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# Intravesical Nanocrystalline Silver Decreases Experimental Bladder Inflammation

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**Purpose:** Interstitial cystitis is a sterile bladder inflammatory disease characterized by pelvic pain, urinary urgency and frequency. Nanocrystalline silver has anti-inflammatory properties, prompting us to investigate its effect in experimental bladder inflammation.

**Materials and Methods:** Nanocrystalline silver (0.01%, 0.05%, 0.1%, 0.5% or 1%) or phosphate buffered saline (Invitrogen™) (0.5 ml) was introduced intravesically in Sprague-Dawley female rat (Charles River Laboratories, Wilmington, Massachusetts) bladders for 20 minutes, followed by vehicle or protamine sulfate (10 mg/ml for 30 minutes) and lipopolysaccharide (Sigma®) (2 mg/ml for 45 minutes). Urine was collected throughout for histamine assay. The catheter was removed, the rat was returned to its cage and 4 hours later it was sacrificed. The bladder was harvested, minced and cultured overnight. The medium was collected for tumor necrosis factor- $\alpha$  assay.

**Results:** Mean  $\pm$  SD total urine histamine increased from  $270 \pm 190$  ng in 4 controls to  $842 \pm 239$  ng after protamine sulfate/lipopolysaccharide and it decreased to  $505 \pm 187$  ng in 6 animals after pretreatment with 1% nanocrystalline silver ( $p = 0.036$ ). Tumor necrosis factor- $\alpha$  release in explant medium increased from  $0.02 \pm 0.03$  pg/mg in 6 controls to  $0.28 \pm 0.15$  pg/mg in 14 animals after treatment with protamine sulfate/lipopolysaccharide and it decreased to  $0.12 \pm 0.11$  pg/mg in 10 animals pretreated with nanocrystalline silver ( $p = 0.009$ ). Nanocrystalline silver was not effective at less than 1% and at 1% alone it released  $0.05 \pm 0.07$  pg/mg tumor necrosis factor- $\alpha$  in 7 rats (vs phosphate buffered saline in 6,  $p = 0.387$ ). Nanocrystalline silver (1%) significantly decreased bladder inflammation and mast cell activation. These effects were apparent even 4 days later.

**Conclusions:** Intravesical administration of nanocrystalline silver (1%) decreased urine histamine, bladder tumor necrosis factor- $\alpha$  and mast cell activation without any toxic effect. This action may be useful for interstitial cystitis.

*Key Words: bladder; inflammation; cystitis, interstitial; silver; tumor necrosis factor-alpha*

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Interstitial cystitis is a sterile bladder disease occurring mostly in women that is characterized by bladder/pelvic pain as well as urgency and frequency of urination in the absence of urinary tract infection.<sup>1</sup> The prevalence of IC was recently estimated to be as high as 464 to 575/100,000 women.<sup>2,3</sup> While symptoms can be quite debilitating, there is no curative treatment currently available.<sup>4,5</sup> A number of immunomodulators have been proposed or studied<sup>6</sup> because many patients with IC have bladder inflammation but the results are still not impressive.<sup>4,7</sup> There have been repeated reports of an increased number of activated mast cells in the bladder of patients with IC,<sup>8</sup> which is the only pathological finding shown to correlate with dry symptoms, specifically nocturia.<sup>9</sup>

NCS particles were recently investigated in many inflammatory processes. NCS (1%) cream significantly decreased swelling and inflammation, including TNF- $\alpha$  gene expression, in an experimental animal model of dermatitis.<sup>10</sup> NCS (1%) was also shown to promote wound healing.<sup>11</sup> Currently NCS is clinically available as an impregnated wound dressing that is used commonly in patients with burns.

We examined the effect of intravesical NCS in an established animal model<sup>12,13</sup> of bladder inflammation using P and bacterial LPS to investigate it as a potential treatment for IC.

## MATERIALS AND METHODS

### Materials

NCS powder (Nucrust, Wakefield, Massachusetts) was prepared by physical vapor deposition using a process of magnetic sputtering, whereby the silver atoms are layered atom by atom in the presence of trace oxygen levels.<sup>14</sup> NCS was dissolved in lactate buffer (pH 4) to a final concentration of 1% in sterile water that was fresh on the day of the experiments. Working dilutions were obtained with PBS.

