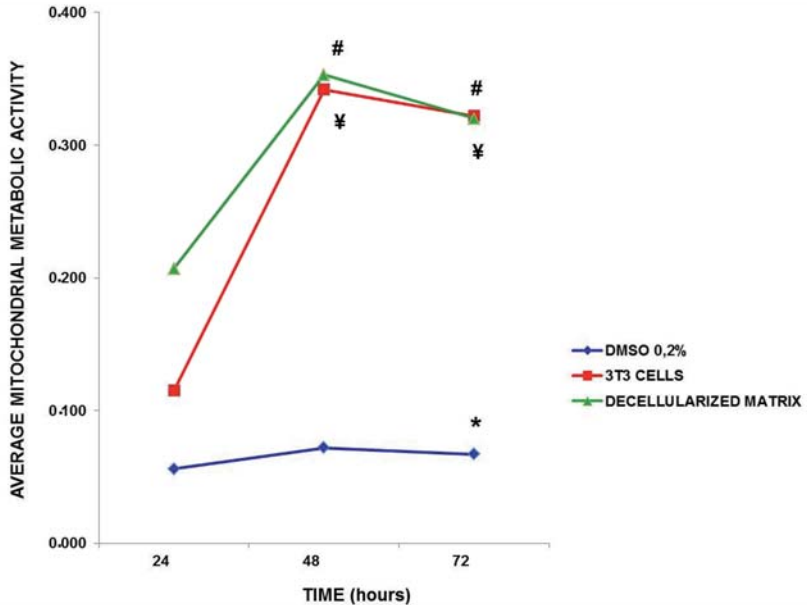
Neutral Red incorporation

The analysis of incorporation of neutral red dye into lysosomes of viable 3T3 cells revealed a group effect (Figure-4; F(5.52)=6.8; p<0.001), showing that the decellularized glans matrices group had significant lower changes in lysosome membranes than the 0.2% DMSO group (cytotoxic group) (p<0.001) and similar to the 3T3 cells, regardless of the exposure over time (Figure-4). The results obtained showed that within 48 hours, the integrity of lysosomes membranes in 3T3 cells that were in direct contact with the decellularized glans matrices samples were better preserved when compared to the other exposure periods.

Quantification of genetic material – Violet Crystal dye

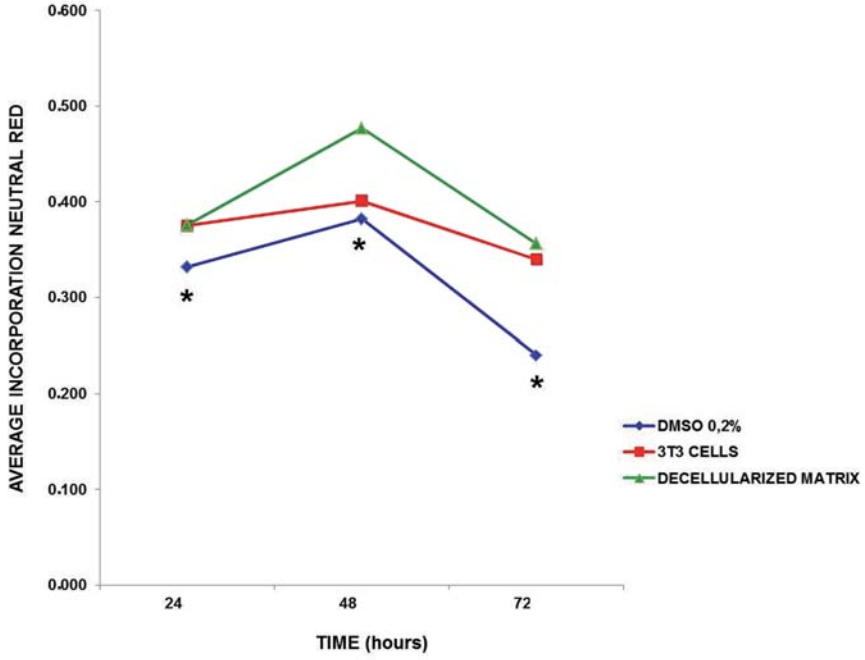
The analysis revealed group effect (Figure-5; F(5.52)=174.9; p<0.0001), showing that in the decellularized glans matrices and the 3T3 cells groups the cell density of viable 3T3 cells was significantly more important and different from the cell density quantified in 0.2% DMSO group (cytotoxic), (p<0.0001). The 3T3 cell group

Figure 3 – Quantification of mitochondrial metabolic activity of 3T3 cells over time "*in vitro*" culture. After 72, the 0.2% DMSO group was statistically different its 24 hour baseline time point. The 3T3 cells and decellularized glans matrices groups showed cellular respiration significantly higher at 48 and 72 hours than when analyzed with 24 hours.



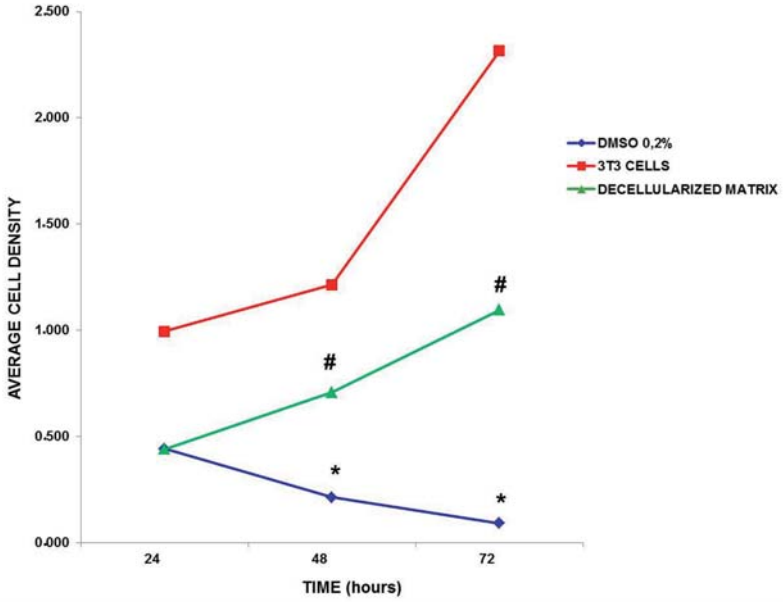
(* p<0.028 compared with respective 24 hour time point); (# ¥ p<0.0001 compared with respective 24 hour time point).

Figure 4 – Incorporation of neutral red dye into lysosomes of 3T3 cells over time "*in vitro*" culture. The 0.2% DMSO group, independent of time, differed statistically from the 3T3 cells and decellularized glans matrices groups.



(* p<0.009 for group 3T3 cells, p<0.001 for group decellularized glans matrices).

Figure 5 – Quantification of cellular density of 3T3 cells over time *in vitro* culture. The 0.2% DMSO group has low cell density at 48 hours and the decellularized glans matrices has high cell density at 72 hours compared to their respective baseline time of 24 hours.



(* p<0.026 for 48 hours; p<0.009 for 72 hours); (# p<0.009 for 48 hours and p<0.006 for 72 hours).

was different from the decellularized glans matrices (p<0.0001), (Figure-5).

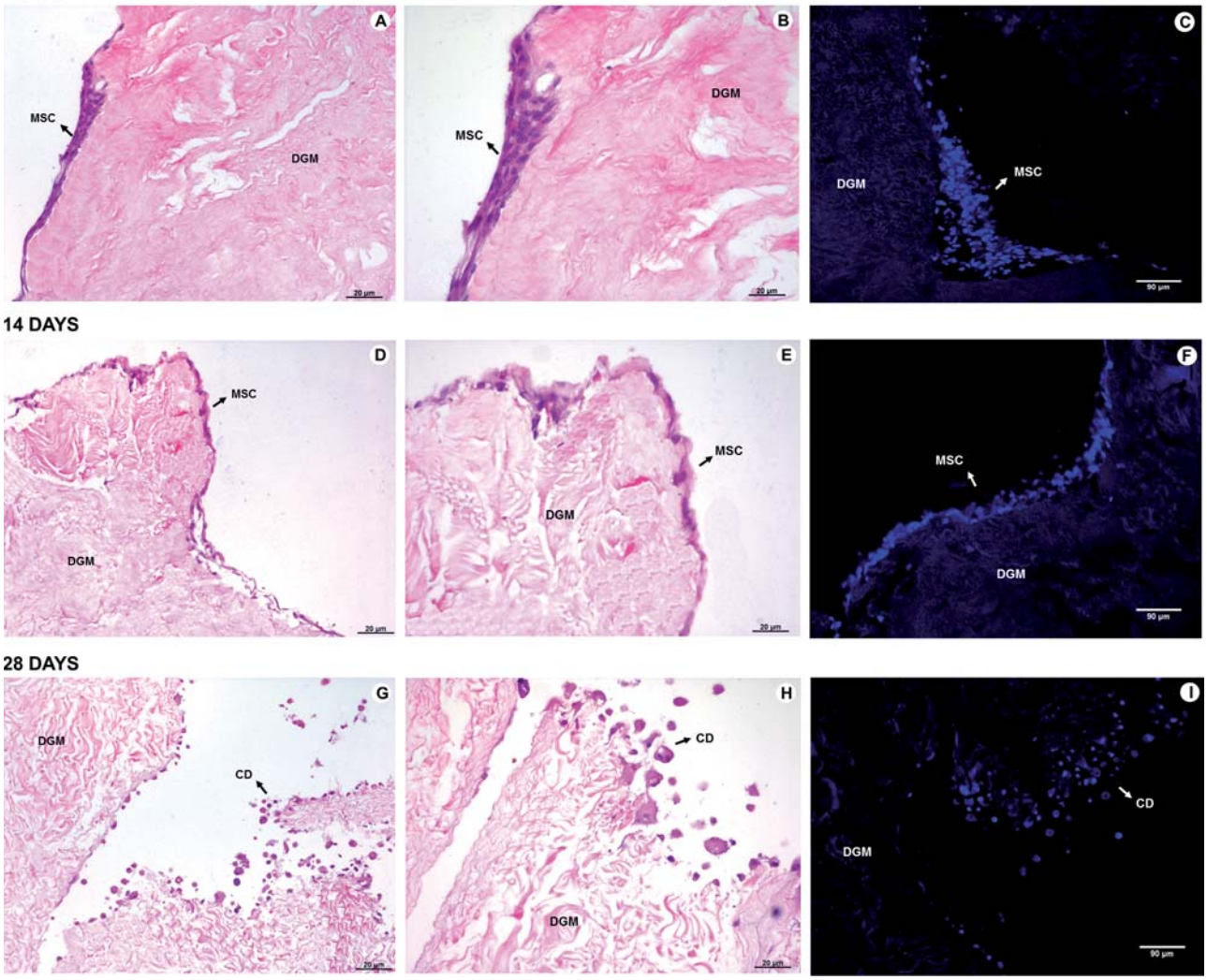
Integration of mesenchymal stem cells

The static seeding of mesenchymal stem cells showed that after 7 and 14 days of in vitro culture the cells remained viable and integrated into the surface of the glans matrices (Figure-6). However, after 28 days the stem cells were no longer viable and only cellular debris were observed (Figure-6).

Biomechanical characteristics

Biomechanical analysis in 7th 14th and 28th days showed that the decellularization process caused a decrease in elastic modulus, which was 1.43Mpa in native tissue, 1.10Mpa in the seeded matrix with 14 days of culture and 0.81MPa in the decellularized matrix. A decrease in the tensile strength of the tissue samples was also observed (2.10MPa in native tissue, 1.70 Mpa in the seeded matrix with 14 days of culture and 1.03 MPa in the decellularized matrix). The specimens from decellularized matrix presented a relative deformation of 1.22, the seeded matrix with 14 days of culture presented a relative deformation of 1.50 while the specimens of native tissue also showed a relative deformation of 1.50 (Figure-7). The average maximum force and final deformation corresponded to 15.94 N and 7.5 mm for the native tissue, 18 N and 7.6mm for the seeded matrix with 14 days of culture and 9.73 N and 6.1 mm for the decellularized glans matrices (Figure-7). A reduction in maximum force required to rupture the tissue was observed at 28 days compared to 7 and 14 days of in vitro culture, which corresponds to the culture time at which cellular debris was found in the matrix (Figures 7 and 8). Overall, among the evaluated parameters, the matrix seeded with mesenchymal stem cells maintained in culture for 14 days showed an intermediate pattern of elasticity modulus and tensile strength between the values obtained with native tissue and the values with decellularized glans matrices.

Figura 6 – Integration of mesenchymal stem cells into decellularized glans matrices. A, B and C correspond to mesenchymal stem cells in the matrices after 7 days of in vitro culture. D, E and F 14 days of in vitro culture, and G, H and I after 28 days of in vitro culture. A, D and G (100X), and B, E and H (200X) - H&E. C, F and I (200X) - DAPI. DGM - Decellularized glans matrices; MSC - Mesenchymal stem cells, CD - Cellular debris.



