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CHRONIC CYCLIC BLADDER OVER-DISTENSION UPREGULATES HYPOXIA-DEPENDENT PATHWAYS

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Abstract

Purpose—Bladder over-distension secondary to anatomic or functional obstruction can eventually lead to pathologic changes, including decreased elasticity and contractile dysfunction. We hypothesize that chronic bladder distension in a murine model will activate hypoxia-dependent signaling pathways despite intermittent relief of the distention.

Methods—Female C57Bl/6 mice were oophorectomized at 5–6 weeks and subjected to urethral catheterization and 90 minutes of bladder distension. Acute and chronic time points were evaluated. Bladder tissue was harvested for H&E staining and for immunohistochemical (IHC) staining with the hypoxia markers, glucose transporter-1 and Hypoxyprobe™-1. Bladder tissue was also harvested for RT-PCR and measurement of oxidative stress. Hypoxia PCR Arrays were performed to determine changes in gene expression. Oxidative stress was measured using F2-Isoprostanes (F2-IsoP). Functional changes in the bladder were evaluated using voided urine blots.

Results—After acute distension and 5 consecutive distensions, bladders exhibited marked inflammatory changes on H&E staining and evidence of tissue hypoxia by IHC. qRT-PCR demonstrated up-regulation of hypoxia and oxidative stress related genes including *Hif1a, Arnt2, Ctgf, Gpx1, and Hmox1*. Measurements of oxidative stress with F2-IsoP did not change. Voided urine blots before and after bladder distension demonstrated marked changes showing an overactive voiding pattern.

Conclusion—Chronic bladder distension is possible in the female mouse and does generate a hypoxic injury characterized functionally by increased voiding patterns. This bladder injury model might more closely replicate bladder dysfunction in patients with poor bladder emptying due to neurologic disease including patients who are noncompliant with intermittent catheterization.

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Keywords

Hypoxia; Bladder dysfunction; Oxidative stress

INTRODUCTION

Understanding the mechanisms that underpin the functional and structural changes occurring in the bladder after obstruction is crucial to developing therapies to improve bladder function. The tissue response to hypoxia has been implicated as a potential mechanism of interest in the obstructed bladder.[1, 2] Hypoxic tissue insult following lower urinary tract obstruction has been linked to bladder pathology including tissue fibrosis, and altered smooth muscle metabolism and contractility.[3–5]

The bladder routinely responds to intermittent physiologic periods of ischemia and reperfusion during voiding.[6, 7] Prolonged and/or chronic deprivation of blood and oxygen with subsequent reperfusion is known to produce reactive oxygen species (ROS). Reperfusion events mediated by ROS may be more damaging than the ischemic event itself.[8] This type of stimulus, termed oxidative stress, can have pathologic ramifications for bladder function.

We previously demonstrated tissue level hypoxia following partial bladder outlet obstruction (pBOO) in a mouse model of urethral ligation.[1, 2] While urethral ligation is often used to study benign bladder obstruction, this model does not completely mimic what urologists observe clinically. We sought to evaluate a different method for achieving cyclic hypoxia followed by reperfusion in the bladder. We hypothesized that chronic over-distension, defined as intermittent periods of prolonged bladder distension, precipitates cycles of oxidative stress injury and creates a hypoxic environment.

MATERIALS AND METHODS

The Vanderbilt University Medical Center Institutional Animal Care and Use Committee approved all animal studies.

General Mouse Procedures

Female C57Bl/6 mice underwent bilateral oophorectomy at 5–6 weeks of age because estrogen can blunt the inflammatory process in the murine bladder.[9] Two weeks later, mice were subjected to either 90 minutes of bladder over-distension or anesthesia (controls) and then sacrificed at acute and chronic time points. Data from multiple organ systems have shown that volatile anesthetics, such as isoflourane, can upregulate hypoxic pathways.[10, 11] Experimental and control bladders were harvested for histologic and molecular analysis and measurement of tissue oxidative stress.

Bladder Distension

Under isoflourane anesthesia, the urethra was sterilely catheterized with a 24-gauge angiocatheter and connected to a gravity-dependent water column filled with sterile 0.9%

saline at 37°C. If pericatheter leakage occurred, the water columns were continuously refilled to maintain appropriate water column height, thus ensuring a constant 60cmH_20 pressure. This distending pressure was selected from our prior experience with murine urodynamics where control mice had an average leak-point pressure ranging from 30–40cmH_20. After 90 minutes, bladders were drained. Acute time points included mice sacrificed immediately following (time 0), 3 (time 3), 6 (time 6), and 24 (time 24) hours after a single distension. Chronic time points included a single daily distension repeated consecutively for 3 or 5 days. Mice in these groups were sacrificed immediately after the last distension (Table 1).

Tissue Preparation, Staining and Immunohistochemistry

In animals used for histology, the dome and trigone were excised after formalin fixation creating a ring of bladder tissue which was dehydrated in 50% ethanol, processed, paraffin embedded, and sectioned at 5μm. Hematoxylin and eosin staining was performed. A single pathologist reviewed all slides and was blinded to study groups. The remaining bladders were stored in RNLater at −20°C (Qiagen, Venlo, Netherlands).

