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ORIGINAL ARTICLE

Correlation of in vivo bladder blood flow measurements with tissue hypoxia

Wei-Yu Lin · Anita Mannikarottu · Suning Li · Yung-Shun Juan · Catherine Schuler · Zeeshan Javed · Jerry Blaivas · Robert M. Levin

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Abstract
Aims Obstructive bladder dysfunction is in part due to reduced blood flow and the resulting ischemia of the bladder smooth muscle and mucosa. Our aim was to determine if the severity and localization of ischemia could be determined by measuring blood flow to the bladder with a non-invasive probe placed on the surface of the urothelium.
Materials and methods Twenty-four adult male rabbits (5 months, 3.5–4.0 kg) were divided into three groups: 1—controls; 2—2 h of bilateral ischemia; and 3—partial outlet obstruction, and were evaluated after 2 weeks. Each rabbit received an intraperitoneal injection of Hypoxyprobe-1. In vivo real-time monitoring of blood flow was measured at five sites within the bladders with a laser Doppler flowmeter.
Results For all groups, the blood flow readings showed no significant differences among the five sites. The ischemic bladders showed significant decreases in blood flow. The obstructed bladders had significantly lower blood flow than the ischemic bladders. The hypoxyprobe studies demonstrated that there was no hypoxia present in the control bladders; the mucosa of the ischemic bladders showed even hypoxia at an intermediate concentration; the obstructed bladders showed dense but even staining.
Conclusion We have demonstrated that we can determine the severity of ischemia by surface measurement of blood flow.

Keywords Bladder · Blood flow · Laser doppler · Hypoxia · Ischemia

W.-Y. Lin
Division of Urology,
Chang Gung Memorial Hospital, Chia-Yi, Taiwan

W.-Y. Lin · A. Mannikarottu · S. Li · Y.-S. Juan · C. Schuler · Z. Javed · R. M. Levin (✉)
Albany College of Pharmacy,
106 New Scotland Ave, Albany, NY 12208, USA
e-mail: levinr@acp.edu

W.-Y. Lin · A. Mannikarottu · Y.-S. Juan · Z. Javed · R. M. Levin
Albany Medical College, Albany, NY, USA

C. Schuler · R. M. Levin
Stratton VA Medical Center, Albany, NY, USA

J. Blaivas
Weill Cornell Medical Center, New York, NY, USA

Introduction

Vascular occlusive disease and concomitant chronic ischemia have been reported to produce smooth muscle dysfunction in several organs including the intestine, stomach and penile erectile tissue [1–3]. Similarly, clinical and basic studies have suggested that ischemia may play a role in bladder dysfunction [4, 5]. The functional effects of decreased blood flow due to total ligation of the vesicle arteries have been described from in vivo and in vitro studies of the rabbit bladder [6]. Atherosclerotic arterial occlusion as well as arterial compression due to outlet obstruction have been shown to cause significant bladder ischemia in the rabbit [7, 8]. Obstruction-associated decreases in contractile function closely correlate with the decreased bladder blood flow [9]. We and several other investigators have shown that partial outlet obstruction of the rabbit, dog, and pig induces a relative reduction in bladder wall blood flow (ischemia) and reduced oxygen tension (hypoxia) during bladder filling and contraction, which is reversed after voiding [10, 11]. In addition, we demonstrated that partial obstruction of the rabbit bladder outlet

caused decreases in mucosal and detrusor smooth muscle blood flow that were directly proportional to the level of contractile dysfunction present [12].

Experimental studies on bladder blood flow use either laser Doppler techniques where the probe is placed superpubically within the detrusor muscle or quantitatively with florescent microspheres. Laser Doppler flowmetry is a safe and reliable method of measuring real-time tissue blood flow at a given point in an organ. It has been widely used in both the clinical and laboratory settings to measure blood flow in a variety of tissues including the skin, gastrointestinal tract, heart, and brain [13–16]. In the bladder, laser Doppler flowmetry can be used as a non-invasive measure of blood flow to the bladder by placing it on the surface of the bladder mucosa through a cystoscope.

Our hypothesis states that if we measure blood flow to the bladder by using a non-invasive probe placed through the urethra on the surface of the urothelium, we can: (1) determine the severity of ischemia, (2) map out the distribution of ischemia within the bladder, (3) follow the progression of ischemia over time, (4) determine the optimal time to offer therapeutic intervention including outlet reducing surgery, and (5) test whether or not ischemia is reversible after various treatments.

