Protective Effects of Inosine on Urinary Bladder Function in Rats With Partial Bladder Outlet Obstruction

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OBJECTIVES To evaluate the protective effects of inosine on bladder dysfunction, nerve density, and oxidative damage after partial bladder outlet obstruction in rats.

METHODS A total of 60 adult male Sprague-Dawley rats were divided into 5 groups. Groups 1-4 underwent bladder outlet obstruction. Groups 1-3 were treated with inosine at 100, 150, or 225 mg/kg/d intraperitoneally and group 4 with saline. Group 5 consisted of sham-operated control rats. At 21 days postoperatively, cystometry were performed in 30 rats (6 per group). In the remaining 30 rats (6 per group), the bladders were excised and used for contractile responses to various stimulations, immunohistochemical examination for protein gene product 9.5 (a neuronal marker) and evaluation of superoxide dismutase activity and thiobarbituric acid reductase substance level.

RESULTS Inosine administration resulted in dose-dependent protective effects on the contractile responses to both field stimulation and carbachol, although the protective responses to KCl was restricted to a greater dose of inosine. A dose-dependent reduction in residual volume was noted in inosine-treated groups at different dosages compared with the saline-treated group. In addition, the protein gene product 9.5-positive nerve density decreased in the saline-treated group but significantly increased in the inosine-treated (225 mg/kg/d) group. Compared with the saline-treated group, significantly enhanced superoxide dismutase activity and a reduced thiobarbituric acid reductase substance level were observed in the inosine-treated group at 150 and 225 mg/kg/d.

CONCLUSIONS These results suggest that inosine has a potential protective effect against partial bladder outlet obstruction-induced bladder dysfunction and oxidative injury in rats.


Partial bladder outlet obstruction (PBOO) is a common clinical problem and can result from benign prostatic hyperplasia, urethral stricture disease, congenital anomalies, or alteration in the urethrovessical reflex.¹ It has been suggested that several pathophysiologic mechanisms might underlie the pathogenesis of long-term PBOO. PBOO results in an immediate increase in urethral resistance, followed by an increase in the bladder wall tension and micturition pressure. These changes eventually result in an increase in bladder wall thickness, changes in detrusor morphology, progressive denervation, detrusor receptor upregulation, and decrease of blood flow to the bladder wall, ultimately leading to ischemia. Several investigators have demonstrated that ischemia is a major factor in the etiology for obstructive bladder dysfunction²,³ and that part of the damage results from the generation of free radicals and the resultant cellular and subcellular membrane peroxidation.⁴

Inosine, a naturally occurring purinergic nucleoside formed from the breakdown of adenosine, has recently been shown to act not only as an anti-inflammatory agent, but also to exert powerful protective effects both in vitro and in vivo.⁵–⁸ Inosine might also enhance endogenous antioxidant systems because the breakdown of inosine yields urate, a scavenger of oxyradicals and peroxynitrite.⁹ However, little information is available about the effects of inosine on changes in the bladder after PBOO in animal studies; thus, it is of interest to determine whether inosine exerts an effect on the urinary bladder after PBOO.
We therefore examined the influence of inosine on the contractile responses, urodynamic parameters, innervation density, and oxidative damage in the bladder of the PBOO rat model. We used these findings to characterize the benefit effects of inosine on nerve degeneration, smooth muscle contractility, the voiding profile, and lipid peroxidation in the bladder after PBOO.

MATERIAL AND METHODS

Operative Procedure
All studies were performed in accordance with the National Institutes of Health guidelines for the care and use of experimental animals, and the corresponding institutional committee approved the study protocol. A total of 60 male Sprague-Dawley rats (250-280 g) were randomly divided into 5 groups. Groups 1-3 consisted of PBOO rats that received inosine at doses of 100, 150, and 225 mg/kg/d, and the dosage was determined from our pilot studies and the results of our previous study. Group 4 consisted of PBOO rats that received normal saline treatment. Inosine (Sigma-Aldrich, St. Louis, MO) and normal saline were given intraperitoneally, starting immediately after PBOO induction. Group 5 consisted of sham-operated rats.

The PBOO model was created according to a previous published report. In brief, the bladder was exposed, and a 20-gauge angiocatheter was placed adjacent to the urethra (with the outside diameter of the catheter 1.1 mm). A 3-0 silk suture was placed snugly around the urethra and catheter below the bladder neck. The catheter was then carefully removed, and the abdominal incision was closed. Three weeks after surgery, cystometry were performed in 30 rats (6 rats per group). The remaining 30 rats were killed, and their bladders were removed and weighed. The bladder tissue was used for the contractility studies, immunohistochemical examination, superoxide dismutase (SOD) activity, and thiobarbituric acid reductase substance (TBARS) level evaluations.

