Isolation of Nanobacteria from Egyptian Patients with Urolithiasis

¹K. Abo-EL-Sooud, ¹M.M. Hashem, ¹A. Ramadan and ²A.Q. Gab-Allaha

¹Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt

²The Holding Company for Biological Products and Vaccines Vacsera, Egypt

Abstract: Background: Recent evidence suggests a role for nanobacteria in a number of human diseases, especially renal stone formation. It has become clear that the pathophysiology of renal stone disease cannot be explained by crystallization processes alone. The crystalline components of urinary tract stones can be classified into several types: calcium oxalate, calcium phosphate, bacterial related, purine, or cystine. **Materials and Methods:** To confirm the data, we searched for nanobacteria from 8 aseptically removed upper urinary tract stones. All the samples were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% γ -irradiated fetal calf serum under cell culture conditions. Scanning electronic microscopy (SEM) images of the spherical units in the carbonate apatite kidney stones were clearly similar in size and morphology. Detection of nanobacteria was made with a Transmission Electronic Microscopy (TEM) of inoculated cells. **Results:** Chemical analysis of liquid fraction of the stones with Energy Dispersive X-Ray spectrum (EDX) revealed a simple profile of carbon, oxygen and sodium at atomic C:O:Na ratios of 42:30:0.3. SEM showed nanoparticles in four of eight stones similar to those recently described. TEM of inoculated 3T6 cell monolayers has shown transient intracytoplasmic vacuolar formations containing 200 to 300 nm particles in cell cultures.

Key words: Nanobacteria, renal stone, 3T6 cells, pathophysiology

INTRODUCTION

Nanobacteria or so called calcifying nanoparticles, are self-replicating bacteria and the smallest described bacteria to date, with dimensions of 20-200 nm in length (Kajander and Ciftcioglu, 1998). Furthermore, these organisms were found to produce a biofilm containing hydroxyl apatite or carbonate, preventing their effective staining. The stimuli for calcium salt deposition in patients with these conditions are unclear but nidi (meaning that biomineralization is taking place out of chemical equilibrium) for precipitation and crystallization are needed even under supersaturation conditions (Carson, 1998). Nanobacteria are carbonate apatite forming, cytotoxic bacteria recently discovered in human and bovine blood and blood products (Kajander *et al.*, 1997; Ciftcioglu *et al.*, 1997; Kajander and Ciftcioglu, 1998). Calcific kidney stones in humans are located on renal papillary surfaces and consist of an organic matrix and crystals of calcium oxalate and/or calcium phosphate (Khan, 1997). It has been stated that the core of 67% of calcium oxalate stones contains calcium phosphate (Abraham and Smith, 1987). Nanobacteria are thought to play an important role in extraskeletal calcifying diseases including stones formation, urolithiasis and polycystic

kidney disease (Kajander *et al.*, 2003). Nanobacteria are phylogenetically close relatives of mineral forming bacteria (Kajander *et al.*, 1997). These particles have been isolated from kidney stones and urine of patients with renal lithiasis (Ciftcioglu *et al.*, 1999), renal fluid taken from patients with polycystic kidneys. It has been speculated that nanobacteria may be the spherical deposits found in the kidneys of patients who suffer from kidney stones (Vogel, 1998; Bradbury, 1998). Two strains, one of *Nanobacterium sanguineum* and the other of *Nanobacterium* sp., were isolated from kidney stones and human and bovine sera, respectively. Phylogenetic analysis based on comparison of 16S ribosomal DNA (rDNA) sequences has placed the nanobacteria isolated from fetal calf serum into the α_2 subgroup of *Proteobacteria* (Kajander *et al.*, 1997), closely related to *Thiobacillus*, a water contaminant and *Agrobacterium* and *Rhizobium* which are plant-associated bacteria. In this study, we provide evidence that nanobacteria can act as crystallization centers (nidi) for the formation of biogenic apatite structures. The mineralization process was studied *in vitro* with one bovine isolate from commercial Fetal Bovine Serum (FBS) and with a human isolate. Present findings are of concern in medicine because nanobacterial bacteremia occurs in humans and nanobacterial nidi might

initiate pathological calcification. The aims of our study were to confirm the presence of nanoparticles in urinary tract stones by morphological evidence with Scanning Electron Microscopy (SEM) and Transmission Electronic Microscopy (TEM) of inoculated cells. Moreover, the chemical analysis was detected by with energy dispersive X-ray (EDX) spectra.

