

Decreased nanobacteria levels and symptoms of nanobacteria-associated interstitial cystitis/painful bladder syndrome after tetracycline treatment

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Abstract

Introduction and hypothesis This study was designed to detect whether nanobacteria (NB) reside in urine and bladder tissue samples of patients with interstitial cystitis/painful bladder syndrome (IC/PBS) and whether antibiotic therapy targeting these organisms is effective in reducing NB levels and IC/PBS symptoms.

Methods Twenty-seven IC/PBS patients underwent cystoscopy. Bladder biopsies and urine samples were obtained and cultured for NB, which were identified by indirect immunofluorescent staining and transmission electron microscopy.

Results Eleven bladder samples showed growth of microbes that were identified to be similar to NB. Homologous study of the 16S ribosomal RNA gene suggested that the NB could be the pathogen. For enrolled 11 patients, NB levels decreased dramatically after tetracycline treatment, and they reported significant reduction in the severity of IC/PBS symptoms.

Conclusions A high prevalence of NB was observed in female IC/PBS, and anti-NB treatment effectively improved the symptoms, which suggest that NB may cause some cases of IC/PBS.

Keywords Bladder biopsies · Interstitial cystitis · Nanobacteria · Painful bladder syndrome

Introduction

Interstitial cystitis/painful bladder syndrome (IC/PBS) is chronic suprapubic/bladder disorders of unknown etiology and exhibits inflammation in bladder tissues [1]. It is a challenging and frustrating problem that affects 10% to 15% of all women [2]. Knowledge of the basic mechanisms causing IC/PBS is fragmentary. Although the hypothesis of some undiscovered infectious agents as the etiology of IC/PBS is controversial, a number of lines of evidence suggest that IC/PBS may be caused by some infectious organisms, and some studies have shown that patients with IC/PBS have a higher prevalence of various organisms in the urine than those without IC/PBS [3, 4].

Nanobacteria (NB) are a newly discovered infectious agent of 100–500 nm in size with a 16S ribosomal RNA (rRNA) gene sequence and slow growth and a doubling time of about 3 days [5]. They are fastidious and difficult to be cultured and detected with standard microbiologic methods [6–9]. In vivo, NB are found to be voided mainly through the urinary system, and they have been isolated within the genitourinary tract, including polycystic kidney disease, renal calculi, and chronic prostatitis [10]. Furthermore, some IC/PBS patients had a marked reduction in the severity of IC/PBS symptoms after doxycycline treatment [11, 12], which may inhibit the growth of NB as well as providing a conventional anti-inflammatory effect [13]. Therefore, this study was designed to detect whether NB reside in the bladder walls of patients with IC/PBS and

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whether antibiotic therapy targeting these organisms is effective in reducing NB growth and IC/PBS symptoms.

Material and methods

Subjects

Twenty-seven consecutive females fulfilling the criteria of the National Institutes of Health, National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK), for IC/PBS [14] were investigated from April 2005 to November 2008. All patients had a history taken and a clinical examination performed. They had been diagnosed more than 1 year previously and had not been treated with either intravesical or oral tetracycline or had any anti-infection therapy within the preceding 1 month. Patients were excluded if they had an abnormal physical examination; could not swallow oral medications, follow instructions or participate for 3 months; or had known renal disease. All patients gave their written informed consent to participate in the investigation, including undergoing cystoscopic examination under anesthesia, and the study was approved by the local Institutional Review Board of our department.

Sample collection

At the beginning of the trial, the 27 patients received a cystoscopy under anesthesia. Urine specimens were obtained via the cystoscope. Bladder biopsies were obtained by cold cup technique from three sites near glomerulations or Hunner's ulcers [1]. The procedures of sample collections were under strict aseptic condition. Parts of the urine cultured on Tryptic Soy Agar plates and M4 medium were used to detect whether subjects had bacterial infection, *Chlamydia*, *Ureaplasma urealyticum*, and *Mycoplasma hominis*, and any patients positive for these organisms were excluded from further study. The remaining urine samples were used for NB culture.