Cystometry
Cystometric studies were performed with the rats under urethane anesthesia (1.0 mg/kg, subcutaneously) according to a previously published report. In brief, cystometry was done using a 24-gauge catheter implanted into the apex of the bladder dome. The catheter was connected by a 3-way stopcock to a pressure transducer (TP-200 T, Nihon Kohden, Tokyo, Japan) and a pump for continuous saline infusion (0.1 mL/min). During infusion, the intravesical pressure and urine volume voided were recorded continuously using a computer interface and appropriate software. The following parameters were evaluated: voided volume, residual urine volume, maximal voiding pressure (peak pressure during voiding), and baseline pressure (lowest pressure after voiding). In each rat, approximately 6-8 voiding cycles were recorded, and the mean values of the voiding were calculated.

Contractility
The contraction of the isolated bladder strips was measured as previously described. In brief, the bladder strips were mounted in a 25-mL organ bath containing Krebs solution and equilibrated with 95% oxygen and 5% carbon dioxide. Transmural nerve stimulation was performed with a field stimulator delivering biphasic square pluses of supramaximal voltage, 50-μs durations, and variable frequencies (2, 8, and 32 Hz). Thereafter, the bladder strip responses to the muscarinic receptor agonist carbachol (20 μM) and the receptor-independent stimulus KCl (120 mM) was determined.

Histologic Examination and Quantification
Serial 14-μm full-layer frozen sections were cut on cryostat and were used for immunohistochemical staining using polyclonal rabbit antibodies to protein gene product 9.5 (PGP9.5, 1:4000 dilution; Ultraclone, UK). The sections were subsequently incubated with peroxidase-labeled antirabbit antibody (Sigma-Aldrich). The primary antibody was omitted on the slides serving as a negative control.

The sections were examined under a microscope. At least 5 nonoverlapping fields of the representative areas were captured in each histologic section. Later, the total number of nerve profiles in all the captured images of each specimen was recorded. The number of nerves per square millimeter random fields (20× magnification) were determined along each transverse section of bladder and averaged for each group. For this purpose, each stained small individual track was counted as a single nerve filament. The observer was unaware of the treatment of groups.

Determination of SOD Activities and TBARS Levels
Bladder tissue homogenates were prepared from different groups. An aliquot of the homogenate and supernatant was stored at −80°C until the determination of SOD enzyme activities and TBARS levels. The total (Cu–Zn and Mn) SOD activity was determined according to the method of Sun et al. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the nitroblue tetrazolium reduction rate. SOD activity was expressed as units per milligram protein. The TBARS levels were measured on homogenate according to the method of Draper and Hadley, and expressed as nanomoles malondialdehyde per milligram of protein.

Statistical Analysis
All data are expressed as the mean ± SD of each experiment. The statistical significance of differences between groups was assessed by analysis of variance followed by Bonferroni analysis, with P < .05 considered significant.

RESULTS

Bladder Weight
The mean bladder mass in different treatment groups are listed in Table 1. No significant difference was found in the bladder mass between the saline-treated and inosine-treated groups at 100, 150, and 225 mg/kg/d.

Contractile Responses
The contractile response of the bladder strips to field stimulation, carbachol, and KCl was determined (Fig. 1). Transmural stimulation of the intrinsic nerves in the muscle strips resulted in a frequency-dependent increase in the tension of the strips (Fig. 1A). The force of contraction was reduced at each frequency in PB00 rats and with a gradual but incomplete recovery after the administration of inosine (Fig. 1A). At the 8- and 32-Hz
frequency stimulations, inosine administration induced a dose-dependent recovery of tension generated by the muscle strips. A significant decrease was found in the contractile response to carbachol and KCl of the muscle strips from saline-treated and inosine-treated groups compared with the sham-operated group (Fig. 1B). Inosine administration resulted in dose-dependent protection of the contractile responses to carbachol (Fig. 1B).

Cystometrography
Significant increases occurred in the voided volume and residual volume in the saline-treated and inosine-treated groups compared with the sham controls (Table 1); however, no differences were noted in the voided volume between the saline-treated group and inosine-treated groups. However, inosine administration resulted in a dose-dependent reduction in the residual volume compared with the saline-treated group (Table 1). In addition, the maximal voiding pressure and baseline pressure in saline-treated group increased significantly compared with sham-operated group. In contrast, inosine treatment resulted in a slight decrease in maximal voiding pressure and baseline pressure compared with the saline-treated group but the decreases were not statistically significant (Table 1).

