Research Article

Preliminary Evaluation of Nanobacteria on Crystal Retention, CaSR, and Claudin-14 Expression in HK-2 Cells

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To evaluate cell morphology, crystal adhesion, cell damage, Calcium sensitive receptor (CaSR), and Claudin protein-14 (Claudin-14) expression at different time intervals and explore the role of nanobacteria in the formation of urinary calculi. In this experiment, HK-2 cells were cocultured with nanobacteria (NB) in the absence or presence of tetracycline (Tet). Cells treated with calcium oxalate monohydrate (COM) crystals were used as a positive control of urinary stone-induced cell damage. After which, cell morphology was evaluated by hematoxylin-eosin staining in comparison to untreated HK-2 cells (negative control). Use different methods to assess cell damage, crystal adhesion, and protein expression. (The degree of cell damage, crystal adhesion, and protein expression were evaluated by various methods). It was found that the degree of cell damage observed in Tet + NB-treated cells was significantly lower than that in NB-treated cells. Lactate dehydrogenase (LDH) leakage was higher in COM-exposed than in control cells (P < 0.05). However, LDH release from both NB- and Tet + NB-treated cells was significantly lower than from COM-treated cells (P < 0.05). The relative expression of CaSR and Claudin-14 proteins was higher in NB, COM, and TET + NB cells than in control cells (P < 0.001) and was lower in Tet + NB than in NB cells (P < 0.01). And P < 0.05 means that the difference was statistically significant, and P < 0.001 means that there was a significant difference between the both things. From the cell morphology, the cell damage in the COM group was greater than that in the NB group, and the cell damage markers in the COM group and the NB group were elevated. NB caused damage to HK-2 cells by inducing lipid peroxidation, and the degree of damage was increased in processing time. The adhesion of HK-2 cells to COM crystals increased after injury and was proportional to the duration of NB coculture. NB upregulated the expression of CaSR and Claudin-14 in HK-2 cells.

1. Introduction

The occurrence of urinary calculi is a common phenomenon worldwide. The average prevalence in China is 6.5%, and the prevalence in some areas is as high as 11.6%. Urinary calculi are among the most common urological conditions in China [1]. Kidney calculi are the most common type of urinary calculi. Urolithiasis is a very complex process. In recent years, an important role of nanobacteria (NB) in the pathogenesis of urinary stones has emerged [2–4]. NB have a diameter of about 80–500 nm and are Gram-negative bacteria. Their shape is mostly spherical or rod-shaped, with a thicker cell wall. The detection rate of NB in blood, urine, and calculi in patients with kidney calculi is above 90% [5–7]. NB have a unique ability to produce hydroxyapatite minerals in the human body and deposit them in tissues and organs. Therefore, they can adhere to the epithelial cells of the collecting ducts and the papillary cells, destroy the membrane structure of the cells, and form apatite crystals as reaction centers, further inducing the formation of calculi [8].

It was reported that, after treatment with traditional extracorporeal lithotripsy, the 5-year recurrence rate in patients with urinary calculi is still as high as 41.8% [9], causing patient suffering and increasing socioeconomic costs [10]. Therefore, the elucidation of the mechanisms responsible for urinary calculi formation and the development of new treatments are
urgent needs. Tetracycline is one of the few antibiotics that can kill NB at physiological concentrations. Shoskes et al. showed that a 3-month treatment with Tet greatly alleviated the symptoms in 80% of patients [11]. However, at present, we do not know exactly how NB play their unique role in the formation of kidney calculi, let alone treatment for NB.

Hypercalcuria is another risk factor for the formation of calcium stones. It is reported that approximately 33% of patients with kidney stones develop urinary calcium reabsorption disorders. The activation of calcium-sensing receptor plays an important role in this process by upregulating, in the thick ascending limb (TAL), the downstream molecule Claudin-14, which in turn inhibits the resorption of calcium.

The purpose of this study was to investigate the mechanism of NB-induced crystal retention in renal tubular epithelial HB-2 cells. An interesting question to be addressed was whether calcium signaling pathways contribute to the development of NB-induced kidney stones. Therefore, the influence of NB on the expression of CaSR and Claudin-14 was investigated.

