

Original Article

Role of tiny nanoparticles in the development of interstitial cystitis in an animal model

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Abstract: The etiology of interstitial cystitis/painful bladder syndrome (IC/PBS) is unknown. Some unknown infectious organism may play a role. We previously detected tiny nanoparticles (NPs) in the bladder of IC/PBS. This study was to examine whether NPs could invade the superficial facet cells of the urinary mucosal epithelium and create an IC/PBS phenotype. NPs were isolated and cultured from bladder biopsies from IC/PBS patients. Eight-week-old female mice were inoculated with 0.2 ml NPs suspension directly into the bladder by transurethral catheterization. Mice were euthanized 5 weeks after instillation. Pathology, mast cells, substance P and gene expression of inflammatory mediators were measured in the bladder. Bladder ultrastructures were observed. For functional analysis, the 24-h micturition frequency and total voided urine of mice were tested. NPs instillation resulted in cystitis with increase in the ratio of bladder to body weight, histological damage, mast cell proliferation, substance P, and bladder-specific elevated gene expression of inflammatory cytokines. NPs in the bladder of nanobacteria-instilled mice were observed by transmission electron microscopy and scanning electron microscopy. Evaluation of 24-h micturition habits of NPs mice showed significantly increased micturition frequency and significantly decreased urine output per micturition when compared with control mice. Therefore, the results in the present study indicate that a systemic inoculation of NPs may have a crucial impact on inducing cystitis mimicking many of the clinical and histopathological characteristics of human IC/PBS, and NPs may be one of important etiological factors for IC/PBS.

Keywords: Interstitial cystitis, nanoparticle, bladder pain, inflammation

Introduction

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a chronic inflammatory disease characterized by nonspecific urinary symptoms of urgency, frequency and nocturnal accompanied by severe bladder pain [1]. To date, no effective clinical or research approaches used for investigating the activity of IC/PBS have been available because of its unknown etiology and lack of well-characterized animal models [1, 2]. Current evidences both from clinical and laboratory studies have confirmed several potential pathophysiologic causes for IC/PBS including bacterial or viral infections, neurogenic inflammation, immunologic factors, alteration of the glycosaminoglycan layer from superficial cells, toxic substances, and activation of mast cells [1-3]. Although ordinary bacteria could not be isolated and cultured from IC/PBS patients,

multiple lines of evidence have suggested that IC/PBS may be caused by some unknown infectious organisms [4, 5]. Recently, nanometer scale bacteria-like organisms (referred to as nanobacteria) have been detected in bladder biopsies and urine samples from some patients with IC/PBS [6-8].

The term 'nanobacteria' as very small bacteria-like organisms were a recently discovered infectious agent with an outlook as nanometer-scale particles from 0.1 to 0.5 μm in size [9]. Under an electron microscope, they look like typical bacteria, and even resemble cells undergoing division and self-replicate [10]. Although, those tiny nanoparticles (NPs) called nanobacteria don't seem to fit scientists' criteria for life, one thing is clear that they're very widespread, occurring in a number of disorders such as osteoarthritis, polycystic kidney disease, aortic

valve calcification, peripheral neuropathy, placental calcification, and so on [11]. Therefore, they have been called NPs and regarded as an infectious cause of a wide variety of diseases, from kidney stones to atherosclerosis—a prospect which now should be tested with the new NPs. The objective of the present study is to elucidate whether NPs are one cause of some cases of IC/PBS, based on whether or not they can invade the superficial facet cells of the urinary mucosal epithelium mimicking many of the clinical and histopathological characteristics of human IC/PBS.