PGP9.5 Immunohistochemical Staining
PGP9.5-immunoreactive nerves were seen mainly in the circular and longitudinal muscle regions of the detrusor and also surrounding small blood vessels (Fig. 2A-C). The distribution of PGP9.5-immunoreactive nerves was similar in all parts of bladder, despite suburothelium or detrusor. In the sham-operated rats, very fine and viscosity appeared nerve fibers distributed throughout the bladder wall (Fig. 2A). However, only a few PGP9.5-immunoreactive nerves were found in the bladder of saline-treated rats. In the 225-mg/kg/d inosine-treated group, the PGP9.5-immunoreactive nerves were significantly more than the saline-treated group (Fig. 2B,C). The average numbers of PGP9.5-immunoreactive nerves of different treatment groups are shown in Figure 2D.

Bladder Oxidative Stress
A significant reduction was found in SOD activity and a significant elevation in the TBARS level in bladder tissue from saline-treated rats compared with sham-operated animals (Table 1). Inosine administration showed a dose-dependent effect in enhancing SOD activity and in diminishing the TBARS level in the obstructed bladder. Compared with the saline-treated group, significant increase in the SOD activity and reduction in TBARS level were noted in the inosine-treated group at 150 and 225 mg/kg/d (Table 1).

COMMENT
Previous reports from animal studies of bladder outlet obstruction report rapid and marked morphologic and

<table>
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<tr>
<th>Group</th>
<th>BW (mg)</th>
<th>VV (mL)</th>
<th>RV (mL)</th>
<th>MVP (cm H₂O)</th>
<th>BP (cm H₂O)</th>
<th>SOD (U/mg protein)</th>
<th>TBARS (nm MDA/mg protein)</th>
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<tr>
<td>Sham operated</td>
<td>154.3 ± 15.3</td>
<td>1.23 ± 0.14</td>
<td>0.04 ± 0.02</td>
<td>3.92 ± 0.14</td>
<td>6.89 ± 1.39</td>
<td>0.46 ± 0.29</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Saline treated</td>
<td>154.1 ± 15.3</td>
<td>1.22 ± 0.14</td>
<td>0.05 ± 0.02</td>
<td>3.91 ± 0.14</td>
<td>6.88 ± 1.39</td>
<td>0.47 ± 0.29</td>
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<tr>
<td>Inosine (mg/kg/d)</td>
<td>100</td>
<td>154.5 ± 15.5</td>
<td>1.23 ± 0.14</td>
<td>0.04 ± 0.02</td>
<td>3.92 ± 0.14</td>
<td>6.89 ± 1.39</td>
<td>0.46 ± 0.29</td>
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<tr>
<td>150</td>
<td>416.4 ± 16.4</td>
<td>1.26 ± 0.17</td>
<td>0.05 ± 0.02</td>
<td>3.93 ± 0.14</td>
<td>6.90 ± 1.40</td>
<td>0.47 ± 0.30</td>
<td>0.01 ± 0.00</td>
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<tr>
<td>225</td>
<td>419.5 ± 19.5</td>
<td>1.27 ± 0.18</td>
<td>0.05 ± 0.02</td>
<td>3.94 ± 0.15</td>
<td>6.91 ± 1.41</td>
<td>0.48 ± 0.31</td>
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*P < 0.05 compared with sham-operated group.
†P < 0.01 compared with sham-operated group.
‡P < 0.05 compared with saline-treated group.
§P < 0.01 compared with saline-treated group.

BW, bladder weight; VV, voided volume; RV, residual volume; MVP, maximal voiding pressure; BP, baseline pressure; SOD, superoxide dismutase; TBARS, thiobarbituric acid reductase substance; MDA, malondialdehyde.

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functional changes in the detrusor muscle, similar to those reported in human clinical studies. Most of these studies reported a decrease in several parameters of detrusor contractility, an increase in postvoid residual volume, and partial denervation of the detrusor, which is probably responsible for the decreased nerve-evoked contractility. In agreement with numerous previous studies, the present study showed a decrease in contractile function, deterioration of bladder function, partial denervation, and oxidative damage in bladders from PBOO rats compared with sham-operated control rats. In the present study, there was a potential weakness of the animal model as a reflection of benign prostatic hyperplasia in humans, as “too acute,” which might not reflect human PBOO.