Immunohistochemistry for tissue-level hypoxia markers, glucose transporter-1 (*Glut-1*: EMD Millipore, Darmstadt, Germany) and Hypoxyprobe™-1 (HPI Inc, Burlington, MA), was performed. We have previously published the use of these markers in our lab.[1, 2] Tissues were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Antigen retrieval was performed by heating with sodium citrate (9 mM, pH 5.0) and endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent (Dako, Carpinteria California). Sections were incubated overnight in a humidified chamber at 4°C with the primary antibody for *Glut-1* and Hypoxyprobe™-1 (both 1:100 dilution). Tissues were visualized, followed by streptavidin horseradish peroxidase conjugated, secondary antibody incubation, and diaminobenzidine development.

Quantitative RT-PCR Array

RNA was isolated from bladder tissue using the RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands). RNA quantity was determined spectrophotometrically and quality verified on 2% agarose gel. cDNA was reverse transcribed using an RT^2 First Strand Kit (SABiosciences, Qiagen). A SABiosciences commercial hypoxia qRT-PCR array (Plate #PAMM-032A, Qiagen) was used to amplify the molecular signature in 5-day distended mice in triplicate. In total, 84 genes were analyzed using SABiosciences qRT-PCR analysis software (sabiosciences.com) and represented by fold changes based on ΔΔCt analysis.

Oxidative Stress Measurement

Oxidative stress was measured using tissue levels of F2-Isoprostane (F2-IsoP). F2-IsoPs are stable end-products of lipid peroxidation created non-enzymatically by free radicals, thus providing an objective measure of oxidative stress. Snap frozen bladder tissue was analyzed by the Vanderbilt University Eicosanoid Core Laboratory. A simplified version of their protocol is: frozen bladder tissue was added to 20ml of ice-cold Folch solution (chloroform +methanol+BHT crystals) and tissue homogenized. For maximal extraction of lipids, the solution stood at room temperature for 1 hour. Aqueous 0.9% NaCl was added and

centrifuged for 10 minutes to separate aqueous and organic layers. The organic layer was removed and evaporated under nitrogen stream until dry. Lipids were resuspended in 4ml methanol containing 0.005% BHT and 4ml aqueous KOH. This was incubated at 37°C for 20 minutes and then acidified to pH3 with 1N HCl and diluted to 80ml with pH3 water. Tissue samples were purified and quantified using gas chromatography and mass spectroscopy. F2-IsoP quantity was reported in nanograms of F2-IsoP per gram of tissue.[12]

Functional Bladder Assessment

To functionally assess the bladder, voided urine blots were obtained in the 5-day distension group before distension on days 1–5 and after distension on days 1–4. Mice were placed individually in metabolic cages with cellulose filter paper (Whatman International Ltd, Grade 3) placed over the metal grate for 2 hours with free access to water and food. Both distensions and urine blots were completed on the same day between 9am-3pm as diurnal variation in mouse voiding patterns has been reported.[13] Urine blots were photographed under ultraviolet light and the area (mm^2) and the number of individual voids was recorded. A standard curve plotting urine volume versus urine blot area was used to estimate voided volumes (r^2=0.996) using Image J software (National Institute of Health).

Statistical Analysis

For qRT-PCR, mean fold change of genes was compared using a Student's t-test of the replicate 2^(−ΔΔCT) values. A p-value ≤0.05 was considered significant. Tissue F2-Isop levels were compared using the Mann-Whitney U test for nonparametric data and p ≤0.05 was considered significant. Mean number of voids and mean urine volume was compared using ANOVA for repeated measures and by Student's t-test.

RESULTS

Gross Pathologic Changes

Bladder weight increased significantly in mice undergoing 5 days of distension. Mean bladder weight in controls was 28.6mg (95% CI, 21.99–35.21) versus 58.6mg (95% CI, 49.24–65.26) in distended mice (p=0.0079). Bladder weight, as indexed by mouse body weight (mg bladder weight/kg body weight), was 1.34 (95% CI, 1.07–1.67) in controls versus 2.08 (95% CI, 2.33–3.28) in experimental animals (p=0.0079).

Histology

On H&E, mice at time 0 demonstrated diffuse hemorrhage in the lamina propria compared to controls (Fig **1**a–b). At time 24, significant inflammatory infiltrate was observed throughout the bladder with edema in the lamina propria. The urothelium demonstrated enlarged nuclei (Fig **1**c). Five-day distension animals demonstrated transmural and serosal inflammation with a marked increase in inflammatory cells. Areas of hemorrhage and necrosis were seen focally (Fig **1**d–e). Tissue level hypoxia was demonstrable using both Hypoxyprobe™-1 and *Glut-1*. Among the acute group, hypoxia was most evident in time 24 animals. After 5 distensions, the urothelium and serosal layers exhibited positive membrane staining for *Glut-1* in areas near hemorrhage (Fig **2**a–b).

