

Use of a Sulfated Chitosan Derivative to Reduce Bladder Inflammation in the Rat

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OBJECTIVES

Interstitial cystitis is a chronic, debilitating disease of the bladder. Treatments using intravesicular inoculation of long-chain polysaccharide formulations, such as hyaluronic acid or anti-inflammatory agents, have been used to some effect. The objective of this study was to test a long-chain polysaccharide derivative of chitosan as a vehicle for delivery of the anti-inflammatory agent 5-aminosalicylic acid (5-ASA) for treatment of inflammation in the bladder.

METHODS

Bladder inflammation was induced in rats by intravesicular inoculation of protamine sulfate and lipopolysaccharide. Groups of rats were randomly assigned to the treated or control groups, which received either the treatment agents or saline 24 hours after induction. The animals were killed 5 days after inoculation, and the bladders harvested for histologic examination of inflammation by a blinded observer. Four parameters of inflammation were measured using a 6-point scale. In another experiment, urinary frequency was measured 4 days after inoculation.

RESULTS

The most potent treatment agent was 3% *N*-sulphonato-*N,O*-carboxymethylchitosan plus 5-ASA, with a mean reduction in inflammation, as measured by histologic examination, of up to 75%. This level of reduction was significantly greater than that seen by treatment with the commercially available product Cystistat. In a separate experiment, 3% *N*-sulphonato-*N,O*-carboxymethylchitosan plus 5-ASA ameliorated the increase in urinary frequency seen in induced, untreated animals.

CONCLUSIONS

The combination of 3% *N*-sulphonato-*N,O*-carboxymethylchitosan and 5-ASA reduced bladder inflammation as measured by histologic examination and by the lower urinary frequency. UROLOGY 70: 1014–1018, 2007. © 2007 Elsevier Inc.

Interstitial cystitis (IC) is a chronic, debilitating disease of the bladder affecting more than 500,000 individuals in the United States, 90% of those being women.¹ It is characterized by urgent, sometimes painful, urination that can be accompanied by diffuse pelvic pain. It has been suggested that the etiology of IC might involve infectious agents, lymphovascular obstruction, neurologic pathologic features, inflammatory conditions, or autoimmune pathologic features.¹ Some researchers have proposed that a disruption in the glycosaminoglycan (GAG) layer of the bladder might allow access of toxic products from the bladder lumen to the epithelial lining, leading to inflammation.² As a result of the uncertain

etiology, diverse presentation, and episodic nature of the disease, treatment continues to be a challenge. IC can be temporarily managed by oral medications such as antispasmodics or synthetic products such as pentosan polysulfate, but these therapies have yielded inconsistent results.² Bladder (intravesicular) instillations have also been used to treat IC, but many patients treated in this manner will have relapses with time and require additional, more frequent, treatments.² Finally, the option of surgery is available, but this is a last resort because of the increased risk of further damage to the bladder and often short-lived effectiveness.²

A number of rodent models of IC are available,^{1,3} with the most commonly used models characterized more by acute inflammation than chronic pathologic features. Common to all the models is the occurrence of bladder inflammation, including edema, fibrosis, inflammatory cell infiltration, epithelial damage, venous congestion, and hemorrhage. In this study, we initiated bladder inflammation with protamine sulfate (PS) and lipopolysaccharide (LPS). This method of induction has the advantage that it also results in the increase in urinary frequency characteristic of the human disease.

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In this study, we used a long-chain polysaccharide that has many qualities similar to hyaluronic acid. Hyaluronic acid is one of the major components of the GAG layer of the bladder, and, as such, researchers have delivered hyaluronic acid intravesicularly in animal models^{4,5} and in humans^{6,7} as a GAG replacement therapy. The polysaccharide used is nontoxic, and, when sulfated to form *N*-sulphonato-*N,O*-carboxymethylchitosan (sNOCC), it easily coats the bladder wall.⁸ We report on our experiments using sNOCC combined with the anti-inflammatory agent 5-aminosalicylic acid (5-ASA) to treat IC in a rat model.

MATERIAL AND METHODS

Animals

Female 8-week old Sprague-Dawley rats (weight 225 to 250 g) were purchased from Charles River (St. Constant, Quebec, Canada) and provided with food and water ad libitum. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council of Animal Care.