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Study received approval from the Tufts-New England Medical Center Institutional Animal Care and Use Committee.

\* Financial interest and/or other relationship with Nucrust Pharmaceutical.

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## Methods

Female Sprague-Dawley rats weighing 175 to 200 gm were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg)/xylazine (4 mg/kg) and catheterized to drain the bladder using a 24 gauge Angiocath™, as reported previously.<sup>15</sup> The rats were divided into 4 groups based on treatment parameters, including group 1—negative controls that received PBS for 95 minutes, group 2—positive controls that received NCS (1%) for 20 minutes, followed by PBS for 75 minutes, group 3—rats with baseline inflammation that received PBS for 20 minutes, followed by P (10 mg/ml) for 30 minutes, followed by LPS (2 mg/ml) for 45 minutes and group 4—the treatment group, which received NCS (1%) for 20 minutes, followed by P for 30 minutes and LPS for 45 minutes.

The dose-response (0.01%, 0.05%, 0.1%, 0.5% and 1%) was carried out with 20-minute pretreatments. The timing of NCS delivery in group 4 was also investigated at 1% based on the findings from the dose-response study. Therefore, CS (1%) was added 20 minutes before P/LPS, together with or 20 minutes after P/LPS. Control rats were treated with PBS with or without NCS (1%) for comparison.

Protamine was instilled before LPS based on prior reports suggesting that its use produces maximal bladder inflammation.<sup>12,13</sup> Each rat was rotated manually sideways 180 degrees every 10 minutes during NCS treatment to allow uniform distribution in the bladder. Urine was recovered at the 20, 50 and 95-minute time points for each condition and samples were pooled and stored at -20C until assayed. After treatment the catheter was removed and the animal was allowed to recover from anesthesia in its cage for 4 hours. It was then sacrificed by CO<sub>2</sub> asphyxiation and decapitation at the time that had previously been shown to provide the maximal TNF- $\alpha$  response.<sup>16</sup> The bladder of each rat was removed, minced into 1 mm<sup>2</sup> pieces (each condition was based on about 70 mg tissue) and cultured overnight in RPMI 1640 (Sigma). Supernatant medium was collected by centrifugation at 300  $\times$  gravity for 20 minutes. It was assayed using enzyme-linked immunoassay kits for TNF- $\alpha$  with a minimum level of detection of 5 pg/ml (R & D Systems®) and for histamine with a minimum level of detection of 0.05 ng/ml (Beckman Coulter, Fullerton, California). The bladder explants were then blotted and weighed. This protocol was approved by the Tufts-New England Medical Center Institutional Animal Care and Use Committee.

## Duration of the Effect of NCS and Assessment of NCS Toxicity

After establishing 1% as the most effective NCS treatment dose we investigated the duration of the inhibitory effect of NCS and any toxic effects that it could have on the bladder. As such, rats were treated with NCS (1%), followed by P/LPS as before and were then placed in metabolic cages (Nalgene® No. 650-0100). Urine was collected every day for 4 days over dry ice and stored at 20C for histamine assay. On day 4 the rats were sacrificed and the bladders were processed for explants and histology.

## Bladder Histology

Bladder tissue from groups 1 to 4 was collected at the end of each treatment period (95 minutes) and immediately fixed in freezing medium (Triangle Biomedical Sciences, Durham,

North Carolina). Cryostat sections (8  $\mu$ m) were prepared, dried and stored at -80C until staining with 0.1% toluidine blue (pH 2) for mast cell and leukocyte identification. Cells were observed under a high power field (40 $\times$  objective) and counted by 2 independent investigators.

## Statistical Analysis

Results are presented as the mean  $\pm$  SD. They were analyzed using the nonparametric Mann-Whitney U test when comparing the effect of P/LPS to control or NCS+P/LPS to P/LPS alone. Some values are based on all experiments performed, including those performed earlier in the course of this study and, hence, the higher number of preparations indicated. Multiple comparisons among groups treated with different concentrations of NCS were done by ANOVA. Significance was considered at  $p < 0.05$ .