NB culture

We used the culture techniques described by Ciftcioglu and Kajander [6–8]. After pretreatment with oscillation, dilution, filtration (pinhole filter, 0.45 and 0.22 μm , Millex; Millipore Carrigtwohill, Cork, Ireland), and centrifugation, the samples were cultured in flasks containing RPMI 1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) with 10% gamma-irradiated fetal bovine serum (Sigma Chemical, St. Louis, MO, USA) and were kept at 37°C (pH 7.4) in 5% $\text{CO}_2/95\%$ air. Sterile normal saline was cultured as the negative control.

NB staining and identification

After 3 to 6 weeks of culture, white granular sediments in the nutritive medium were harvested by centrifugation at 20,000 g for 45 min at 4°C, washed with phosphate-buffered saline (PBS), and used for further study. Smears were made and blocked with normal goat serum, incubated with 1:10 dilution of mouse monoclonal antibody 8D10 against NB (IgG1 subclass, NanoBac Oy, Kuopio, Finland) overnight then incubated with a 1:500 dilution of tetramethyl rhodamine isothiocyanate labeled goat antimouse IgG (Chemicon, Temecula, CA, USA) for 1 h. Diamidino phenylindole was used to label the nucleus. PBS was used to replace 8D10 antibodies as the negative control. A Zeiss LSM 510 meta confocal laser scanning microscope (CLSM, Carl Zeiss, Jena, Germany) was used for immunofluorescent staining (IIFS)-CLSM.

For transition electron microscopy (TEM), the bacterial sediments were placed on 200 mesh copper grids, negatively stained with 2% phosphotungstic acid for 30 s and then subjected to TEM.

Homologous study

The method for the homologous study was as described in previous study of NB in chronic pelvic pain syndrome [9]. Genomic DNA was isolated from NB cultures using the FastDNA spin kit (BIO101, USA). The 16S gene sequences of NB were amplified using the following primers: (a) 5'-AACGAACGCTGGCGGCAGGC-3' and (b) 5'-CACCC-CAGTCGCTGACCC-3'. After purification with a gel extraction kit (Viogene, Taiwan), the amplified fragment was ligated into pGEM-T vector systems (Promega, Madison, WI, USA) for cloning into ECOS competent cells (Yeastern Biotech, Taipai, Taiwan). The inserted fragment in the recombination plasmid was sequenced using the primers TAF (5'-CAAGGCGAT-TAAGTTGGGTA-3') and TAR (5'-GGAATTGTGAGC-GATAACA-3') and then compared with its homologous genes using the Genebank database and the Basic Local Alignment Search Tool. Criteria for identification [15] are as follows: A >99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate to the species level; a 97% to 99% identity in 16S rRNA gene sequence was the criterion used to identify an organism at the genus level; and <97% identity in 16S rRNA gene sequence was the criterion used to define potentially new bacterial species.

Treatments and assessments

To improve compliance, a combination of intravesical and oral tetracycline (500 mg/day, 3 months) [16] was given to

Table 1 Microbiological results

| Urine samples (<i>n</i> =27) | Positive for NB in bladder tissues (<i>n</i> =13) | Negative for NB in bladder tissues (<i>n</i> =14) |
|-------------------------------|--|--|
| Gram positive cocci | 0 (13) | 0 (14) |
| Gram negative rods | 1 (13) | 1 (14) |
| <i>Chlamydia</i> | 0 (13) | 0 (14) |
| <i>Ureaplasma urealyticum</i> | 0 (13) | 2 (14) |
| <i>Mycoplasma hominis</i> | 1 ^a (13) | 0 (14) |
| Positive for NB | 7 ^a (13) | 0 (14) |

NB nanobacteria

^a One patient with positive for both *Mycoplasma hominis* and NB in urine samples

those patients with cultures positive for NB. Intravesical instillations were performed twice a week, totally six times. An 8-Fr catheter was used for the intravesical instillations. After the catheter was placed in the urethra, the bladder was drained, and a solution of tetracycline (500 mg mixed with 30 ml sterile normal buffered saline) was instilled, retained for a minimum of 30 min to a maximum of 60 min, and voided. The patients initially negative for NB but positive for other microorganisms were treated according to the results of the antibiotic susceptibility test.