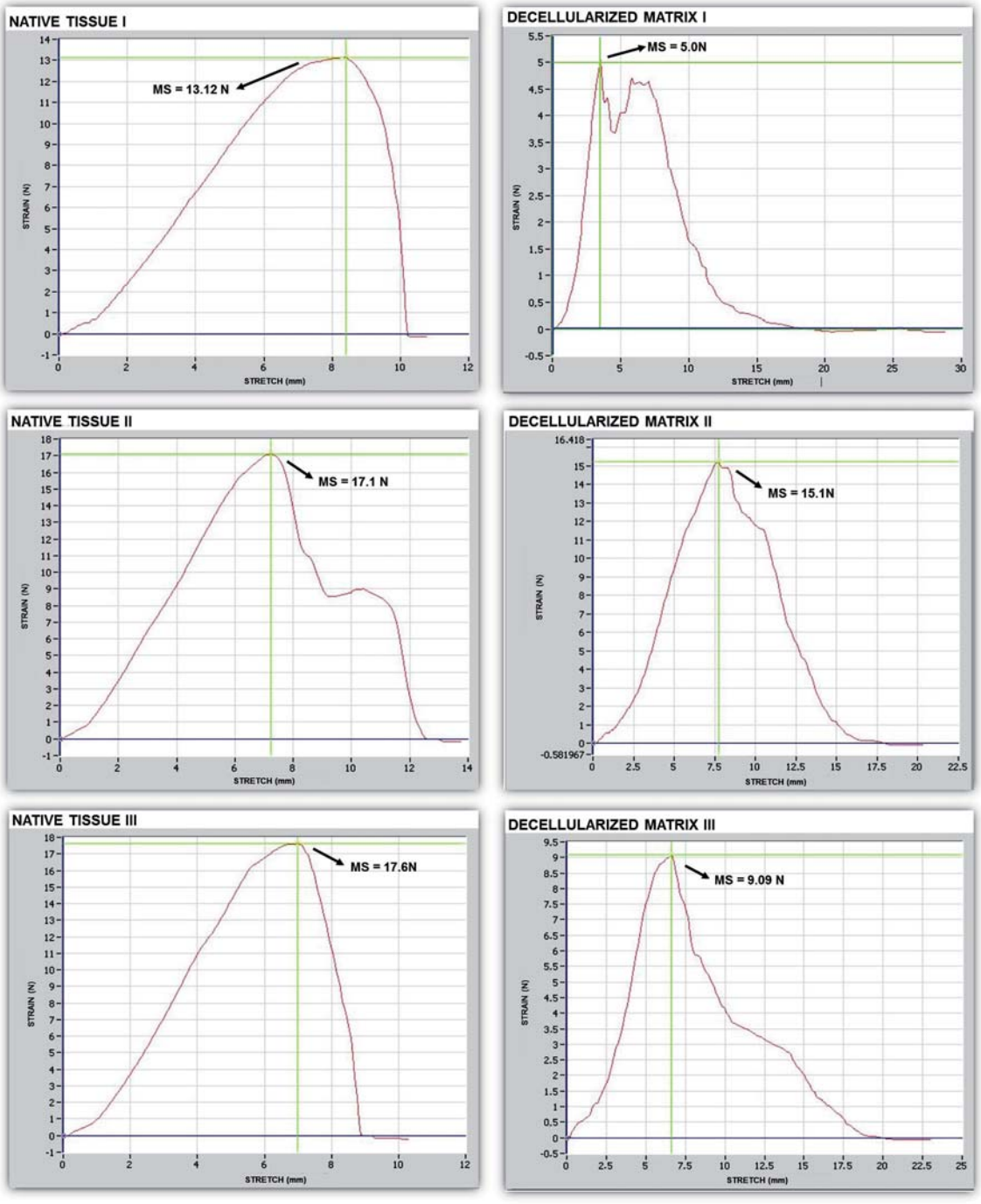
DISCUSSION

Diseases that compromise the integrity of the glans may result in aesthetic and functional changes (1). The greatest challenges when glans reconstruction is required are the limited availability of biocompatible grafts, due to its complex anatomical structure and its particular function during sexual intercourse.

Currently, the techniques for glans reconstruction are restricted to the use of autologous tissue from non-urological origin. The use of these tissues may have potential consequences such as immunological rejection, infection, or functional incompatibility between the native tissue and the implant (7). Thus, tissue engineering is a new alternative for replacing all or part of the glans (7-12). Extracellular matrices of natural origin have potential biological recognition to promote the development of new tissues (11-21).

In this study, decellularized glans matrices, after tissue processing to remove cellular components, preserved the tissue architecture and retained the biological and biomechanical properties.

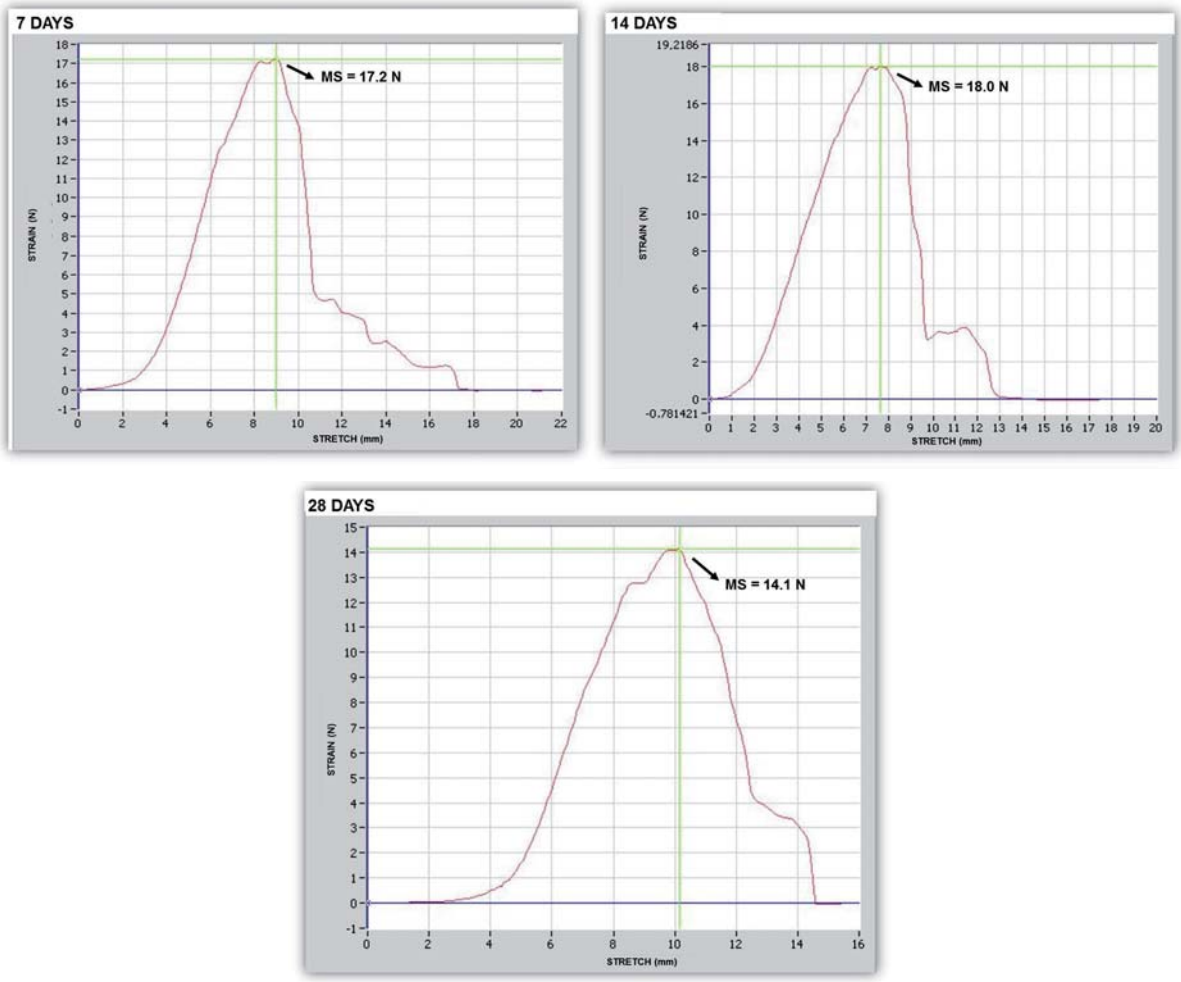
Figure 7 – Biomechanical testing of the native tissue and decellularized glans matrices samples.



Analyses of the glans matrices seeded with mesenchymal stem cells exhibited the integration of these cells into the matrices, and its viability in vitro for two weeks.

We sought to establish a protocol for the decellularization of the glans matrices from cadaver that promoted minimal changes in tissue properties. The protocol described by Kwon et al.

Figure 8 – Biomechanical testing of the glans matrices after seeding with mesenchymal stem cells, and in vitro culture for 7, 14 and 28 days.



MS = Maximum Strain; **N =** Newton; mm = millimeters

(22) was modified, and histological studies of the decellularized tissue showed effectiveness in achieving complete removal of the cellular tissue components, as well as the preservation of tissue architecture. Similarly processed natural acellular matrices from collagen have been extensively used in urogenital reconstruction (23).

Ciapetti et al. (6) showed that the potential cytocompatibility of a biomaterial must be evaluated using different quantitative methods, since the biomaterials exhibit different rates of cytotoxicity and, in addition, damages can occur in a specific structure or function of the cell. Similarly Minnem et al. (24) showed that the biocompatibility constitutes the largest problem in the development of new biodegradable materials in tissue engineering. In this experiment, we evaluated the biocompatibility of the decellularized glans matrices after 24, 48, and 72 hours of exposure to the 3T3 cells using three different methods, and found that the functionality of the mitochondria of 3T3 cells and the structural integrity of the lysosomes membranes were preserved. We also observed a high density of viable 3T3 cells. The cytotoxicity of decellularized glans matrices was therefore considered low, suggesting that they may be used for penile reconstruction.

Decellularized glans matrices and the matrices seeded with mesenchymal stem cells were also evaluated for the preservation of their biomechanical properties. The tests revealed that the elasticity modulus, tensile strength, and relative deformation of the decellularized tissue decreased compared to the native tissue. The matrices seeded with mesenchymal cells and maintained in culture for 14 days exhibited tensile modulus and tensile strength parameters values between those obtained for the native tissue and decellularized glans matrices, suggesting that once reimplanted, these matrices could be incorporated and maintain proper tension.

In this study static seeding of mesenchymal stem cells was performed on acellular glans matrices with the aim of analyzing the biological interactions between the transplanted cells and biomaterial. The matrices seeded with cells were kept in culture for 7, 14 and 28 days in order to verify the viability and integration of the stem cells to the matrices over time. It was observed that after 7 and 14 days in culture the mesenchymal stem cells remained viable and integrated into the glans matrices. After 28 days, however, only cellular debris was found, partially integrated into the matrices, suggesting that the in vitro viability of mesenchymal stem cells decreases over time. This occurs possibly due to a lack of essential supplements into culture medium, responsible for promoting the proliferation and viability of cells in culture for extended periods.

This experiment suggests that the decellularized glans matrices may be able to provide an environment conducive to the integration and viability of mesenchymal stem cells in vitro for up to 14 days, maintaining biological properties after the decellularization process. Furthermore, the mesenchymal stem cells seeded on glans matrices remained viable up to 14 days.

Further studies are needed to develop methods to obtain a higher density of mesenchymal stem cells integrated into the glans matrices and verify the tissue remodeling and reconstruction process of these matrices seeded with mesenchymal stem cells after implantation in laboratory animals.

This study haas some limitations as the reduced casuistic, the fact that is “in vitro” and the low density seeding method. Further studies are suggested in small and large animal models demonstrating how this matrix integrates “in vivo”. The vascular supply is another big challenge. Vascularization is one of the major issues in regenerative medicine. The use of decellularized tissue with preservation of the vascular pedicle and vascular growth factors like VEGF and FGF are some promising methods for overcoming this issue.

CONCLUSIONS

The decellularization process did not alter the biological and biomechanical characteristics of the human glans matrices. When these matrices were seeded with non-autologous mesenchymal stem cells, they were able to maintain the stem cells integrity and vitality *in vitro* for up to 14 days.

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Luiz G. Freitas Filho, MD
Rua Coronel Lisboa 667
Disciplina de Cirurgia Pediátrica São Paulo
São Paulo, SP, 04020-041, Brasil
E-mail: luizfreitasepm@gmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 1002-1007, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2013.00186

Histological changes caused by meclofenamic acid in androgen-independent prostate cancer tumors: evaluation in a mouse model

Iván Delgado-Enciso [1,2], Alejandro D. Soriano-Hernández [1], Alejandrina Rodriguez-Hernandez [1], Héctor R. Galvan-Salazar [1,2], Daniel A. Montes-Galindo [1], Rafael Martinez-Martinez [1], Laura L. Valdez-Velazquez [4], Rafael Gonzalez-Alvarez [1], Francisco Espinoza-Gómez [1], Oscar A. Newton-Sanchez [1], Agustín Lara-Esqueda [5], Jose Guzman-Esquivel [1,3]

[1] School of Medicine, University of Colima, Colima, México; [2] Instituto Estatal de Cancerología, Servicios de Salud del Estado de Colima, Colima, México; [3] Hospital General de Zona Nº1 del IMSS, Colima, México; [4] Chemical Sciences School, University of Colima, Colima, México; [5] Servicios de Salud del Estado de Colima, Colima, México

ABSTRACT

Meclofenamic acid is a nonsteroidal anti-inflammatory drug that has shown therapeutic potential for different types of cancers, including androgen-independent prostate neoplasms. The antitumor effect of diverse nonsteroidal anti-inflammatory drugs has been shown to be accompanied by histological and molecular changes that are responsible for this beneficial effect. The objective of the present work was to analyze the histological changes caused by meclofenamic acid in androgen-independent prostate cancer. Tumors were created in a nude mouse model using PC3 cancerous human cells. Meclofenamic acid (10 mg/kg/day; experimental group, n=5) or saline solution (control group, n=5) was administered intraperitoneally for twenty days. Histological analysis was then carried out on the tumors, describing changes in the cellular architecture, fibrosis, and quantification of cellular proliferation and tumor vasculature. Meclofenamic acid causes histological changes that indicate less tumor aggression (less hypercellularity, fewer atypical mitoses, and fewer nuclear polymorphisms), an increase in fibrosis, and reduced cellular proliferation and tumor vascularity. Further studies are needed to evaluate the molecular changes that cause the beneficial and therapeutic effects of meclofenamic acid in androgen-independent prostate cancer.