2. Materials and Methods

2.1. Clinical Data of Patients. Patients (n = 10) (average age 49.2 ± 14) with nephrolithiasis were enrolled for sample collection (Urine is more likely to reflect the environment of NB-induced urinary calculi, and more conducive to NB bacteria growth). They had not been treated with drugs or undergone surgery. The inclusion criteria were (1) diagnosis of upper urinary tract stones; (2) male patients; (3) no antibiotics used before surgery. I have unified the diagnosis of the selected patients with the inclusion criteria, and all patients were from the Department of Urology, the First Affiliated Hospital of Shihezi University School of Medicine. Prior written and informed consent was obtained from every patient and the study was approved by the ethics review board of the First Affiliated Hospital of Shihezi University School of Medicine.

2.2. Culture and Identification of NB. Urine samples (2 ml) were collected from patients with nephrolithiasis and diluted five times with saline under aseptic conditions. After centrifugation at 4°C for 45 min (14,000 × g), the pellet was resuspended in 2 ml saline and filtered with a 0.45 μm filter. The filtered sample was diluted 5 times with saline and centrifuged at 4°C for 45 min (14,000 × g). Next, the pellet was resuspended in 1 ml of saline and filtered with a 0.22 μm filter. Then, 1 mL of the filtered sample, containing NB, was collected and transferred to a cell culture flask containing Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, USA), 10% FBS (Hyclone, USA), and 1% HEPES (Hyclone, USA). The NB were cultured at 37°C and 5% CO2 for 5 to 7 days. Inverted phase contrast microscopy was used to monitor NB growth. NB was collected by centrifugation at 14,000 × g for 20 min at 4°C and washed three times with sterile water. The calcium-specific von Kossa staining and scanning transmission electron microscopy were used to identify NB.

2.3. Cell Culture and Treatments. HK-2 cells were obtained from Wuhan University Collection Center and cultured in 10% fetal bovine serum 1640 medium, at 37°C, 5% CO2. Cells were divided into four different subpopulations corresponding to the following treatments: control, NB, COM, and Tet + NB. Control cells were cultured with medium only. For NB exposure, HK-2 cells were cultured with an NB suspension (OD = 0.7) and culture medium. COM cells (positive control) were cultured with a 5 mmol/L suspension of calcium oxalate monohydrate crystals (COM) (Sigma, USA) and culture medium. Tet (tetracycline) + NB cells were treated with 5 mg/L Tet and an NB suspension (OD = 0.7). The cells were cultured for 6 h, 12 h, and 24 h.

2.4. HE Staining. After treatments, slides in 12-well plates were removed, rinsed 3 times with PBS, and fixed with 95% alcohol for 20 min. After washing with PBS 3 times, the cells were stained with Harris hematoxylin for 1 min and then rinsed with tap water for 2 min. Then, it was differentiated for 5 s in 1% hydrochloric acid and stained with eosin for 5 min. Finally, after gradient alcohol dehydration and xylene transparency, a neutral resin was used to seal the slides and cell morphology was observed under an optical microscope.

2.5. Detection of H2O2, MDA, LDH, Na+/K+ ATPase, and Ca2+/Mg2+ATPase Activity. After treatments, the cell supernatants were collected and the content of H2O2, malondialdehyde (MDA), and LDH were measured according to the instructions provided in the relevant kits (H2O2/MDA/LDH assay kits, Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

The activities of Na+/K+ ATPase and Ca2+/Mg2+ATPase were measured by an ATPase kit (colorimetric method; Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

2.6. Laser Confocal Microscopy for Detection of Crystal Retention. HK-2 cells were seeded in a 24-well plate containing clean coverslips. After 6, 12, and 24 h of cocultivation, a COM suspension was added at a final concentration of 200 mg/L per well and gently shaken for 5–10 s, so as to allow for proper contact between the COM crystals and the bottom cells. After 3 min, the liquid above the cover glass was moved and the coverslips rinsed three times with saturated sodium oxalate solution. The coverslips were removed from the wells, fixed in 4% paraformaldehyde for 20 min, washed in PBS twice for 5 min, and shaken in a rocker. Then, 50 μL of 5 mg/L phalloidin-FITC were added and incubation was performed in 20°C in the dark for 20 min. After 2 PBS rinses, 5 min each, the slides were observed with an LSM800 microscope (Carl Zeiss, Germany) with 488 nm and 633 nm excitation light, followed by image acquisition.