Materials and methods

Generation and preparation of NPs

As previously described [6-8], NPs were isolated and cultured from bladder biopsies obtained by cold cup technique from IC/PBS patients. Briefly, after being oscillated, diluted, filtrated (using pinhole filter, 0.45 and 0.22 μm , Millex; Millipore Carrigtwohill, Cork, Ireland), and centrifugated, samples were routinely cultured in flasks containing serum-free RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) medium at 37°C (pH 7.4) in 5% CO_2 /95% air. After 5 weeks of inoculation, cultures were harvested by 30-min centrifugation at 20,000 and resuspended in 10 ml PBS (pH 7.2) to prepare NPs suspension under the same ionic condition as normal saline (1 McFarland U) for further experiments.

Mice infections with NPs

All animal protocols were conducted following the National Institutes of Health guidelines for housing and care of laboratory animals and performed in compliance with institutional regulations after pertinent review and approval by the Animal Studies Committee at the Third Military Medical University. Female Sprague-Dawley mice (200-230 g) were obtained from Harlan Sprague-Dawley Inc. By referring to Hannan's chronic and recurrent urinary tract infection [12], the mouse model of chronic cystitis was built with NPs. Briefly, 7-8 week old female mice were inoculated with 0.2 ml NPs suspension directly into the bladder by transurethral catheterization. Normal saline were infused instead as controls. All mice were raised under the same conditions and were euthanized by asphyxiation with carbon dioxide

(CO_2) followed by cervical dislocation 5 weeks after NPs infusion.

Histological staining

Bladders (ten mice in each group) were harvested, weighed, fixed in 10% buffered formaldehyde for 24-48 h, and then embedded in paraffin. The paraffin blocks were cut into 3- μm thick pieces and then stained with hematoxylin and eosin.

Acidified toluidine blue staining

To visualize mast cells, tissue sections were deparaffinized, rehydrated, and then stained with acidified toluidine blue by following the described protocol [13].

Bladder ultrastructural study

For transmission electron microscopy (TEM), fresh bladder tissue was cut into 1-2 μm pieces and fixed overnight in 2.5% glutaraldehyde. They were embedded on a membrane coated copper screen and stained with 3% phosphotungstic acid for 1-2 min before being viewed on a Tecnai 10 transmission electron microscope (Philips, Eindhoven, The Netherlands) with an 80 kV working voltage. For scanning electron microscopy (SEM), fresh bladder tissue was cut into small blocks and analyzed as described in reference [14]. Briefly, tissue was fixed for 1 hour with 2.5% glutaraldehyde and dehydrated in an ethanol gradient, followed by cryodesiccation and metal plating. Samples were observed under a KYKY-EM3200 scanning electron microscope (KYKY Technology Development, Beijing, People's Republic of China) using an accelerating voltage of 30 kV.

Mediator assay

Total RNA was extracted from bladder tissues, converted to cDNA, and analyzed for gene expression by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using the following primer pairs: tumor necrosis factor (TNF) α , sense, CAAAGGGAG-AGTGGTCAGGT, antisense, ATTGCACC TCAGG-GAAGAGT; interleukin (IL) 1 β , sense, GAGTGTG-GATCCC AAGCAAT, antisense, AGACAGGCTTG-TGCTCTGCT; iNOS, TTGGGTCTTGTTAGCCTAGT C, antisense, TGTGCAGTCCCAGTGAGGAAC; β -actin, sense, GGTCATCACTA TTGGCAACG, antisense, ACGGATGTCAACGTCACT.