Quantitative RT-PCR Array

After 5 days of distension, qRT-PCR demonstrated upregulation of key hypoxia and oxidative stress related genes. Significant fold changes were noted for *Hif1a* (2.6 fold), *Arylhydrocarbon receptor nuclear translocator2 (Arnt2)* (4.07 fold), *Connective tissue growth factor (Ctgf)* (20.8 fold), *Glutathione peroxidase-1 (Gpx1)* (1.6 fold), and *Hemoxygenase 1 (Hmox1)* (4.8 fold) (Fig **3**a–b). Significant upregulation of *Collagen 1 alpha 1* (9.8 fold) and the inflammatory gene *Interleukin-6* (145 fold) was noted.

Oxidative Stress

In the acute phase, F2-IsoP levels increased with a slight peak at time 0 before returning to normal by time 24 (Fig 4a). After 3 and 5 days of consecutive over-distension, F2-IsoP levels were unchanged compared to controls (Fig 4b–c).

Bladder Function

Voided urine blots changed significantly over the course of 5 distensions characterized by increased voiding with gross hematuria (Fig 5). On days 1 and 4 prior to distension, the mean number of voids per animal was 2.8 ± 1.4 and increased to 21.2 ± 16.7, respectively (p=0.06). Mean volume per void decreased from 85.1μl ± 56.9 on day 1 pre-injury to 40.9μl ± 29.8 on day 4 pre-injury (p=0.37) (Fig 6).

DISCUSSION

We theorized that poor bladder emptying leads to over-distension, thus hypothesizing that repetitive and intermittent over-distension creates a hypoxic environment leading to oxidative stress events. We designed a mouse model of bladder distension to mimic a clinical scenario where a patient might experience periods of prolonged over-distension. A perfect example is a myelomeningocele patient that is noncompliant with intermittent catheterization.

Intermittent hypoxia may be a normal part of bladder function. Unlike the kidney, which maintains regions of low oxygen tension (pO2) under normal conditions (<10mmHg),[14] the bladder muscle and mucosa maintain estimated levels of at least 25mmHg.[15, 16] Several studies agree that brief periods of ischemia with subsequent reperfusion occur during and immediately after voiding in the healthy bladder. It is theorized that compression of intramural blood vessels creates this imbalance in bladder oxygen supply.[17] Once the normal bladder empties, blood flow and thus oxygen delivery may actually rebound beyond pre-filling levels.[6] These cyclic episodes of ischemia are likely brief (<30 seconds) and infrequent. While a compliant bladder is able to manage these small, intermittent periods of hypoxia, an obstructed bladder may lose the ability to compensate. The duration of post-contraction ischemia in the experimentally obstructed bladder may last up to 5 minutes.[3]

Using a model of chronic bladder distension, we induced tissue level hypoxia, infiltration of inflammatory cells, increases in hypoxia-related gene transcription, and demonstrable changes in bladder function. Upregulation of several specific hypoxia-related genes suggests a hypoxic response to chronic distension. *Hif1a* is the key monomer in the dimeric

transcription factor Hypoxia Inducible Factor 1 (HIF1), which regulates cell response to hypoxia. Under hypoxic conditions, *Hif1a* accumulates and translocates to the nucleus to interact with *Hif1b*, forming functional HIF1.[18] *Arnt2* complexes with *Hif1a* in the nucleus and may aid transcriptional activity of HIF1.[19] *Ctgf* is a profibrotic gene transcriptionally induced in renal epithelial cells by direct interaction between HIF1 and the *Ctgf* promoter.[20] Hypoxia is also thought to induce collagen 1 transcription via HIF1. We found *Collagen 1 alpha 1* mRNA to be elevated.

Initially, we were uncertain if repeated catheterization and distension would be possible without causing urethral or bladder perforation. These adverse events were uncommon. More commonly, ~20% of mice leaked around the catheter after 3 days of chronic distension, thus requiring frequent refilling of the distending water column to ensure a consistent pressure of 60cm H2O. In addition, maintenance of mouse body temperature was critical as early on several mice died from hypothermia (data not shown). With further refinement, this model could be applied to various aspects of bladder research, including: children with voiding dysfunction and postponement, early changes following bladder obstruction, mechanisms underlying hydrodistension for interstitial cystitis and chronic pelvic pain, and therapies to control bladder inflammation.

Measuring free radicals directly is daunting and various biomarkers have been used to quantify oxidative stress. Isoprostanes are a unique class of compounds now considered the “gold standard” for identifying oxidative stress caused by non-enzymatic free radical peroxidation of arachadonic acid.[21] These molecules are similar in structure to prostaglandins and were discovered and characterized at our institution and various classes exist. One specific class, F2-IsoP, is detectable in all human tissues and fluid by mass spectroscopy. This isomer is a chemically stable metabolic end product and is readily quantifiable, thus an attractive marker of lipid peroxidation and *in vivo* oxidative stress.[22] F2-IsoP may also mediate oxidative damage. Known bioactivity includes platelet activation and vascular smooth muscle contraction by ligand-activation of the thromboxane receptor.[23]