Test Agents

sNOCC in citrate buffer was provided by Kytogenics Pharmaceuticals (Dartmouth, Nova Scotia, Canada). 5-ASA with a nominal particle size of 11 μm was purchased from Sigma Chemical (St. Louis, Mo) and used at a concentration of either 2 mg/mL or 20 mg/mL. The hyaluronic acid was purchased from Sigma Chemical and used as a 1% solution in phosphate-buffered saline (PBS). Cystistat (800 $\mu\text{g/mL}$ sodium hyaluronate) is a commercially available prescription preparation produced by Bioniche (Belleville, Ontario, Canada).

Intravesicular Inoculation of PS and LPS

The rats were anaesthetized with sodium pentobarbital (65 mg/kg). The surface area around the urethral opening was swabbed with 70% isopropyl alcohol and Betadine. A sterile catheter (PE-50) was inserted into the bladder through the urethral opening. The rats were inoculated with 1 mL of PS (10 mg/mL, Sigma Chemical) in PBS (Sigma Chemical) using the catheter. After 45 minutes, the bladders were emptied, washed with PBS, and given 1 mL LPS (750 $\mu\text{g/mL}$, Sigma Chemical) for 30 minutes. During the treatment period, the catheters were occluded to prevent loss of the solution in the bladder.

Intravesicular Treatment with Test Agents and Assessment

After PS/LPS inoculation, the animals were randomly assigned to the treatment groups. One group received PBS and served as the untreated control group. The other groups were treated with the various test agents. The test agents were administered, by way of an intravesicular catheter (PE-50), in a volume of 1 mL, 1 day after PS/LPS inoculation. The catheters were occluded and left in the bladder for 1 hour before being removed. The treatment agents were not extracted. The animals were killed 5 days after inoculation using an overdose of sodium pentobarbital. The bladders were then removed and fixed in 10% formalin for conventional histologic examination. Paraffin sections (5 μm) were cut and stained with Harris' hematoxylin-eosin. The entire section was examined for signs of inflammation, with three sections per slide assessed. The parameters of inflamma-

tion measured were venous congestion, edema, cellular infiltration, and epithelial damage. The parameters were measured separately by a blinded observer using a 6-point scoring system. Venous congestion was scored as 0 for no dilated vessels; 1 for focal, small dilated vessels, 2 for diffuse, small dilated vessels; 3 for diffuse, small dilated vessels with rare large dilated vessels; 4 for diffuse, small dilated vessels with focal large dilated vessels; and 5 for diffuse small dilated vessels with diffuse large dilated vessels. Edema was scored as 0 for no edema; 1 for focal mild edema; 2 for focal moderate edema; 3 for focal marked edema; 4 for diffuse marked edema; and 5 for diffuse marked edema with architectural distortion. Cellular infiltration was scored as 0 for no cellular infiltration; 1 for focal mild lymphoid infiltration; 2 for diffuse mild lymphoid infiltration; 3 for diffuse moderate lymphoid infiltration; 4 for diffuse moderate lymphoid infiltration with one or two dense lymphoid aggregates; and 5 for diffuse moderate lymphoid infiltration with three or more dense lymphoid aggregates. Epithelial damage was scored as 0 for no injury; 1 for minimal intraepithelial lymphoid infiltration with intracellular edema; 2 for mild intraepithelial lymphoid infiltration with epithelial hyperplasia; 3 for marked intraepithelial lymphoid infiltration; 4 for marked intraepithelial lymphoid and neutrophilic infiltration with focal necrosis and excoriation; and 5 for ulceration. A total inflammatory score was calculated for each rat by summing the scores of the four inflammatory parameters. A mean total inflammatory score was also calculated for each group.

In one experiment, the animals were placed in a metabolic cage overnight 4 days after induction. The animals had access to water but not food. Each cage was equipped with a screen floor and a urine collection device that was computer monitored. Continuous computer monitoring of the weight of the urine collection bucket allowed identification of both timing and volume of each void. A simple analysis of the voiding pattern was performed using the number of voids during the 12-hour dark period.

Experimental Plan

Study 1: Evaluation of sNOCC Combined with 5-ASA. Four groups of 10 rats were induced with PS/LPS. After 24 hours, group 1 received PBS, group 2 received 20 mg/mL 5-ASA alone, group 3 received 3% sNOCC alone; and group 4 received 3% sNOCC with 20 mg/mL 5-ASA. The bladders were harvested 4 days later.