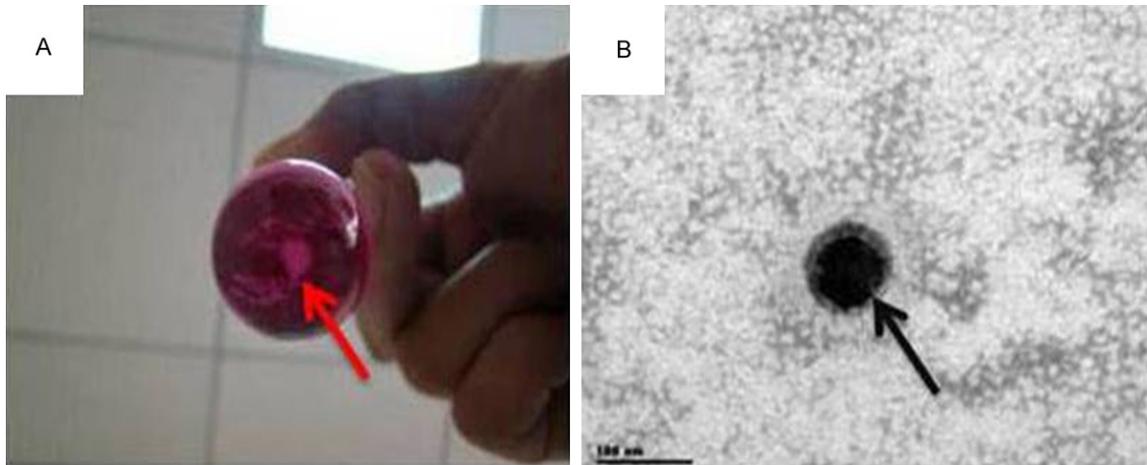


Figure 1. Characterization of NPs cultured in vitro. A. Positive culture showing white granular sediments firmly adherent to the bottom of flask (red arrow) after 4-6 weeks incubation. B. TEM showed that the cultured microbes were spheroid and about 80 nm in size with an apparently thick cellular wall (black arrow), which are homoplastic features to those of NPs described in previous studies.

Micturition behavior observation by urinary frequency-volume assessment

The 24-h micturition and drinking habits of mice were tested as described previously [15]. At 5 weeks after injection, the bladder function in mice was studied with 24-h micturition habits with 12 h of light and 12 h of dark cycles by using metabolic cages. Urine volumes and urinary frequency in the light and dark periods were recorded consistently. In order to prevent faeces from interfering with measurement of urine output, mice were firstly received a residue-free diet (Lactaid brand whole milk, lactose-free) for 24 h and then the liquid diet. In the whole test period, mice had free access to the liquid diet and light-dark cycle times were recorded. A mouse micturition chamber used to measure urinary output in real time was custom built by Medical Associates Inc. (St. Albans, VT, USA). The key feature of the micturition chamber was a wire mesh bottom, which was designed to collect urine droplets unobstructedly. Directly below the bottom opening was a balance, of which the data port was connected to a data acquisition system. The collection changes of weight were recorded at a sampling speed of four times/s (Origin 7.5, OriginLab Corporation, Northampton, MA, USA). The whole system (chamber and balance) was installed in a Plexiglas outer casing, which served to decrease evaporation and the effect of air draft.

Measurement of substance P (SP) using immunohistochemistry staining

After deep anesthesia, the rats were killed and bladders were removed, fixed in 4% paraformaldehyde, and then embedded in paraffin. Bladder blocks were cut into 3- μ m thick pieces for immunohistochemical staining, which was performed according to the instruction of the SP-9001 Histostain TM-Plus kits (Zhongshan Golden Bridge Biotechnology, China). Primary antibodies used were rabbit-anti-rat SP (Zhongshan Golden Bridge Biotechnology).

Statistical analysis

Results are presented in the form of mean \pm standard error. Statistical analysis was performed with the unpaired student t test or with a one-way analysis of variance with the Tukey post hoc test. Data were processed using the SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). Significance was accepted at a *P* value of less than 0.05.

Results

Characterization of NPs cultured in vitro

NPs were isolated from IC/PBS bladder tissues with growth of white granular sediments in cultures (**Figure 1A**), which existed as spheroids with approximately 100-200 nm in size exhibited by TEM (**Figure 1B**).