## RESULTS

### Total Urine Volume

The total urine volume collected from each group was 1.46  $\pm$  0.31 ml in 5 group 1 rats (PBS, negative control), 1.6  $\pm$  0.30 ml in 5 group 2 rats (1% NCS, positive control), 2.16  $\pm$  0.50 ml in 9 group 3 rats (P/LPS, baseline inflammation) and 1.79  $\pm$  0.55 ml in 8 group 4 rats (NCS [1%] plus P/LPS, treatment) ( $p = 0.249$ ). There were statistically significant differences between baseline inflammation and the 2 control groups, ie for PBS vs P/LPS and NCS (1%) vs P/LPS ( $p = 0.016$  and 0.043, respectively). Comparisons between all other groups did not reveal any significant disparity. The slighter higher volume in the P/LPS was considered when calculating total urine histamine.

### Effect of NCS on Total Histamine Levels in Urine

Total histamine in urine increased from 270  $\pm$  190 ng in 4 bladders treated with PBS alone to 842.5  $\pm$  239 ng in 4 P/LPS treated animals ( $p = 0.009$ , fig. 1, A), thereby establishing the inflammatory baseline to which treatment with NCS would be compared. The total histamine level in animals treated only with NCS (1%) was 495  $\pm$  51 ng, which was not statistically different from that in the 4 animals in the PBS group ( $p = 0.062$ ). Total histamine in animals pretreated with NCS (1%) decreased by 40% from 842.5  $\pm$  239 ng to 505.2  $\pm$  187 ng compared to the P/LPS group in 6 preparations ( $p = 0.036$ , fig. 1, A).

### Effect of NCS on Histamine Release From Bladder Explants

Histamine release, now expressed as ng/mg of bladder tissue explants, increased from 0.36  $\pm$  0.08 ng/mg in the 6 controls treated with PBS alone to 0.79  $\pm$  0.29 ng/mg in the 5 rats in the P/LPS group ( $p = 0.007$ , fig. 1, B). Histamine release in 5 animals treated only with NCS (1%) was 0.45  $\pm$  0.25 ng/mg, which was not statistically different from that in the 6 treated with PBS alone ( $p = 0.423$ ). Histamine release decreased from 0.79  $\pm$  0.29 to 0.60  $\pm$  0.13 ng/mg in 4 rats pretreated with NCS (1%). This difference was not statistically significant compared to that in rats treated with P/LPS ( $p = 0.147$ , fig. 1, B).