2.7. Observation of Cell Crystal Retention. Cells were seeded in 24-well plates containing clean coverslips in RPMI 1640 medium supplemented with PBS and EGF and then treated
with NB or COM in medium. After 6, 12, and 24 h of treatment, the COM suspension was added to each well (200 mg/L) and incubations continued for 3 min. Then cells were rinsed 3 times with a saturated calcium oxalate solution. After fixation with 4% paraformaldehyde for 20 min and incubation with phalloidin-FITC for 20 min, the coverslips were examined with a laser scanning confocal microscope with 488 nm and 633 nm excitation light to evaluate the crystal retention by cells.

2.8. The Detection of CaSR and Claudin-14 mRNA Expression in HK-2 Cells by Quantitative Real-Time PCR. HK-2 cells were seeded in 6-well plates, and total RNA was extracted by Trizol method after coculture for 6, 12, and 24 h, and the purity and concentration were determined. The extracted RNA was reverse-transcribed into cDNA and then subjected to CaSR and Claudin-14 and PCR detection of the GAPDH gene. The reaction conditions were set according to the kit instructions. The relative expression levels of the genes of CaSR and Claudin-14 were obtained by GAPDH correction. The primer sequences are shown in Table 1.

2.9. Western Blotting. Lysates were prepared from the treated cells using a RIPA lysis buffer. The protein samples were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. Next, 5% nonfat dried milk was used to block the nonspecific binding sites at 20°C. Subsequently, the membrane was incubated with a primary antibody overnight at 4°C. After four 5-min washes with TBST, the membrane was incubated with the secondary antibody conjugated with horseradish peroxidase for 2 h at 20°C. The signals were captured using an ECL advanced system. The bands were quantified through Quantity One software and normalized to GAPDH.

3. Statistical Analysis

SPSS 24.0 statistical software (SPSS Inc., Chicago, IL) was used for statistical analysis. Measured data were expressed as mean ± standard deviation (SD). One-way ANOVA was used to compare the effects of the different treatments, followed by LSD-t test. One-way ANOVA was used for quantitative comparison between multiple groups, and the S-N-K method was used for pairwise comparison. The test level α = 0.05, and the statistical graph was drawn using GraphPad Prism 6.

4. Results

4.1. Impact of Treatments on Cell Morphology. HE staining was performed to evaluate the morphology of HK-2 cells after the different treatments. HE staining results are shown in Figure 1. The control cells exhibited a uniform morphology, clear nuclei, dense cytoplasm, and no obvious abnormalities. The number of COM-treated cells was significantly reduced, and the nuclei were loose. Some of the nuclear membranes were dissolved and the nucleoli were not visible. After 6 h of coculture with NB, some cells exhibited enlarged cell bodies, loose nuclei, and blurred nuclear membranes. Finally, most of the cells that had been exposed to Tet + NB were morphologically regular, dense, and presented clear nuclei. Some cells showed cell body swelling and a loose nucleus.

4.2. ATPase Activity, LDH, H2O2, and MDA in the Medium of HK-2 Cells. As shown in Table 2, after 12 h and 24 h of incubation in the presence of NB and COM, the activities of Na+/K+ ATPase and Ca2+/Mg2+ ATPase in HK-2 cells were significantly lower than in the control cells (P < 0.05). Notably, both enzyme activities were significantly higher in the Tet + NB-treated cells, compared to the NB-treated cells, at all time points (P < 0.05).

As shown in Table 3, at each time point, the LDH release from the COM-exposed cells was significantly higher than from control cells (P < 0.05). The LDH released from the NB- and Tet + NB-exposed cells was lower than from the COM-treated cells (P < 0.05). After 12 h and 24 h, the content of H2O2 and MDA in the extracellular medium of NB- and COM-treated cells was significantly higher than in that of the control cells at the corresponding time points (P < 0.05). On the other hand, the release of H2O2 and MDA from the Tet + NB-treated cells was significantly lower than from the NB-treated cells (P < 0.05).

4.3. Assessment of Crystal Adhesion by Laser Scanning Confocal Microscopy. To observe the adhesion of crystals to cells, a confocal microscope was used. As shown in Figure 2, after coculture for 6 hours, a small amount of crystal adhesion was observed in the NB group and the COM group, and no crystal adhesion was observed in the blank group. After 12 h of coculture, the crystal adhesion of the NB group and COM group increased significantly compared with 6 h, and there was no significant change in the blank group. After 24 h of coculture, the crystal adhesion of the NB group and COM group was further increased than that of 12 h, and there was no significant change in the blank group. At all time points, Tet + NB treated crystal adhesion was significantly reduced compared to NB treated cells.

