ORIGINAL ARTICLE

A new experimental model for inducing interstitial cystitis by oxidative stress using bladder instillation of a nitric oxide donor gel


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KEYWORDS
Interstitial cystitis; Nitric oxide; In vivo model

Abstract

Purpose: The aim of this study is to develop a new experimental model of inducing interstitial cystitis (IC) through vesical instillation of a polymeric solution containing the NO donor S-nitrousglutathione (GSNO) and to compare it to the experimental interstitial cystitis induced by vesical instillation of protamine and potassium chloride.

Materials and methods: For that purpose 40 female Wistar rats were used, divided into four groups: (1) saline solution + GSNO; (2) saline solution + polymeric solution (without GSNO); (3) protamine sulphate + KCl; (4) protamine sulphate + GSNO. The rats received one application (5 animals) or 3 applications (5 animals) of the corresponding substance through intravesical instillation, and after 6 days (5 animals) or 9 days (5 animals) they were euthanized and their bladders were removed for macroscopic evaluation and histological study.

Results: In the macroscopic evaluation we observed edema and hyperemia of the mucosa in 2 (22%) of the animals in group 1, in 0 (0%) of the animals in group 2, in 10 (100%) of the animals in group 3, and in 5 (50%) of the animals in group 4. In the protamine + KCl group and in saline + GSNO similar effects were observed on the bladder wall. The animals in group 2 (saline + polymeric) showed vascular congestion, significantly smaller than the rest after 9 days instillations ($P = 0.0035$). Significant increased fibrosis was observed after instillations in groups 3 and 4, after 6 days ($P = 0.3781$) and 9 days ($P = 0.0459$), respectively, when compared to control (group 2). All groups presented neutrophilic infiltrate of variable intensity 6 days after instillations ($P = 0.7277$). After 9 days, there was a regression of the infiltrate, with no evidence of accentuated neutrophilic reaction in all the groups ($P = 0.2301$).

Conclusion: The inflammatory response to bladder instillation of an aqueous solution of S-nitrousglutathione was very similar to that induced by bladder instillation of protamine and KCl.

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Introduction

Interstitial cystitis (IC) is a condition characterized by bladder pain, urinary urgency, frequency and nocturia. The International Continence Society (ICS) prefers the term painful bladder syndrome defined as the supra-pubic pain related to the bladder filling, and with other symptoms such as increased frequency (day and night) in the absence of urinary tract infection or some other obvious disease. It is known that IC affects both men and women, but it is predominantly present in women (around 90% of all the patients). It has been known that IC affects both men and women, but it is predominantly present in women (around 90% of all the patients).

The main problem for the patients is how disease impacts their quality of life. The regulation of the nitric oxide synthase enzyme (NOS) in the urine was suggested as an important factor in the immune response of IC. Other theories include a possible infectious origin, neurogenic inflammation and histamine-induced generalized visceral hypersensitivity caused by abnormalities in the immune or neuroendocrine system.

A new experimental model using oxidative stress would be a major advantage for understanding this idiopathic condition. Moreover, it would allow for the experimental evaluation of new treatments. The objective of this study is to present a new experimental model of IC induction by oxidative stress using a nitric oxide donor gel.

Materials and methods

We studied the effects of a polymeric aqueous solution of the copolymer poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) Pluronic F127, containing S-nitrosglutathione (GSNO) as a nitric oxide donor on the bladder wall of Wistar rats. GSNO is an endogenous S-nitrosthioil that acts as a carrier and donor of NO, increasing its half-life. This project was developed at our institution, after approval by the Research Ethics Committee, protocol 1296-1. The rats were accommodated in cages containing five animals each, under ideal feeding, temperature, humidity and light conditions.

A pilot study including 20 Wistar rats was carried out as a training process for bladder catheterization in the animals. The sample size included 40 female Wistar rats aged
3 months. The nitric oxide donor chosen for this experiment was the 5-nitroso-glutathione (GSNO), produced and donated by the Institute of Chemistry of the State University of Campinas. GSNO was synthesized by reacting equimolar amounts of glutathione with sodium nitrite in aqueous hydrochloric acid (HCl 0.5 M), stirring it in an ice bath for 40 min. The final solution was precipitated with acetone, filtered and washed with cold water and acetone. The precipitate formed was dried for 24 h. The GSNO obtained was stored in the freezer (−20 °C) and protected from light.

Preparation of the solution containing NO Gel Pluronic F-127 (25 wt%) in water containing GSNO (100 μM) was prepared as described previously. Pluronic F-127 solid was added to cold water (5 °C). This solution was then left at 5 °C for 12 h to reach equilibrium dissolution of the polymer. Appropriate volume of aqueous solution of GSNO (0.35 mM) was added to the solution of Pluronic F-127 by stirring in an ice bath to complete homogenization of the solution. The animals were anesthetized by injection of sodium thiopental in the dorsal vein of the tail and then placed in the supine position, so that antisepsis could be performed with PVPI-lode.