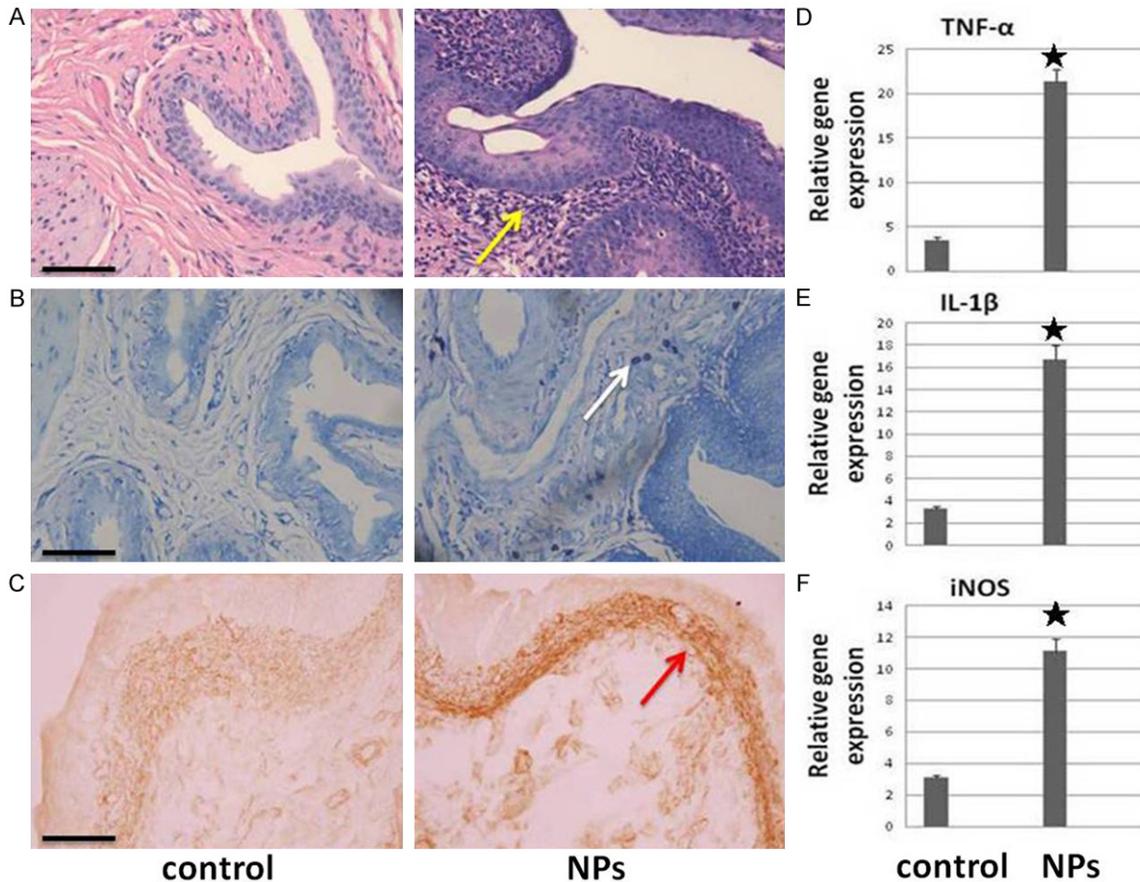


Figure 2. Bladder-specific molecular and immunocytochemical analysis in mice 5 weeks after instillation with NPs. A. HE in group of NPs-instilled mice showed extensive perivascular leukocytic infiltration within submucosa (yellow arrow) not evident in control mice. B. Acidified toluidine blue staining showed a predominance of mast cells in the bladder infiltrates of mice instilled with NPs (white arrow) not evident in control group. C. Immunostaining with SP antibody showed a predominance of the SP-containing nerve fibres in bladder infiltrates of mice instilled with NPs (red arrow), but no evidence of SP-containing nerve fibres in control group. Solid bar = 100 μ m for all tissue sections. D-F. Quantitative reverse transcription analysis showed significantly elevated gene expression levels of TNF- α , IL-1 β , and iNOS in the bladder of mice instilled with NPs compared with tissues taken from control mice (* P < 0.005 for all). Relative quantitation of gene expression was determined as the ratio of individual gene expression to β -actin in individual tissue by the method of comparative threshold cycle.