ARTICLE INFO

Key words:
Prostatic Neoplasms; Meclofenamic Acid; Therapeutics; Anti-Inflammatory Agents

Int Braz J Urol. 2015; 41:1002-7

Submitted for publication:
October 19, 2013

Accepted after revision:
June 06, 2014

INTRODUCTION

Prostate cancer (PCa) is a worldwide public health problem and is the first cause of death by cancer in men over fifty years of age (1). The growth of this neoplasia is generally dependent on androgen stimulation, although at a given moment it can proliferate in a hormone-independent manner. Androgen-independent prostate cancers are the most aggressive and difficult tumors to control, causing the majority of deaths by this neoplasia (2-5). For these reasons new treatments that can help PCa patients, especially those with androgen-independent tumor, are necessary.

Recent data show inflammation to be a critical component in the origin, proliferation, and

dissemination of different cancers, including PCa (6, 7), and thus the effect of anti-inflammatory drugs, particularly nonsteroidal anti-inflammatory drugs (NSAIDs), has been studied with great interest. Fenamates are a group of NSAIDs that stand out for their strong anti-inflammatory properties upon COX enzyme inhibition. At the same time they are very effective inhibitors of aldo-keto reductases (AKR), especially the AKR1C subfamily members. The inhibition processes of both COX and AKR1C are involved in the NSAID antitumor effect, and thus fenamates show great potential in the treatment of cancer (8, 9).

Of the fenamates studied in PCa, meclofenamic acid is the one with the greatest therapeutic effect (10). It has shown a high degree of cytotoxicity for both androgen-dependent and androgen-independent PCa. In addition, it has been confirmed in a nude-mouse model of human androgen-independent prostate cancer that meclofenamic acid at non-toxic doses (10 mg/kg/day/25days) significantly reduces tumor growth, prolongs survival, and is even capable of generating total tumor regression in up to 25% of mice treated (10).

Therefore, it is of interest to study the mechanisms by which meclofenamic acid produces therapeutic effects in PCa. The objective of the present work was to analyze the histological changes caused by meclofenamic acid on PCa tumors (nude-mouse model) as a possible first step towards understanding the drug's antineoplastic effects.

MATERIALS AND METHODS

Prostate cancer cell line PC3 was employed in this study. PC3 does not respond to androgens, glucocorticoids, epidermal growth factor (EGF), or fibroblastic growth factor (FGF) (11). Cell lines were maintained in DMEM medium (Sigma, St. Louis, MO, USA) and supplemented at 10% (v/v) with fetal bovine serum (FBS) (GIBCO). They were incubated at 37°C, 5% CO_2, and 97% relative humidity. Drug was obtained from SIGMA-ALDRICH (Belgium) with 98% purity. Meclofenamic acid was dissolved in alcohol at 70% to generate a 440mM stock.

Nude-Mouse model of human prostate cancer

A xenotransplanted murine model harboring prostate tumors was created by subcutaneous injection of $1X10^6$ prostate PC3 cells at the dorsum. The mouse strain used in these experiments was Foxn1nu (6-to 8-week-old males from Harlan Mexico, Mexico City). Once tumors reached an approximate diameter of 4 mm, mice were divided into two groups. For 20 days, a single application per day of meclofenamic acid at a volume of 100 µL was intraperitoneally administered to one group of mice at doses of 10 mg/kg/day (n=5) and a second group received saline solution (PBS) (n=5). Mice were euthanized one day after the end of treatment (day 21) and tumors were extracted and histologically processed and analyzed. It has previously been reported that treatment with meclofenamic acid for 25 days significantly reduced tumor growth and on occasion produced complete tumor regression (10). The purpose of the present study was not to evaluate treatment effectiveness, and so treatment was given for only 20 days in order to avoid any total tumor regression and consequent tumor histological analysis loss. Animals were handled according to institutional guidelines and to the Mexican Official Norm regulating laboratory animal use (11, 12).

Tumor histological analysis

Neutral buffered formalin-fixed tumor tissue was embedded in paraffin. Tissue sections (5 µm) were prepared using a microtome and mounted on slides. Proliferation and angiogenesis markers were evaluated by immunostaining for Ki-67 (clone MIB-1) and CD34 (clone QBEnd 10), respectively, as previously described (13). DAKO (CA, USA) brand antibodies were used.

Proliferation marker was assessed by counting the number of nuclei with positive stain for Ki-67 and total number of cancer cells at x100 magnification in three representative regions of each tumor. Results were expressed as the proportion of cells that stained positive over the total number of cells. In each tumor, microvessel density was assessed by counting the number of microvessels at x400 magnification in three fields with the highest vascularization. Results were expressed as mean number of microvessels per field. For

statistical analysis, fifteen tumor data per group were obtained by analyzing three representative regions of each of five tumors per group.

Since it has previously been demonstrated that there can be an increase in fibrosis in prostate tumors after different treatments (14, 15), Masson's trichrome stain was also carried out to detect collagen. A commercial Dakocytomation (Artisan) Kit was used according to the manufacturer's instructions (16). Fibrosis was considered to be 1) focal, when it covered less than 10% of the tumor area and presented as isolated bands, 2) moderate, when it covered 10-75% of the tumor area and presented as localized fibrotic zones, and 3) extensive, if it presented in generalized form covering more than 75% of the tumor area.

Statistical analysis

After further normal data distribution corroboration (by Kolmogorov-Smirnov test), Student t test was used to compare microvessel density and Ki-67 marker of tumors treated with meclofenamic acid and saline solution. Statistical significance was interpreted at values of $P<0.05$ using the MedCalc program (version 8.1.0.0 for Windows; Mariakerke, Belgium).

RESULTS

At day 21 from beginning of treatment, proliferation and angiogenesis markers (Ki-67 and CD34) and degrees of fibrosis were analyzed in five tumors from the PBS group (control) and from the 10 mg/kg/day meclofenamic acid group. Initially, general histological tumor characteristics were qualitatively analyzed. Tumors treated with PBS had greater hypercellularity, greater nuclear polymorphism, and a greater number of atypical mitoses than the tumors treated with meclofenamic acid (Figure-1). In regard to quantitative analyses supported by immunohistochemical techniques, there was significant reduction in proliferation (Ki-67) and vascularization (CD34) markers (Table-1) in tumors of mice treated with meclofenamic acid.

In addition, Masson's trichrome stain showed differences among the groups in regard to degree of fibrosis. Fibrosis was focal in control

Figure 1 - Histological slices representative of PC-3 prostatic tumors stained with Masson´s trichrome. Collagen fibers are blue, as signaled by arrows. Tumors treated with meclofenamic acid (A: X200 and B: X400) present with fibrosis (blue color) clearly covering a large portion of the tumor, whereas tumors treated with PBS (controls) only present with isolated collagen fibers (C: X200 and D: X400). Clear differences in cellularity and nuclear polymorphism are also observed.

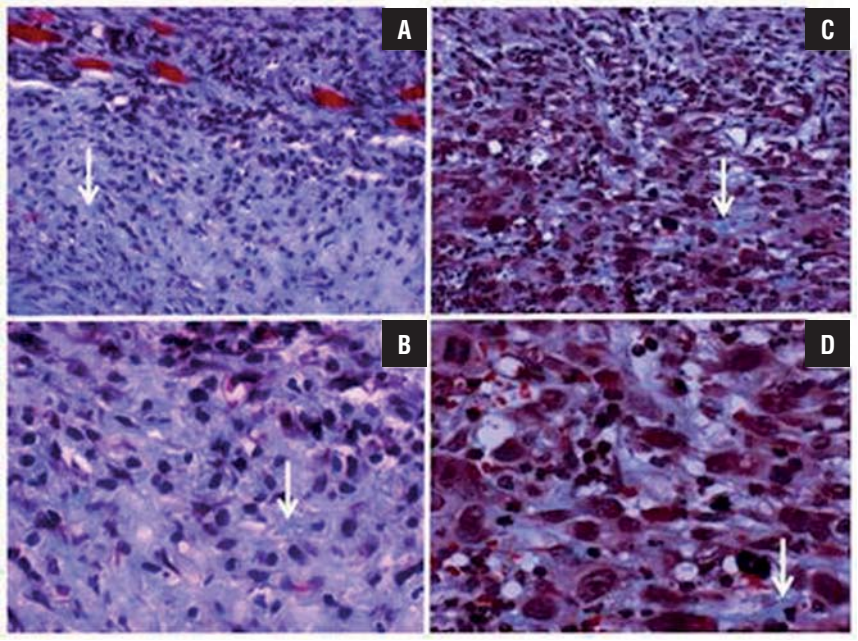

Table 1 - Immunohistochemical markers detected in tumors in the nude-mouse model of human prostate cancer.

Marker	Control	Meclofenamate*	P
Ki-67**	22.0±4	13.9±6.6	0.002
CD34***	10.8±2.4	8.5±2.1	0.006

*10 mg/kg/day dose
**Positive cell percentage (mean±standard deviation)
*** microvessels at x400 magnification per field (mean±standard deviation)

tumors and was moderate in tumors treated with meclofenamic acid. This result was constant in all five tumors analyzed in each group (Figure-1).

DISCUSSION

Treatment with meclofenamic acid produces significant histological changes that can be considered beneficial and they are concordant with antitumor effects previously reported for this drug. In an androgen-independent PCa mouse model, Soriano-Hernandez et al. (2011) showed that 25 days of treatment with meclofenamic acid at 10 mg/kg/day significantly reduced tumor growth, prolonged survival and was even able to generate total tumor regression (10). However, the histological mechanisms or changes associated with this beneficial therapeutic effect were not established.

The present study showed that meclofenamic acid caused histological changes denoting less tumor aggression (less hypercellularity, fewer atypical mitoses, and fewer nuclear polymorphisms). It quantitatively confirmed that treatment increased fibrosis, reduced cellular proliferation (Ki-67 reduction) and possibly reduced angiogenesis since it significantly reduced tumor vascularity.

There is evidence in clinical or preclinical trials that NSAIDs can reduce cellular proliferation (determined by means of the Ki-67 marker) in tumors of breast, tongue, cervix, thyroid, and liver (17-21). In different tumor tissues, including PCa, elevated levels of COX-2 have been shown to induce cell proliferation (22, 23), presenting a parallel overexpression of COX-2 and Ki-67 (24, 25). This elevation of COX-2 has also been associated with lower survival rate (24). In addition elevated levels of ARK1C in PCa have been detected and its reduction, through iRNA, causes a decrease in cell proliferation (26, 27). Meclofenamic acid is a potent simultaneous inhibitor of the COX-2 and AKR1C enzymes, which can explain the significant reduction in cellular proliferation in tumors in the mice treated in the present study.

There are different reports regarding the influence of NSAIDs on tumor vasculature. A study in a human breast cancer model showed that COX-2 inhibition by means of celecoxib administration for 7 days significantly reduced microvessel density (28% reduction on average), confirming that angiogenesis had been inhibited (28). Various NSAIDs can inhibit tumor growth by drastically reducing their vascularization (29). This effect is generated when endothelial cell apoptosis is induced (30), or through the reduction of vascular endothelial growth factor (VEGF) levels - one of the principal angiogenesis inducers (31-33). In addition it has been reported that AKR1C3 overexpression promotes angiogenesis and aggressiveness of PCa cells (PC3), suggesting that AKR1C3-inhibition would reduce angiogenesis. This concurs with the significant decrease in vascularity (21% reduction on average) caused by meclofenamic acid (COX-2 and ARK1C inhibitor) in the PCa (PC3 tumors) model in the present study, even though the molecular mechanism responsible for it was not determined.

Also the clear increase in fibrosis in the treated tumors could be a reflection of therapeutic effectiveness. Fibrosis is a parameter that is not often evaluated after PCa treatments. However, it has been observed in tumors in patients with favorable progression after brachytherapy (15) or minimally invasive treatments such as high-intensity focused ultrasound (34). Fibrosis has also

been detected in nonmalignant residual prostatic tissue after hormone treatment in humans (35).

In conclusion, meclofenamic acid caused histological changes that indicated decreased tumor aggression, increased fibrosis, and cellular proliferation and vascularity reduction in androgen-independent prostate tumors, lending support to its great therapeutic potential in regard to this neoplasia. Further studies are needed to evaluate the molecular changes that produce meclofenamic acid's histological and therapeutic effects in PCa.

ACKNOWLEDGEMENTS

This study was funded by the Fondo Mixto CONACYT- Gobierno de Colima, 2008-C01-83189 and the Fondo Ramon Alvarez Buylla de Aldana (Universidad de Colima).

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Jose Guzman-Esquivel, MD
Hospital General de Zona Nº1 del IMSS,
Av. De los Maestros 149, colonia Centro,
CP 28000. Colima, México
FAX: + 52 312 312-2121
E-mail: enicetoeto@hotmail.com

ORIGINAL ARTICLE

Vol. 41 (5): 1008-1013, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0470

Anti-inflammatory effects of royal jelly on ethylene glycol induced renal inflammation in rats

Zeyneb Aslan [1], Laçine Aksoy [1]

[1] *Department of Chemistry, Biochemistry Division, Faculty of Science and Arts, Afyon Kocatepe University, Afyonkarahisar, Turkey*

ABSTRACT

Objective: In this study, anti-inflammatory effects of Royal Jelly were investigated by inducing renal inflammation in rats with the use of ethylene glycol. For this purpose, the calcium oxalate urolithiasis model was obtained by feeding rats with ethylene glycol in drinking water.