**Group distribution**

Instillation was performed according to animal division in four groups, as shown in Table 1. In all groups, the substances were infused intravesically (Fig. 1), until bladder overflow was achieved (mean of 1 mL). The substances were kept in the bladder until the next micturition of the animals.

- **Group 1.** Ten rats underwent two sessions of bladder instillation of saline solution 0.9% at 0.04 ml/min, with a 24-h interval between them, until bladder overflow was achieved; they were then injected with GSNO solution in 3 doses, with a 2-day interval between each dose; after that they were euthanized.

- **Group 2.** Ten rats underwent two sessions of bladder instillation of saline solution 0.9% at 0.04 ml/min, with a 24-h interval between them until bladder overflow was achieved; they were then injected with excipient solution (only polymeric solution without GSNO) in 3 doses, 2 days between each dose and after that they were euthanized.

- **Group 3.** Ten rats underwent two sessions of bladder catheterization using protamine solution (30 mg/ml) and KCl 300 mM 0.04 ml/min, with a 24-h interval between them until bladder overflow (when the maximum bladder capacity was obtained); they were then injected with excipient solution (only polymeric solution without GSNO) in 3 doses, with a 2-day interval between each dose; after that they were euthanized.

- **Group 4.** Ten rats underwent two sessions of bladder catheterization using protamine solution (30 mg/ml) and KCl 300 mM 0.04 ml/min, until bladder overflow with a 24-h interval between them (when the maximum bladder capacity was obtained); they were then injected with GSNO solution in 3 doses, with a 2-day interval between each dose; after that they were euthanized.

**Analysis**

Half of the rats of each group were euthanized with a lethal dose of anesthetic on day 6, and the other half after 9 days. Skin and muscle layers were dissected and the abdominal wall was opened. The bladder was identified and removed (Fig. 2). After opening the bladder, it was put on a stick to be fixated in formaldehyde for 24 h and then in ethylc alcohol 70%, before being embedded in paraffin and analyzed for the presence of IC. Histological slides were made with histological cuts 3–4 μm wide and HE stained. The pathological study was based on the following classification according to the therapeutic groups (Tables 2 and 3).

The histological slides were analyzed by a pathologist, who received them identified only by numbers, with no indication of the groups. All the parameters of the table were analyzed and the data were analyzed statistically. Statistical analysis was performed with assistance of the Department of Statistics of the Research Board of the Faculty of Medical Sciences of the State University of Campinas. A descriptive analysis was conducted using frequency tables for categorical variables. For comparison of proportions, the Fisher exact test was used and the level of significance was 5%.

**Table 1** Time table of groups 1–4.

<table>
<thead>
<tr>
<th>Time</th>
<th>T = 1</th>
<th>T = 2</th>
<th>T = 3</th>
<th>T = 6</th>
<th>T = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1. Saline solution + GSNO</td>
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<tr>
<td>Day</td>
<td>Instillation</td>
<td>Saline solution</td>
<td>Saline solution</td>
<td>GSNO</td>
<td>GSNO</td>
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<tr>
<td>Group 2. Saline solution + polymeric solution</td>
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<tr>
<td>Day</td>
<td>Instillation</td>
<td>Saline solution</td>
<td>Saline solution</td>
<td>Polymeric solution</td>
<td>Polymeric solution</td>
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<td>Group 3. Saline solution + protamine and KCl</td>
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<tr>
<td>Day</td>
<td>Instillation</td>
<td>Saline solution</td>
<td>Saline solution</td>
<td>Protamine</td>
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<tr>
<td>Group 4. Protamine and KCl + GSNO</td>
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<td></td>
</tr>
<tr>
<td>Day</td>
<td>Instillation</td>
<td>Protamine</td>
<td>Protamine</td>
<td>GSNO</td>
<td>GSNO</td>
</tr>
</tbody>
</table>

In T = 6, five animals were euthanized.
Figure 1  The rats undergo tricotomy and asepsis (A). The urethra is shown with tweezers (B). The catheter is inserted carefully (C). Instillation of the liquid according to each group (D).

Figure 2  Skin is incised (A). Muscle layer is dissected (B). Opening of the abdominal wall (C). Identification and removal of the bladder (D). Opening the bladder (E). The bladder is put on a stick to be fixated in formaldehyde (F).

Results

Macroscopically we observed edema and hyperemia of the mucosa in 2 (22%) of the animals in group 1, in 0 (0%) of the animals in group 2, in 10 (100%) of the animals in group 3, and in 5 (50%) of the animals in group 4. In the protamine + KCl group and in saline + GSNO group, similar effects were observed on the bladder wall. A pathologist performed the microscopic evaluation following the parameters cited in Table 3.