4.4. The Expression of CaSR and Claudin-14 in HK-2 Cells. qRT-PCR and western blotting assays were employed to detect CaSR and Claudin-14 in HK-2 cells. The relative levels of mRNA are shown in Figure 3. At 6, 12, and 24 h, the treatment with both NB and COM resulted in a significant increase in CaSR and Claudin-14 mRNAs as compared to control cells (P < 0.001). As shown in Figure 4, Western blotting confirmed a remarkably higher level of both proteins under the treatment with both NB and COM (24; P < 0.001). Both protein levels were lower in the TET + NB-treated than in the NB-treated cells (P < 0.01).

5. Discussion

The etiology of urinary calculi is very complex, involving genetic, environmental, and dietary factors, among others. It has been reported that calcium-containing stones account...
for the majority of urinary calculi, and about 33% of patients have abnormal urinary calcium metabolism [12]. In recent years, a damaging effect of calcium oxalate monohydrate (COM) on animal renal tubular epithelial cells has been reported [13, 14]. For example, Li et al. [15] have found that after the addition of 5 mmol/L calcium oxalate monohydrate (COM) crystals to cultured renal tubular epithelial cells from Wistar rats, a large amount of cell-adhered crystals could be observed. (# therefore, in this study, calcium oxalate monohydrate (COM) treatment (5 mmol/L) was used as a positive control. Nanobacteria (NB) are commonly found in human blood, urine, tissues, and organs and form a calcium phosphate shell.

The results of this study are consistent with previous reports [16], demonstrating that calcium oxalate monohydrate (COM) crystals cause more damage to cell membranes than other groups. HE staining showed that the degree of the cell damage caused by Nanobacteria (NB) was higher than that associated with cell exposure to TET + NB. Although, in HK-2 cells, as assessed by transmission electron microscopy and by the detection of cell damage markers. After cell injury, the degree of crystal adhesion was much higher in HK-2 cells cocultured with NB than in the Tet + NB-treated cells. Tet prevents the Nanobacteria (NB) effects on this process, suggesting that the dominant role in cell damage and subsequent crystal

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Table 1: Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’TATGCCTCCTCCAGCAGACT3’</th>
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<tr>
<td>CaSR</td>
<td>5’TTCAGCGGAAATACTCGAT3’</td>
<td>122</td>
</tr>
<tr>
<td>Claudin-14</td>
<td>5’CTCCTGAAAGGGCTCTGGA3’</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>5’AGGCAGGAGATGACCATGAG3’</td>
<td>132</td>
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<tr>
<td>GAPDH</td>
<td>5’ACGGCAAAGTACACGCGCACG3’</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>5’CGACATCTCAGCACCAGCATC3’</td>
<td>129</td>
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Table 2: Comparison of Na+/K+ATP and Ca2+/Mg2+ATPase activities in HK-2 cells after the different treatments (n = 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>6h Na+/K+ATP</th>
<th>12h Na+/K+ATP</th>
<th>24h Na+/K+ATP</th>
<th>6h Ca2+/Mg2+ATP</th>
<th>12h Ca2+/Mg2+ATP</th>
<th>24h Ca2+/Mg2+ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.74 ± 3.50</td>
<td>12.88 ± 1.80</td>
<td>3.40 ± 1.43</td>
<td>9.91 ± 2.80</td>
<td>11.84 ± 2.38</td>
<td>6.26 ± 1.26</td>
</tr>
<tr>
<td>NB</td>
<td>9.73 ± 1.90</td>
<td>6.56 ± 1.59*</td>
<td>0.60 ± 0.40*</td>
<td>8.22 ± 2.52</td>
<td>3.78 ± 0.78*</td>
<td>1.45 ± 1.50*</td>
</tr>
<tr>
<td>COM</td>
<td>7.16 ± 2.03*</td>
<td>2.01 ± 1.07*</td>
<td>0.45 ± 0.30*</td>
<td>5.25 ± 1.86</td>
<td>2.48 ± 1.61*</td>
<td>0.25 ± 0.17*</td>
</tr>
<tr>
<td>TET + NB</td>
<td>11.37 ± 2.08</td>
<td>10.45 ± 2.04*</td>
<td>2.85 ± 0.60*</td>
<td>7.81 ± 2.86</td>
<td>7.31 ± 2.70*</td>
<td>3.85 ± 0.75*</td>
</tr>
</tbody>
</table>

Note. *Compared with control cells, P < 0.05. #Compared with NB-treated cells, P < 0.05.