Materials and Methods: The rats were divided in five study groups. The 1st group was determined as the control group. The rats in the 2nd group received ethylene glycol (1%) in drinking water. The rats in the 3rd group were daily fed with Royal Jelly by using oral gavage. The 4th group was determined as the preventive group and the rats were fed with ethylene glycol (1%) in drinking water while receiving Royal Jelly via oral gavage. The 5th group was determined as the therapeutic group and received ethylene glycol in drinking water during the first 2 weeks of the study and Royal Jelly via oral gavage during the last 2 weeks of the study.

Results: At the end of the study, proinflammatory/anti-inflammatory cytokines, TNF-α, IL-1β and IL-18 levels in blood and renal tissue samples from the rats used in the application were measured.

Conclusion: The results have shown that ethylene glycol does induce inflammation and renal damage. This can cause the formation of reactive oxygen species. Royal Jelly is also considered to have anti-inflammatory effects due to its possible antiradical and antioxidative effects. It can have positive effects on both the prevention of urolithiasis and possible inflammation during the existing urolithiasis and support the medical treatment.

ARTICLE INFO

Key words:
Calcium Oxalate; Inflammation; royal jelly [Supplementary Concept]; Ethylene Glycol; Cytokines

Int Braz J Urol. 2015; 41: 1008-13

Submitted for publication:
September 19, 2014

Accepted after revision:
December 04, 2014

INTRODUCTION

Urinary system stone disease is a frequent disease that can adversely affect the quality of life. Various factors such as genetic factors, characteristics of the residential area and their effects on the metabolism, dietary habits, smoking, alcohol use, air pollution and stress are considered to trigger stone formation. In the urinary system stone disease, genetic factors have a 25% effect on individuals. Urinary system stone disease is three times more common in men than in women. Although it can be seen at any age, its frequency increases with age and it is most commonly seen in individuals between the ages of 30 and 60 (1-4). India and European and Middle Eastern countries are determined as the stone zone and Turkey is one of these countries in which the urinary system stone disease is endemic (5). Fluid intake has a significant effect on stone formation. Leading to increased concentration in the urine content, urine volume decreasing with decreased water consumption affects stone formation.

Increased water consumption accelerates the urinary system cycle and increases urine volume and therefore, reduces stone formation (6).

ESWL (Extracorporeal shock wave lithotripsy) is the most frequently used method for the prevention of stone formation and the growth of the existing stone and litholysis, if applicable. The reliable technology and therapeutic effect of ESWL rendered it the most preferred method. However, the success of the therapy varies with the location and structure of the stone and the presence of anatomical anomalies. Despite yielding high disintegration levels, the ESWL method carries some risks. In the long term, increase in systemic blood pressure, decrease in the renal function and increase in the recurrence of calcium oxalate urolithiasis occur. Some experimental studies determined that the ESWL method causes acute and chronic damages to kidneys and some of these damages are irreversible. Patient selection is crucial to the success of the ESWL method. Applications on pregnant women and short children and applications with the presence of contraindications to anesthesia, untreated urinary system infections, active tuberculosis infection, stenosis of the urinary tract, obesity or skeletal abnormalities are difficult (6, 7).

Due to its attributed extraordinary biological properties, Royal Jelly is a significant commercial attraction for the health researches and the food industry. It is used in a wide variety of industries such as the drug, food and cosmetic industries. Royal jelly is a substance secreted from the hypopharyngeal and mandibular glands of 5-15 days old worker honey bees. It is produced in the glands of adult worker bees fed with honey and pollen (8-10).

The aim of this study was to obtain ethylene glycol induced urolithiasis in rats and to investigate the proinflammatory and anti-inflammatory effects of Royal Jelly on ethylene glycol induced urolithiasis and inflammation due to renal tubular damage by measuring cytokine levels.

MATERIALS AND METHODS

Chemicals

Ethylene glycol used to induce urolithiasis was obtained from Merck (Darmstadt, Germany). Royal Jelly was directly supplied from a natural honey producer (Kahramanmaraş, Turkey).

Stone-forming rat model and experimental design

Before the initiation of the research, ethics committee approval dated 26/09/2013 no. AKÜHADYEK-284-13 was obtained from Afyon Kocatepe University Animal Ethics Committee. 35 male Sprague-Dawley strain rats weighing 300-380 g were used in the study. For 4 weeks, rats were kept in 12 hours ideal lighting and at the appropriate temperature and fed with standard pellet rat feed (Bilyem, Ankara, Turkey). Rats were divided into 5 groups. Group 1, Control group (n=7): Fed with standard feed and drinking water. Group 2: EG Group (n=7): Fed with standard feed and drinking water containing 1% ethylene glycol. Group 3: RJ group (n=7): Daily fed with 100mg/kg Royal Jelly by using oral gavage. Group 4: RJ+EG group (n=7): Received 1% ethylene glycol containing drinking water. Additionally, these rats were daily fed with 100mg/kg Royal Jelly via oral gavage. Group 5: EG+RJ group (n=7): Received 1% ethylene glycol containing drinking water for the first 2 weeks. In the last 2 weeks, rats were fed with 100mg/kg Royal Jelly via oral gavage. EG represents the urolithiasis group; RJ+EG represents the prophylactic group; EG+RJ represents the therapeutic group.

Evaluation of Total Protein Levels

The total protein levels of tissue homogenates were measured with ELISA (BIOTEK ELx800) using commercial kits (Fluka 51254). The Bradford method was used in the measurement. Coomassie Brillant Blue G-250, the dye used in this method, has a negative charge and binds to the positive charges on the surface of protein. The dye has blue and red forms. Protein binding turns the blue form into the red form. The absorbance of the resulting complex was measured at 630nm (11).Total protein results were used to calculate the levels of the parameters analyzed in kidney tissue.

Evaluation of TNF-α, IL-1β, IL-18 Cytokine Levels

Inflammation markers (TNF-α, IL-1β, IL-18) were measured in renal homogenates and plasma samples by using commercial kits. The

kits used in the measurement have the following brand, reference and lot numbers: TNF-α (eBioscience, Ref: BMS622, Lot: 91475038), IL-1β (eBioscience, Ref: BMS630, Lot: 87225015), IL-18 (Novex, Ref: KRC2341, Lot: 130401/A). Cytokine levels were given as pg/mL for plasma and pg/mg-protein for renal homogenates.

Statistical analysis

The data were expressed as the mean±standard deviation (SD). Statistical comparisons were performed using the appropriate ANOVA model with Duncan post-hoc tests. Differences with $p<0.05$ were considered significant. The SPSS 15.0 package for Windows was used for the statistical analysis.

RESULTS

Plasma and renal tissue TNF-α Levels

Based on the plasma TNF-α values shown in Table-1, the value of the control group did not significantly differ from the value of the Royal Jelly group ($p>0.05$), whereas it differed significantly from the other groups ($p<0.05$). The TNF-α values of the EG and EG+RJ groups were significantly the highest ($p<0.05$). The TNF-α value of the RJ+EG group was significantly lower than those of the EG and EG+RJ groups ($p<0.05$), whereas it was significantly higher than the values of the control and the RJ groups ($p<0.05$).

Plasma and renal tissue IL-1β Levels

Based on the plasma IL-1β values shown in Table-2, the value of the control group was significantly lower than the other groups ($p<0.05$). The IL-1β value of the EG group was significantly the highest ($p<0.05$). The IL-1β value of the RJ group was significantly higher than the values of the RJ+EG and EG+RJ groups ($p<0.05$).

Based on the renal IL-1β values; the value of the control group was significantly lower than the other groups ($p<0.05$). The difference between the values of the RJ group and RJ+EG groups were not significant ($p>0.05$), whereas their values were significantly lower than those of the control and EG+RJ groups ($p<0.05$). The IL-1β value of the EG group was significantly the highest ($p<0.05$) (Table-2).

Plasma and renal tissue IL-18 Levels

Based on the plasma IL-18 values shown in Table-3, the IL-18 value of the control group was significantly the lowest ($p<0.05$). The values of the RJ group and EG+RJ groups were not significantly different from each other ($p<0.05$), whereas their values were significantly lower than the value of the RJ+EG group ($p<0.05$). The IL-18 value of the EG group showed a significantly higher increase ($p<0.05$).

The analysis of the renal IL-18 values showed that only IL-18 value of the EG group was significantly higher than the other groups ($p<0.05$) (Table-3).

DISCUSSION

Inflammation is a natural defense response of the organism against stimuli, such as trauma, infectious agents, toxic and chemical substances,

Table 1 - Plasma and renal tissue TNF-α Levels.

Group	Plasma TNF-α (pg/mL)	Kidney TNF-α (pg/mg-protein)
Control	43.78±3.81[b]	413.26±17.78[c]
EG	75.54±9.34[a]	469.28±28.96[a]
RJ	49.96±2.65[b]	343.16±24.28[b]
RJ+ EG	59.07±5.79[c]	361.68±15.18[b]
EG+RJ	81.27±5.24[a]	474.14±15.87[a]

a, b, c, d: Differences between the groups that are coded with different letters in the same column are significant ($p<0.05$). EG represents the group administered with ethylene glycol; Royal Jelly represents the group solely administered with Royal Jelly; RJ+EG represents the group simultaneously administered with Royal Jelly and ethylene glycol; EG+RJ represents the group administered with Royal Jelly after inducing urolithiasis with ethylene glycol.

Table 2 - Plasma and renal tissue IL-1β Levels.

Group	Plazma IL-1β (pg/mL)	Kidney IL-1β (pg/mg-protein)
Control	99.17±2.79[b]	1252.19±90.42[b]
EG	165.33±10.25[a]	2162.49±54.93[a]
RJ	113.17±5.12[c]	1559.00±99.30[d]
RJ+ EG	146.67±10.42[d]	1488.46±117.48[d]
EG+RJ	138.00±7.72[d]	1361.47±73.01[c]

a, b, c, d: Differences between the groups that are coded with different letters in the same column are significant (p<0.05). EG represents the group administered with ethylene glycol; Royal Jelly represents the group solely administered with Royal Jelly; RJ+EG represents the group simultaneously administered with Royal Jelly and ethylene glycol; EG+RJ represents the group administered with Royal Jelly after inducing urolithiasis with ethylene glycol.

Table 3 - Plasma and renal tissue IL-18 Levels.

Group	Plasma IL-18 (pg/mL)	Kidney IL-18 (pg/mg-protein)
Control	2285.30±103.57[b]	980.02±42.16[b]
EG	3577.76±135.95[a]	1258.35±89.96[a]
RJ	2572.73±121.82[c]	1050.45±86.33[b]
RJ+ EG	3111.12±71.80[d]	1063.43±92.30[b]
EG+RJ	2683.17±109.96[c]	1037.43±75.48[b]

a, b, c, d: Differences between the groups that are coded with different letters in the same column are significant (p<0.05). EG represents the group administered with ethylene glycol; Royal Jelly represents the group solely administered with Royal Jelly; RJ+EG represents the group simultaneously administered with Royal Jelly and ethylene glycol; EG+RJ represents the group administered with Royal Jelly after inducing urolithiasis with ethylene glycol.

physical and surgical operations. Ethylene glycol is a highly preferred agent to induce nephropathy in experimental animal studies. The administration of ethylene glycol results in intrarenal CaO_x deposition and acute renal damage. Various studies have shown that CaO_x deposition causes renal inflammation (12).

Proinflammatory cytokines are released at the onset of the inflammation and they increase the release of other cytokines and activate inflammatory cells. They are essential to the initiation and sustenance of immune response. Main proinflammatory cytokines in human immune response are TNF-α, IL–1 and IL-18 (13). TNF-α is a cytokine produced mainly by monocytes/macrophages, although it can be produced by Kuppfer cells, skin keratinocytes, brain glial cells, T lymphocytes and B lymphocytes. Various tissues such as liver, muscle, intestinal and lung tissues have high-affinity receptors for TNF-α. It is primarily released and also the strongest among the proinflammatory cytokines as a mediator in host response. It stimulates mononuclear phagocytes and other cells to produce IL-1, IL-6 and chemokines (14, 15). IL-1β is a proinflammatory cytokine that has effects similar to those of TNF-α. TNF-α increases the synthesis and release of IL-1α and IL-1β which are released from macrophages and endothelial cells and coded by different genes. IL-1β is responsible for the metabolic and physiological effects of circulatory TNF-α. It has a role in the local and systemic effects of acute and chronic inflammation (16). IL-18 is an IFN-γ producing proinflammatory cytokine. IL-18 is released from renal tubular cells and macrophages. It functions as a mediator in acute renal damage. Studies have shown that IL-18 levels increase in the proximal tubular epithelium in the case of renal damage and acute renal failure (17).

Apitherapy means the use of bee products for medical purposes. In the recent years, there is a growing interest, especially in the food industry,

in the functional foods which are claimed to have benefits for human health. The benefits of consumed food on human health are globally accepted. Today, widely known bee products, honey, propolis and Royal Jelly have a significant place among the functional foods (18).

Many studies on the effects of apitherapeutical products on inflammation induced by various causes were conducted. Khayyal et al. (19) considered propolis as an anti-inflammatory agent due to its inhibition of platelet aggregation and eicosanoid synthesis and investigated its effects on edema and arthritis induced inflammation. The researchers have found that it shows anti-inflammatory effects depending on the dosage. Another study to investigate the anti-inflammatory effects of honey on inflammation due to intestinal diseases showed that application of honey is as effective as treatment with prednisolone. However, it was stated that further studies are required to clarify the mechanism (20). In another study, paw edema was induced in rats and the effects of ethanolic extract of pollen were investigated. It was reported that pollen shows anti-inflammatory effects by reducing NO production and COX-2 activity (21). Similar to those studies, the TNF-α values in our study (Table-1) showed that the administration of EG increases TNF-α values in plasma and kidneys. In plasma and kidney, the TNF-α values of the RJ+EG group showed that Royal Jelly has preventive effects on inflammation.