Our interest in oxidative stress stems from attempts to better quantify tissue response to pBOO. We found incremental and significant increases in tissue F2-IsoP levels over time in 4, 8, and 16-week animals following pBOO (unpublished data). In the current study, F2-IsoP levels appeared to be elevated at time 0 with a return to control levels by 24 hours. These data are how we chose our time points for distending bladders in a consecutive daily manner. In 3 and 5-day distension animals, no F2-IsoP increase was noted; however an oxidative stress event may still have occurred. We theorize that chronic distension increased the transcription of genes critical to cellular detoxifying mechanisms. *Gpx1*, an abundant cellular enzyme containing selenium, is critical to detoxifying hydrogen peroxide free radicals and was increased 2-fold.[24] *Hmox*1, an inducible enzyme that plays a role in cellular free radical scavenging, was increased > 5-fold. In cardiac studies, heterozygous loss of *Hmox1* resulted in greater susceptibility to ischemia/reperfusion injury.[25]

Over-distension has been shown to result in contractile and metabolic dysfunction of the bladder.[26, 27] Previous investigators utilized single bladder distensions as a model of acute urinary retention, but no reports of chronic distension models are known. Following a single

bladder distension, a burst in ROS has been documented in conjunction with an increase in pro-apoptotic gene transcription.[26] Prolonged distension can result in injury to the neural pathways responsible for micturition, reduce bladder elasticity, alter the biochemical and neuronal responsiveness of the bladder, and lead to impaired micturition.[28] We utilized voided urine blots, which have been shown to correlate with cystometric studies, as a measure of bladder function.[29] Following 5 days of distension, mice developed progressively overactive voiding patterns with frequent, small volume voids. Pre- and post-distension voiding patterns worsened over time, yet the bladder displayed an ability to compensate overnight between distension insults. This appeared to wane until an apparent plateau was reached at day 4. We suspect this was a result of inflammation in the submucosal layers of the bladder, but the role of smooth muscle contractility alteration or changes in neuronal sensory response cannot be excluded. We cannot speculate how hypoxic injury may have affected neural pathways in the bladder; however, new research suggests that the urothelium functions as a sensory neural network all across the bladder lumen.[30] Since the urothelium clearly demonstrated hypoxia, it is plausible that urothelial hypoxia may be driving the altered voiding pattern.

CONCLUSION

Experimental chronic over-distension of the mouse bladder creates a hypoxic environment that may mimic clinical disease states. Further studies are needed to demonstrate a true pathological injury but preliminary results from a relatively simple technique are promising. The application of this model will aid our investigation into the mechanisms by which hypoxia can alter bladder structure and function.

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ABBREVIATIONS

Arnt2 Arylhydrocarbon Receptor Nuclear Translocator 2

F2-IsoP	F2-Isoprostane
Glut-1	Glucose Transporter 1
Gpx1	Glutathione peroxidase 1
H&E	Hematoxylin and Eosin
Hif1a	Hypoxia inducible factor 1 alpha
HIF1	Hypoxia inducible factor 1
Hmox1	Hemoxygenase 1
IHC	Immunohistochemistry
pBOO	Partial Bladder Outlet Obstruction
qRT-PCR	Quantitative real time polymerase chain reaction
ROS	Reactive oxygen species