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Figure 1: HE staining of HK-2 cells after the different treatments (200x).
<table>
<thead>
<tr>
<th>Groups</th>
<th>H$_2$O$_2$</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>MDA</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>LDH</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
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<tbody>
<tr>
<td>Control</td>
<td>17.85 ± 6.11</td>
<td></td>
<td></td>
<td></td>
<td>8.31 ± 3.33</td>
<td>7.73 ± 0.27</td>
<td>8.21 ± 0.29</td>
<td>7.99 ± 0.31</td>
<td>972.01 ± 207.51</td>
<td>597.40 ± 124.4</td>
<td>884.50 ± 210.23</td>
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</tr>
<tr>
<td>NB</td>
<td>20.86 ± 3.25</td>
<td></td>
<td></td>
<td></td>
<td>17.30 ± 2.49*</td>
<td>22.13 ± 5.70*</td>
<td>18.40 ± 0.73*</td>
<td>16.47 ± 2.28*</td>
<td>25.70 ± 1.47*</td>
<td>1363.00 ± 441.30</td>
<td>1124.35 ± 292.84</td>
<td>1418.79 ± 264.00</td>
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<tr>
<td>COM</td>
<td>15.60 ± 2.39</td>
<td></td>
<td></td>
<td></td>
<td>17.88 ± 3.27*</td>
<td>20.60 ± 3.40*</td>
<td>25.46 ± 4.29*</td>
<td>29.50 ± 5.72*</td>
<td>61.74 ± 13.50*</td>
<td>1581.23 ± 279.00*</td>
<td>2392.08 ± 734.78*</td>
<td>2009.19 ± 754.95*</td>
</tr>
<tr>
<td>TET + NB</td>
<td>13.59 ± 3.54</td>
<td></td>
<td></td>
<td></td>
<td>10.72 ± 1.79*</td>
<td>10.81 ± 1.38*</td>
<td>8.46 ± 2.09*</td>
<td>9.27 ± 1.36*</td>
<td>9.41 ± 2.30*</td>
<td>713.36 ± 388.01*</td>
<td>738.81 ± 410.49</td>
<td>809.98 ± 196.22</td>
</tr>
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</table>

Note. *Compared with control cells, *P* < 0.05. #Compared with NB-treated cells, *P* < 0.05.
adhesion may be played by the bacterial body itself rather than the calcium phosphate shell.

Cell damage is a key factor in the formation of stones, and the exposure of basement membranes can attract crystal adhesion. Necrotic cell debris can further exacerbate the formation of stones, leading to the development of kidney stones [17, 18]. It has been reported that calcium oxalate monohydrate (COM) crystals cause damage renal tubular epithelial cells by inducing lipid peroxidation reactions that generate large amounts of reactive oxygen species [16, 19]. Thamilselvan et al. [20] observed that the extracellular concentration of LDH and MDA was significantly increased after culturing LLC-PK1 cells with 500 mg/L calcium oxalate monohydrate (COM) crystals for 4 h. However, antioxidants within a certain concentration range can effectively inhibit lipid peroxidation [20], thus reducing the damage to cells caused by reactive oxygen species. In this study, the release of H$_2$O$_2$ and MDA was increased after cell exposure to COM and NB. On the other hand, the release of malondialdehyde (MDA) and H$_2$O$_2$ was not significantly increased by Nanograde hydroxyapatite (nHAP) treatment and was lower in the Tet + NB-treated than in the NB-treated cells. Thus, lipid peroxidation may be one of the mechanisms by which NB damage HK-2 cells and Tet inhibited this process.

Tet is one of the few antibacterial agents that can kill NB at physiological concentrations. It penetrates the calcium crust of NB and thus exerts its antibacterial action. The efficacy of Tet in the treatment of NB-associated chronic prostatitis and interstitial cystitis [20] has been reported. Hu Weiguo et al. [3] established a rat kidney stone model by tail vein injection of NB and found that Tet gavage reduced, in 24 h, urinary Lactate dehydrogenase (LDH) and the number of renal tubular crystals, suggesting an inhibiting effect on the formation of kidney stones. To date, however, there is still no evidence of such an effect from in vitro experiments.