In the study to investigate effects of propolis on stress-induced immunosuppression, IL-1β and IL-6 cytokine levels were examined. The researchers reported that propolis cannot substitute for inhibitory effects on proinflammatory cytokines but partially regulates TLR2 mRNA expression and balances inhibition of TLR-4 expression in stressed rats (22).

The IL-1β values in our study showed that the urolithiasis group had an increased release of the cytokine when compared to the control group. When the RJ+EG and EG+RJ groups are compared with the urolithiasis group, it is possible to say that Royal Jelly has anti-inflammatory effects. In renal tissue, the anti-inflammatory effect of Royal Jelly is higher in the therapeutic group. Renal IL-1β values show that Royal Jelly has an effect on EG-induced inflammation (Table-2).

Parikh et al. (23) published a study about cytokine IL-18 as an early diagnostic biomarker of acute renal damage. In their study on mice, Faubel et al. (24) determined that cisplatin induced renal damage is associated with the increase in IL-1β and IL-18 cytokines.

The IL-18 values in our study clearly showed the effect of exposure to ethylene glycol on cytokine release. In the plasma, the effect of Royal Jelly was higher on the therapeutic group than the control group. Data on renal tissue show that the release of IL-18 in this tissue is remarkably inhibited by Royal Jelly. Due to the insignificant differences from the control group, the values of the Royal Jelly, RJ+EG and EG+RJ groups support this assessment (Table-3).

CONCLUSIONS

Various studies showed that oxidative stress occurs in patients with kidney stones. CaO_x exposure causes oxidative damage by reactive oxygen species such as superoxide and H_2O_2. The produced ROS activate several signaling pathways.

This study has shown that inflammation can be induced by ethylene glycol. The favorable effects of Royal Jelly on renal damage due to inflammation are presented. The resulting renal damage is associated with oxidative stress. Antioxidants in Royal Jelly prevent ROS production and support the antioxidant system. Hence, Royal Jelly is considered to show anti-inflammatory effects by affecting the signaling pathways. Further cell culture studies will help to clarify the mechanism.

CONFLICT OF INTEREST

We declare that, this study was financially supported by Scientific Research Projects Committee (project number: 13.FENBIL.30), Rectorate of Afyon Kocatepe University, Afyonkarahisar, Turkey.

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Correspondence address:
Laçine AKSOY, PhD
Afyon Kocatepe University,
Faculty of Science and Arts,
Department of Chemistry, 03200, Afyonkarahisar, Turkey
Fax: +90 272 228 13 39
e-mail: lacinetur@aku.edu.tr

SURGICAL TECHNIQUE

Vol. 41 (5): 1014-1019, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2015.0007

A Novel method of ensuring safe and accurate dilatation during percutaneous nephrolithotomy

Tarun Javali [1]; Amey Pathade [1]; H. K. Nagaraj [1]

[1] *M.S. Ramaiah Hospital, Bangalore, India*

ABSTRACT

Objective: To report our technique that helps locate the guidewire into the ureter enabling safe dilatation during PCNL.
Materials and Methods: Cases in which the guidewire failed to pass into the ureter following successful puncture of the desired calyx were subjected to this technique. A second guidewire was passed through the outer sheath of a 9 Fr. metallic dilator cannula, passed over the first guidewire. The cannula and outer sheath were removed, followed by percutaneous passage of a 6/7.5 Fr ureteroscope between the two guidewires, monitoring its progress through both the endoscopic and fluoroscopic monitors. Once the stone was visualized in the calyx a guidewire was passed through the working channel and maneuvered past the stone into the pelvis and ureter under direct endoscopic vision. This was followed by routine tract dilatation.
Results: This technique was employed in 85 out of 675 cases of PCNL carried out at our institute between Jan 2010 to June 2014. The mean time required for our technique, calculated from the point of introduction of the ureteroscope untill the successful passage of the guidewire down into the ureter was 95 seconds. There were no intraoperative or postoperative complications as a result of this technique. Guidewire could be successfully passed into the ureter in 82 out of 85 cases.
Conclusions: Use of the ureteroscope introduced percutaneously through the puncture site in PCNL, is a safe and effective technique that helps in maneuvering the guidewire down into the ureter, which subsequently enables safe dilatation.

ARTICLE INFO

Key words:
Nephrostomy, Percutaneous; Ureteroscopy

Int Braz J Urol. 2015; 41: 1014-9

Submitted for publication:
March 24, 2015

Accepted after revision:
May 13, 2015

INTRODUCTION

Establishing an effective and safe percutaneous access is the cornerstone in performing a successful and uneventful percutaneous nephrolithotomy. After successful puncture of the desired calyx, passage of the guidewire through the calyx into the pelvis and ureter provides the most secure position to proceed with tract dilatation. Failure to pass the guidewire through the punctured calyx down into the ureter may arise in some cases due to large stone bulk in the calyx or stone blocking the infundibulum. Dilatation over a guidewire coiled in a calyx vis-à-vis a guide wire successfully passed down a ureter is tenuous, with chances of slippage/dislodgement during dilatation, resulting in loss of access. The objective of the present study is to report our technique that helps locating the guidewire into the ureter enabling safe dilatation.

MATERIALS AND METHODS

All patients underwent pre-operative evaluation with USG KUB, intravenous pyelography,

urine culture and routine blood tests. PCNL was carried out in prone position under general anesthesia. Retrograde ureteric catheterization and fluoroscopy helped in defining the calyceal anatomy. Puncture was performed with an 18 G PCN puncture needle using the Bull's eye technique. Free flow of urine/saline confirmed successful puncture. A Terumo guidewire was then maneuvered to pass it through the punctured calyx down into the ureter. Cases in which this was successful proceeded with routine tract dilatation using telescopic metal dilators. In cases where the guidewire coiled in the calyx itself and failed to advance into the pelvis and ureter, we performed our technique (described below) to ensure safe location of the guidewire before proceeding with routine tract dilatation (Figures 1 and 2).

A 9 Fr. metallic dilator cannula with outer sheath was passed over the guidewire under fluoroscopic guidance into the calyx. The outer sheath was advanced into the calyx followed by removal of the inner cannula. A second guidewire was passed through the outer sheath, which was then withdrawn. An 6/7.5 Fr. semirigid ureteroscope was passed percutaneously through the puncture wound between the two guidewires, monitoring its gradual progress via both the endoscopic as well as the fluoroscopic monitors, till the semirigid ureteroscope entered the correct calyx and the stone was visualized. The two guidewires served as "safety wires", comparable to "tramtrack" or "railway lines", between which the semirigid ureteroscope was gradually passed, taking care that the two guidewires were always in vision. The ureteroscope was not passed over one of the guidewires or through the outer sheath as the former may result in buckling of the guidewire and the later may hinder the maneuverability of the semirigid ureteroscope. A third guidewire was then passed through the working channel of the semirigid ureteroscope, and under direct vision, maneuvered by the side of the stone, through the infundibulum, into the pelvis and ureter. The first two guidewires were then removed and routine tract dilatation was then carried out over the guidewire that was located into the ureter, after withdrawing the semirigid ureteroscope. The rest of the procedure was then carried out in the usual manner.

RESULTS

Between January 2010 to June 2014, a total of 675 PCNLs were carried out at our institute. Consultants or final year urology residents under supervision performed all procedures. We had to resort to our technique in 85 of these cases (12.5%). Table-1 gives the patient data analysis of these 85 cases. The mean time required for our technique was calculated from the point of introduction of the semirigid ureteroscope untill the successful passage of the guidewire down into the ureter. In three patients, with calyceal and tightly impacted pelviureteric junction stones, after puncture of the lower calyx, the guidewire could not be passed into the ureter and was instead coiled into the superior calyx. In all these three patients, initial attempt with the traditional approach resulted in failure to advance the guidewire beyond the punctured calyx itself. In the remaining 82 cases, the guidewire could be successfully moved out of the punctured calyx into the ureter. There were no intraoperative or postoperative complications in the 85 patients in whom this technique was employed.

DISCUSSION

The three most important steps in performing PCNL are:

1. Accurate puncture
2. Passage and location of the guidewire into the pelvicalyceal system in such a way as to prevent slippage (the most ideal being passage of guidewire into the ureter)
3. Dilatation

Current literature on PCNL emphasizes a lot on steps 1 and 3 (1-7). Our technique ensures location of the guidewire into the ureter after a successful puncture to enable safe dilatation and highlights the importance of step 2. This was a single arm, single institution retrospective series of an alternative method of placing a guidewire down the ureter during percutaneous access, in instances where fluoroscopic placement was unsuccessful.

Table 1 - Patient Data.

Characteristics	Data
Total number of PCNLs	675
No. in which our technique was used; (%)	85 (12.5%)
Mean age; (range)	45.5 yrs (16-71 yrs)
Previous open surgery	12
Puncture	
Superior calyx	2
Middle calyx	32
Lower calyx	51
Mean time taken to park the guidewire into the ureter with our technique ;(range)	95 secs (45- 135secs)
Mean fluoroscopy time from insertion of ureteroscope to passage of guidewire into ureter	8.8secs (6-14secs)
Total time from puncture to sheath placement	210 secs (125-280 secs)
Mean fluoroscopy time from puncture to insertion of sheath	15.5 secs (12-22 secs)
Complications	nil

Figure-1a - Plain X ray KUB showing inferior calyx and pelvic stone.

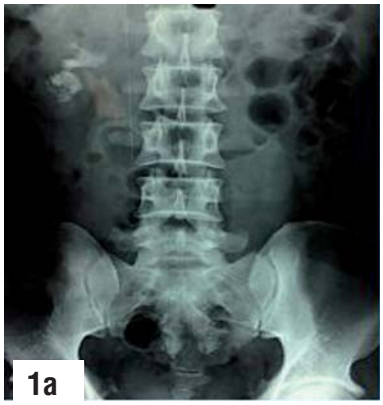
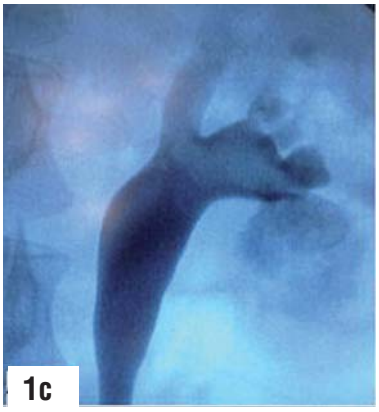


Figure-1b - Retrograde Pyelogram in prone position, prior to puncture.



Various devices and alternative techniques have been described in literature in order to obtain a better puncture and to pass a perfect guidewire (8). Retrograde nephrostomy by Lawson's procedure was reported in 1980. Grass et al. (9) described flexible ureteroscopy for assisting antegrade percutaneous renal access under fluoroscopic control in difficult cases. Bader et al. (1) reported their experience with the "all–seeing needle" as an optical puncture system in PCNL for achieving op-

Figure-1c - Lower calyx puncture, guidewire coiled in lower calyx. Guidewire could not be located beyond the infundibulum of the punctured calyx. Dilatation at this stage would be tenuous with the possibility of slippage of guidewire resulting in loss of access.

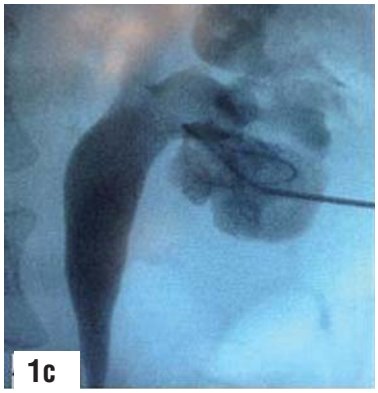


Figure-1d - Second guidewire coiled into the lower calyx through the outer sheath of the puncture cannula.

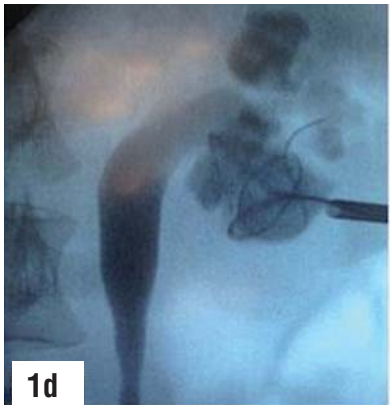
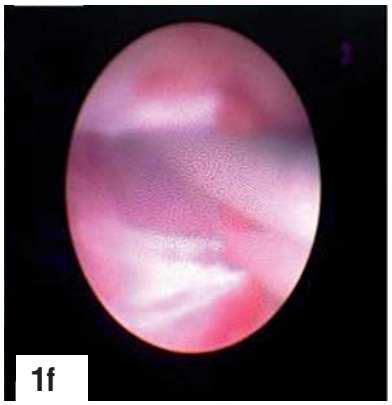


Figure-1e - Ureteroscope being passed percutaneously through the puncture site.

Figure-1f - Ureteroscope passed between the two guidewires.



timal renal access. The micro-optics of 0.9mm and 0.6mm diameter with integrated light lead was passed through the working channel of a 4.85 Fr. access needle, which was connected to an irrigation system for better intraoperative view. By this technique the punctured calyces and calculi could be visualized in all 15 patients prior to placement of guidewire and tract dilatation. Chen et al. (10) described a novel device called 'sonic flashlight' to visualize and guide the needle during renal access. This technique is a renal-time tomographic reflection that generates a virtual anatomically scaled image. The demonstration of the sonographic image appears from the tip of the transducer. The authors claimed that this technique could facilitate safe renal access for complicated cases. Other techniques described include a computer assisted gantry system (4) and mobile augmented reality for computer assisted PCNL (5).