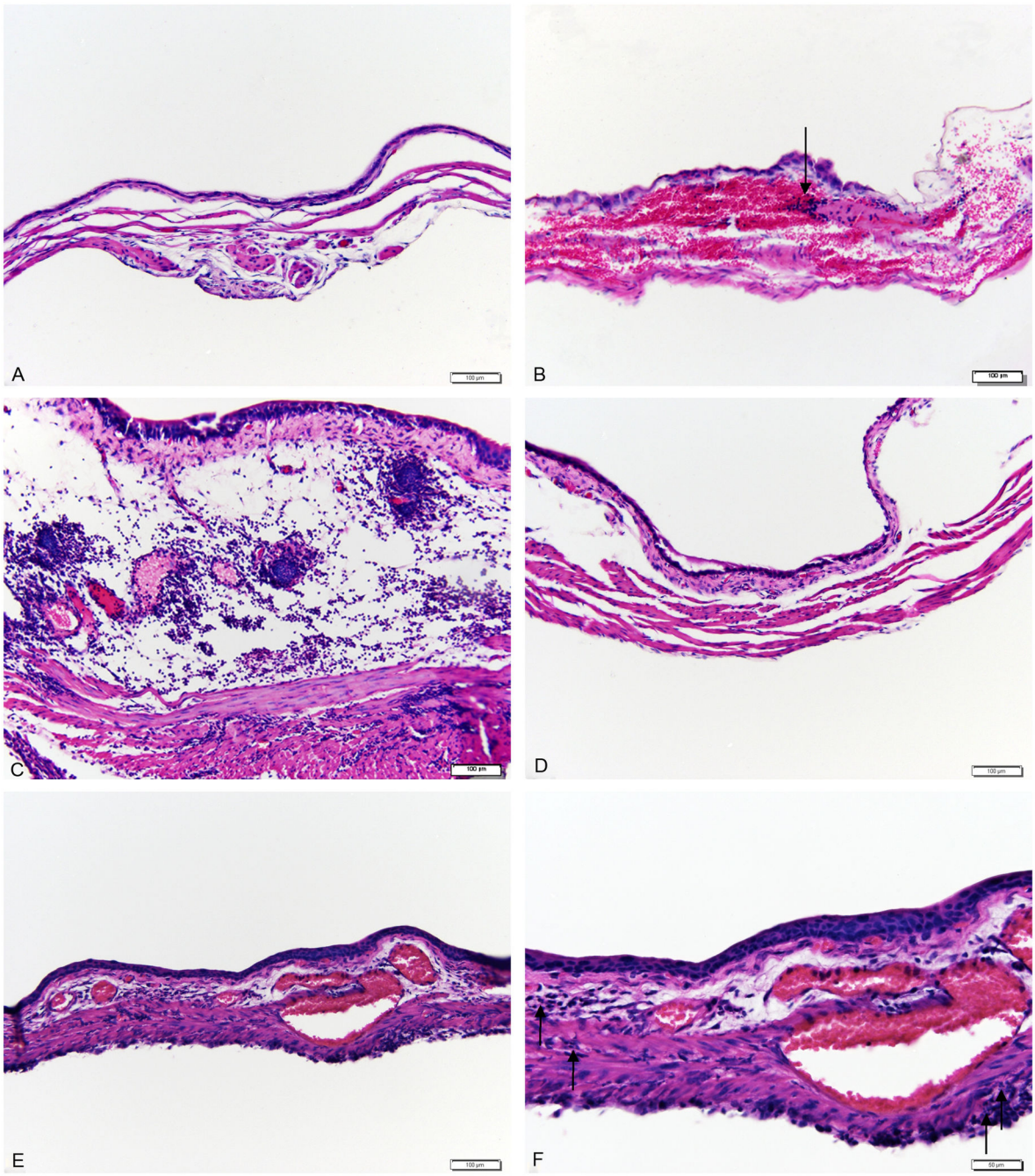

Figure 1. H&E staining

H&E staining in bladders of control and experimental mice. **1**A) Acute control mouse shows quiescent urothelium without evidence of inflammation. **1**B) Subepithelial hemorrhage noted with margination of neutrophils (arrow) suggesting acute inflammation in time 0 mouse following single distension. **1**C) Time 24 animal demonstrating diffuse, transmural inflammation with edema within the lamina propria, and evidence of urothelial hyperplasia. **1**D) 5-day distension control bladder with normal appearance. **1**E) 5-day chronic distension animal demonstrating transmural acute inflammation. All images reproduced from 200×.