Bladder specific inflammatory response in mice instilled with NPs

Histological analysis of bladder tissue 5 weeks after instillation with NPs showed extensive cystitis with significantly leukocytic infiltration within submucosa (**Figure 2A**, right) that was not shown in any bladder sections taken from control mice instilled with controls (**Figure 2A**, left). Acidified toluidine blue staining showed that the bladder-infiltrating cells were predominantly mast cells (**Figure 2B**, right) and that such mast cell infiltration was few shown in bladders from control mice (**Figure 2B**, left). SP, a neuropeptide transmitter, has been shown to

be responsible for the supra-pubic pain appearing during the IC/PBS disorder. In this study, the SP-containing nerve fibres in NPs-instilled mice were denser than in controls in the submucosa (**Figure 2C**). RT-PCR analysis revealed a significant (P < 0.001 for all) mRNA level upshift of the inflammatory cytokines TNF- α , IL-1 β , and iNOS in the bladder tissue of NPs instilled mice compared with the control tissue (**Figure 2D-F**).

Bladder ultrastructural observations in mice instilled with NPs

TEM observations of control mice revealed normal bladder submucosal structures with regu-

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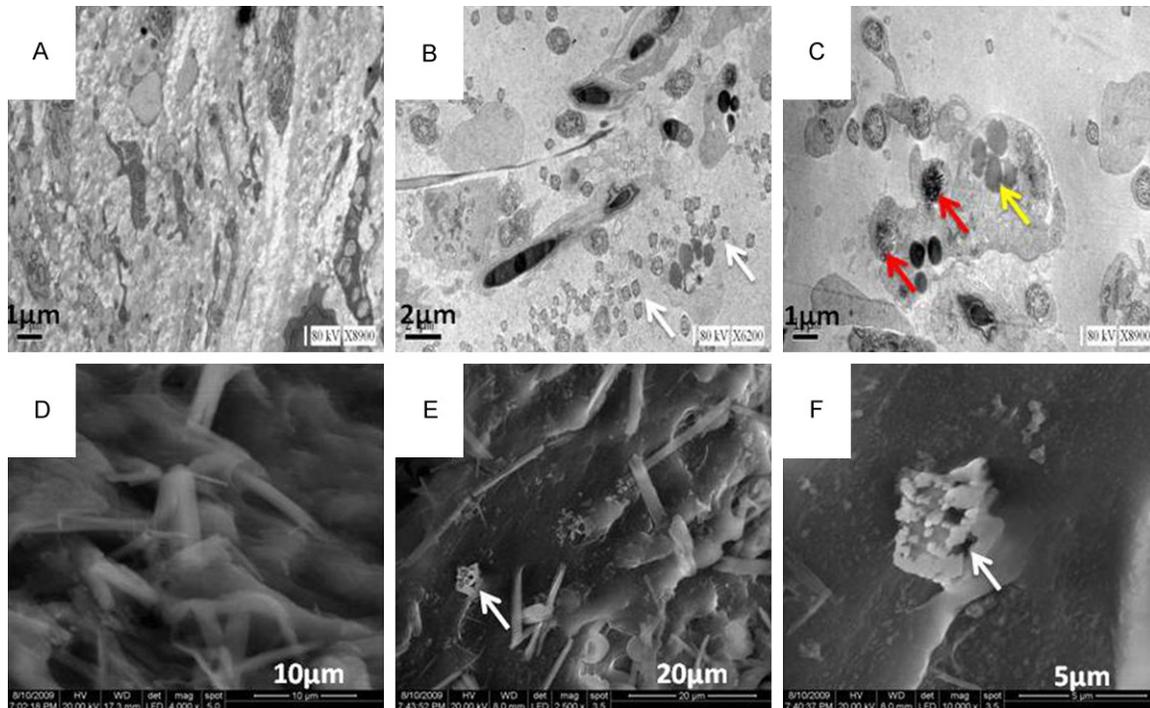