MATERIALS AND METHODS

Stones: Eight urinary tract stones were collected from male and female patients hospitalized in the Kasr El Aini, Cairo University, Egypt. Stones were demineralized in 1M HCl and then neutralized (Folk, 1993), centrifuged at 14,000 X g for 15 min and the pellets used for Immuno-fluorescence Staining (IIFS) and Transmission Electronic Microscopy (TEM). Part of the pellets were suspended in Dulbecco-Vogt's modification of Eagle's medium DMEM, sterile-filtered and cultured in (DMEM) supplemented with γ -FBS (Sera-Lab, Crawley Down, Sussex, U.K.) under nanobacterial culture conditions. One fragment of each stone was preserved for culture analysis and the other fragment was used to determine its chemical structure by with Energy Dispersive X-ray (EDX) spectra (Blijenberg *et al.*, 1997). Hydroxyapatite (No-H-0252; Sigma) was used as a reference. Hydroxyl and carbonate groups in the apatite minerals were detected (National Research Institute, Dokki-Egypt) following the standard method. Chemical analysis of demineralized stone was carried out as described previously by Hyvonen *et al.* (1986).

Nanobacterial culture: The cultures were prepared using strict aseptic techniques in a cell culture facility. Nanobacterial samples were filtered through 0.2 mm filters before culturing. Subcultures were made using γ -FBS as a culture supplement. FBS and nanobacteria were γ -irradiated, when indicated; at a minimum dose of 30 kGy given at room temperature during about 16 h. Subculturing of nanobacteria in Serum-free (SF) DMEM was performed with monthly passages. SF nanobacteria attach firmly to the bottom of the culture vessel. These cultures were passaged or harvested with a rubber scraper. Cultures were established on Loeffler medium supplemented with 10% conditioned medium from nanobacterial culture and DMEM replaced water in the formula (Nash and Krenz, 1991). The incubation period was 6 weeks under cell culture conditions. Only pure nanobacterial cultures were used. Control experiments were performed to determine whether spontaneous crystallization could occur in a culture medium. The samples were viewed under Light microscopy with Differential Interference Contrast (DIC) optics.

Preparation and Infection of 3T6 Cells: 3T6 cells (ATCC CCL 96) were cultured on coverslips as described before (Ciftcioglu and Kajander, 1998). SF nanobacterial cultures were scraped and 100-mL portions were added to the cell cultures and incubated for 24 h in the incubator. Only DMEM was added to the control experiments. TEM and IIFS were used for the observation of the cell-SF nanobacteria interaction.

Electron microscopy and Energy Dispersive X-ray microanalysis (EDX): For negative staining, nanobacteria were isolated by centrifugation at 40,000 X g for 1 h directly from FBS diluted 1:5 in PBS. A carbon-coated 400 mesh copper grid was placed on a drop of the suspension of nanobacteria in Phosphate Buffered Saline (PBS) for 1 min, washed with water and stained on a drop of 1% phosphotungstic acid for 90 sec. Scanning Electron Microscopy (SEM) and Transmission Electronic Microscopy (TEM) were performed by a BX-51 optical microscope (Olympus) and dark-field condenser (CERBE). Images were acquired by using a KY-F55 color camera (JVC). The topographic features of the nanobacteria were investigated with a scanning electron microscope equipped with EDX as described previously (Suzuki *et al.*, 1997).

RESULTS

The clinical specimens of kidney stones, were cultured in DMEM medium for six weeks and examined macroscopically once a week for the appearance of a white biofilm or floccules adhering to the culture flask. After incubation, the mineralized biofilm was visible to the naked eye in 60% of the sample studied. Macroscopic nanobacterial colonies on DMEM medium were stony, grayish brown and penetrated the medium layer and attached to the bottom of the culture vessel after 6 wk of culture. Light microscopy with DIC optics revealed revealed nanobacteria coated in needle-like apatite crystals similar to the hydroxyapatite crystals (Fig. 1a, b).

Scanning electron microscopy images of the spherical units in the carbonate apatite kidney stones were clearly similar in size and morphology to the mineralized forms of nanobacteria cultured under SF conditions showed similar characteristics (Fig. 2a, b). To visualize the presence of nanobacteria all the "mineralized" cultures were scraped off, harvested by centrifugation and reexamined by SEM. As expected, SEM analysis revealed clusters of spherical particles displaying morphological properties typical of nanobacteria. Spherical coccoid particles were observed which

Fig. 1: (a, b) Nanobacterial stony colonies penetrated through the medium forming stony needle-like crystal deposits

Fig. 3: SEM of (a) negative culture derived from control FBS diluted to 10% in DMEM (b) Nanobacteria obtained by incubating FBS diluted to 10% in DMEM showing colonies of coccoid bacteria-like formations grouped together in coarse clusters. Their diameter was between 200 and 300 nm. Bar, 1 μ m. Magnification, X 40,000