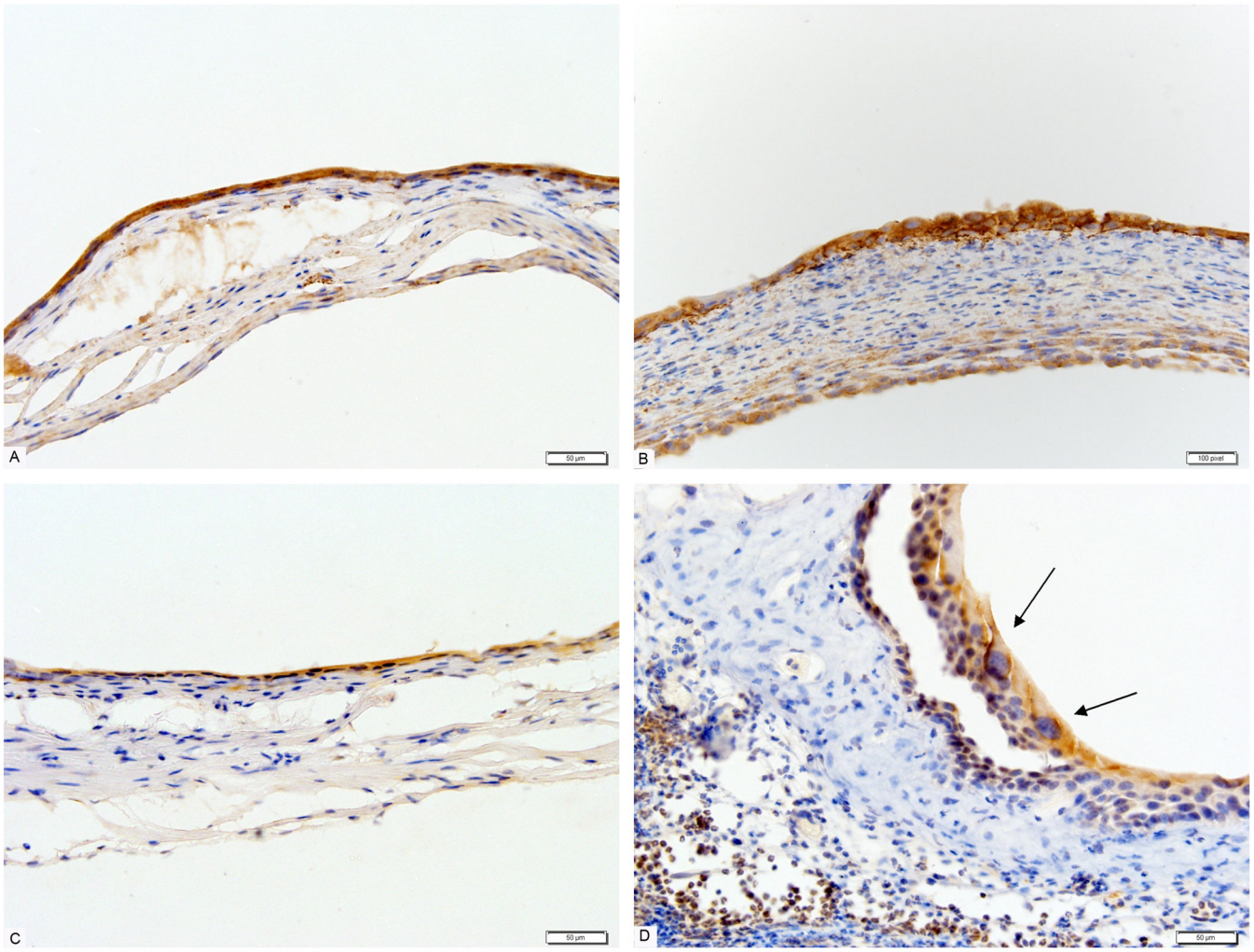

Figure 2. IHC for hypoxia markers

Glut-1 and Hypoxyprobe™-1 staining. **2**A) *Glut-1* antibody staining in a control mouse for the chronic distension group with non-specific tissue staining is seen primarily in the urothelium. **2**B) 5-day distension animal with intense tissue staining for *Glut-1* seen focally within the urothelium which demonstrates area of positive membrane staining (top portion of tissue) with additional areas of membrane staining in mesothelial cells along the serosa (lower edge of tissue). **2**C) Nonspecific Hypoxyprobe™-1 staining within the urothelium of an acute control animal. **2**D) Positive Hypoxyprobe™-1 staining in time 24 animal with intense staining seen particularly in the inflammatory cells in the bottom left. The urothelium has stained as well. Note reactive nuclei of the two urothelial cells (arrows). All images reproduced from 400×.

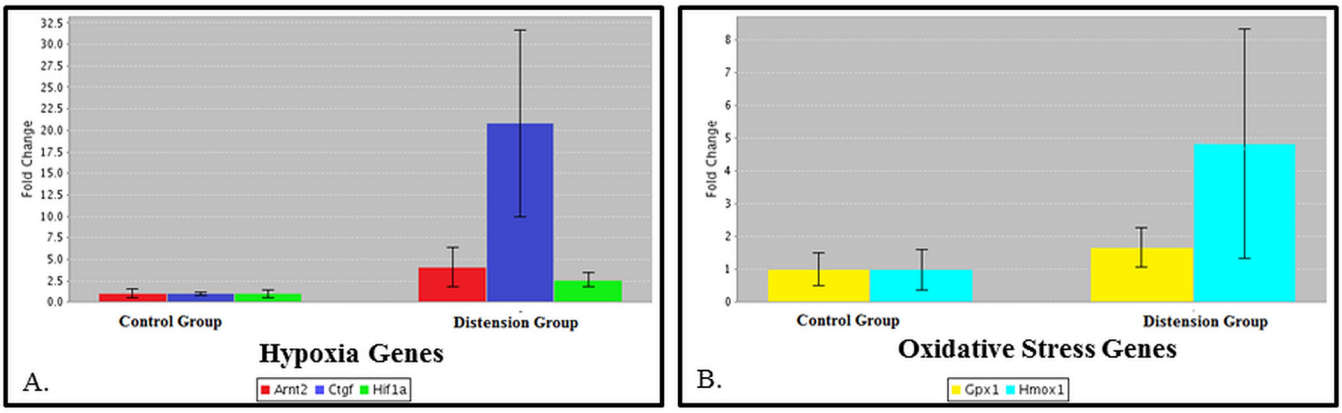

Figure 3. Hypoxia Gene Array

qRT-PCR results in 3 control mice and 3 5-day distension mice. **3A**) A significant upregulation of genes associated with hypoxia observed (*Hif1a*, *Ctgf*, and *Arnt2*) ($p < 0.05$). **3**B) Genes critical to clearance of ROS also significantly upregulated (*Gpx1* and *Hmox1*) ($p<0.05$).