Microscopically, there was no significant difference between the groups regarding vascular congestion in the animals on day 6 ($p=0.6329$). After 9 days, vascular congestion was significantly reduced in group 2 (saline solution + excipient) when compared to groups 1, 3 and 4 ($p=0.0035$) (Table 4). No difference was observed between the groups regarding fibrosis on day 6 ($p=0.3781$). After 9 days, fibrosis was significantly higher in group 4 (protamine + GSNO) when compared to groups 1 and 2 ($p=0.0035$) (Table 5).
Neutrophilic infiltrate of variable intensity was observed in all groups after 6 days ($p = 0.7277$). A tendency towards regression was observed after 9 days, without reaching differences of statistical significance between groups ($p = 0.2301$). A tendency towards regression was also observed in all groups regarding edema, without significant differences either ($p = 0.8096$ at 6 days; $p = 0.2478$ at 9 days). The animals euthanized on day 6 did not present any significant differences regarding lymphomonocitary infiltrate ($p = 0.7253$); however, on day 9 there was a significant difference between the groups ($p = 0.0459$). In the group treated with GSNO + saline solution, infiltrate was more extensive than in the rest. Differential presence of mast cells in the infiltrate was not observed among groups.

**Discussion**

Interstitial cystitis is a condition that deserves clinician attention, especially due to the lack of reliable criteria for diagnosis. Classically reported models do not reproduce the inflammation status properly, mostly because its cause is unknown. Previous studies found higher levels of NO in these patients; however, it is very difficult to collect material from patients in clinical trials because it causes great discomfort. A study was performed on patients in which 25 mL of air were introduced in the bladder by means of a catheter and the level of NO was compared to that in the air in the room. The study found significant differences, proving that the patients with cystitis presented more NO than healthy individuals.

Experimental models of IC described in the literature are scarce. The first projects to develop interstitial cystitis in animals used acetic acid, cyclophosphamide, lipopolysaccharides, protamine sulphate and vanilloid receptor agonists. These substances did not produce the effects intended, either because they damaged the bladder too much, or because they were not reliable. Fraser et al. sought to develop a model that would reliably mimic the acute phase of this debilitating disease. To this end, they combined protamine sulphate (PS) treatment, thought to breakdown urothelial umbrella cell barrier function, and physiologic concentrations of potassium chloride (KCl) in an open cystometrogram animal model. Female Sprague-Dawley rats were anesthetized and transurethral continuous cystometry was performed with normal saline and 100 mmol/L KCl or 0 mmol/L KCl as control. Either 10 or 30 mg/mL PS was then added to the control solution for a 30-min period (for modest and severe urothelial barrier...
breakdown, respectively), after which the control solution was continued for 1–2 h. Bladder contraction amplitudes, durations, frequency and intercontractile intervals were recorded and analyzed. There were no differences between the saline 100 mmol/L KCl or 500 mmol/L KCl control periods, indicating that barrier function in these animals was not affected by the physical preparation. Of the treatments tested, 100 mmol/L KCl with 30 mg/mL PS and 500 mmol/L KCl (the physiologic concentration of rat urine) with either 10 or 30 mg/mL PS produced reliable irritation, which continued up to 2 h after cessation of PS administration. These results indicate that the historical use of normal saline, instead of the more physiologic 500 mmol/L KCl for cystometry, greatly biased our understanding of the function of the lower urinary tract in animal models of IC; and modest, non-cytotoxic insults to urothelial barrier function can result in dramatic irritative responses, given the proper physiological conditions.8

In 2007, an experimental model of IC with intraperitoneal administration of cyclophosphamide (CP) was described. This anti-tumor agent is metabolized into acrolein in the kidney and accumulates in the bladder to produce toxic effects, ultimately resulting in visceral pain. This substance produces histological damage only in the urothelium when dissolved in 0.9% saline and administered intraperitoneally (i.p.). Vehicle or CP was administered (200–400 mg/kg, i.p.), and spontaneous and evoked pain behaviors were evaluated simultaneously in the same mice.9

Another experimental study was performed in cats that already presented idiopathic IC. In some cats there is naturally an idiopathic form of IC that provokes all the characteristics of human IC, including symptoms. The animals had history of pollakiuria, hematuria and micturition in inadequate locations. After the study of NO levels in both control and IC bladders, it was observed that the NO levels in the IC bladders were higher.10 Other experimental models of IC include the use of attenuated pseudorabies virus (Bartha PRV), an α-herpes virus that is taken up by neurons and undergoes retrograde transport and viral replication within the central nervous system9,10 and IC induction with substance P and lipopolysaccharides that induce the release of histamine and cytokine by the bladder. The probable relation between NO and the inflammatory process related to IC brings an experimental model that is closer to reality with the use of a NO donor gel to start up the inflammatory process. The inflammatory response to bladder instillation of an aqueous solution of S-nitrosglutathione (GSNO) was very similar to that induced by bladder instillation of PS and KCl. Therefore, instillation of GSNO can be considered a new model for experimental induction of interstitial cystitis in rats.

Conflict of interest
The authors declare to have no conflict of interest.

References