

Nanobacteria May Be Linked to Testicular Microlithiasis in Infertility¹

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ABSTRACT

Testicular microlithiasis (TM) in infertility is an uncommon pathologic condition of unclear etiology, which is characterized by calcium deposits within the seminiferous tubules. Nanobacteria (NB), as novel microorganisms mediating tissue calcification, have been discovered in some diseases. In this study, we hypothesized that NB may participate in the pathogenesis of TM, particularly in infertility. Seventeen infertility patients with TM detected by scrotal color Doppler ultrasonography and 17 infertility patients without TM as controls were enrolled in the study. The NB were isolated and cultured from semen samples and urine samples. After 3-6 weeks of culture, 10 of 17 (58.8%) semen samples and 2 urine samples from infertile patients with TM showed the growth of white granular microbes which firmly attached to the bottom of the culture flask and were visible to the naked eyes. In the control group, only 1 of 17 (5.9%) semen samples from infertile patients without TM showed the growth of white granular microbes. The cultured microbes were identified by indirect immunofluorescent staining (IIFS), transmission electron microscope (TEM), and 16s rRNA gene expression. IIFS and TEM revealed NB to be coccoid and 100-500 nm in diameter. The BLAST result revealed that the 16s rRNA gene sequence from the cultured microbes was 97%, the same to that of the known NB with identity (97%). Our results showed that NB may be linked to the development of TM, which may provide a potential target for the diagnosis and treatment of infertility with TM.

Keywords: *infertility; nanobacteria; semen; testicular microlithiasis.*

INTRODUCTION

Testicular microlithiasis is a rare pathologic condition which is usually discovered incidentally during testicular ultrasound evaluation for other conditions, such as infertility (Aizenstein et al, 1998; Sakamoto, et al, 2006; Qublan et al, 2007). Microliths are randomly scattered throughout the seminiferous tubules of testicular parenchyma and cause infertility in some men by seminiferous tubule obstruction (Smith et al, 1999). No treatments have been found to eradicate microliths effectively. The etiology of microlithiasis and the mechanism of formation remain unclear. They have been presumed to be formed by the precipitation of degenerating intratubular cells and calcification of the glycoprotein material (Ganem et al, 1999; De Jong et al, 2004). Interestingly calcification is the mineral formed by nanobacteria (NB), newly discovered microorganisms implicated in biomineralization in the kidney (Kajander et al, 2001,) and blood vessels (Miller et al, 2004). NB have been discovered in some diseases including periodontal diseases (Ciftçioğlu et al, 2003), urolithiasis (Ciftçioğlu et al, 2005), malignant microcalcifications in breast cancer (Altundag et al, 2006), aortic valve calcification (Bratos-Pérez et al, 2008), and human arthritic synovial fluid (Tsurumoto et al, 2008).

In this prospective study, we determined whether NB participate in the pathogenesis of TM. Semen and urine samples after pretreatment were cultured according to the culture methods for NB. The acquired microbes from the culture flasks were identified by indirect immunofluorescent staining (IIFS), transmission electron microscope (TEM), and 16s rRNA gene expression.

MATERIALS AND METHODS

Subjects and Sample Collection

This study included consecutive male patients diagnosed with infertility referred to our clinic for andrologic evaluation from September 2006 to December 2008. Infertility was defined as the inability to achieve pregnancy after 12 months of contraceptive-free intercourse. All patients provided a relevant history and underwent a clinical examination. Ultrasound examination and semen analysis were done in 832 patients and testicular volume was calculated from the formula of

an ellipsoid (length × width × height × 0.52). All subjects recruited in this study were free of bacterial cystitis and epididymitis, and had no anti-infection therapy within 3 months prior to the semen analysis. Patients were excluded from study when they had a history of hemospermia, seminal vesicle tumors, testicular tumors, atrophic testes, testicular torsion, and testicular trauma. Seventeen infertile patients with TM (multiple foci < 2-3 mm in diameter in testicular parenchyma with sonography) were recruited. Seventeen healthy men with infertility without TM were enrolled as the control group. Table 1 lists the baseline demographics and clinical characteristics of the subjects. All enrolled patients were given written-form consent to the investigation and the study was approved by our Institutional Review Board.