The O'Leary–Sant Symptom Index, Problem Index (OSPI, including its components: ICSI and ICPI) and the Pain, Urgency, Frequency Symptom Scale (PUF) were used at the beginning and the end of the trial to assess any changes in symptoms [17] for patients with positive NB cultures. All enrolled patients received a follow-up and presented for a posttreatment evaluation, as requested. Patients who were subjectively free of symptoms after treatment and remained so were considered cured. Those reporting less frequency, urgency, dysuria, or suprapubic

discomfort after treatment were considered improved. Failure was defined as no subjective improvement in symptoms.

Statistical analysis

All data are expressed as mean±SD. The significance of differences between sample means and between rates was determined using Student's *t* test and chi-square test, respectively. Data were processed using the SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). A *P* value of less than 0.05 was considered significant.

Results

Enrolled patients and their characteristics

As shown in Table 1, of the 27 patients, 13 had bladder tissue with growth of white granular sediments in NB cultures; of

Table 2 Baseline demographics and clinical characteristics of the enrolled patients

| Characteristics | Enrolled patients (<i>n</i> =11) |
|----------------------------------|-----------------------------------|
| Age (years) | 27–62 (mean 41.81, SD 10.68) |
| Duration of symptoms (months) | 13–67 (mean 30.72, SD 15.84) |
| Antibiotic allergy | Penicillin (1) |
| Presence of Hunner's ulcers | 3 |
| Uroflow volume (ml) | 101–180 (mean 137.81, SD 22.96) |
| Bladder capacity (ml) | 97–172 (mean 131.91, SD 25.12) |
| O'Leary–Sant total score (0–36) | 21–33 (mean 27.81, SD 4.28) |
| O'Leary–Sant ICSI score (0–20) | 12–18 (mean 15.18, SD 2.04) |
| O'Leary–Sant ICPI score (0–16) | 7–15 (mean 12.63, SD 2.50) |
| PUF total scores (1–35) | 18–34 (mean 25.55, SD 4.63) |
| PUF symptom scores (1–23) | 10–23 (mean 16.63, SD 3.80) |
| PUF bother scores (0–12) | 6–11 (mean 8.91, SD 1.30) |
| Pain (1–9) ^a | 5–9 (mean 6.82, SD 1.17) |
| Urgency score (1–5) ^b | 2–5 (mean 4.18, SD 0.98) |
| Urinary frequency | 12–21 (mean 16.72, SD 3.68) |

SD standard deviation, PUF Pain, Urgency, Frequency Symptom Scale

^a Score 1, no pain; score 9, severe pain

^b Score 1, no urgency; score 5, severe urgency

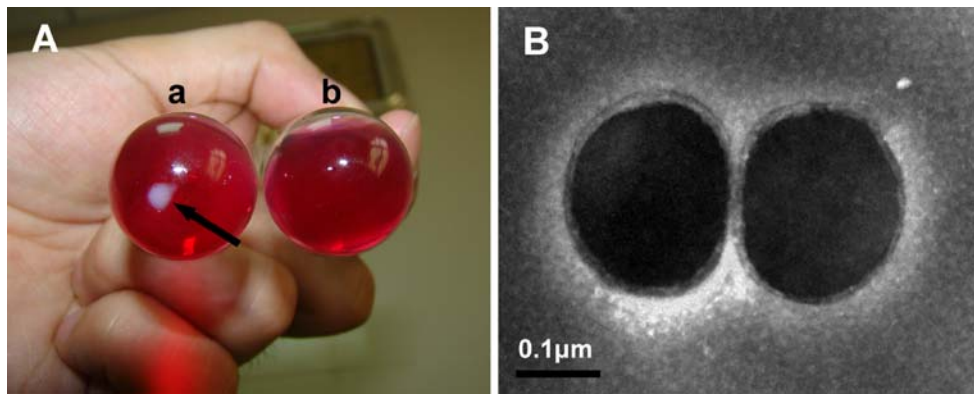


Fig. 1 Morphologic observation of NB cultured in vitro. **a** Positive culture (flask *a*) showing a mineralized biofilm (arrow), finely granulated and firmly adherent to the bottom of flask; negative culture (flask *b*). Both flasks were incubated for 3–6 weeks. **b** TEM revealed

that the cultured microbes were spheroid and 200 nm in diameter with an apparently thick cellular wall, which are similar features to those of NB described in previous studies

whom, seven also had sediments in urine NB cultures. Of the 13 patients, one also had a positive culture for gram-negative rods and one for *Mycoplasma hominis*, and they were excluded from further study. The remaining 11 subjects received tetracycline treatment. Their baseline demographics and clinical characteristics are listed in Table 2.