In order to test the first three elements of our hypothesis and provide proof of concept, we have used the Oxylab System (Oxford Optronix Ltd., 19–21 Central 127, Milton Park, Oxford OX14 4SA, UK) to measure blood flow from the bladder mucosal surface. These results are then compared with the results from quantitative immunohistochemical measurements of hypoxia using hypoxyprobe-1 immunohistochemistry.

Materials and methods

All studies were approved by the Institutional Animal Care and Use Committee of the Stratton Veterans Administration Medical Center.

Twenty-four adult male White New Zealand rabbits (5 months old, 3.5–4.0 kg) were divided into three equal groups: group 1 were control rabbits receiving sham surgery; group 2 received bilateral ischemia for 2 h; group 3 received partial outlet obstructions and allowed to recover for 2 weeks.

All rabbits were sedated with ketamine–xylazine (25–6 mg/kg) and surgical anesthesia was maintained with isofluorane (2%). Each bladder was catheterized per urethra with an 8 French Foley catheter and the urethra exposed through a midline incision. The base-proximal urethra was then freed of fat and connective tissue. We manually compressed the bladder to empty any remaining urine in all animals to insure that overdistension did not occur.

In the ischemia experiment, bilateral vesicle vessels were dissected away from the bladder and clamped with microvascular clamps at the point they become attached and enter the bladder for 2 h.

For obstruction, a ligature was tied around the catheterized urethra. The ligature was tied without any space between the ligature and urethra, but did not visually compress the urethra. For consistency, the same surgeon performed all surgeries. This surgeon has performed hundreds of similar partial outlet obstructions, and it is her extensive experience with these models that allows them to be consistent.

The sham group received similar surgery without either clamping the vessels or placing the ligature. Finally, the incision was closed in layers and the catheter removed. The obstructed and half the sham rabbits allowed to recover for 2 weeks. The second half of the sham group were maintained anesthetized for 2 h similar to the ischemia group. All rabbits were maintained on a ventilator during surgery and during the ischemic period. Two hours before euthanasia, the obstructed rabbits were anesthetized, placed on a ventilator to insure consistent tissue oxygenation, and injected with 2-ml hypoxyprobe-1, iv. The ischemic and 2-h sham rabbits received hypoxyprobe-1 immediately following clamping of the vessels or exposing the urethra in the 2-h sham group.

After the 2 h, the bladder was exposed and a small opening was made in the base wall. The bladder, including the anterior wall, dome, posterior wall, and bilateral wall, was visualized using a laser Doppler scanning imaging system (Moor Instruments, UK). Blood flow to the mucosa (and possibly the underlying detrusor) was measured in five areas (dome, anterior wall, posterior wall, dorsal and ventral walls) by gently applying the flow probe to the surface of the mucosa without inserting it into the bladder wall. Each area was marked with a O-O silk ligature tied on the serosa over the five areas. The probe (1 mm in diameter) was scanned by a beam of light from a stable helium neon laser ($\lambda = 632.8$ nM, visible red) that uses a moving mirror to execute a raster pattern over the tissue. Incident light is scattered by moving blood cells, then it is redirected by a mirror system back to the detectors. The scattered laser light is frequency shifted (according to the Doppler effect). The average Doppler frequency shift is directly proportional to the average speed of the blood cells. The bladder image was outlined, and this area was then analyzed for blood flow using an LDI software package (Moor Instruments, UK). Blood flow is given in "perfusion units". Although these arbitrary units represent the direct readings from the LDI, they are linear with blood flow and the relative change is proportional to the difference between control and experimental values.

Because surface measurements are prone to movement artifacts, measurements were obtained only from readings

that were stable for at least 1 min. Typically about 2 min were required to obtain stable readings free of movement artifacts. The penetration of the blood flow probe depends on the tissue density and has been estimated to be approximately 1–2 mm; and thus it would be expected to include underlying detrusor in areas where the mucosa is thin. This however would no way compromise the study since it is the idea that blood flow readings from the luminal surface can be used as a marker for dysfunction; and not just mucosal blood flow.