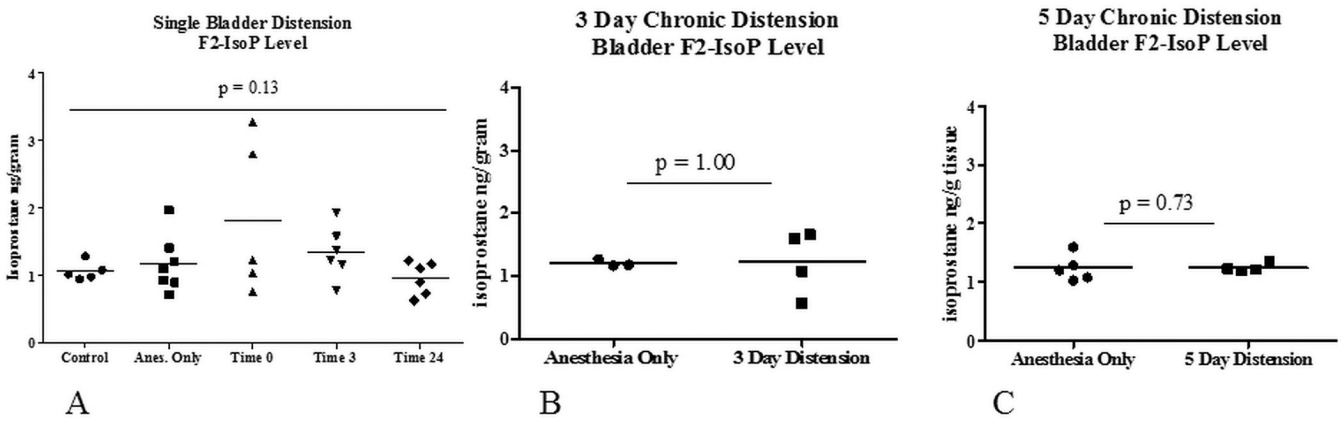

Figure 4. Measurement of Oxidative Stress

F2-IsoP measurements in acute and chronic distension animals. Horizontal lines represent group means. Fig 5A mild elevation in F2-Isop at time 0 with return to control levels by 24 hours. 1 way ANOVA revealed no statistical significance. Fig **4**B and **4**C shows identical F2-IsoP levels after 3 and 5 days of bladder distension compared to controls.

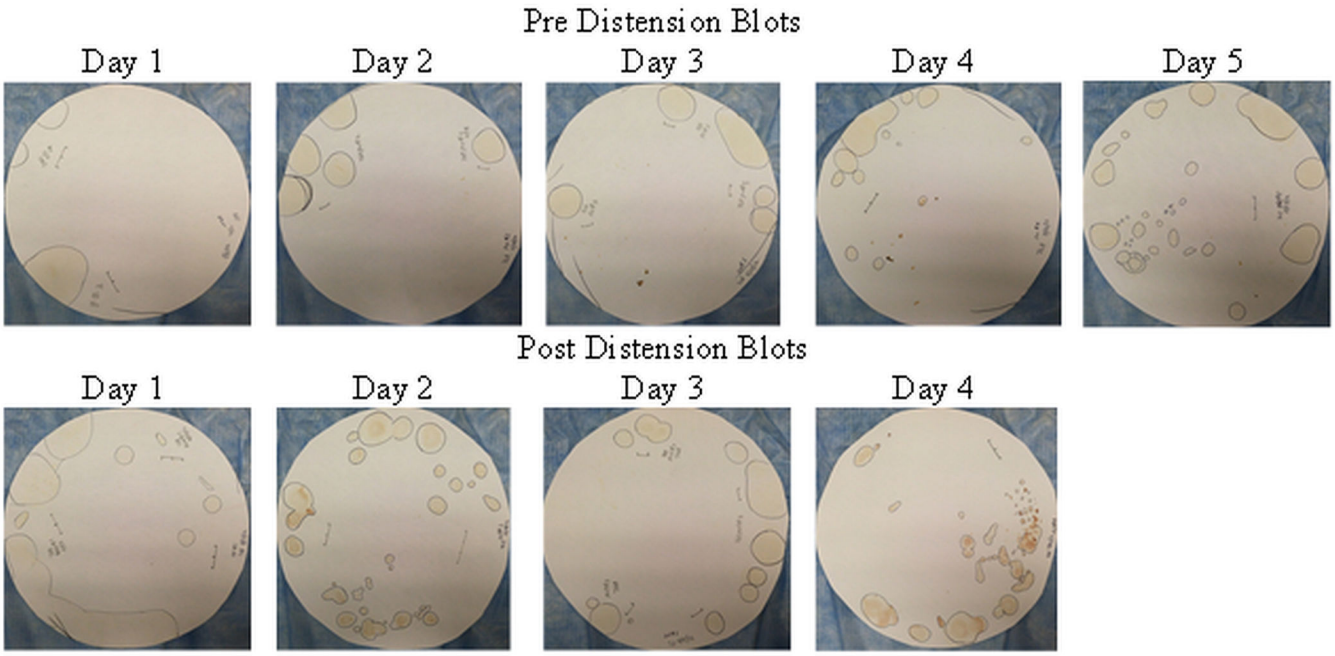

Figure 5. Images of Voided Urine Blots

Urine blots before and after distension in a single representative mouse. The bladder was removed immediately following the last distension on day 5 preventing post-distension urine blot.