Morphologic observation and identification of NB

Because of NB's unusual properties [6–9], they are difficult to be stained with commonly used dyes and are best observed by TEM. In our study, after 3 to 6 weeks of culture, the 11 bladder tissues and six urine samples with pure growth of NB had white granular sediments firmly attached to the bottom of the culture flasks, and these were visible to the naked eye (Fig. 1a). The microbes isolated from these sediments were clustered and of different sizes

but all less than 500 nm when studied with IIFS-CLSM (Fig. 2). TEM revealed that the microbes were spheroid or coccobacillary, with crystals around the bacterial body (Fig. 1b). These morphologic and distinctive features are the same as those of NB described in previous studies [6–8]. The negative control showed no microbe growth. The results indicate that the microbes found in the bladder of IC/PBS patients were similar to NB.

Homologous study

NB belong to the alpha-2 subgroup of Proteobacteria based upon their 16S rRNA gene sequence [6]. In order to discuss whether the NB detected in the IC/PBS bladder tissues were the pathogen, we performed homologous study of the 16S rRNA gene. After DNA sequencing for the inserted fragment in the recombinant plasmid, the target sequence

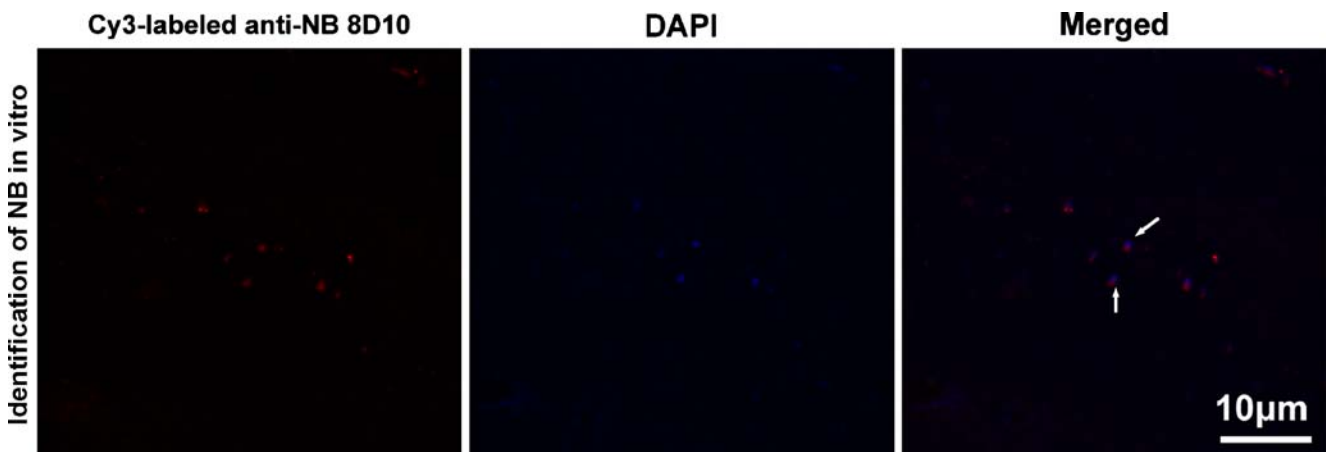


Fig. 2 Identification of the microbes cultured in vitro with IIFS-CLSM. The cultured microbes stained with Cy3-labeled anti-NB 8D10 were found clustered with different sizes (100–500 nm), which were positive NB (arrow)

Table 3 Decreasing levels of nanoparticles after anti-NB treatment

| | Before treatment | After treatment | <i>P</i> value ^a |
|---------------------------------|------------------|-----------------|-----------------------------|
| Bladder tissues (<i>n</i> =11) | 11 (11) | 3 (11) | <0.01 |
| Urine samples (<i>n</i> =11) | 6 (11) | 0 (11) | <0.01 |

^a Chi-square test.

of 1,400-bp length was obtained and compared against the Genebank database. The BLAST result showed that the observed sequence was 97% similar to the 16S rRNA gene of NB (Genebank accession number, X98419) with a score of 2,480, identity 97%, and E-value of 0. This implies that the cultured NB belong to the alpha-2 subgroup of Proteobacteria and could be the pathogen.