The procedures of sample collections, including urine and semen, from 34 subjects were under strict aseptic conditions. A portion of the urine and semen specimens cultured on Tryptic-Soy Agar plates was used to detect whether subjects had bacterial infection, who, if positive, were excluded from further study. The remaining urine and semen samples were used for NB culture.

Culture of NB

According to the culture techniques of Ciftçioğlu and Kajander (Ciftçioğlu et al, 1998; Kajander et al, 1998), after semen and urine samples were pretreated with oscillation, 1:3 dilution in a sterile glass with sterile water, they were demineralized by adding 1 M HCl, which was subsequently neutralized with 1 M NaOH. The resultant was filtered (pinhole filter, 0.45µm and 0.22 µm, Millex; Millipore Carrigtwohill, Cork, Ireland), centrifuged at 20,000 g for 45 min, after which the supernatant fluids were removed and the remnant 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 kept at 37°C (pH 7.4), in 5% carbon dioxide/95% air. Sterile normal saline was cultured as the negative control.

Identification of Cultured Microbes

Smears made of isolated culture pathogenic bacteria for IIFS were air dried and fixed in methanol (-20°C) before blocking with 2% goat serum PBS. The sections were incubated in a humidified chamber for 24 hours with mouse monoclonal antibody 8D10 against NB (NanoBac Oy, Kuopio, Finland) used at 1:10 dilution, after washing in PBS, and then with a 1:500 dilution of tetramethyl rhodamine isothiocyanate (TRITC) labeled goat anti-mouse IgG (Chemicon, Temecula, CA, USA) for 1 hour. Diamidino phenylindole (DAPI) was used to label the nucleus. Phosphate-buffered saline 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 IIFS-CLSM.

For TEM, the microbes were placed on 200 mesh copper grids, negatively stained with 2% phosphotungstic acid for 20-30 seconds, and then evaluated by TEM.

16s rRNA Gene Expression

Genomic DNA was acquired from NB cultures using the FastDNA spin kit (BIO101; USA) and 16s rRNA gene sequences of NB were amplified using the following primers: A, 5'-AACGAACGCTGGCGGCAGGC-3' and B, 5'-CACCCCAGTCGCTGACCC-3'. After the amplified fragment was purified with a gel extraction kit (Viogene, Taiwan), and then ligated into pGEM-T vector systems (Promega, Madison, WI, USA) to clone in ECOS competent cells (Yeastern Biotech, Taipai, Taiwan). The inserted fragment in the recombination plasmid was sequenced using the primers, TAF (5'-CAAGGCGATTAAGTTGGGTA-3') and TAR (5'-GGAATTGTGAGCGATAA CA-3'), and then compared with homologous genes according to the Genebank database using the BLAST tool.

Statistical Methods

All data are expressed as the mean \pm SD. The chi-square test and t-test were performed using the SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). A P value < 0.05 was considered significant.

RESULTS

Morphologic Characteristics of the Cultured Microbes

After 3-6 weeks of culture, we found a total of 10 semen samples from 17 infertile patients with TM had the growth of microbes, which sedimented to the bottom of the culture flask and were white granular to the naked eyes. Two urine samples from 17 infertile patients with TM and 1 semen samples from 17 infertile patients without TM also showed the growth of microbes. No microbes grew in urine samples from 17 infertile patients without TM. At the same time, the negative controls showed no microbial growth.