Our technique is easy to learn and inexpensive as no sophisticated instruments are required. Furthermore, no complications related to our tech-

Figure-2a - The progress of the ureteroscope is monitored simultaneously through both the endoscopic and fluoroscopic monitors untill the stone is visualized. Third guidewire is passed through the working channel of the ureteroscope and is maneuvered to bypass the stone under vision. Note that the previously passed guidewire, which was assumed to be coiled in the calyx fluoroscopically, had in fact perforated the mucosa. Dilatation over this guidewire would have been hazardous.

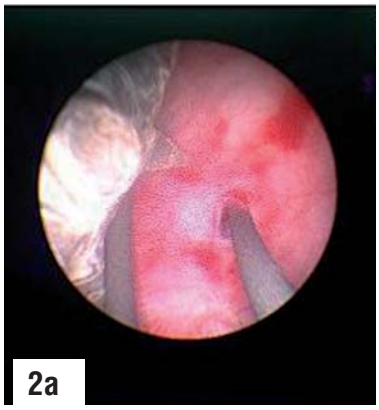
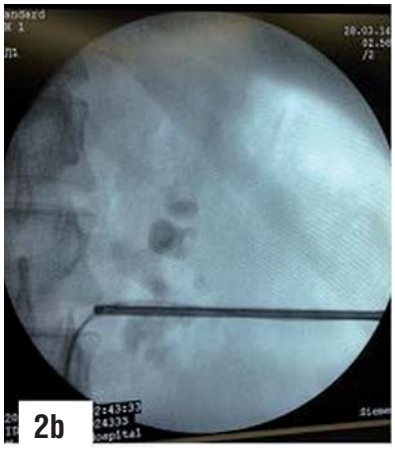


Figure-2b - Guidewire has been successfully passed down into the ureter.

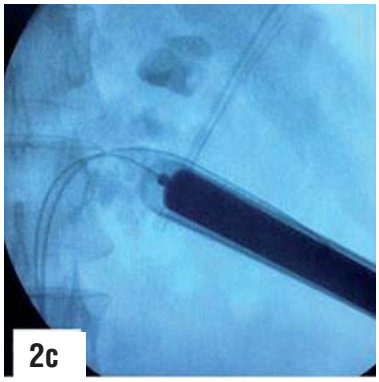


nique were observed, reiterating that it is safe to instrument a newly created tract into the kidney.

CONCLUSION

Use of the ureteroscope passed percutaneously through the puncture site in PCNL is a safe and effective technique that helps in maneuvering the guidewire down into the ureter, which subsequently enables safe dilatation.

Figure-2c - Routine dilatation is carried out over the newly passed guidewire to enable passage of amplatz sheath of desired size.



ABBREVIATIONS

USG KUB = Ultrasonogram kidney-ureter-bladder
PCNL = Percutaneous Nephrolithotomy
PCN = Percutaneous Nephrostomy

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Tarun Javali, MS, M.Ch
Assistant Professor, Urology
M.S. Ramaiah Hospital, Bangalore, India
E-mail: tarunjavali@gmail.com

CHALLENGING CLINICAL CASES

Vol. 41 (5): 1020-1026, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0002

Transanal Minimally Invasive Surgery (TAMIS) to Treat Vesicorectal Fistula: A New Approach

Marcos Tobias-Machado [1,2], Pablo Aloisio Lima Mattos [1,3], Leonardo Oliveira Reis [4,5], César Augusto Braz Juliano [1,3], Antonio Carlos Lima Pompeo [3]

[1] Programa de Cirurgia Urológica Minimamente Invasiva, Departamento de Urologia, Faculdade de Medicina do ABC, Santo André, São Paulo, Brasil; [2] Seção de Uro-oncologia, Departamento de Urologia, Faculdade de Medicina do ABC, Santo André, São Paulo, Brasil; [3] Departamento de Urologia, Faculdade de Medicina do ABC, Santo André, São Paulo, Brasil; [4] Divisão de Urologia da Faculdade de Ciências Médicas da Universidade de Campinas, UNICAMP, Campinas, Brasil; [5] Faculdade de Medicina - Divisão de Urologia do Centro de Ciências da Vida, Pontifícia Universidade Católica de Campinas (PUC-Campinas), Brasil

ABSTRACT

Purpose: Vesicorectal fistula is one of the most devastating postoperative complications after radical prostatectomy. Definitive treatment is difficult due to morbidity and recurrence. Despite many options, there is not an unanimous accepted approach. This article aimed to report a new minimally invasive approach as an option to reconstructive surgery.

Materials and Methods: We report on Transanal Minimally Invasive Surgery (TAMIS) with miniLap devices for instrumentation in a 65 year old patient presenting with vesicorectal fistula after radical prostatectomy. We used Alexis® device for transanal access and 3, 5 and 11 mm triangulated ports for the procedure. The surgical steps were as follows: cystoscopy and implant of guide wire through fistula; patient at jack-knife position; transanal access; Identification of the fistula; dissection; vesical wall closure; injection of fibrin glue in defect; rectal wall closure.

Results: The operative time was 240 minutes, with 120 minutes for reconstruction. No perioperative complications or conversion were observed. Hospital stay was two days and catheters were removed at four weeks. No recurrence was observed.

Conclusions: This approach has low morbidity and is feasible. The main difficulties consisted in maintaining luminal dilation, instrumental manipulation and suturing.

ARTICLE INFO

Key words:
Fistula; Postoperative Complications; Natural Orifice Endoscopic Surgery

Int Braz J Urol. 2015; 41: 1020-6

Submitted for publication:
January 02, 2014

Accepted after revision:
June 08, 2014

INTRODUCTION

Vesicorectal fistula consists of an abnormal communication between the bladder urothelium and rectal mucosa, which represents a devastating condition. Diverticulitis, Crohn's disease and cancer are the most common etiologies (1-5). Vesicorectal fistula is an extremely rare complication following radical prostatectomy.

Patients may present with irritative urinary symptoms, urinary tract infection, pneumaturia, fecaluria and tenesmus (1, 6, 7). Cystoscopy and tomography are the most accurate tests to confirm the diagnosis (1, 7). Non- surgical watchful waiting is

an option in selected cases (8). Drug therapy such as antibiotics, intravenous total parenteral nutrition and bowel rest may be used in patients with few symptoms and non-toxemic and non-malignant etiology (9). Proximal colostomy and urinary catheterization are options in poorly responsive or very symptomatic patients. Definitive treatment aiming to separate the organs and close the defect with preservation of function is recommended in the absence of infection or obstruction. Partial resection and interposition of omentum between suture lines may be required. It can be done in stages. (1, 7, 10).

Definitive treatment is challenging due to the morbidity of the classic techniques and the high recurrence rate. Currently we prefer traditional approaches like transanal, transabdominal, trans-sphincteric and transperineal (11). However, there is not an universally accepted approach. Techniques described for minimally invasive repair such as laparoscopic transperitoneal approach can reduce the morbidity of treatment and was recently described with good results (12-14).

These evidences have motivated us to evaluate new approaches as options to treat vesicorectal fistulas in selected cases. This article aims to describe and evaluate the results of a new minimally invasive approach to treat vesicorectal fistula.

MATERIALS AND METHODS

A 65 year old patient developed a vesicorectal fistula in the first 20 days after radical prostatectomy to treat prostate cancer. The diagnosis was confirmed by cystoscopy and CT scan, which showed the fistula in the trigone. Conservative treatment was attempted with high absorption diet, suprapubic cystostomy and proximal colostomy, but the treatment failed after two months.

We performed Transanal Minimally Invasive Surgery (TAMIS) with miniLap devices for instrumentation. Initially, the patient was positioned in lithotomy and cystoscopy was made for implant of a hydrophilic guide wire through fistula to facilitate identification and dissection. We did not position ureteral catheters but it can be made to improve safety.

The patient was placed in the jack-knife position with the buttocks apart. We used Alexis® (Applied Medical System) device for 2.5-6 cm size incisions to configure the TAMIS platform. The device was inserted into the anal canal and rectum. A silicone glove was fixed in the outer ring of the device and the self-retaining design held the anal canal open, allowing access to the operative field (Figure-1). We positioned three triangulated ports (one 11 mm port for rigid endoscope 0 degrees, one 5 mm port for ultrasonic scalpel and one 3 mm port for minilap devices). We kept the pneumorectum around 15 mmHg.

The fistula was identified 5 cm from the anal verge. The fistula tract was excised with ultrasonic scalpel. The guidewire was removed only at the end of excision (Figure-2). The bladder wall was closed with 3-0 Vicryl in a running suture. The space between the bladder wall and rectal wall was filled with fibrin glue (Figure-3). The rectal wall was closed with 3-0 Vicryl in a running suture (Figure-4). Finally we maintained a urethral catheter 18 Fr and cystostomy 20 Fr.

RESULTS

The operative time was 240 minutes for TAMIS with 120 minutes for reconstruction.

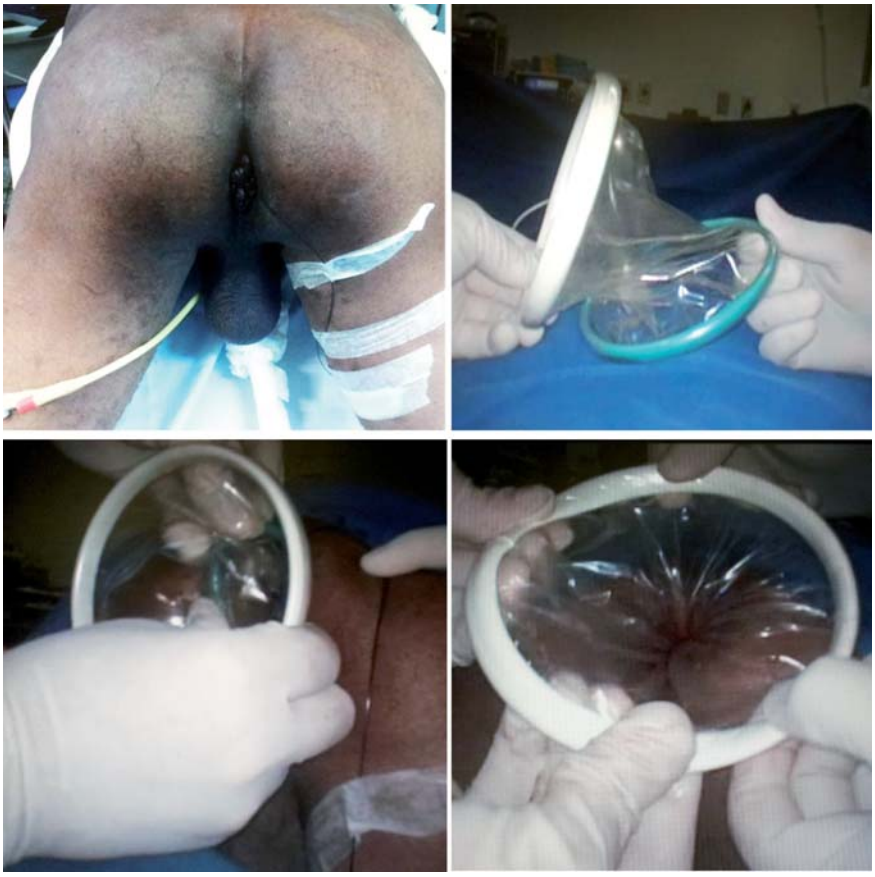
There was no conversion, perioperative or postoperative complications in the procedure, including bleeding and rectal perforation. The hospital stay was two days.

The full return to daily activities was in two weeks. Cystoscopy was performed after four weeks of surgery and it revealed no signs of fistula. After four months of follow-up no recurrence was observed. There was no stenosis of anal canal and patient is defecating and voiding normally.

DISCUSSION

There is no consensus about the better approach to treat recto-urinary fistula. Conventional open surgery remains the preferred choice, however it has some limitations (15-17). Trans-abdominal and posterior approaches have the disadvantages of large incisions to achieve good access. Transperineal and transanal approaches are

Figure 1 - Transanal access by Alexis® device.

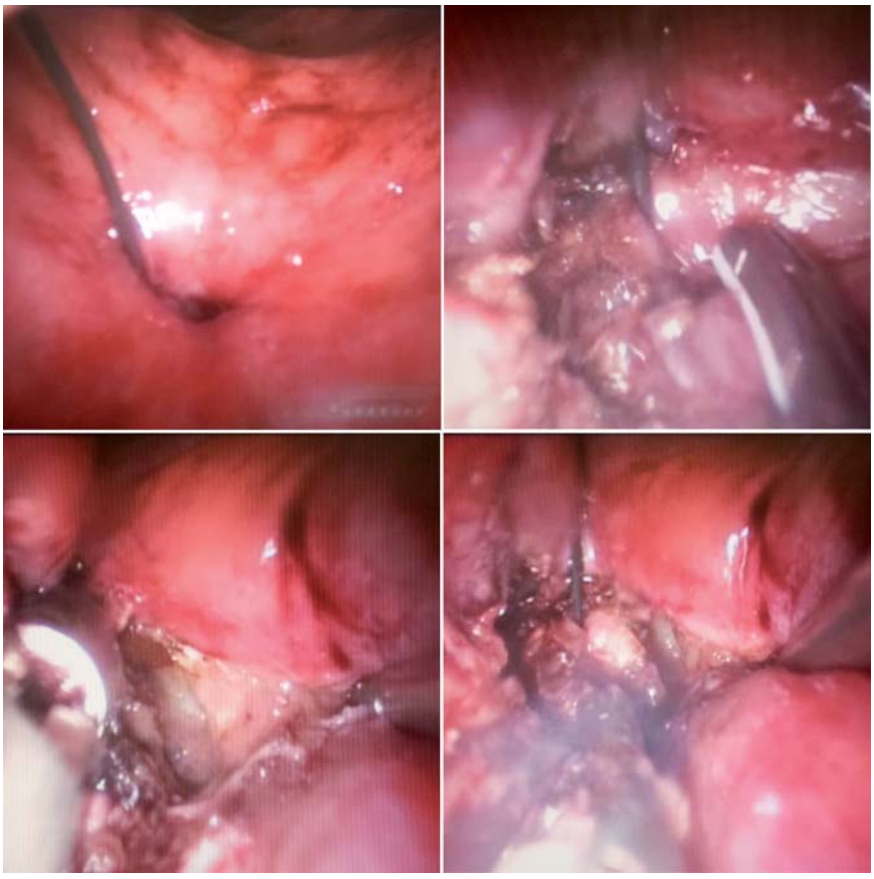


less invasive but limited visualization and small working space can difficult a good repair. Laparoscopic transperitoneal surgery was introduced in 2003. With an excellent magnification the posterior bladder wall is opened including the fistula tract to achieve good visualization and dissection. The advantages are fast recovery with preliminary good results (14). The refinement of this technique using robotic assistive technology was also described with good results (18).