Study 2: Comparison of sNOCC Plus 5-ASA to Cystistat. Three groups of 8 rats were induced with PS/LPS. After 24 hours, group 1 received PBS, group 2 received 3% sNOCC plus 5-ASA (2 mg/mL), and group 3 received Cystistat. The bladders were harvested 4 days later.

Study 3: Evaluation of pH Change in sNOCC on Efficacy. The sNOCC solutions were tested at pH 7.2 and 5.2 (approximate pH of the bladder). Three groups of 10 rats were induced with PS/LPS as described above. After 24 hours, group 1 received PBS, group 2 received 3% sNOCC (pH 5.2) plus 5-ASA, and group 3 received 3% sNOCC (pH 7.2) plus 5-ASA (20 mg/mL). The bladders were harvested 4 days later.

Study 4: Evaluation of Urinary Frequency in Animals Treated with sNOCC Plus 5-ASA. Three groups of 8 animals were used. Group 1 received no PS/LPS induction. Groups 2 and 3 were induced with PS/LPS. After 24 hours, group 3

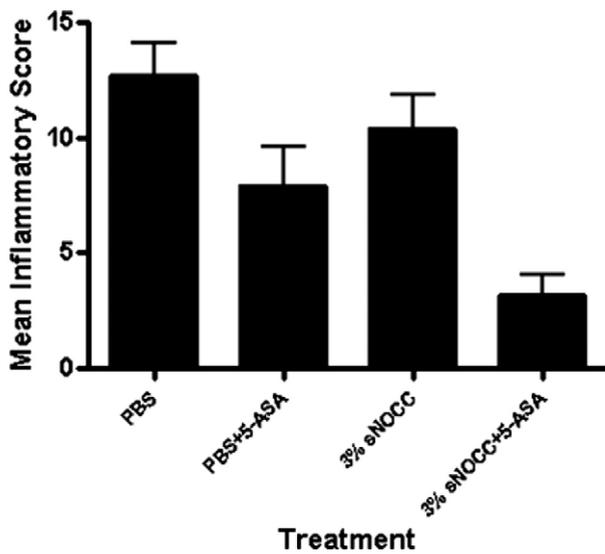


Figure 1. Evaluation of sNOCC combined with 5-ASA. 5-ASA was combined with various agents and mean inflammation recorded. Inflammation scoring done using 6-point score of each of four parameters representative of IC: venous congestion, edema, cellular infiltration, and epithelial damage. Mean inflammatory severity shown for control treatments (PBS, PBS plus 5-ASA, and 3% sNOCC) and test agent with 5-ASA (sNOCC versus 3% sNOCC plus 5-ASA). Data shown as mean \pm standard error of mean; $n = 10$.

received 3% sNOCC plus 5-ASA (2 mg/mL). On day 4, urinary frequency was monitored.

Statistical Analysis

One-way analysis of variance with the Kruskal-Wallis test, followed by Dunn's multiple comparison test was used for all four experiments. $P < 0.05$ was considered significant.

RESULTS

Bladder Inflammation

As expected, induction using PS/LPS resulted in significant inflammation in the bladder. The edema, venous congestion, and cellular infiltration scores showed a range of severity, with some animals exhibiting scores of up to 5 for some or all of these parameters. The scores for epithelial damage were routinely in the 1 to 2 range, with rare scores of 3, very rare scores of 4, and no scores of 5 seen in the induced animals. As a consequence, epithelial damage was less important to the cumulative scores than the other parameters measured.