Identification of Cultured Microbes

NB are difficult to be stained with common dyes. Mouse monoclonal antibody 8D10 against NB has been successfully used for the detection and identification of NB (Ciftçioğlu et al, 1998; Kajander et al, 1998). In this study, IIFS-CLSM showed that the cultured microbes were clustered with different sizes and about 100-500 nm in diameter (Fig.1). TEM revealed that the microbes were spheroid with a black coat and crystals around the bacterial body (Fig.2). These distinctive features coincided with those of NB described in other studies (Ciftçioğlu et al, 1998; Kajander et al, 1998). From these results, we concluded that the cultured microbes from semen samples of infertile patients were NB.

16s rRNA Gene Expression

NB have a 16s rRNA gene sequence (European Molecular Biology Laboratory database, X98418 and X98419) and they belong to the alpha-2 subgroup of Proteobacteria based upon their 16s

rRNA gene sequence (Ciftçioğlu et al, 1998). The alpha-2 subgroup of Proteobacteria contains bacteria which are able to penetrate into eukaryotic cells. Analysis of the 16s rRNA gene sequence has been successfully used for the identification of NB and their pathogenicity (Kajander et al, 1998; Wood et al, 2006). In this study, we obtained the target sequence of 1400 bp length after DNA sequencing for the inserted fragment in the recombinant plasmid. According to 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 2480, identity 97%, and E-value of 0, which implied that the cultured microbes were NB and could be causative agents.

DISCUSSION

Calcium phosphate has associated with many diseases, but formation mechanisms remain speculative. NB are the smallest cell-walled bacteria, discovered in human and cow blood (Miller et al, 2004). NB cannot grow with standard culture methods or be detected with standard procedures (Cisar et al, 2000; Drancourt et al, 2003; Benzerara et al, 2006). In this study, the microbes were successfully cultured with cell culture serum from 58.8% semen samples of infertile patients with TM and identified as NB by using IIFS-CLSM, TEM, and 16s rRNA gene expression. IIFS-CLSM clearly showed that NB were very small, different size with 100-500 nm in diameter and mostly clustered. TEM exhibited microbes to be spheroid with a black coat and crystals around the bacterial body. The sequencing of 16s rRNA gene of cultured microbes implied that they could be causative agents. Obviously, these results indicate that NB may reside in the testicular architecture of infertile patients with TM.

Whether NB could be one cause of TM and participate in the formation of TM may be a worthy topic in need of discussion. NB are novel Gram-negative microorganisms with small sizes (100-500 nm), able to form calcium phosphate crystals at neutral pH (Miller et al, 2004). There is evidence supporting the notion that NB could be causative agents. For example, they are able to grow and exert cytotoxic effects (Hjelle et al, 2000; Ciftçioğlu et al, 2007). Now, NB have been

found in periodontal diseases (Ciftçioğlu et al, 2003; Demir et al, 2008), urolithiasis (Ciftçioğlu et al, 2005), aortic valve calcification (Bratos-Pérez et al, 2008), human arthritic synovial fluid (Tsurumoto et al, 2008), and have been proved to participate in the clinical pathological process of those diseases. In this study, we successfully isolated and cultured NB from semen samples of infertile patients with TM, which have been found to be composed of calcium phosphate and degenerating intratubular cells in the seminiferous tubules (De Jong et al, 2004). Based on the above data, we hypothesize that NB may participate in the pathogenesis of TM particularly in infertility. Apatite produced by NB may play a key role in the formation of TM by making a central calcium phosphate deposit around which other components are collected. In this study, we cannot confirm where NB came from with the presented data. *In vivo*, NB are voided mainly through the urinary system and have been isolated within the genitourinary tract, including polycystic kidney disease, renal calculi, and chronic prostatitis (Wood et al, 2006). Therefore, we infer that NB in urine may reflux into the seminiferous tubules and survive there. Further studies are needed to elucidate the potential mechanism by which NB originate in infertile patients with TM.

