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Original Research Article

Establishment of a male Wistar rat model of nanobacteria-induced kidney stones

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Abstract

Purpose: *To establish a male Wistar rat model of nanobacteria (NB)-induced kidney stones.*
Methods: *Sixty male Wistar rats were randomly divided into control group (NC group) given caudal vein injection of saline + saline gavage, and NB-induced stone group (NBS group) given caudal vein injection of NB + saline gavage.*
Results: *Compared with NC, serum creatinine, blood uric acid, urea nitrogen and urinary calcium levels in NBS group increased between weeks 3 and 8 ($p < 0.05$). Kidney index (kidney weight/body weight ratio) in the NBS group was higher than that in NC group from weeks 8-10. At week 8, urine pH and serum phosphorus in NBS group were higher than those in NC group ($p < 0.05$). Between weeks 6 and 7, serum calcium in NBS group was higher than that in NC group ($p < 0.05$). Calcium crystals in NBS rats were distributed mostly in the distal and proximal convoluted tubules. However, no such crystals were observed in NC rats. Similarly, no such pathological changes were seen in the renal tissue of NC group. Calculus analysis showed that stone formation was higher in NBS group than in NC group ($p < 0.05$). There was no significant difference in micro-CT between the two groups ($p > 0.05$).*
Conclusion: *The successful establishment of the Wistar rat kidney stone model using NB cultured from urine of upper urinary tract stone patient is potentially useful for further etiological studies on kidney stone formation.*

Keywords: *Kidney stone, Nanobacteria, Kidney index, Urine specific gravity, Calcium crystals*

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INTRODUCTION

Kidney stone disease is a frequently-occurring disease of urology, and also a prominent social public health problem. It constitutes a heavy burden on human health and national public healthcare. In recent years, due to the development of minimally invasive technology, great progress has been made in the treatment of kidney stones, although the postoperative recurrence rate is still high. Thus, there is need for more research on the etiology of kidney stones. At present, kidney stones are difficult to clear. Studies involving animal experiments have shown that NB are associated with the formation of kidney stones [1,2]. In addition, it has been reported that ipsilateral urine from ipsilateral kidney stone patients was able to culture

nanobacteria, while the contralateral culture was negative [3]. Thus, it was suggested that NB infection and kidney stones may be related.

Nanobacteria (NB) were discovered and named by the Finnish scientist Kajander and his colleagues. With diameter between 0.1 ~ 2 μm, NB are currently the smallest living body entities known [4]. Due to the fact that NB have unique mineralization ability, they have attracted a lot of research attention all over the world. Studies have shown that NB have a close relationship with genito-urinary stones, polycystic kidney disease, polycystic liver, calcified aortic stenosis, atherosclerosis, softening spots, scleroderma, arthritis, cataracts, pineal calcification and tumors. In some people, *in vivo* organization of pathological calcification and the development of sclerosis occurred [5].

The present study was carried out to further investigate the relationship between NB and kidney stones by establishing a Wistar rat model of NB-induced kidney stones. This was with a view to providing a basis for animal experiments on the study of the etiology of kidney stones.

EXPERIMENTAL

Animals and materials

Sixty (60) SPF level male Wistar rats about 6 weeks old (mean weight = 200 ± 20 g) were purchased from the Experimental Animal Center of Xinjiang Medical University [Certificate No. SCXK (Xin) 2013-0001]. The rats were acclimatized (15 to 25 ^{o}C, 10 to 20 %) and fed for 1 week, prior to randomly dividing them into two groups: normal control group (NC group, n = 30) and NB-induced stone group (NBS group, n = 30). The basal diet was purchased from Experimental Animal Center of Shihezi University. The main instruments and equipment used, and their makers (in brackets) were: electronic balance (LT1000B, Jiangsu Changshu City Large Instrument Limited Liability Company), automatic Biochemical Analyzer (Modual DPP, Germany Roche); multifunctional optical microscope and image acquisition system (BX40, Japan Olympus); Infrared Spectroscopy Analyzer (LIIR-20, Tianjin Lan Mode); transmission electron microscope (JEOL-1230 Electronics, Japan JEOL), and microcomputer tomography technology (Microcomputed Tomography, Micro CT) (Skyscan1176, Belgium Antwerpen).

The research was approved by the Animal Ethical Committee of Tongji Hospital (approval no. 20171233) and perform according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [6].