Our recent studies were designed to determine whether inosine had a protective effect against bladder contractile and biochemical dysfunctions using a model of PBOO. The data have clearly demonstrated that inosine, administered systematically, and in a dose-dependent manner, ameliorated the impaired bladder contractile function and normalized the partial denervation of detrusor in the PBOO rats. It has been shown that nerve degeneration was one of the first degenerative changes after the onset of PBOO, which correlates well with a selective loss in nerve-mediated contraction. This is in agreement with

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**Figure 1.** (A) Effects of inosine on isolated bladder strips in response to field stimulation. (B) Effects of inosine on bladder muscle strips in response to carbachol and receptor-independent stimulus, KCl. Each bar indicates mean ± SD of 6 individual rats. **P < .01 vs sham-operated rats; *P < .05, **P < .01 vs saline-treated rats.

**Figure 2.** Immunohistochemical staining of protein gene product 9.5 (PGP9.5) in rat bladders. (A) Sham-operated, (B) saline-treated partial bladder outlet obstruction (PBOO), and (C) inosine-treated PBOO rats (225 mg/kg/d). Arrows indicate PGP9.5-immunoreactive nerve bundles or varicosities. (D) Nerve density in bladders from rats with different treatments. Each bar indicates mean ± SD of 6 individual rats. **P < .01 vs sham-operated rats; *P < .05 vs saline-treated rats.
our results that very sparse PGP9.5-immunoreactive nerves and impaired nerve-mediated contraction were observed in the bladder of saline-treated animals. However, inosine treatment induced a marked protective effect against bladder denervation in the PBOO rats. To further confirm the beneficial effects of inosine on bladder dysfunction in PBOO rats, in vivo cystometry was performed. Inosine administration clearly showed dose-dependent reduction effects on residual volume generation induced by PBOO. It is possible that this effect on bladder dysfunction might due to the amelioration of the negative functional changes of the bladder smooth muscle associated with PBOO.

Inosine is considered an inactive metabolite in most biologic systems, but recently, evidence from our group and others have demonstrated that inosine could elicit protection, immunomodulatory, and regeneration after various injuries. Our previous studies have demonstrated that inosine could significantly reduce the spread of secondary degeneration after spinal cord injury in adult rats and showed a beneficial effect on retinal ganglion cell survival in adult rats.

Several investigators have shown that PBOO in different species induces a relative reduction in blood flow to bladder tissue during and immediately after voiding, resulting in partial ischemia and hypoxia. After bladder emptying, the blood supply recovers, allowing reperfusion. Although this occurs in normal bladders, this phenomenon is significantly exaggerated in the obstructed bladder. Both bladder smooth muscle and autonomic nerves appear to be the most vulnerable to the ischemic injury in normal tissue in related to outlet obstruction. Previous studies demonstrated that reperfusion (reoxygenation) generates free radicals that result in lipid peroxidation of cell membranes, which can result in significant cellular and subcellular membrane dysfunction, leading to the progressive destruction of nerves (denervation), mitochondrial damage, and muscle contractile dysfunction. Additional evidence for the role of oxidative injury in obstructed bladder dysfunction comes from the beneficial effects of a variety of antioxidants in the treatment of bladder dysfunction in various PBOO animal models.

Inosine has been shown to play a potential therapeutic role in the treatment of tissue damage caused by ischemia-reperfusion. In an effort to better understand the mechanisms by which inosine protects PBOO-induced damage to the bladder, oxidative stress injury was evaluated by measuring SOD activity and TBARS levels in the bladder, because SOD and TBARS are well-established and reliable markers for quantifying oxidative damage in the bladder tissue. Inosine administration induced a dose-dependent elevation in SOD activity, as well as a decrease in TBARS values in the obstructed bladder. These results indicated that inosine is protective in PBOO-induced oxidative injury to the bladder, at least in part, by its antioxidant and antifree radical effects. This is in agreement with a recent study showing that inosine could prevent oxidative damage to DNA, decrease the generation of reactive oxygen species, and protect mice against γ-radiation-induced death. The particular mechanism underlying the antioxidative stress effects of inosine on PBOO would be the subject of future studies. Inosine is a safe, naturally occurring purine, which appears to be nontoxic to humans, even when ingested at high doses. The clinical application of inosine treatment could represent a novel approach for improving bladder function after PBOO.

CONCLUSIONS

The results of the present study have demonstrated the effectiveness of inosine as a protective therapy in a rat model of acute PBOO. Administration of inosine is shown to ameliorate the bladder contractile dysfunction, protect against nerve degeneration, and decrease oxidative damage associated with PBOO. However, the physiologic and pharmacologic role of inosine in the bladder remains unclear and warrants additional study.

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References