Study 1: Effect of sNOCC Plus 5-ASA on Reducing Bladder Inflammation

In this study, we compared the effects of 3% sNOCC and 3% sNOCC plus 5-ASA on bladder inflammation after the initiating stimulus. The data in Figure 1 show that 3% sNOCC alone did not reduce bladder inflammation but that 3% sNOCC plus 5-ASA significantly reduced the mean inflammatory score. This formulation decreased the severity of inflammation by 75% compared with the

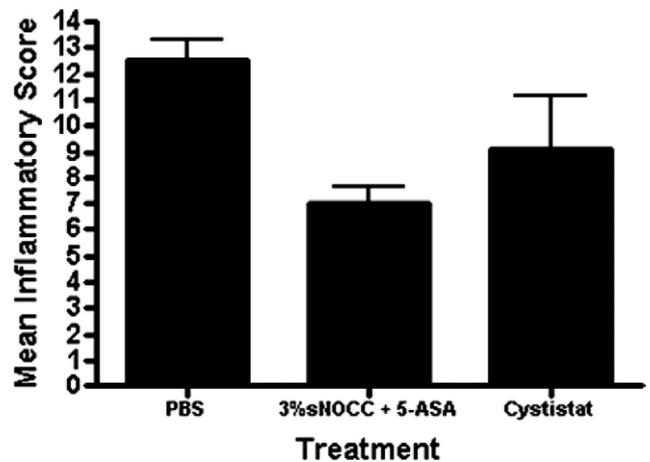


Figure 2. Comparison of sNOCC plus 5-ASA with Cystistat. Test agent 3% sNOCC plus 5-ASA compared with commercially available product Cystistat. Both groups were compared with PBS control group. Mean inflammatory score represented by mean \pm standard error of mean; $n = 8$.

PBS control. This was highly significantly different from the controls when tested by analysis of variance followed by a multiple comparison test ($P < 0.001$).

Study 2: Comparison of 3% sNOCC Plus 5-ASA and Cystistat

The data from Study 2 demonstrated that the animals treated with 3% sNOCC plus 2 mg/mL 5-ASA showed less inflammation than animals treated with Cystistat (Fig. 2). sNOCC plus 5-ASA was able to reduce the mean inflammatory score by 49% compared with the controls. This decrease was significant ($P < 0.05$). Although this 5-ASA concentration was 10-fold lower than that used in Study 1, when used in conjunction with 3% sNOCC, it still showed highly significant efficacy. Cystistat treatment did not significantly decrease inflammation compared with the PBS control ($P > 0.05$).

Study 3: Effect of Changing pH

When the pH of the 3% sNOCC solution was increased to 7.2, the efficacy of the 3% sNOCC plus 5-ASA treatment was ablated (Fig. 3). The results for the pH 7.2 group were not significantly different statistically from those of the control group as assessed by one-way analysis of variance followed by Dunn's multiple comparisons test.

Study 4: Effect on Urinary Frequency

Urinary frequency was calculated by a computer analysis program using the number of significant increases in the weight of the urine collection bucket. As shown in Figure 4, the control animals, which were not induced with PS/LPS and thus had no inflammatory disease, showed a mean urinary frequency of 11 voids per night (11.1 ± 1.2). The rats induced with PS/LPS but that had received no treatment agent had a significantly greater ($P < 0.05$) mean urinary frequency (20.5 ± 2.4 voids per night)

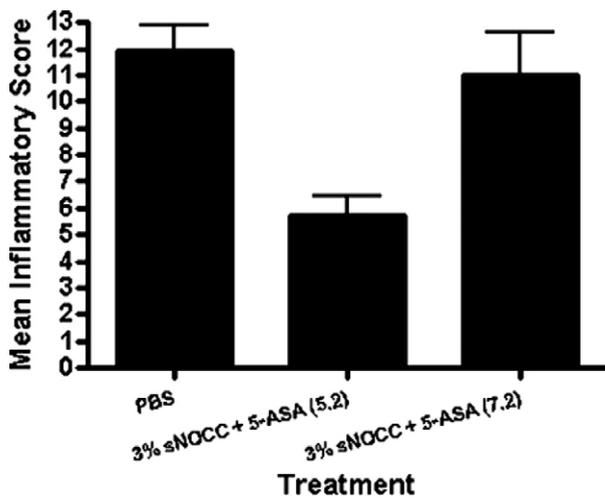


Figure 3. Efficacy of sNOCC with pH change. Mean inflammatory score measured for animals treated with 3% sNOCC plus 5-ASA at two different pH levels (pH of 5.2 versus 7.2). Efficacy of both groups compared with efficacy of PBS control. Data shown as mean \pm standard error of mean; n = 10.