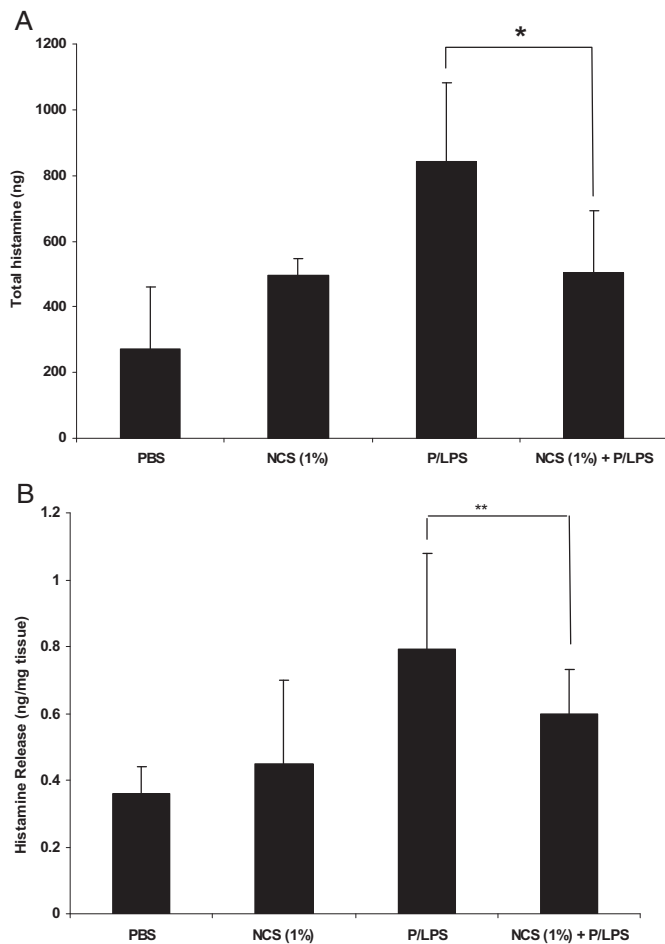


FIG. 1. Effect of NCS on P/LPS induced total urine histamine in 4 PBS, 4 NCS (1%), 4 P/LPS and 6 NCS (1%) plus P/LPS treated rats (A). Asterisk indicates  $p = 0.036$ . Effect of NCS on P/LPS induced rat bladder explant histamine in 6 PBS, 5 P/LPS, 4 NCS (1%) plus P/LPS and 5 NCS (1%) treated rats (B). Double asterisks indicate  $p = 0.007$ .

### Effect of NCS on TNF- $\alpha$ Release From Bladder Explants

TNF- $\alpha$  from bladder explants increased from  $0.02 \pm 0.03$  pg/mg in 6 rats in the PBS only control group to  $0.28 \pm 0.15$  pg/mg in 14 P/LPS treated rats (fig. 2). TNF- $\alpha$  was  $0.05 \pm 0.07$  pg/mg in the 7 rats treated with NCS (1%) alone ( $p = 0.387$ ). TNF- $\alpha$  release decreased by 57% from  $0.28 \pm 0.15$  to  $0.12 \pm 0.11$

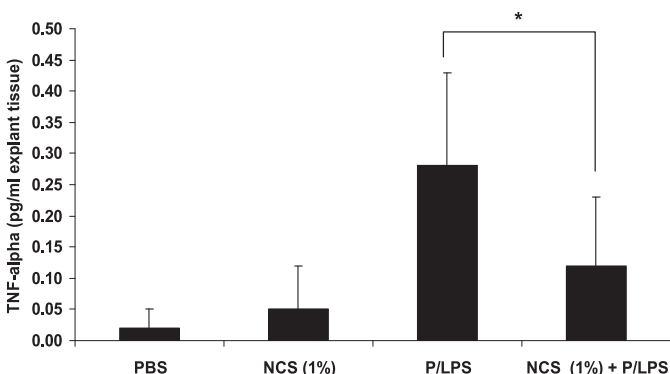


FIG. 2. Effect of NCS (1%) on P/LPS induced TNF- $\alpha$  release from bladder explants in 6 PBS, 7 NCS, 14 P/LPS and 10 NCS plus P/LPS treated rats. Asterisk indicates  $p = 0.009$ .

pg/mg in the NCS (1%) group compared to the P/LPS group in 10 preparations ( $p = 0.009$ , fig. 2).

### Timing of NCS Administration

We then tested the effect of NCS (1%) added at different times with respect to P/LPS. When NCS was instilled 20 minutes before P/LPS, it decreased total urine histamine from  $928 \pm 556$  ng in 9 rats to  $353 \pm 198$  ng in 7 ( $p = 0.017$ ), which was a 62% inhibition (fig. 3, A). Addition at the same time as P/LPS or after P/LPS had no effect (fig. 3, A).

When NCS (1%) was administered 20 minutes before P/LPS instillation, TNF- $\alpha$  release decreased from  $0.28 \pm 0.15$  pg/mg bladder tissue in 14 rats to  $0.12 \pm 0.11$  pg/mg bladder tissue in 10 ( $p = 0.009$ ), which was a 57% inhibition (fig. 3, B). Administration of NCS concurrently in 4 rats with