Figure 3. Bladder ultrastructural observations in mice 5 weeks after instillation with NPs. A-C. TEM revealed the features and distribution of NPs. A. Well-formed cell junctions with no NPs were observed in controls. B. Bladders from mice instilled with NPs showed a loose submucosal structure and many globoid or racket-shaped particles 50 to 200 nm in size in cytoplasm (white arrows). C. Collagen-containing particles of digested NPs at different infection stages were observed in the cytoplasm of fibroblasts (red arrows). Some collagen fibrils were present in the cytoplasm space (yellow arrows). D-F. SEM showed bladder ultrastructural structures. D. Well-formed cell junctions were observed and cell surface was smooth with no globoid or racket-shaped particles in control. E, F. Cell surface was disordered and particulate NPs clustered at cell surface with the size of 50 to 200 nm in diameter were found (white arrows).

larly arranged and well-formed cell junctions (**Figure 3A**). In contrast, a distinct loosening of the submucosal structure of the urinary bladders from mice instilled with NPs was clearly displayed. Many globoid or racket-shaped particles in cytoplasm were only observed in this group (**Figure 3B**), but not present in any of the controls, indicating that the particles were nanobacteria. Moreover, numerous particles of digested NPs with remodeled collagen fibrils were present in the cytoplasm of the cells (**Figure 3C**). By using SEM, particulates, altered architecture and significant globoid NPs were observed on the detrusor smooth muscle cell surface from mice instilled with NPs (**Figure 3E, 3F**) and that such particulate or globoid NPs were not shown in bladders from control mice (**Figure 3D**).

IC/PBS phenotype in mice instilled with NPs

Functional analysis indicated that 5 weeks after instillation with NPs, mice had significant-

ly increased urinary frequencies (62.33 ± 3.445 vs. 38.33 ± 4.967 ; $P < 0.05$; **Figure 4C**) and significantly reduced mean urine outputs per void (0.34 ± 0.051 vs. 0.63 ± 0.043 ; $P < 0.05$; **Figure 4D**). In addition, NPs-instilled mice reported significantly increased ratios of bladder weight (in milligrams) to body weight (in grams) (**Figure 4A**), a measure of organ inflammation and damage (0.67 ± 0.057 vs. 0.52 ± 0.041 ; $P < 0.003$; **Figure 4B**). **Figure 5** show the urinary frequency volume chart results of individual mice instilled with NPs and controls, respectively.

Discussion

IC/PBS is a chronic inflammation of the submucosal and muscular layers of the bladder [16, 17]. In the past 20 years, at least 16 animal models have been developed to help identify underlying possible pathophysiological mechanisms for IC/PBS, including occult infection, neurogenic, autoimmune, vascular or lymphatic