Immediately following the blood flow study, each bladder was excised, rinsed in ice-cold saline, weighed, and immediately placed in fixative (10% phosphate-buffered formalin). After approximately 10-h fixation, the bladder was opened and one full thickness sample was taken from each of the five sites for immunohistochemical analysis. The distribution and density of hypoxia in each section was determined by hypoxyprobe-1 immunohistochemistry [17, 18]. Each tissue section was embedded in paraffin and 5 µm sections taken and stained with the mouse monoclonal antibody supplied with the Hypoxyprobe-1 kitHypoxyprobe 1 is a chemical that freely distributes to all tissues in the body but covalently binds only to tissues where the oxygen tension is below 10 mm Hg (hypoxic tissue). Within 2 h the unbound hypoxyprobe is washed out of the tissues and eliminated by the kidney. The "Hypoxyprobe Kit" contains the chemical Hypoxyprobe-1 and the antibody that binds specifically to hypoxyprobe-1, thus allowing imaging the tissues using standard immunohistochemistry.

It was important to fix the bladder intact so that we could be sure of taking the proper areas for histological evaluation; thus it was not possible to take isolated strips for contractile studies. However, bladder function can be estimated by bladder weight [12], thus the bladder weight can give a good estimation of the contractile dysfunctions within the models.

The level of hypoxia for the mucosa and muscle was quantitated by image analyses of the stained areas. The density of the hypoxyprobe staining was based on digital analyses of the slides. Specifically for each tissue, five different areas of each tissue section were analyzed and averaged. The optical density of control slides was 0 since there was no staining nor was there any counter-stain. The range of densities was from 0 (clear) to 5.0 (black).

Statistics

One value from each section for blood flow was obtained from each rabbit and the mean ± SEM for the group was calculated ($N = 4$). For the histological studies, quantitative values were obtained from eight different areas of each section of bladder evaluated, averaged and the mean used as one value for that section. Thus, for each section of bladder only the mean density was used. Thus, there was an $N = 4$ for the histological values for each section of the group. The statistics were performed using analyses of variance followed by the Bonferonni test for individual differences. A $P < 0.05$ was required for statistical significance.

Results

Bladder weights for the groups were: sham: 4.0 ± 0.1; bilateral ischemia: 5.1 ± 0.3*; obstructed: 10.4 ± 1.1*×; * = significantly different from sham; × = significantly different from ischemic, $P < 0.05$.

Figure 1 shows the blood flow for the control, 2-h ischemic bladders, and the 2-week obstructed bladders. The readings were very consistent and they showed no significant differences among the control readings of the five regions of the bladder. Two hours of ischemia caused a significant (48%) decrease in blood flow to all areas of the bladder. Two weeks of partial outlet obstruction caused a further decrease in blood flow (15% of control) to the mucosa significantly greater than ischemia. The magnitude of the decrease was approximately the same for all five areas for both ischemic and obstructed bladders.

Figure 2 shows the density of hypoxyprobe staining of the bladder mucosa from the various bladder locations. The density of the staining was similar for all areas within the same bladder. Control bladders showed no staining. The density of staining was significantly greater for the obstructed bladders than for the ischemic bladders.

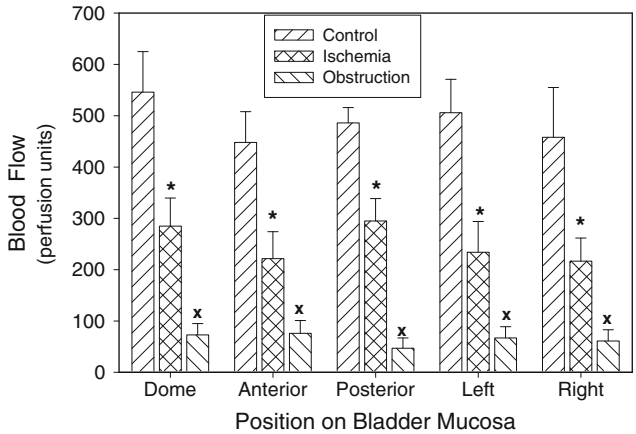
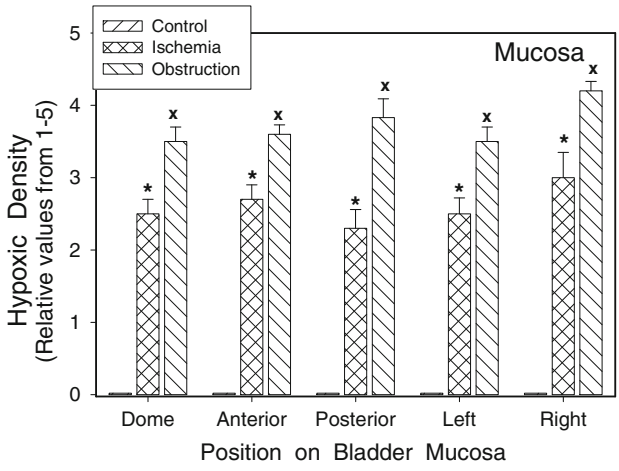