Culture and identification of nanobacteria

Patients with upper urinary tract calculi with no previous medication or surgical history were selected from Department of Urology, The First Affiliated Hospital of Shihezi University. Mid-stream morning urine samples (5-mL) were collected from the patients after careful cleaning of the urethral meatus. The urine was diluted double its volume with normal saline and centrifuged for 10 min at 3000 rpm. The supernatant was discarded; 1 mL of the sediment was thoroughly mixed, filtered through 0.45 um and 0.22 um filter paper, and cultured with 3 ml DMEM (high glucose) and FBS (heat-inactivated, American Gibco Company) at 37 ℃ under 5 % CO_2 atmosphere. The culture medium was changed once every 28 - 30 days. Then, 0.9 % NaCl was used (as negative control) to repeat the above procedure. The growth curve was observed and recorded every week; contaminated specimens were discarded, and samples were transferred to EP tubes.

The EP tubes were centrifuged at 12 000 rpm for 30 min, and the supernatant was discarded. White precipitates were observed at the bottom of the EP tubes. Then, 1.5 mL of PBS was added to each EP tube, followed by thorough mixing. These steps were carried out in triplicate. Using a microplate reader, the absorbance of NB was standardized to a value of 1.00 at 650 nm. The bacterial suspension concentration was 25 McFarland [7]. An inverted microscope was used to study the morphological features of the NB. Similarly, calcium alizarin red staining and electron microscopic study were done. Finally, a Wistar male rat kidney stone model was established using the cultured NB.

Establishment of animal model

Sixty (60) rats were randomly divided into control group (NC group) which were injected through the caudal vein with normal saline (1.2 ml), and NB-induced stone group (NBS group) which were injected through the caudal vein with 1.2 mL of NB suspension (absorbance value of 1.00). There were 30 rats in each group. The animals had free access to standard diet and tap water.

General condition of rats and determination of renal volume ratio

For 10 consecutive weeks, activities like feeding and other behaviors were observed, and body

weight was recorded. Three rats from each group were sacrificed weekly. Bilateral kidney were collected, weighed and renal volume was calculated as shown in equation 1:

$$RV\ (\%) = \frac{(KW) \times 100}{(BW)} \quad \ldots\ldots\ldots(1)$$

where RV is renal volume, KW is kidney weight and BW is body weight.

Specimen collection

Using metabolic cage method, 24-h urine was collected 24 h before the rats were sacrificed. The abdomen was opened under 10 % chloral hydrate intraperitoneal anesthesia (4 ml/kg), the kidney and inferior vena cava were identified, and 3 - 4 ml of venous blood sample was collected and sent for biochemical analysis. The bilateral kidney was excised, washed, weighed and preserved in liquid nitrogen prior to histology (H&E staining). Micro-CT scan was also performed. About 0.5 g kidney tissue was obtained using a thick needle, dried and sent for stone analysis.

Measurement of indicators

The gross morphology, as well as color and size were recorded. Crystals were observed under the anatomical microscope. The specimen were embedded, and 5 um thick cross-sectional section was cut and fixed, followed by H & E staining. The formation of the crystal and pathological changes in the kidney were observed under the light microscope. Crystal formation in kidney was graded according to standard kidney crystallization classification as described earlier [8]. Furthermore, urea, creatinine, uric acid, serum calcium, serum magnesium, phosphorus, 24-h urine volume, urine specific gravity, urinary pH, and urinary calcium were measured using Automatic Biochemical Analyzer. Micro-CT scan was used to study stone formation, and stone components were analyzed with Infrared Spectroscopy Automatic Analyzer.

Statistical analysis

The collected data are expressed as mean ± standard deviation (SD). Statistical analysis was carried out with SPSS17.0. Variance of repeated measures was used to analyze data at different time periods, while independent *t*-test was used for group comparison. Comparison between groups at the same time period was done using one-way analysis of variance (ANOVA). SNK-q test was used to compare between the two groups. Enumerated data were compared between the two groups using χ^2 test. Crystal formation was compared with rank sum test, Kruskal and Wallis test (the significant level was $\alpha = 0.05$).

RESULTS

Characteristics of culture and NB

The culture medium appeared turbid at 6 – 8 weeks, and white flocculate precipitate was visible at 8 weeks. Under the inverted microscope, NB were found to grow in clusters, with some adhering to the wall of the container. The NB were seen as solid oval particles with diameters of 100 - 300 nm under the electron microscope, and they were stained brown with alizarin red stain (Figure 1).