In the current decade, transluminal procedures have been developed to manage selected cases of urologic pathologies. The potential advantages include less effects of carbonic gas, no mobilization or contact with other organs and reduction of entrance ports. The main disadvantages consist in the small working space that requires more skills to reconstructive procedures. Transvesicoscopic surgery has been reported to treat bladder foreign body, lithiasis, diverticula, ureteral reimplantation and vesicovaginal fistula with excellent results (19-21).

Transanal Minimally Invasive Surgery (TAMIS) is a variant of Natural Orifice Endoscopic Surgery (NOES). Presented in 2009, this surgical platform uses access devices that traditionally are used for single site laparoscopy. The most used devices are the SILS™ Port, Alexis Wound Protector/Retractor and the GelPOINT Path Trans anal Access Platform. The chosen device is inserted into the rectum. When pneumorectum is established, the surgical field is then increased considerably and gives to the surgeon the ability to expand their skills to include procedures from the distal rectum to the mid and proximal rectum. This platform uses standard laparoscopic or minilap instrumentation.

Figure 2 - Vesicorectal fistula is excised.

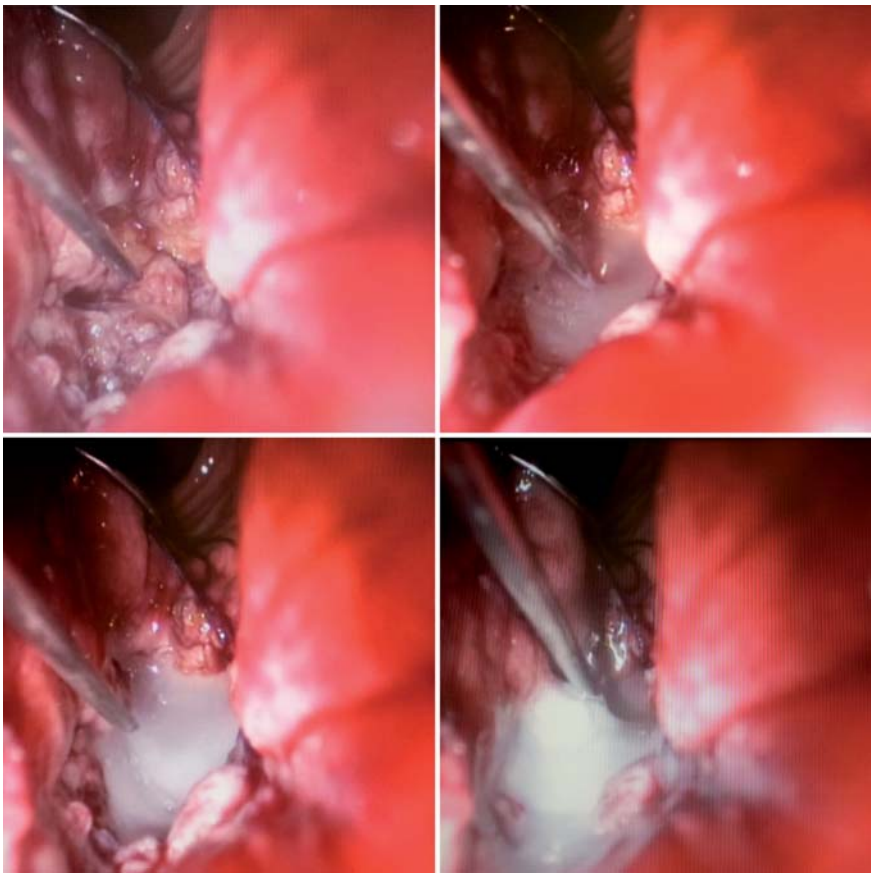


TAMIS was initially described for treatment of benign lesions. After that, treatment of malignant lesions was also described. We observed a growing acceptance in the use of TAMIS to approach anorectal fistulas and tumors at early grades with good results (22-24). Albert and cols. performed a retrospective analysis of 50 patients with benign and malignant rectal lesions treated with TAMIS at a tertiary referral center. All procedures were made without conversion to other approaches and 68% of patients were discharged on the day of surgery. Only 6% were found to have microscopically positive margins. No long-term complications were observed.

TAMIS platform is versatile and there are some applications beyond local excision. There are descriptions of rectourethral fistula repair, ligation of rectal Dieulafoy's lesion and extraction of foreign body. Atallah and cols. performed TAMIS to treat a man with rectourethral fistula after cryoablation treatment for prostate cancer. A follow-up enema demonstrated resolution of the fistula (23). Gómez et al. comment in a letter to the editor of Actas Urologicas Espanõlas (in press) one case in which TAMIS was utilized to repair an uretrorectal fistula using Gelpoint device. The procedure was successful, achieving a good exposition to 2-layer repair and hemostasis, according to the authors (25).

These works motivated us to propose this new approach to treat vesicorectal fistula and evaluate its results. In our report the duration of surgery was 240 minutes, with 120 minutes for reconstruction. We believe that the limited experience with access and non-availability of a material specifically developed for these new approaches is

Figure 3 - Injection of fibrin glue between vesical and rectal wall.



still an obstacle to overcome and make surgery times prolonged when compared to conventional invasive procedures.

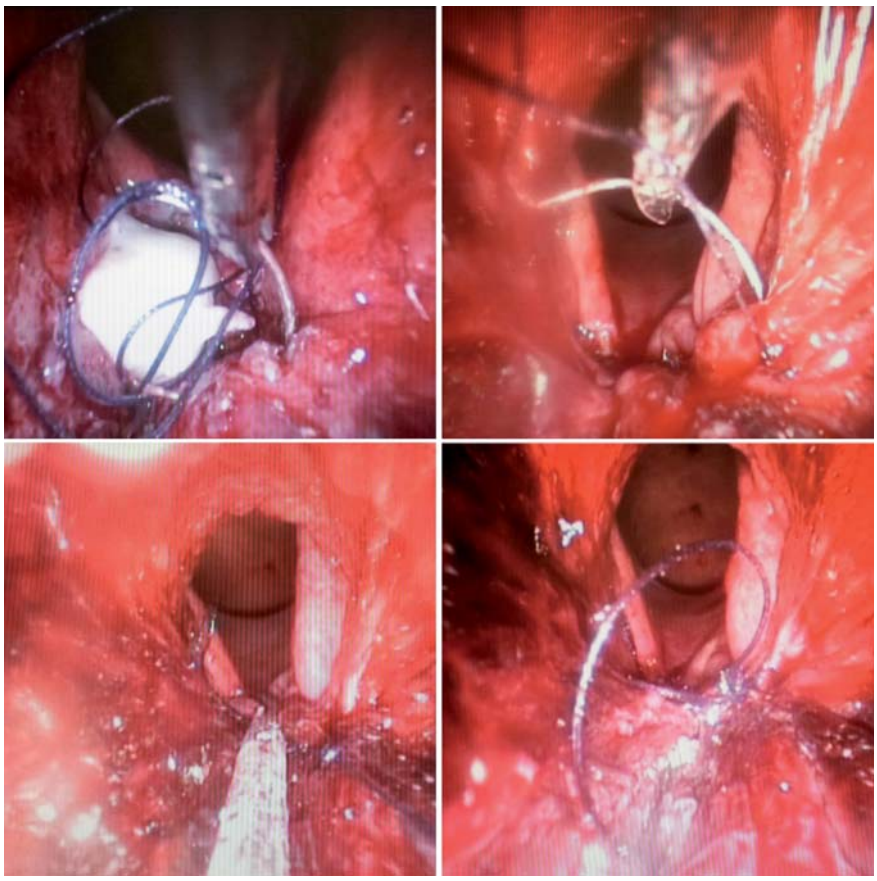
In our procedure the greatest difficulties were maintaining luminal dilation, the instrumental manipulation and intraoperative suture. Nevertheless, the length appears to be similar to trans-peritoneal laparoscopic approaches already described. No complications were observed. The procedure was completed without conversion, and intraoperative bleeding was negligible.

Despite the limitations, careful magnified dissections and subsequent repairs were the elements that allowed a better control and a minimized risk of perioperative complications and conversion.

One of the most feared troubles in the repair of vesicorectal fistulas is the loss of functionality due to rectal morbidity of most techniques traditionally used, with the emergence of problems such as anal stenosis and fecal incontinence. None of these postoperative complications were observed in our report, even with little experience with the new method. Minimally invasive surgery done by an expert professional is less aggressive, reduces the risk of complications and may reproduce the results of traditional techniques.

The length of hospital stay in our report was 2 days. This result was even slightly better than some series in the literature and reinforces the potential of minimally invasive surgery in decreasing the morbidity (14,18,26,27). Although with a short follow-up, the preliminary result is encouraging and shows compliance with the findings in the literature (14,26).

Figure 4 - Rectal wall closed.



CONCLUSIONS

Transanal Minimally Invasive Surgery (TAMIS) to treat vesicorectal fistula is feasible and seems to have lower morbidity when compared with more traditional techniques. It is effective and can be offered as an option by experienced laparoscopic surgeons to patients. The greatest difficulties were maintaining luminal dilation, instrumental manipulation and intraoperative suture.

ABBREVIATIONS

TAMIS = Transanal Minimally Invasive Surgery.
NOES = Natural Orifice Endoscopic Surgery.

ACKNOWLEDGEMENTS

To Language Editing Facility - Faculty of Medical Sciences, University of Campinas, UNICAMP

CONFLICT OF INTEREST

None declared.

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Correspondence address:
Pablo Aloisio Lima Mattos, MD
Rua Veridiana, 115 / 13
São Paulo, SP, 01238-010, Brazil
Fax: + 55 11 3996-0045
E-mail: palmattos@hotmail.com

Vol. 41 (5): 1027-1029, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0639

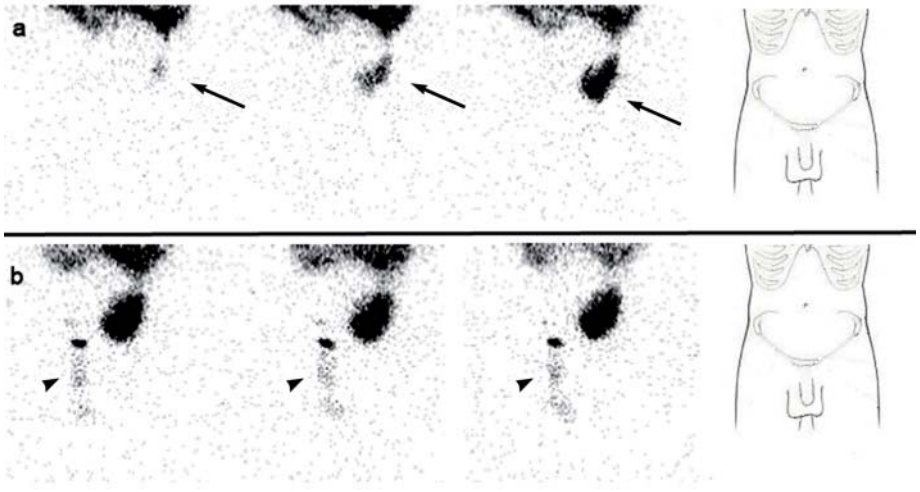
Early peritoneal-scrotal leakage in a patient submitted to peritoneal dialysis demonstrated by dynamic peritoneal 99mTc-Phytate scintigraphy

Andrés Martínez-Esteve [1], Francisco Javier García-Gómez [1], Juan Ignacio Cuenca-Cuenca [1], Juan Luis Tirado-Hospital [1]

[1] *Departamento de Medicina Nuclear, Hospital Virgen del Rocío Universitario, Sevilla, España*

A 77 year old male with history of renal lithiasis leading to right nefrectomy and end stage renal disease (ESRD) secondary to possible vascular nephropathy and focal segmental glomerulosclerosis diagnosed in 2009, started ambulatory peritoneal dialysis (APD) due to clinical decline. Insufficient drainage of the peritoneal dialysis solution with progressive bilateral testicular edema (more severe in right side) was observed from the first APD session. As a consequence, the patient needed temporarily hemodialysis. In order to diagnose a patent peritoneal-vaginal duct, a dynamic 99mTc-Phytate scintigraphy was performed after introduction of peritoneal dialysis solution (600, 1200 and 2000 mL) labelled with 74 MBq of 99mTc-Phytate in the abdominal cavity. A total of 60 images (30 seconds per image) were acquired and a hypogastric uptake of radiotracer was observed (Figure 1 - Panel a). Right inguinal and scrotal uptake was observed only after per-

Figure 1 - Dynamic peritoneal 99mTc-Phytate scintigraphy. Panel a: Images acquired during the filling of peritoneal dialysis solution (600, 1200 and 2000 ml) labeled with 74 MBq of 99mTc Phytate, revealing a hypogastric uptake of radiotracer (arrow), not reaching the inguinal canal or the scrotal area. On the right side, anatomical reference contour. Panel b: Inguinal and right scrotal uptake of radiotracer (head arrow) after Valsalva's maneuver. On the right side, anatomical reference contour.



forming the Valsalva's maneuver (Figure 1 - Panel b). Delayed sectorial images (Figures 2 and 3) were acquired following the peritoneal drainage period (250 seconds per image), revealing the presence of peritoneal dialysis solution in both inguinal canal and right scrotal area (Arrow and head arrow). Due to the peritoneal-vaginal leakage an inguinal hernioplasty with raquideal anesthesia was performed.