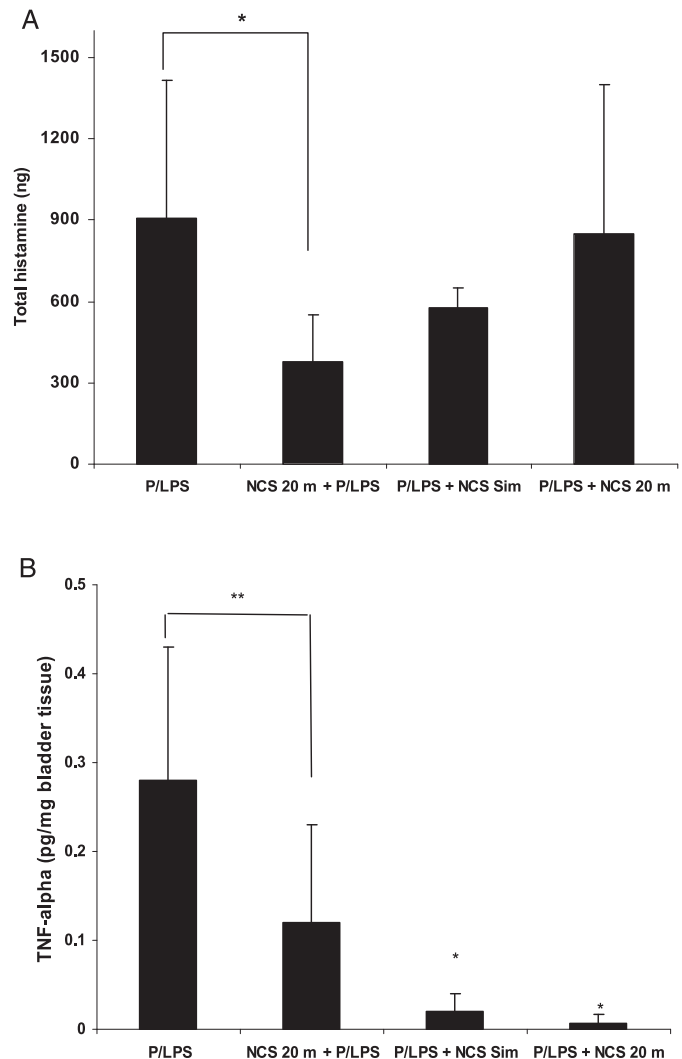


FIG. 3. Timing of NCS (1%) administration on P/LPS induced total urine histamine on 13 P/LPS, 7 NCS for 20 minutes plus P/LPS (equal to 20-minute pretreatment with NCS [1%] before P/LPS), 3 P/LPS plus NCS with simultaneous addition (*Sim*) of P/LPS and NCS and 3 P/LPS plus NCS for 20 minutes (equal to P/LPS followed by treatment with NCS for 20 minutes) treated rats (A). Asterisk indicates  $p = 0.017$ . TNF- $\alpha$  release in medium from bladder explants in 14 PBS, 10 NCS before P/LPS (equal to 20-minute pretreatment with NCS before P/LPS), 4 P/LPS plus NCS with simultaneous addition of P/LPS and NCS and 5 P/LPS followed by NCS for 20 minutes treated rats (B). Single asterisk indicates  $p = 0.004$ . Double asterisks indicate  $p = 0.0009$ .

P/LPS and 20 minutes after P/LPS in 5 also decreased TNF- $\alpha$  levels in bladder tissue to below detectable levels ( $p = 0.0009$ , fig. 3, B). There was no statistically significant difference between these treatments.

### Dose-Response Investigation of NCS

A dose-response study was done using pretreatment with several concentrations of NCS for 20 minutes. Except for 1% NCS all other concentrations used did not inhibit total urine histamine release or bladder explant TNF- $\alpha$  release compared with that of P/LPS instillation alone (fig. 4).

### Effect of NCS on Bladder Inflammation and Mast Cells

Treatment with P/LPS induced extensive bladder inflammation, including an increase in mast cells from  $2 \pm 1$  to  $8 \pm 3$  per high power field in 3 preparations ( $p = 0.03$ ). Inflammatory cells were apparent as small blue cells and blue exudates, while mast cells were distinguished as large blue-pinkish cells (fig. 5, A). Mast cells in the P/LPS treated group were activated/degranulated (greater than 70%), as judged by the absence of clear surface margins, lack of homogeneous cell staining and pinkish/violet granular material outside the cell perimeter (fig. 5, A and B).