Fig. 1 Blood flow for the five areas from the control, 2-h ischemic bladders, and the 2-week obstructed bladder. *Bar* indicates standard error of the mean. Each *bar* is the mean ± SEM of eight individual rabbits. *Asterisks* significantly different from control ($P < 0.05$). *Multiplication sign* significantly different from muscle ($P < 0.05$)



* = significantly different from control, $p < 0.05$
x = significantly different from ischemia and control, $p < 0.05$

Fig. 2 Density of hypoxyprobe staining of the bladder mucosa of the various bladder locations. *Asterisks* significantly different from control ($P < 0.05$). *Multiplication sign* significantly different from both control and ischemia ($P < 0.05$)

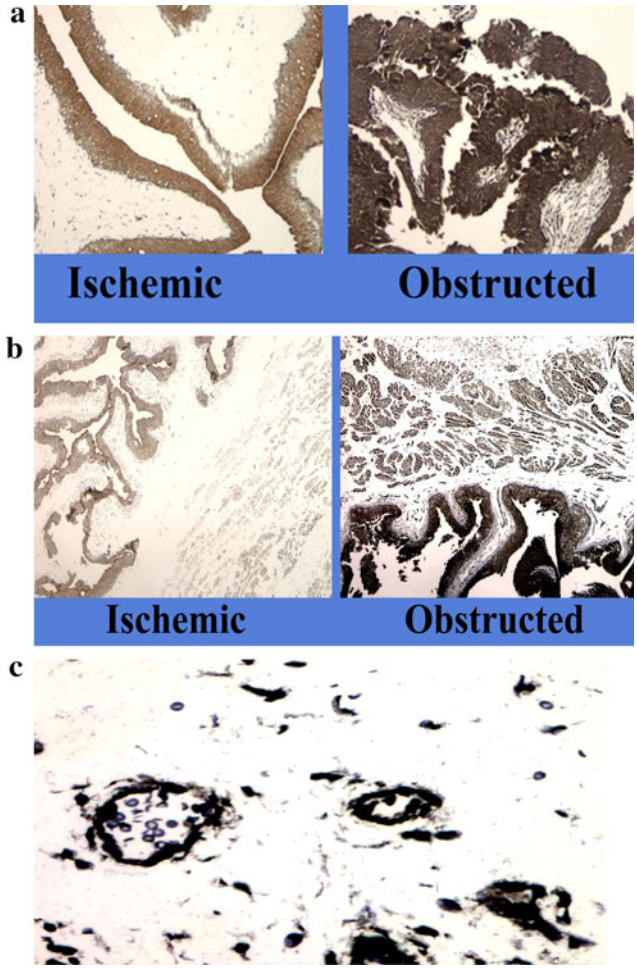


Fig. 3 **a** Representative hypoxyprobe staining of ischemic and obstructed bladders. **b** Representative histological sections of ischemic and obstructed bladders showing detrusor smooth muscle involvement. **c** Representative hypoxyprobe staining showing vascular endothelial staining in the obstructed bladder

Figure 3a shows representative hypoxyprobe staining of both ischemic and obstructed bladders. The obstructed bladders showed much denser staining than the ischemic bladders. The muscle, however, showed uneven and localized staining that could not be quantitated. Figure 3b shows sections of ischemic and obstructed bladders that have clear staining of the muscle. In viewing all the slides, it was obvious that the obstructed bladders had both denser muscle staining, and more discrete areas of muscle stained than the ischemic bladders. These results indicate that the level of mucosal hypoxia is directly related to the density and distribution of hypoxia within the underlying muscle.