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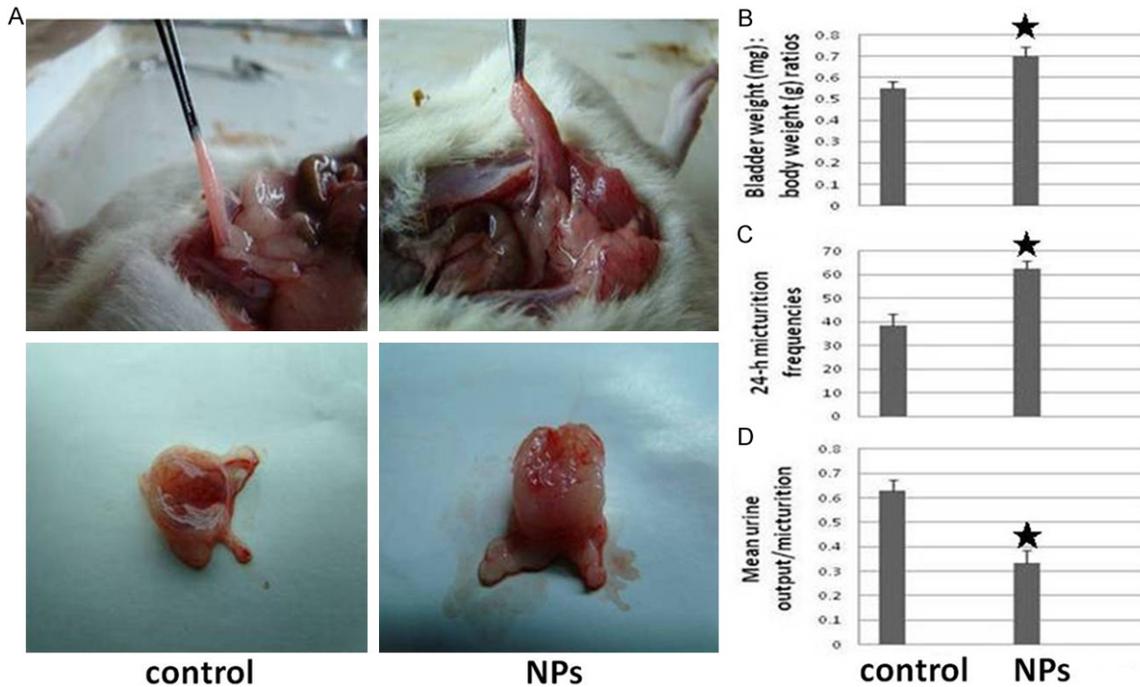


Figure 4. Increased bladder weight and bladder dysfunction in mice 5 weeks after instillation with NPs. A. Compared with control mice (left panel), NPs induced bladder inflammation characterized by vasodilation, petechial hemorrhage and edema (right panel). In addition, the bladders of mice that received instillation of NPs were significantly greater than those of mice instilled with wild-type fetuin-A. B. Bladder weight (milligrams) to body weight (g) ratios were significantly higher ($*P < 0.05$) in mice instilled with NPs compared with control mice. C. 24-hour micturition frequencies were significantly higher ($*P < 0.05$) 5 weeks after instillation with NPs compared to control mice. D. Inversely, mean urine output/micturition was significantly lower ($*P < 0.05$) in mice instillation with NPs compared to control mice. All values represent the mean plus or minus the standard error.

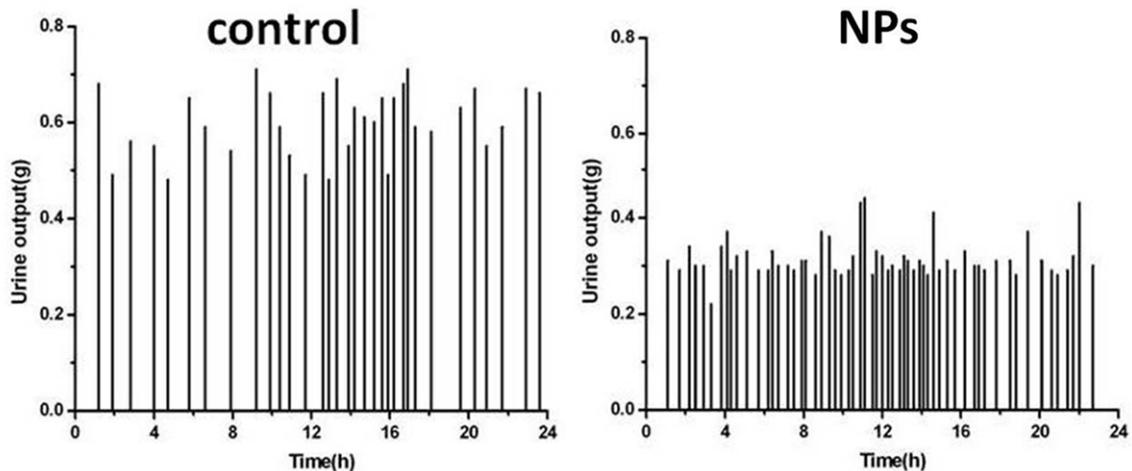


Figure 5. Representative the urine production and frequency from mice in control group (upper panel) and group instilled with NPs (lower panel). Each vertical line demonstrates a micturition event, and the height of each vertical line indicates the urine production measured in grams.