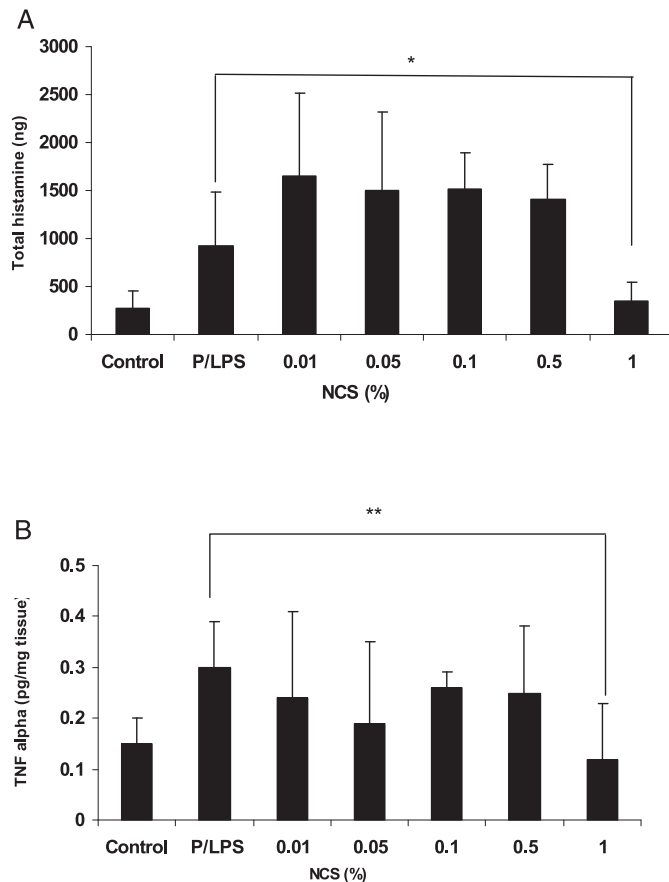


FIG. 4. Dose-response effect of NCS pretreatment for 20 minutes, followed by P/LPS for 75 minutes on total urine histamine using NCS at 0.01% in 6, 0.05% in 3, 0.1% in 3, 0.5% in 3 and 1.0% in 7 rats (A), and explant TNF- $\alpha$  release using NCS at 0.01% in 6, 0.05% in 6, 0.1% in 3, 0.5% in 3 and 1.0% in 10 rats (B). Results were compared to those in 6 PBS controls and 10 P/LPS treated rats with baseline inflammation. Single asterisk indicates  $p = 0.0174$ . Double asterisks indicate  $p = 0.0008$ .

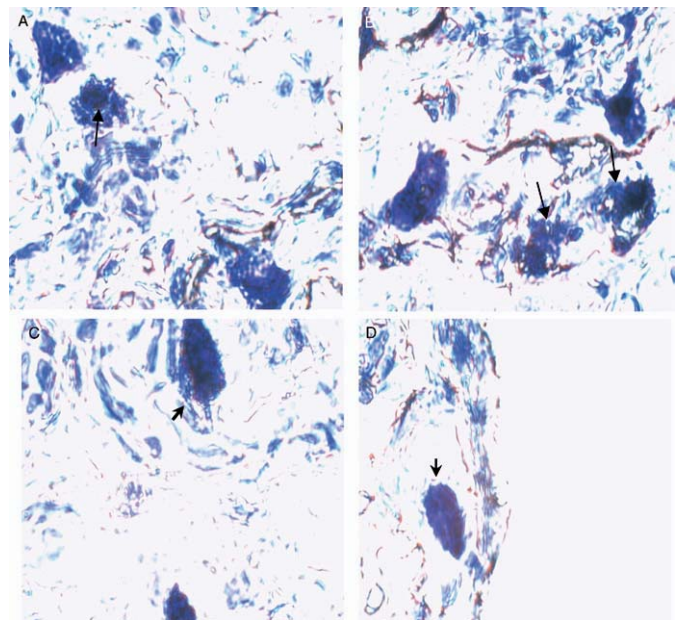


FIG. 5. Photomicrographs show bladder sections from 3 rats treated intravesically with P/LPS with degranulated mast cells (arrows) (A and B) and from 3 treated with NCS (1%) followed by P/LPS with intact mast cells (arrowheads) (C and D). Acidified toluidine blue stain, reduced from  $\times 400$ .