In this study, in vitro coculturing NB and HK-2 cells revealed that Tet inhibited NB-induced injury to HK-2 cells and reduced crystal retention after injury. These results further confirmed the effectiveness of Tet in the prevention and treatment of kidney stones and emphasized its potential relevance for both diagnosis and treatment of nephrolithiasis in clinical practice.

Calcium sensitive receptor (CaSR), a member of the G protein-coupled receptor family, commonly expressed in organs such as the parathyroid, gastrointestinal tract, and kidney, plays an important role in the regulation of calcium secretion in the kidney [21]. The paracellular calcium reabsorption in TAL is tightly regulated by Calcium sensitive receptors (CaSR) in response to circulating calcium levels [22]. Increases in urinary calcium levels as a consequence of hypercalciuria can activate Calcium sensitive receptors (CaSR) to inhibit calcium-sensitive potassium channels and reduce calcium reabsorption [23]. Calcium-sensitive receptor (CaSR) overexpression increases the sensitivity to extracellular calcium and disrupts calcium metabolism, eventually resulting in the formation of kidney stones. Calcium sensitive receptor (CaSR) and Claudin protein-14 (Claudin-14).

A recent large cohort study of patients with kidney stones has identified Claudin protein-14 (Claudin-14) as a major risk gene for hypercalciuria stones through genome-wide association analysis [24]. Gong et al. [25] demonstrated that calcium sensitive receptor (CaSR) regulates the expression of Claudin protein-14 (Claudin-14) in TAL through microRNA (miR-9 and miR-374)-mediated gene silencing. Claudin protein-14 (Claudin-14) inhibits the permeability of paracellular cation channels made of Claudin-16 and Claudin-19, which, in turn, regulate the metabolism of urinary calcium. When the level of urinary calcium increases, the Calcium sensitive receptor (CaSR)
and Claudin protein-14 (Claudin-14) axis is activated, inhibiting the reabsorption of calcium and ultimately resulting in hypercalciuria. Another study of the same group further described a CaSR-NFATc1-microRNA-Claudin protein-14 (Claudin-14) signaling pathway tightly modulating the metabolism of calcium in the kidney. Notably, the knockout of Claudin protein-14 (Claudin-14) abolished the transport of calcium induced by calcium sensitive receptor (CaSR), indicating that Claudin protein-14 (Claudin-14) is required for calcium sensitive receptor (CaSR)-regulated calcium metabolism in the kidney [26]. A previous study from our laboratory also demonstrated that calcium
sensitive receptor (CaSR) induces the formation of stones by activating Claudin-14 in a calcium oxalate rat model [27]. Enhanced activity of calcium sensitive receptor (CaSR) and Claudin protein-14 (Claudin-14) regulatory channels may play an important role in the formation of calculi by nanobacteria [28].

However, to date, the relationship between NB and the CaSR-Claudin-14 axis has not been explored. In this study, HK-2 cells were cocultured with NB or exposed to calcium oxalate monohydrate (COM) crystals, revealing that both the mRNA and protein expression of CaSR and Claudin-14 were significantly upregulated under both conditions, compared to control cells, indicating that the CaSR-Claudin-14 axis may be involved in the process of NB-induced crystal adhesion in HK-2 cells.

In summary, NB may damage HK-2 cells by inducing lipid oxidation. The extent of damage and crystal retention is proportional to the duration of NB action. NB may lead to the formation of kidney stones through the following mechanism: after the damage of tubular epithelial cells by NB infection, crystals adhere to the exposed basement membrane. The CaSR-Claudin-14 axis may be involved in the process of crystal adhesion, which leads to stone formation in conjunction with NB and necrotic cell debris. To some extent, one experiment is to judge whether tetracycline has certain positive and negative effects on HK-2 cells on the basis of previous studies, and the results show that tetracycline can reduce the damage of NB to HK-2 cells [29].

**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CaSR</td>
<td>Calcium sensitive receptor</td>
</tr>
<tr>
<td>Claudin-14</td>
<td>Claudin protein-14</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>COM</td>
<td>calcium oxalate monohydrate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>TAL</td>
<td>thick ascending limb</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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**Data Availability**

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethical Approval**

This study was ethically approved by Medical Ethics Committee of the First Affiliated Hospital of Shihezi University Medical College (A-2019-037-01).

**Disclosure**

Gang Xu and Biao Qian and should be considered co-first authors.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors’ Contributions**

The two first authors contributed equally to this work.

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**References**