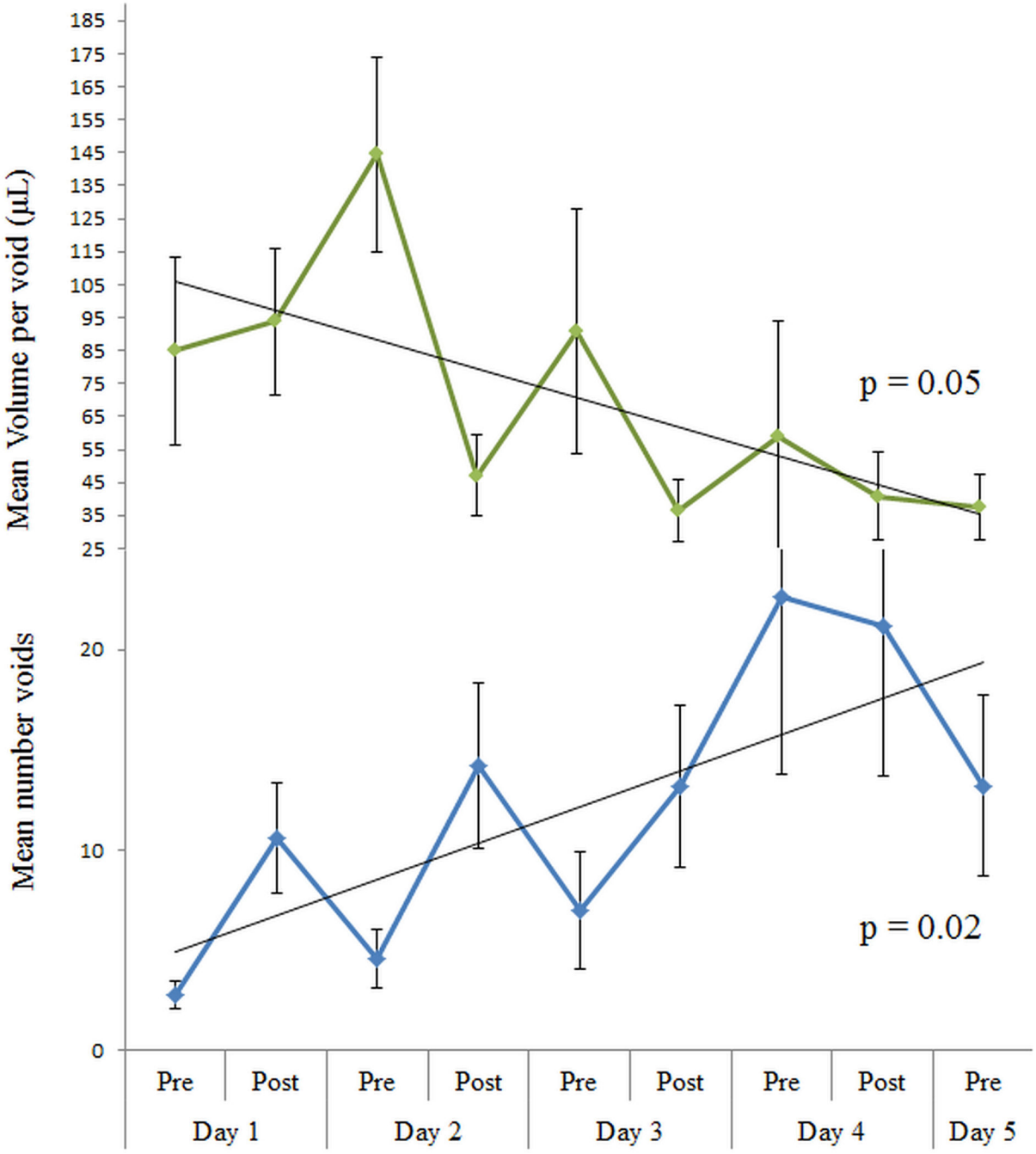

Figure 6. Analysis of Voided Urine Blots – 5 day Chronic Distension

Mean number of voids (lower portion of graph) and mean volume per void (upper portion of graph) over two hours before and after distension in 5 experimental mice. Means analyzed using one-way ANOVA for repeated measures.

Table 1

Distribution of Mice in Distension Experiments

Mice for acute time points were sacrificed after a single distension and variable time of recovery denoted as Time 0 for sacrifice after no recovery period, Time 3 for sacrifice after 3 hours of recovery, etc. Control mice for acute time points included mice undergoing no anesthesia or 90 minutes of anesthesia without distension. Control mice for chronic time points underwent daily consecutive administration of anesthesia (3 days and 5 days, respectively) without distension. Mice undergoing distension for chronic time points underwent a single daily distension on consecutive days and were sacrificed immediately after the last distension.

	Acute Time Points Total N = 49					Chronic Time Points Total N = 39		
Measurement	**Control**	**Time 0**	**Time 3**	**Time 6**	**Time 24**	**Control**	**3 Days**	**5 Days**
Total No./time Point	**15**	**8**	**9**	**8**	**9**	**20**	**7**	**12**
F2-Isoprsostane	12	5	6	5	6	9	4	4
Histology/IHC	3	3	3	3	3	3	--	3
Voided Urine Blots	--	--	--	--	--	7*	--	8*
PCR Array	--	--	--	--	--	8	3	5

* Mice used for multiple measurements.