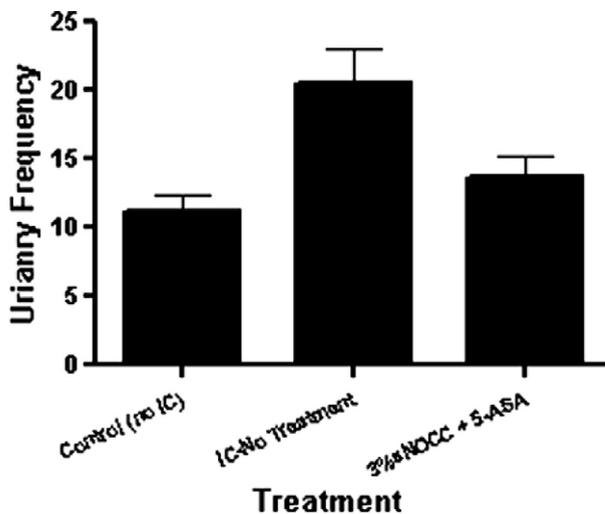


Figure 4. Efficacy of sNOCC to reduce urinary frequency. Urinary output of animals treated with 3% sNOCC plus 5-ASA compared with that of animals with no disease. PS/LPS-induced animals compared with uninduced controls. Frequency of urination represented by mean \pm standard error of mean; n = 8.

compared with uninduced controls. In contrast, the PS/LPS-induced rats treated with sNOCC plus 5-ASA had mean urinary frequency levels similar to those of the uninduced controls (13.6 ± 1.6 voids per night; $P > 0.05$). These data have demonstrated that the animals treated with 3% sNOCC plus 5-ASA had a significant resolution of their disease.

COMMENT

Currently, a number of medications are available for IC, but all have demonstrated limited efficacy. Some oral medications, such as pentosan polysulfate sodium, act

ostensibly to repair the breakdown of the GAG layer, the damage of which has been implicated in inflammation.⁹ Tricyclic antidepressants and antispasmodics focus on pain and urination frequency, and antihistamines act on mast cell involvement.^{2,6,10} Intravesicular therapies are also available. Dimethyl sulfoxide acts primarily as an anti-inflammatory agent,¹¹ and intravesicular hyaluronic acid (Cystistat) has been suggested to restore the GAG layer.^{2,10} Bladder distension, often in conjunction with lidocaine, has provided short-term relief.

The data we have presented have shown that using 3% sNOCC in combination with 5-ASA reduces inflammation and urinary frequency in a rat model of bladder inflammation. This effect was not seen using 3% sNOCC alone. This model is not identical to chronic human IC in that the model is one of short-term acute inflammation in the bladder. Nevertheless, the findings in such models are generally thought to be helpful in addressing the problems associated with chronic inflammation in the bladder in humans.

We suggest that sNOCC is able to effectively coat the bladder wall because of its viscous nature. This might, to some extent, ameliorate the symptoms associated with a reduced GAG layer. However, it is most likely that the primary effect of sNOCC is to allow a close approximation of the 5-ASA with the epithelium, thus enhancing efficient transepithelial transport of 5-ASA into the bladder lamina propria.

At a pH approximating neutral, 5-ASA is more soluble. However, its ability at this pH to reduce inflammation in the bladder is unknown. Therefore, 3% sNOCC was prepared at a pH of 7.2, such that 5-ASA would easily solubilize. When tested in our model, the data revealed that the solution at this pH was ineffective. We speculated that the now-soluble 5-ASA might reprecipitate in the bladder shortly after introduction as the solution slowly reaches the bladder pH of 5.0. This reprecipitation might have reduced the bioavailability of 5-ASA. Additional experimentation will provide evidence consistent, or inconsistent, with this hypothesis. A solution of pH 5.2 was also tested in combination with 5-ASA. Although the 5-ASA was less soluble, the efficacy of 3% sNOCC plus 5-ASA to reduce bladder inflammation was greater.

Taken together, these data have demonstrated that 3% sNOCC (pH 5.2) with 20 mg/mL 5-ASA can be used to reduce the inflammation and increased urinary frequency seen in a rat model of IC. This intravesicular combination therapy was superior to intravesicular, hyaluronic acid treatment (Cystistat). Thus, 3% sNOCC plus 5-ASA could be a promising candidate for development as a possible therapeutic modality for bladder inflammation in humans, including the treatment of inflammatory IC.

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