# Chronic Cyclic Bladder Over Distention Up-Regulates Hypoxia Dependent Pathways

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**Purpose:** Bladder over distention secondary to anatomical or functional obstruction can eventually lead to pathological changes, including decreased elasticity and contractile dysfunction. We hypothesized that chronic bladder distention in a murine model would activate hypoxia dependent signaling pathways despite intermittent relief of distention.

**Materials and Methods:** Female C57Bl/6 mice were oophorectomized at age 5 to 6 weeks and underwent urethral catheterization and 90-minute bladder distention. Acute and chronic time points were evaluated. Bladder tissue was harvested for hematoxylin and eosin, and immunohistochemical staining with the hypoxia markers *Glut-1* (EMD Millipore, Merck, Darmstadt, Germany) and Hypoxyprobe™-1. Bladder tissue was also harvested for real-time polymerase chain reaction and oxidative stress measurement. Hypoxia polymerase chain reaction arrays were done to determine changes in gene expression. Oxidative stress was measured using F2-IsoP. Functional bladder changes were evaluated using voided urine blots.

**Results:** After acute distention and 5 consecutive distentions, bladders showed marked inflammatory changes on hematoxylin and eosin staining, and evidence of tissue hypoxia on immunohistochemistry. Quantitative real-time polymerase chain reaction revealed 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 distention showed marked changes with an overactive voiding pattern.

**Conclusions:** Chronic bladder distention is possible in the female mouse. It generates hypoxic injury, as characterized functionally by increased voiding patterns. This bladder injury model might more closely replicate bladder dysfunction in patients with poor bladder emptying due to neurological disease, including those noncompliant with intermittent catheterization.

**Key Words:** urinary bladder, oxidative stress, anoxia, gene expression, urinary bladder neck obstruction

**Abbreviations and Acronyms**

*Arnt2* = arylhydrocarbon receptor nuclear translocator 2

*Ctgf* = connective tissue growth factor

F2-IsoP = F2-isoprostane

*Glut-1* = glucose transporter 1

*Gpx1* = glutathione peroxidase 1

HIF = hypoxia-inducible factor

*Hmox1* = hemoxygenase 1

pBOO = partial bladder outlet obstruction

qRT-PCR = quantitative real-time polymerase chain reaction

ROS = reactive oxygen species

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UNDERSTANDING the mechanisms that underpin functional and structural changes in the bladder after obstruction is crucial to developing therapy to improve bladder function. The tissue response to hypoxia is implicated as a potential mechanism of interest in the obstructed bladder.[1,2] Hypoxic tissue insult after lower urinary tract obstruction is linked to bladder

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*Distribution of 49 and 39 mice in distention experiments at acute and chronic time points, respectively*

| Time Points | No. Mice | No. F2-IsoP | No. Histology-Immunohistochemistry | No. Voided Urine Blots | No. qRT-PCR Array |
|---|---|---|---|---|---|
| Acute: | | | | | |
| Control | 15 | 12 | 3 | — | — |
| Time 0 | 8 | 5 | 3 | — | — |
| Time 3 | 9 | 6 | 3 | — | — |
| Time 6 | 8 | 5 | 3 | — | — |
| Time 24 | 9 | 6 | 3 | — | — |
| Chronic: | | | | | |
| Control | 20 | 9 | 3 | 7* | 8 |
| 3 Days | 7 | 4 | — | — | 3 |
| 5 Days | 12 | 4 | 3 | 8* | 5 |

* Mice used for multiple measurements.

pathology, including tissue fibrosis, and altered smooth muscle metabolism and contractility.[3–5]

The bladder routinely responds to intermittent physiological 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 ROS. Reperfusion events mediated by ROS may be more damaging than the ischemic event.[8] This type of stimulus, termed oxidative stress, can have pathological ramifications for bladder function.

We previously studied tissue level hypoxia after 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. Thus, we evaluated a different method to achieve cyclic hypoxia followed by reperfusion in the bladder. We hypothesized that chronic over distention, defined as intermittent periods of prolonged bladder distention, would precipitate cycles of oxidative stress injury and create 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 age 5 to 6 weeks because estrogen can blunt the inflammatory process in the murine bladder.[9] Two weeks later the mice underwent 90 minutes of bladder over distention or anesthesia (controls). They were then sacrificed at acute and chronic time points. Data on multiple organ systems show that volatile anesthetics such as isoflurane can up-regulate hypoxic pathways.[10,11] Experimental and control bladders were harvested for histological and molecular analysis, and measurement of tissue oxidative stress.

### Bladder Distention

Under isoflurane 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 37C. If pericatheter leakage occurred, the water columns were continuously refilled to maintain appropriate water column height, ensuring a constant 60 cm $H_2O$ pressure. This distending pressure was selected from our prior experience with murine urodynamics in which control mice had an average leak point pressure of 30 to 40 cm $H_2O$. After 90 minutes, the bladders were drained. Acute time points included mouse sacrifice immediately after (time 0), and 3 (time 3), 6 (time 6) and 24 hours (time 24) after a single distention. Chronic time points included a single daily distention repeated consecutively for 3 or 5 days. Mice in these groups were sacrificed immediately after the last distention (see table).

### Tissue Preparation, Staining and Immunohistochemistry

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

We performed immunohistochemistry for the tissue level hypoxia markers *Glut-1* and Hypoxyprobe-1 (NPI Inc., Burlington, Massachusetts). We previously reported the use of these markers at our laboratory.[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 at pH 5.0). Endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent (Dako®). Sections were incubated overnight in a humidified chamber at 4C with the primary antibody for *Glut-1* and Hypoxyprobe™-1 (each 1:100 dilution). Tissues were visualized, followed by streptavidin-horseradish peroxidase conjugated, secondary antibody incubation and diaminobenzidine development.

### qRT-PCR Array

RNA was isolated from bladder tissue using the RNeasy® Plus Mini Kit. RNA quantity was determined spectrophotometrically and quality verified on 2% agarose gel. cDNA was reverse transcribed using an $RT^2$ First Strand Kit (SABiosciences™). A hypoxia qRT-PCR array (plate