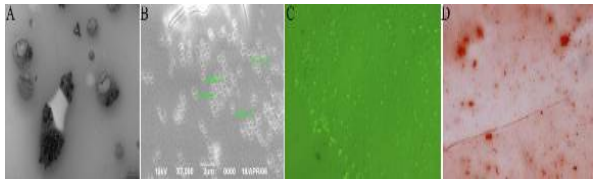

Figure 1: Characteristics of NB. A: Transmission electron microscopic image of NB (×18000), B: Scanning electron microscopic image of NB (×7000), C: Phase contrast microscopic image (×100), and D: Alizarin red staining (×200)

General condition of male Wistar rats

No rats died during the experiment. From 3 to 10 weeks, rats in the NC group ate well and were quiet and gentle, but rats in NBS group were active and restless.

Gross and microscopic features

The kidney in NC group was smooth, bright or dark red. The renal cortex was reddish brown, and the medulla was white in color. Renal papilla were visible, and renal pelvis and calyces appeared normal. No obvious crystals were observed under dissecting microscope. In the early 1 - 3 weeks, there were no significant differences between rat kidneys in the two groups. Starting from week 4, kidneys in NBS group grew larger than those in NC group, the medulla was light red, and cortico-medullary structure was intact with normal renal pelvis. After six weeks, cortico-medullary structure in majority of the NBS group rats was still clear, but few scattered light yellow particles with sand-like feel to when touched were seen in the renal tissue. Crystals were observed fanning out from the renal pelvis under a dissecting microscope (Figure 2).

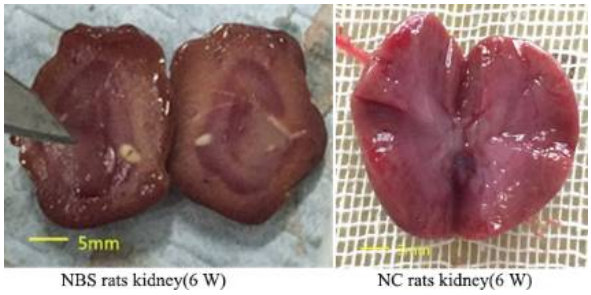

Figure 2: Cross-section of rat kidneys in both groups at 6 weeks

Blood and urine parameters

During the first 8 weeks, serum creatinine, uric acid, urea nitrogen and urinary calcium levels in the NBS group increased to varying degrees and dropped slightly at weeks 9 - 10. Compared to the NC group, the levels of serum creatinine (weeks 4 - 8), blood uric acid (weeks 3 - 8), and urea nitrogen and urine calcium (weeks 3 - 9) in the NBS group were high ($p < 0.05$). No significant differences in serum levels of creatinine, uric acid, urea nitrogen and urinary calcium in the remaining weeks were observed (Figure 3). From weeks 8 – 10, renal volume in the NBS group was higher than that in the NC group ($p < 0.05$). Week 8 urine pH, and weeks 6 - 7 serum calcium in the NBS group were higher than the corresponding values in the NC group ($p < 0.05$). Serum calcium, magnesium, phosphorus, 24-h urine volume, urine pH, kidney index, and urine specific gravity were comparable between the NBS and NC groups during the remaining weeks ($p > 0.05$).

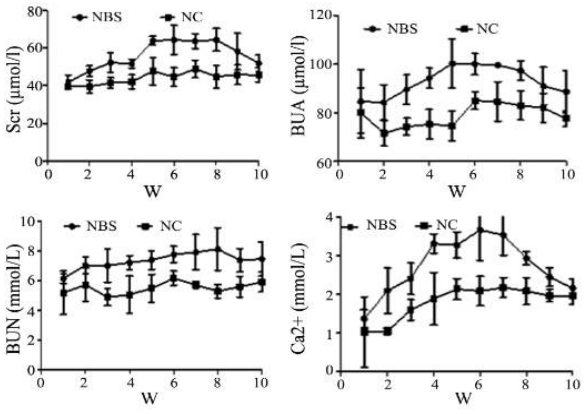

Figure 3: Serum creatinine, uric acid, blood urea nitrogen and Ca^{2+} at different time points. Values are presented as mean ± SD

Crystallization in rat kidney

In NC group, there was no crystal formation. In the first 3 weeks, there was no obvious crystallization in the NBS group, but bright crystals were observed from the fourth week. The crystals were distributed mainly in the distal and proximal tubules, and around the glomeruli. The crystals were irregular and scattered. Few crystals were connected to each other in clusters or piled together. The degree of stone formation was high in NBS group but negligible in NC group ($p < 0.05$). These results are shown in Table 1.