Pretreatment with NCS (1%) greatly decreased inflammation, as documented by fewer lymphocytes and exudates as well as a decrease in the number of mast cells to  $2 \pm 2$  in 3 preparations ( $p = 0.045$ ) and the extent of degranulation (less than 20%) (fig. 5, D).

### Duration of the Inhibitory Effect of NCS

To investigate the duration of the NCS effect rats were treated with NCS (1%), followed by P/LPS as described and then placed in metabolic cages. Urine was collected every day for 4 days over dry ice and stored frozen for histamine assay. On day 4 the rats were sacrificed and the bladders were processed for bladder explants, as before. There was no increase in urine histamine under any condition on any of the 4 days that it was collected (results not shown). TNF- $\alpha$  release from bladder explants was not detectable in the 3 controls, while it was  $0.34 \pm 0.27$  pg/mg after P/LPS in 3 rats and below the detection level in rats treated with NCS (1%).

There was no apparent toxic effect of NCS (1%) on the histology of the normal rat bladder up to 1 week following intravesical administration (results not shown).

### DISCUSSION

Our results show that short intravesical treatment with NCS (1%) significantly decreased P/LPS induced urine histamine, bladder explant TNF- $\alpha$  and bladder inflammation, including mast cell accumulation and degranulation. A threshold inhibitory concentration of NCS was found to be 1%. Interestingly TNF- $\alpha$  release from bladder explants was inhibited whether NCS (1%) was added before, together or after P/LPS, although this was not true for urine histamine. These results suggest that NCS can inhibit histamine release only immediately following pretreatment but it can inhibit delayed TNF- $\alpha$  release even if given after P/LPS.

NCS (1%) added to normal bladders did not appear to alter bladder histology at up to 4 days. Our findings are supported by recent reports showing that NCS (1%) could inhibit TNF- $\alpha$  expression in a model of allergic contact dermatitis.<sup>10</sup> NCS (1%) has also been shown to inhibit metalloproteinases and promote healing.<sup>11,17</sup>

Our results may be relevant to IC. P/LPS is increasingly used to induce inflammatory cystitis and this animal model is commonly used to investigate bladder pathogenetic processes that may be relevant to IC.<sup>12,13</sup> For instance, it was used to show that LPS induced cystitis involves mast cells and neuropeptides<sup>18</sup> as well as test the effect of a novel inflammatory peptide.<sup>16</sup> More recently the same model was used to show that the immunomodulatory molecule IPD (suplatast tosilate) could decrease experimental cystitis.<sup>15</sup>

The current results do not provide information on the direct target(s) of NCS. Urothelial cells are activated in IC and they would be quite susceptible to intravesical NCS treatment.<sup>4,19</sup> Another target could be bladder mast cells, which are increased and activated in IC.<sup>8</sup> Mast cells are necessary for allergic reactions, during which they secrete vasoactive and nociceptive molecules as well as numerous cytokines.<sup>20</sup> Mast cells are also involved in innate and acquired immunity as well as in neuro-inflammatory conditions affected by stress.<sup>20</sup>

The prevalence of IC was recently shown to be 464/100,000 in the 40 to 59-year age group in Austria<sup>2</sup> and 575/100,000 in an office setting in the United States.<sup>3</sup> Symptoms are quite debilitating and they severely affect quality of life but, nevertheless, current oral and intravesical treatments for IC are not curative.<sup>1,5,6</sup> Intravesical NCS may be used together with other modalities at least in patients with IC who have documented bladder inflammation with or without mastocytosis.

## CONCLUSIONS

Intravesical administration of NCS (1%) significantly decreased P/LPS induced rat total urine histamine and bladder explant TNF- $\alpha$  release. NCS also decreased bladder mast cell infiltration and degranulation. As such, we believe that intravesical instillation of NCS may in the future provide an alternative or additive treatment for IC.

### Abbreviations and Acronyms

IC	=	interstitial cystitis
LPS	=	lipopolysaccharide
NCS	=	nanocrystalline silver
P	=	protamine sulfate
PBS	=	phosphate buffered saline
TNF	=	tumor necrosis factor

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