The role of TM in producing symptoms and disease is controversial, particularly because of the high prevalence in some asymptomatic men. However, there is a clear correlation between TM and infertility. As with seminiferous tubule calculi, the key factor in causing infertility should be obstruction, which may bring secondary inflammation, increase intraseminiferous pressure, and affect the testes blood-supply. Such a mechanism could explain why antibiotic procedures may provide improvement in sperm quality. However, therapy does not always enhance the probability of conception (Dohle et al, 2005). NB are self-propagating calcifying macromolecular complexes and resistant to most antibiotics. No other bacteria are as resistant to elimination as NB (Ciftçioğlu et al, 2003), which are the best inhibited by tetracycline (Miller et al, 2004). Therefore, conventional clinical treatments could not eradicate NB. Inflammation and development of calcification caused by NB in the area of seminiferous tubules could affect persistently sperm quality and cause infertility.

Color Doppler ultrasonography has become a valuable method for diagnosing scrotal abnormalities in the infertile population (Sakamoto, et al, 2006; Qublan et al, 2007), which increases the frequency of diagnosing TM. In infertile patients undergoing scrotal ultrasonography, the incidence of TM varies from 1.5% to 2.8% (Ganem et al, 1999; Qublan et al, 2007). In this study, 17 patients were found to have TM from 832 infertile patients (2.0%), which coincided with the above incidence. However, our results (Table 1) indicated that the incidence of epididymal cysts in the TM group was a bit higher than in the control group. We suggest that NB in the TM group may cause some cases of epididymal cysts and further study about the association between NB and epididymal cyst will be needed. Other studies have shown a known association between testicular tumors and TM (von Eckardstein et al, 2001; Ringdahl et al, 2004; Miller et al, 2007). All patients enrolled in our study were informed about monthly self-examinations. Patients also received physical examination and testicular tumor marker evaluation at 3-month intervals and scrotal ultrasonography at 6-month intervals in the follow-up period.

Although we detected NB in semen samples of infertile patients with TM in this study, a clear relationship between NB and infertility with TM could not be drawn from our data presented. Hence, more work needs to be done in this area. Specifically, anti-NB treatment for infertile patients with TM is recommended. Our hypothesis, if proven with evidence-based clinical trials, may have significant impact on the pathogenesis and treatment for those infertile patients with TM.

CONCLUSIONS

Self-replicating calcific nanometer-scale particles, similar to those described as NB from other calcific human tissues, can be cultured and identified from semen samples of infertile patients with TM. These data implicate a possible link between the presence of NB and the development of TM. However, whether NB contribute to the pathogenesis of the disease or are only innocent bystanders need to be clarified in further studies.

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FIGURE LEGENDS

Fig. 1. Identification of the microbes cultured *in vitro* with IIFS-CLSM. The cultured microbes stained with Cy3-labeled anti-NB 8D10 were clustered in different sizes (100-500 nm), which were positive for NB.

Fig. 2. Morphologic observation of the microbes cultured *in vitro* with TEM. The cultured microbes were spheroid, 100 nm in diameter, and had a few crystals around the bacterial body, which were similar to those of NB described in previous studies.

Table 1. Baseline demographics and clinical characteristics of the subjects

	the TM group (n=17)	the control group (n=17)	<i>P</i> value*
Age (years)	31.1 ± 3.6	28.1 ± 1.7	0.1
Duration of infertility (months)	30.7 ± 5.6	29.5 ± 3.7	0.14
Testicular volume† (cm ³)			
Right	9.0 ± 1.9	9.2 ± 1.8	0.32
Left	8.6 ± 1.6	8.9 ± 1.5	0.24
Abstinence before semen analysis (days)	5.9 ± 1.1	6.0 ± 1.0	0.34
Semen volume (mL)	3.0 ± 0.29	3.1 ± 0.23	0.24
Sperm count (million/mL)	39.2 ± 20.6	43.5 ± 22.1	0.30
Total sperm count (million)	92.5 ± 36.7	113.8 ± 42.8	0.06
Motility (%)	29.1 ± 12.1	35.1 ± 11.2	0.08
Cryptorchidism	2	1	
Varicoceles	5	6	
Hydroceles	0	0	
Epididymal cysts	7	2	
Seminal vesicle cysts	0	0	
Chronic pelvic pain syndrome/prostatitis	3	2	
Seminal vesiculitis	0	0	
Obstructive azoospermia	0	0	