Table 1: Grading of crystallization in the two groups

Group	N	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	%
NBS	21	10	7	3	1	0	52.4
NC	30	30	0	0	0	0	0#

#$P < 0.05$, compared with group NBS

Pathological changes

During the establishment of the animal model, neither crystal deposition nor pathological change was observed in the NC group. From the third week, the renal tubules in NBS group showed mild dilation, with occasional tubular epithelial shedding or infiltration of renal interstitium by lymphocytes. During weeks 5 – 10, frozen renal sections from NBS group showed varying sizes of transparent crystals either as a group or dispersed into groups. Most of the crystals were deposited in tubules and appeared as irregular fragments, some in collecting ducts and in the interstitium, and occasionally in the glomerulus (Figure 4).

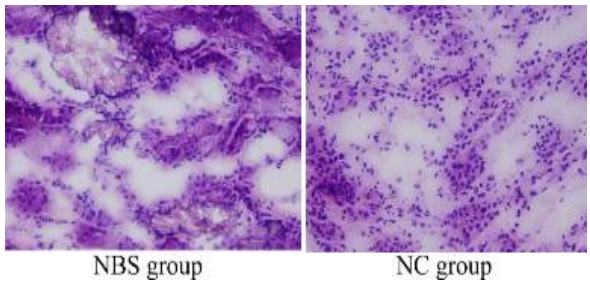

Figure 4: Frozen sections of each group (H & E, ×200)

Micro-CT scan

The NC group micro-CT scan showed normal renal contour and smooth surface with no suspected high density shadow. After week 5, the kidneys in the NBS group showed stone-like high density area. A total of 4 such cases were observed. Although there were more stone-like high density areas in the NBS group than in NC group, the difference was not statistically significant ($p > 0.05$). These results are shown in Figure 5.

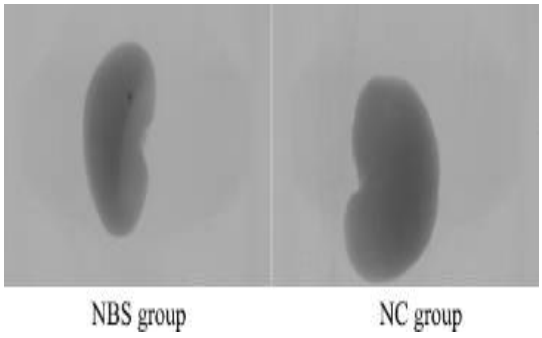

Figure 5: Micro-CT image of rat kidney in each group

Stone analysis

A single case of calcium oxalate stones (6.6 %), and 1 case of magnesium ammonium phosphate stones (3.3 %) were seen in the NC group, amounting to 6.7 % overall stone incidents. In the NBS group, there were 3 cases of calcium oxalate stones (9.9 %), 3 cases of calcium oxalate and calcium phosphate stones (9.9 %); 2 cases of calcium oxalate + magnesium ammonium phosphate stones (6.6 %); 3 cases of magnesium ammonium phosphate stones (9.9 %), 1 case of calcium phosphate stones (3.3 %), and 1 case of uric acid stones (3.3 %). These amounted to 43.3 % overall incidents of stones. Thus, stone formation was significantly higher in the NBS group than in the NC group ($p < 0.05$).

DISCUSSION

The ultrastructure of NB was discovered as cell culture contaminants by a Finland scientist Kajanderand and his colleagues [9]. Nanobacteria (NB) are gram-negative, spherical or near spherical structures with diameters of 100 - 500 nm, covered with an apatite hard shell. They are devoid of capsule or flagella but are capable of self-replication. They are the smallest known cell walled bacteria, and are difficult to visualize using conventional methods due to their ultrastructure. Thus, phase contrast microscopy, calcium staining or electron microscopy are used to study these ultra-micro bacteria [10]. They are the only known organisms that can generate hydroxyapatite in the human body, and they have been confirmed to exist in human urine, blood and different tissues and organs [11].

Nanobacteria (NB) are involved in pathological calcification [12]. Renal stone animal models have been successfully established by intravenous injection of NB [13,14]. In a preliminary experiment in which NB was cultured in the urine of patients harboring upper urinary tract calculi, there was a significantly high presence of stones [3]. These findings strongly suggest a close association between NB and stone formation. In the present study, urine samples were collected from urinary calculi patients, and NB were isolated and cultured. Stone formation was evaluated in rats injected with NB suspension after observation for 10 weeks according to the procedures described earlier [15,16].

It was found that after 5 – 6 weeks, micro-CT analysis, crystallization score and stone formation were higher in NBS group than in NC group. However, there were no significant differences in micro-CT analysis results, probably because crystallization due to NB is a slow process. Specimens in which stones were not detected by micro-CT analysis were considered as false negative. Further biochemical analysis showed that serum creatinine, blood uric acid, urea nitrogen and urinary calcium levels of NBS group from weeks 3 - 8 increased gradually and were significantly higher than those of NC group. After 9 - 10 weeks, serum creatinine, blood uric acid, urea nitrogen and urinary calcium levels in each group dropped, and no significant differences were observed between the two groups. These results indicate that NB-induced stone formation is a gradual process, and that there exists certain defense mechanisms against that pathological process.