Decreasing levels of NB after anti-NB treatment

About 1 month after finishing tetracycline treatment, the 11 patients received a second cystoscopy under anesthesia. The same methods as above were used to obtain bladder tissues and urine samples, which were cultured for NB after pretreatment. After 6 weeks of culture, only three bladder tissues had growth of NB, and no NB were found in the urine samples (Table 3). This implies that NB infection existed in the bladder tissues of patients with IC/PBS and that tetracycline treatment effectively reduced this infection.

Evaluation of therapeutic efficacy

All 11 patients successfully completed their treatment and follow-up schedules. At the end of trial, the total OSPI scores decreased significantly from 27.82 ± 4.29 to 16.73 ± 4.50 ($P < 0.01$), and PUF total scores decreased significantly from 25.55 ± 4.63 to 16.27 ± 3.49 ($P < 0.01$), indicating IC/PBS symptom relief (Fig. 3a). After anti-NB treatment, the median follow-up was 8 months (range 6–15). Four of 11 subjects (36.35%) considered themselves cured, six (54.55%) reported subjective improvement, one (9.10%) reported no change, and no subjects had worsening symptoms (Fig. 3b). These results suggest that tetracycline therapy could significantly relieve the

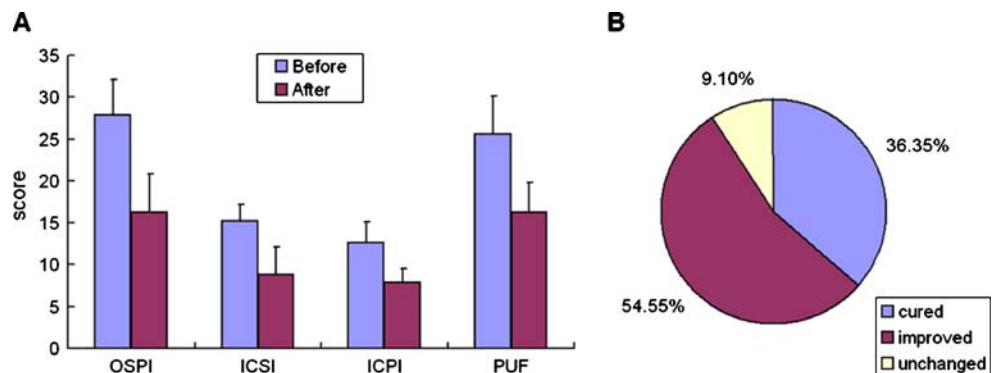
symptoms of IC/PBS and effectively improve the quality of life of IC/PBS patients.

Discussion

IC/PBS is chronic suprapubic/bladder disorders of unknown etiology and exhibits inflammation in bladder tissues [1]. Gram-negative bacteria with a 16S rRNA gene were detected by Dominique et al. in bladder tissues from IC patients using a sensitive and specific nested PCR method, and these were believed to be dormant microbes [3]. The present study used the culture techniques for NB described by Ciftcioglu and Kajander [6, 7]. Our results demonstrate that nanobacterial infection do exist in most bladder biopsies (11/27) and some urine samples (6/27) of patients with IC/PBS. IIFS-CLSM and TEM revealed the microbes to be spheroid or coccobacillary and 100–500 nm in diameter, with a black coat and crystals around the bacterial body. The presence of 16S rRNA gene in the cultured microbes was verified by amplification, cloning, and sequencing and proved to be the pathogen responsible for the symptoms of IC/PBS. These unique observed characteristics are consistent with those of other studies on NB [6–9].