The peritoneal-scrotal dialysate leakage is the major non-infectious catheter-related complication in patients receiving APD, caused by increased intraperitoneal pressure and the loss of integrity of the peritoneal membrane, although the most common cause is fluid extravasation from an indirect hernial sac or patent peritoneal-vaginal duct (1). Pleural, abdominal or genital dialysate leaks tend to develop during the first year of APD, while early leaks after catheter insertion are usually observed in the first 30 days of APD (2).

Several diagnostic methods including intraperitoneal infusion of contrast, abdominal x-ray, computed tomography or peritoneal scintigraphy are employed when the leak diagnosis is uncertain. The peritoneal scintigraphy has proven to be a useful tool for the diagnosis of the peritoneal leakage (2,3), being an effective method for identifying structural abnormalities and localization of the source of the leak.

Figure 2 - Sectorial peritoneal 99mTc-Phytate scintigraphy. Panel a: Post-filling period image revealing the right inguinal and scrotal uptake of radiotracer (head arrow). Panel b: Post peritoneal lavage image, showing the appearance of left inguinal canal uptake of radiotracer (arrow).

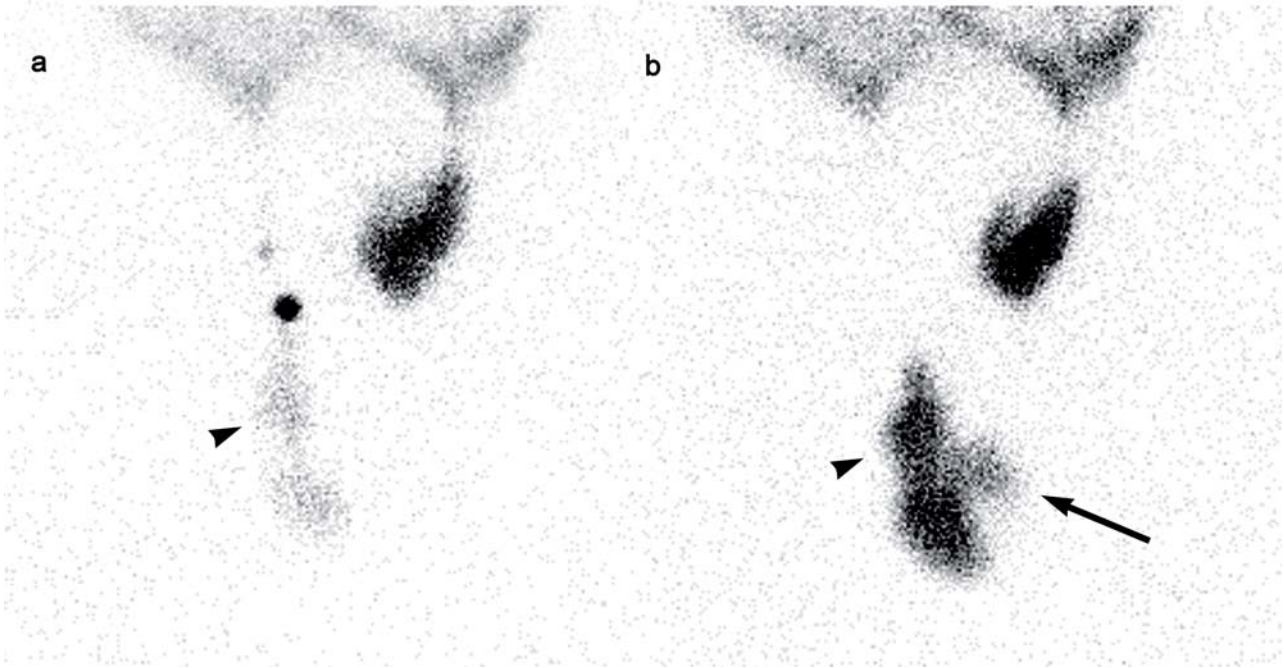
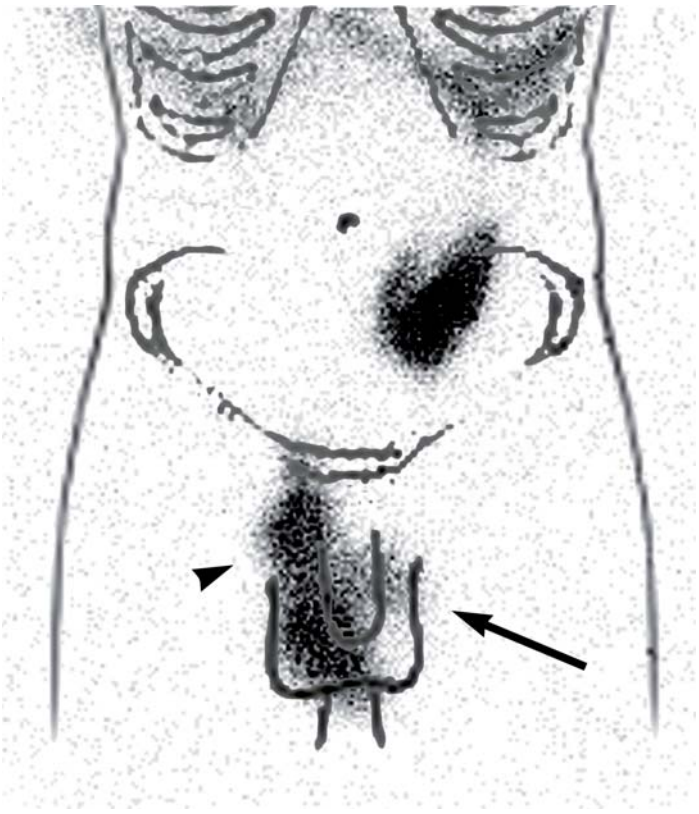


Figure 3 - Sectorial peritoneal 99mTc-Phytate scintigraphy post-peritoneal lavage image over anatomical reference contour showing the right inguinal and scrotal uptake of radiotracer (head arrow) and left inguinal uptake (arrow).



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Correspondence address:
Andrés Martínez Esteve, MD
Departamento de Medicina Nuclear
Hospital Virgen del Rocío Universitario
Sevilla, 41013, España
Telephone: + 34 63 545-0488.
E-mail: andres.martinez.esteve@gmail.com

ARTICLE INFO

Int Braz J Urol. 2015; 41: 1027-9

Submitted for publication:
December 11, 2014

Accepted after revision:
May 12, 2015

VIDEO SECTION

Vol. 41 (5): 1030-1031, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2014.0391

Laparoscopic repair for vesicouterine fistulae

Rafael A. Maioli [1], André R. S. Macedo [1], André R. L. Garcia [1], Silvio H. M. de Almeida [1], Marco Aurélio Freitas Rodrigues [1]

[1] *Disciplina de Urologia, Departamento de Cirurgia da Universidade Estadual de Londrina. Paraná, PR, Brasil*

INTRODUCTION

Vesicouterine fistulae (VUF) comprise 2-9% of all cases of urogenital fistulae. Only 5% of patients respond to conservative management, whereas other patients require definitive surgical repair (1). Although laparoscopy provides better visualization through magnification, this technique is difficult to learn. We hypothesized that the experience gained through other pelvic laparoscopic procedures, such as ureteral re-implantation, correction of vesicovaginal fistulae, and radical prostatectomy, may enhance the ability of urologists to perform this procedure with traditional laparoscopic intracorporeal suturing, without the need for any special tools.

Objective: The purpose of this video is to present the laparoscopic repair of a VUF in a 42-year-old woman, with gross hematuria, in the immediate postoperative phase following a cesarean delivery. The obstetric team implemented conservative management, including Foley catheter insertion, for 2 weeks. She subsequently developed intermittent hematuria and cystitis. The urology team was consulted 15 days after cesarean delivery. Cystoscopy indicated an ulcerated lesion in the bladder dome of approximately 1.0cm in size. Hysterosalpingography and a pelvic computed tomography scan indicated a fistula.
Materials and Methods: Laparoscopic repair was performed 30 days after the cesarean delivery. The patient was placed in the lithotomy position while also in an extreme Trendelenburg position. Pneumoperitoneum was established using a Veress needle in the midline infra-umbilical region, and a primary 11-mm port was inserted. Another 11-mm port was inserted exactly between the left superior iliac spine and the umbilicus. Two other 5-mm ports were established under laparoscopic guidance in the iliac fossa on both sides. The omental adhesions in the pelvis were carefully released and the peritoneum between the bladder and uterus was incised via cautery. Limited cystotomy was performed, and the specific sites of the fistula and the ureteral meatus were identified; thereafter, the posterior bladder wall was adequately mobilized away from the uterus. The uterine rent was then closed using single 3/0Vicryl sutures and two-layer watertight closure of the urinary bladder was achieved by using 3/0Vicryl sutures. An omental flap was mobilized and inserted between the uterus and the urinary bladder, and was fixed using two 3/0Vicryl sutures, followed by tube drain insertion.
Results: The operative time was 140 min, whereas the blood loss was 100ml. The patient was discharged 3 days after surgery, and the catheter was removed 12 days after surgery.
Discussion: Laparoscopy has advantages over open surgery in that it is associated with less pain, shorter length of hospital stay, better cosmesis, quicker recovery, and equal efficacy. Although cases of VUF are rarely noted, the laparoscopic skill obtained through other urological procedures suggest, that laparoscopic repair may be the procedure of choice for such cases (2). The reported operative time for the laparoscopic repair of VUF in the literature varies between 140 and 220 min (3). However, laparoscopic techniques should be considered as a mode of abdominal access and should not influence the method of surgical repair. Surgical success should depend on the adherence to good technique rather than the approach. Hence, this method appears to be a viable alternative for surgeons experienced with laparoscopic suturing techniques.
Conclusion: Laparoscopic repair appears to be a viable alternative for surgeons experienced with laparoscopic suturing techniques.

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Correspondence address:
Silvio Henrique Maia de Almeida, MD
Rua Francisco Marcelino da Silva, 270
Londrina, PR, 86047-160, Brasil
E-mail: salmeida@sercomtel.com.br

ARTICLE INFO

ARTICLE INFO Available at: http://www.brazjurol.com.br/videos/september_october_2015/Maioli_1030_1031video.htm

Int Braz J Urol. 2015; 41 (Video #8): 1030-1

Submitted for publication:
August 04, 2014

Accepted after revision:
March 30, 2015

EDITORIAL COMMENT

This video nicely depicts the laparoscopic repair of a vesicouterine fistula. Vesicouterine fistulae are uncommon and have been estimated to account for only 1% to 4% of all genitourinary fistulae. (1) Conservative measures are usually attempted first. They include bladder decompression with an indwelling catheter and medical management to induce amenorrhea. (2) In select patients cystoscopic fulguration of the vesicular portion of the fistula has also been proven to be effective. (3) Laparoscopic and robotic repairs have been reported as alternatives to open surgery. Regardless of technique, adherence to the principles for genitourinary fistula repair will maximize the chances for success. Wide exposure and excision of scar tissue around the fistula; tension-free closure of the wound in multiple layers; and utilization of an omental or myouterine flap are all possible with minimally invasive techniques. (4, 5)

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Hubert S Swana, MD
Nemours Children's Hospital, Orlando
13535 Nemours Parkway
Orlando, Florida, 32827, USA
Fax: + 1 407 650-7266
E-mail: hswana@nemours.org

LETTER TO THE EDITOR

Vol. 41 (5): 1032-1032, September - October, 2015
doi: 10.1590/S1677-5538.IBJU.2015.0240

Re: Bilateral isolated epididymal agenesis in a 32 year old man

Yadollah Ahmadi Asr Badr [1], Reza Sari Motlagh [1], Ehsan Sepehran [1]

[1] *Department of Urology, Tabriz University of Medical Sciences, Iran*

Vol. 41 (2): 379-381, March - April, 2015

To the editor,

In last issue of the Journal, Badr, et al (1) described a case report of 32 years old otherwise healthy man with bilateral epididymal agenesis. In medicine, agenesis refers to the failure of an organ to develop during embryonal time due to the absence of primordial tissue. Since secondary epididymal atrophy in this case could not be rolled out the term agenesis is inadequate and misleading. Particularly, because atrophic cauda epididymis of the left testis shown in the figure 2. Furthermore, cystic fibrosis carriers of one copy of the genetic mutation are usually healthy but may also be infertile due to undiagnosed secondary malformation of the Wolffian duct. Therefore, term epididymal atrophy instead agenesis would be more appropriate.

Reference

1. Badr YA, Motlagh RS, Sepehran E. Bilateral isolated Epididymal Agenesis in a 32 year old man. Int Braz J Urol. 2015;41:379-81.

Faruk Haziselimovic, MD
Bahnhofplatz 11 4410 Liestal Liestal 4410
Switzerland
liestal@kindermedizin-zentrum.ch
Fax: +41 619 279-099

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▪ Sabiston DC: Textbook of Surgery. Philadelphia, WB Saunders. 1986; vol. 1, p. 25.

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