Except for a brief period when serum calcium, phosphorus and urine pH were moderately higher in the NBS group than in NC group, serum calcium, phosphorus, magnesium and 24-h urine pH were comparable between the two groups. Thus, the increases in levels of the biochemical parameters may not be strongly related to NB infection. At weeks 8-10, the renal volume ratio of the NBS group was significantly higher than that of NC group, suggesting that stone formation in NB model is a slow process which causes mild morphological changes in the kidney.

Studies have shown that injury to renal tubular epithelial cells (HK-2 cells) may play an important role in the formation of kidney stones [17,18]. At the initial stages of crystal formation, damage to HK-2 cells may promote the formation of calcium oxalate stones. Oxalate and oxalic acid damage HK-2 cells by inducing lipid peroxidation [19,20]. Under the influence of various stimuli and interaction between macromolecules, the crystals rapidly adhere to the damaged cell membrane, thereby initiating the reaction cascade of stone formation [21].

Studies have demonstrated that HK-2 cells are impaired at the early stage of stone formation [22,23]. The primary urinary crystals passing

down the renal tubule easily adhere to the damaged epithelium, resulting in crystal accumulation and stone formation. The damage to HK-2 cells causes numerous changes at the cellular level. These include morphological changes, disruption of tight junctions and asymmetry of the phospholipid bilayer; loss of membrane polarity and damage to cellular defense mechanism [24]. These changes enhance the adhesion of crystals and the formation of kidney stones. The newly-formed stones cause mechanical damage and ischemic hypoxia which increase the risk of renal damage [25]. This was responsible for the increases in serum creatinine, blood uric acid, urea nitrogen and urinary calcium in the NBS group.

In a study in which HK-2 and NB were co-cultured [26], it was demonstrated that NB damaged the HK-2 cells, and the injury was aggravated over time, resulting in increased crystal adhesion. The crystal deposition not only causes renal tubular obstruction, but can also lead to tubular dilatation, necrosis and interstitial nephritis [27,28]. Moreover, necrotic exfoliation and cast facilitate the nucleation and growth of the crystals. The pathological changes in the renal tubules become prominent with increase in crystal formation. This is consistent with the findings in the present study.

A study has reported that injection of 99mTc-labeled NB into rabbits induced hydroxyapatite deposits which damaged the collecting duct epithelial cells, and that the damaged sites served as the nucleation surface for initiation and propagation of further crystal deposits [29]. Thus, it can be inferred that NB has renal affinity which results in tubular epithelial damage and lead to pathological calcification. Therefore, NB-induced damage promotes the adhesion and deposition of calcium crystals in the renal tubules, and it is the major determining factor in stone formation.

Micro-CT is widely used in the study of bone structure [30]. The traditional pathological technology is concerned with composition and changes in organs and tissues at the micro level, while micro-CT is a kind of imaging technology which emphasizes the description and representation of the three-dimensional space structure. Application of micro-CT in the study of renal calculi in rats provides useful and comprehensive data on reaction sites, macro stones in calyces, including their sizes and shapes, as well as stone density. NB-induced stone formation has unique biochemical nature and its mechanism needs further in-depth study. In the present study, micro-CT and stone analysis were used for the first time as *auxiliary tools* for verifying the success of renal stone model.

The pathogenesis of urolithiasis is a complex process which is yet to be fully understood, although NB has been proposed as one of the contributing factors. An NB-induced Wistar rat model of renal calculi was successfully established in this study. This model is time-consuming, when compared to the traditional ethylene glycol kidney stone model, and it generates relatively low amount of crystals [31, 32]. The rate of stone formation was low and the pathological changes were mild because the newly-formed crystals in the renal tubules did not aggregate or form a mass. Thus, most of the biochemical indices showed minimum or no changes, thereby making it difficult to establish the model. However, the mineralization process of NB mimic the normal physiological changes that lead to kidney stone formation in humans.

CONCLUSION

This study has successfully established a kidney stone model which can serve as a reliable and effective tool in basic research for investigating the etiology of kidney stones disease.

DECLARATIONS

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Conflict of interest

All authors declare no financial or non-financial competing interests.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Jihong Liu conceived and designed the study. Biao Qian, Gaurab Pokhrel, Qinzhang Wang and Jihong Liu collected and analyzed the data, while Biao Qian wrote the manuscript.

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