NB are the smallest known self-replicating microbes. They have avoided detection and scientific study until recently. Because no nucleic acids have yet been detected, controversy remains over whether NB are living microorganisms or merely abiotic microbes [3, 18–20]. Even Raoult and Young et al. thought NB were mineral-fetuin complexes or pleomorphic mineralo-protein complexes [21, 22]; nevertheless, they could not exclude the possibility that NB are pathogenic microorganisms. Some proteinaceous

Fig. 3 **a** OSPI and PUF scores before and after anti-NB treatment. **b** Percentage of the 11 subjects cured, improved, or failure after treatment with 6 to 15 month follow-up



agents with no nucleic acids have been proved to be infectious particles, such as prions [23]. NB may be another proteinaceous infectious particle and belong to the family of prions.

There is suggestive biochemical and molecular evidence supporting an infectious nature for NB: They are able to grow and exert cytotoxic effects [24, 25], and their growth is best inhibited by tetracycline [15]. In the clinical situation, NB may initiate kidney stone formation [8]. They have been found in periodontal disease [26], calcified human valves [5], and synovial fluid [27] and have been shown to participate in the clinical pathological process of those diseases.

Although we could not determine the nature of the NB because of our presently limited data, some results of this study may provide a more plausible explanation for the unusual properties of NB. First, the NB cultured from our IC/PBS bladder tissues *in vitro* were able to grow, could be subcultured, and were identified as having the 16S rRNA gene, which implies that they could be the pathogen [6, 7]. Second, NB are self-replicating calcifying nanoparticles and are resistant to most antibiotics except tetracycline [15]. The mechanism of whereby tetracycline inhibits the growth of NB is unknown. In the present study, subjects receiving tetracycline therapy reported symptomatic improvement, and the levels of the NB decreased significantly. We therefore propose that NB may be infectious agents that participate in the clinical pathological process of IC/PBS, and they should not be excluded as a potential etiopathologic agent in IC/PBS.

Given that only a few oral antibiotics can reach the bladder and that NB are difficult to be eliminated, the subjects were treated with both oral tetracycline and intravesical instillation to increase local concentrations of the drug, in accordance with polyomavirus-associated chronic interstitial cystitis treatment guidelines [28]. In order to exclude the therapeutic efficacy of single hydrodistention, only 30 ml sterile normal buffered saline was used for intravesical treatment.

Our open, prospective, unblinded, and uncontrolled study indicated that a combination of intravesical and oral tetracycline therapy was effective in decreasing nanobacterial infection and improving IC/PBS symptoms. Intravesical and oral tetracycline treatment was well tolerated, with no observed adverse effects. After a median follow-up of 8 months (range 6–15 months), four of 11 subjects (36.35%) considered themselves cured and six (54.55%) reported subjective improvement. Only one subject reported no change. We speculate that those patients positive for NB after treatment may be insensitive to tetracycline and could potentially be treated with other antibiotics, such as doxycycline.

We cultured our urine samples for the most frequent pathogens isolated from the urogenital tract. We recom-

mend performing these tests to exclude a plethora of other pathogens potentially involved, allowing a better therapeutic approach for IC/PBS patients. In our study, five patients had negative cultures for NB but positive findings for other agents, and they reported a statistically significant improvement in symptoms after appropriate therapy (data not shown).

NB have a strong association with biomineralization [29] but, interestingly, the IC/PBS bladder tissues in our study were largely spared from calcification, presumably owing to local upregulation of counterregulatory inhibitors, such as some regulatory protein excreted by the urothelium and/or the changing urine pH [30].

Although a definite and safe method for the treatment of IC/PBS requires further identification, we suggest that attempted anti-NB treatment with intravesical and oral tetracycline is appropriate for IC/PBS patients in whom other pathogenic agents are not identified. A limitation of this study was the small sample size. Therefore, we recommend that more studies are needed to test the same measures in a larger sample size in other research centers, and further work is required to better define the character of NB and their role in IC/PBS pathogenesis. If additional evidence accumulates to show that NB cause some cases of IC/PBS, further investigation of anti-NB treatment would be warranted.

Conclusions

Despite our study's limitations, particularly the lack of a placebo control, our results imply that nanobacterial infection may account for some cases of IC/PBS. Therefore, we recommend culturing and treating NB in IC/PBS patients after excluding anatomic, neurologic, or other infectious etiologies.

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Conflicts of interest None.

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