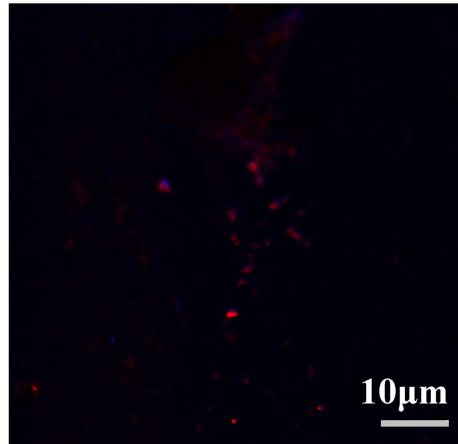
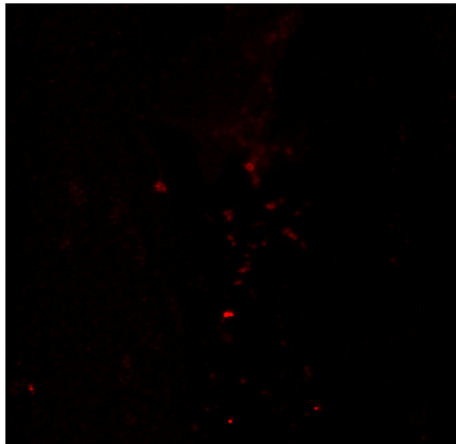
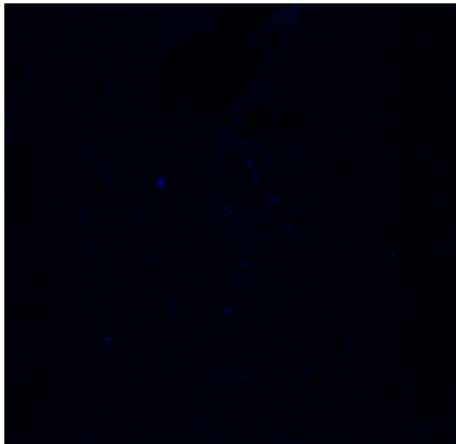
* Student's *t* test.

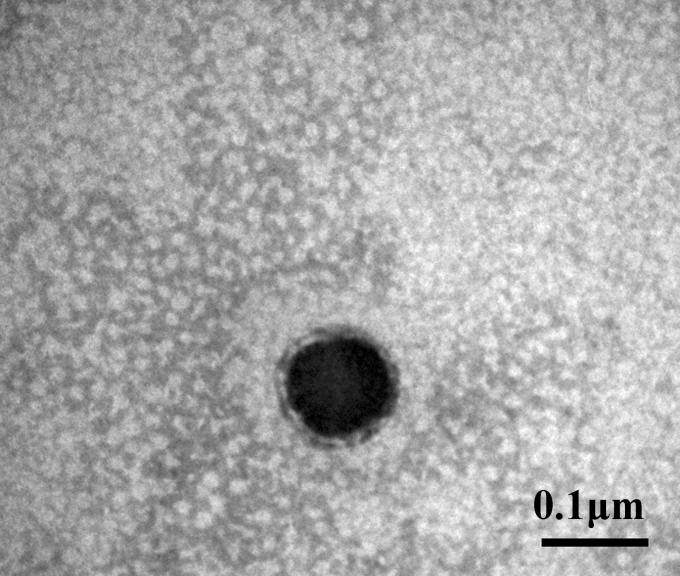
† Obtained by ultrasonography using approximation for an ellipsoid (volume= 0.52 × length × width × depth).

DAPI

Cy3-labeled anti-NB 8D10

Merged





0.1 μm