It was clear that staining of the vascular endothelium correlated directly with the level of staining of the mucosa (Fig. 3c).

Discussion

In the current study, we demonstrated that (1) both bilateral ischemia and partial outlet obstruction resulted in significant decreases in blood flow, (2) the magnitude of the decrease was significantly greater for the obstructed bladders than for the ischemic bladders, (3) the level of ischemia was similar for the five areas of the bladder we monitored, and (4) the magnitude and distribution of the mucosal hypoxia paralleled the decrease in blood flow. In addition, the magnitude of the hypoxia in the detrusor smooth muscle after obstruction was greater in density and distribution than after ischemia. Lastly, vascular endothelium showed fairly uniform hypoxia staining in both ischemic and obstructed bladders.

Laser Doppler flowmetry provides an accurate and convenient method of estimating microcapillary blood flow in various organs and surgical applications and has a good correlation with isotopic methods [19]. It also has the advantage of permitting continuous on-line monitoring of blood flow under various conditions in various location.

We measured blood flow from the surface of the mucosa rather than inserting the probe directly into the bladder wall. Motion artifacts are the major drawback of the present Doppler system [20]. Because surface measurements are prone to motion artifacts, measurements were obtained only from readings that were stable for at least 1 min. Reproducible recording could be obtained after some practice with the instruments. There was no regional variation in perfusion in our open surgical method.

Because of the technology of the PO_2 sensor in the Oxylab probe, it was not possible to receive consistent oxygen readings when the probe was placed on the surface of the urothelium. The probe has to be fully within the tissue for accurate readings [20]. Thus, although the combination probe can monitor both blood flow and PO_2 within bladder tissue, only blood flow can be monitored from the mucosal surface.

The original idea was to measure the blood flow through a pediatric cystoscope. Although the diameter of the probe would allow the probe to go through a pediatric cystoscope, there was a luer-lock on the end of the probe that prevented us from using a cystoscope. In conjunction with Oxylab, this problem has been solved. Our next study will first determine if the severity of obstructive bladder dysfunction in rabbits can be determined from cystoscopic measurements of blood flow, and if we can follow the progression of obstructive dysfunction.

The data from the hypoxyprobe staining confirms that there is a direct correlation between reduced blood flow and the level of hypoxia. In addition, both blood flow and hypoxyprobe studies clearly show that the mucosa responds evenly to ischemia and obstruction whereas the underlying muscle is unevenly affected. Although the detrusor showed focal hypoxia, the density and distribution of smooth muscle hypoxia was directly linked to the density of mucosal hypoxia; i.e., the greater the level of hypoxia within the muscle, the denser and wider was the distribution of hypoxia in the smooth muscle.

In order to fix all areas of the bladder, no physiological studies were performed. Prior publications however, have clearly demonstrated that 2 weeks obstruction will produce contractile dysfunctions more severe than 2-h bilateral ischemia [21, 22]. In addition, the bladder weights is an estimate the level of contractile dysfunction [23]. In this study, the bladder weight of the ischemic group was significantly higher than the sham group; and the obstructed group was significantly higher than both the sham and the ischemic group indicating a greater level of contractile dysfunction in the obstructed group.

In summary, we have a clear indication that mucosal surface blood flow measurements are accurate and show that ischemia can be easily detected using laser Doppler methodology. In addition, the magnitude of the decrease in blood flow correlated with both the density of mucosal hypoxia, and the density and distribution of the focal areas of hypoxia within the detrusor. We know from blood flow studies using fluorescent microspheres that bilateral ischemia mediated by clamping only the two major arteries reduces blood flow to the bladder by approximately 50%. This is consistent with our findings here.

In regard to our original hypotheses, this study confirms that we can (1) determine the level of ischemia from mucosal surface readings of blood flow; (2) that outlet obstruction results in a fairly uniform hypoxia of the mucosa, and focal areas of hypoxia within the detrusor; and (3) by using the laser Doppler fiber through a cystoscope we can follow the progression of obstructive-mediated ischemia over time.

Our next study will use this technology to determine when intervention is necessary, and follow the course of therapeutic intervention such as the use of antioxidants and natural products which have been demonstrated to be able to reverse both obstructive and ischemic bladder dysfunction [24, 25].

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Conflict of interest statement None declared.

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