disorders; mast cell activation; damage to the glycosaminoglycan layer; and the occurrence of toxic substances in the urine [15-18]. However,

there was no adequate animal model that could manifest the three major symptoms of IC/PBS, including increased urinary frequency, decrea-

sed urine outputs per void and chronic pelvic pain.

NPs, new agents of emerging infectious diseases, are mainly discharged from the body in urine [19]. In the earlier reports, NPs were detected and cultured from IC/PBS bladder tissues and urine samples [14-16], which encouraged us to develop a more targeted NPs attack directed against bladder tissue. In this study, we infused the NPs suspension into the female mouse bladder in retrograde fashion, and pathological changes of the mouse resulting from NPs infection in the bladder tissue were observed. Phenotypical characterization of our current model showed that 5 weeks after instillation with NPs, mice did exhibit increased urination frequencies, decreased urine outputs per void (urgency), which reproduced two of three of the major symptoms of IC/PBS. In addition, NPs-instilled mice reported significantly increased ratios of bladder weight to body weight, which is a reliable indicator of organ inflammation and damage. These results indicated that NPs-instilled mice induced a functional and histological disorder characterized by inflammation confined to the bladder that consistently leads to bladder dysfunction similar to that observed in the patients with IC/PBS. In controls, however, no significant pathological changes were observed.

Our data also indicated that the bladder dysfunction occurring in NPs-induced cystitis was due to inflammatory cell infiltration of the bladder tissue. We verified bladder inflammation by revealing increased expression of proinflammatory cytokines and the presence of clusters of mast cells in the bladder following acidified toluidine blue staining, all of which have been as a central role in the pathogenesis and pathophysiology of IC/PBS [20]. Besides those, adequate evidences have demonstrated the presence of neurological lesions in patients with IC/PBS [21]. SP, as a neuropeptide transmitter, contributes to the pathophysiology of pain and inflammation, and is responsible for the suprapubic pain appearing during the IC/PBS disorder. We presented evidence of pelvic pain by revealing upregulated expression of SP in the submucosa (not in the detrusor) the same as the report [22], reproducing the other symptoms of the major three of IC.

In order to better understand the course of NPs invading the superficial facet cells of the urinary mucosal epithelium, bladder ultrastructural study was performed. A loose submucosal structure and many globoid or racket-shaped particles 50 to 200 nm in size in cytoplasm under TEM. Cell surface was disordered and particulate nanobacteria clustered at cell surface with the size of 50 to 200 nm in diameter were found under SEM. These illustrations further proved that human NPs did invade the superficial facet cells of the urinary mucosal epithelium.

To rule out of the possibility of false positives of human NPs cultured from bladder samples, two efforts were made in this study. First, all experimental operations and testing instruments were under strict aseptic conditions. Second, the supernatants of human NPs cultures were filtered with a 0.22- μ m minipore filter, which could remove the common bacteria, fungi, and mycoplasma. However, our experimental study also has several limitations. First, changes in the quality of the cultures or formulations of viable NPs colonies in each flask were not controlled, which might have contributed to the variability in responses among animals. Second, the action of NPs with no cholesterol in the diet is unknown. Third, it must be kept in mind that our conclusions derived from an animal model of cystitis might not be identical to what occurs in humans; therefore, further studies must be confirmed by clinical studies.

In summary, the data in the present study revealed that a cystitis mimicking many of the clinical and histopathological characteristics of IC/PBS was generated in the NPs-induced mouse. NPs may be one of important etiological factors for IC/PBS.

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Disclosure of conflict of interest

None.

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