Fig. 2: Details from a fractured renal stone (hydroxyapatite) from SEM analysis Magnification, X 15,000

were grouped in coarse clusters and bound together to a mineral structure (Fig. 3a, b and 4a, b). These spherical units were similar in size and morphology. The size of these particles varied between 200 and 300 nm and they appeared to have developed in stone cavities. Their rough surfaces were illustrated with hairy apatite layer surrounding a central cavity. TEM of inoculated 3T6 cell monolayers has shown transient intracytoplasmic vacuolar formations containing 200 to 300 nm particles in cell cultures (Fig. 5a, b). The chemical compositions and their percentage of distribution among the screened kidney stones and the Energy-dispersive X-ray EDX spectrum of nanobacteria cultured from the liquid fraction of the stones revealed a simple profile of carbon, oxygen and sodium at atomic C:O:Na ratios of 42:30:0.3 (Fig. 6). No phosphorus was detected that might indicate the presence of hydroxyapatite. Therefore, hydroxyapatite may not be a necessary component of nanobacteria because it appears to deposit onto nanobacteria only under particular circumstances.

Fig. 4: TEM of (a) Negative culture and (b) Nanobacteria with an electron-dense core after incubation in DMEM for 1 month

Fig. 5: TEM analysis of renal stone-inoculated 3T6 monolayers. (a) Free 3T6 cells and (b) Nanoparticles inside vesicles in 3T6 cells

Fig. 6: EDX spectrum analysis in SEM of nanobacteria obtained by incubating the soluble liquid fraction of the stones reveals the absence of calcium and phosphate peaks

DISCUSSION

We have tried to demonstrate the presence of nanobacteria in renal stones by using SEM and TEM of inoculated 3T6 cell monolayers. These approaches were previously reported with success (Kajander and Ciftcioglu, 1998; Ciftcioglu *et al.*, 1999). We observed spherical nanoparticles grouped in clusters binding to the mineral surface and cavities of renal stones. Two mechanisms have been proposed for calculi formation: the development of calculi attached to papillary epithelium and the development of calculi in cavities without any attachment to urothelium (Grases *et al.*, 1998). The small apatite units were observed in all kidney stones in different proportions. We also observed similar formations in *in vitro* nanobacteria culture under SF conditions. Nanobacteria first adhere to the surface of the culture vessel and then create "cave-like" apatite fortresses with a concave face (Kajander and Ciftcioglu, 1998). Additionally, SEM images proved that nanobacterial mineralization takes place via the formation of several thin mineral layers the same as in kidney stone formation. There have been several studies on the possible mechanisms of crystal aggregate formation following the initial nucleation of crystals from supersaturated urine (Mandel, 1996). Renal tubular fluid in the distal nephron is supersaturated with calcium and oxalate ions that nucleate to form the most common crystal, calcium oxalate monohydrate, in renal stones. Urine supersaturation values correlated well with stone composition (Asplin *et al.*, 1998). Intracellular assemblies similar to nanoparticles were transiently observed by TEM in 3T6 cells inoculated with material derived from renal stones (Kajander and Ciftcioglu, 1998; Kajander *et al.*, 1997). The structures observed have been

routinely considered as artifacts, as are the structures we observed when using TEM on renal stone material-inoculated 3T6 cells. The apatite produced by nanobacteria is biogenic because it is formed in a carbon-containing biomatrix, forms small spherical units of apatite in nanoscale crystal size (that are very resistant to acid hydrolysis) and can be formed at non saturating concentrations of calcium and phosphate. In this respect, Ciftcioglu *et al.* (1997) has proven that all kinds of nanobacterial forms are internalized by many types of mammalian cells and once internalized, they are cytotoxic. In a rabbit experiment, Akerman *et al.* (1997) showed that ^{99m}Tc -labeled nanobacteria, injected intravenously, had a tissue specific distribution with a major accumulation in the kidneys and subsequently in urine. The presence of live excreted nanobacteria in urine is proof that these organisms may be involved in the kidney stone formation. Ciftcioglu *et al.* (1999) propose a new theory to explain the formation of human kidney stones: Nanobacteria may act as nuclei for kidney stone formation.

CONCLUSION

This theory is supported by the following findings:

- 97.2% of the analyzed kidney stones contained nanobacteria
- Almost all kidney stones have apatite as a component (Mandel, 1996)
- Nanobacteria are the only known organisms in the human body that produce apatite and accumulate in the kidney (Akerman *et al.*, 1997)
- Nanobacteria isolated from human kidney stones produced stones in culture. The availability of the nanobacterial strain for scientific investigators would be an important step in making progress in our research on nanobacteria

ACKNOWLEDGMENT

This study was funded by Cairo university, Project No, 3/5 2009 Application of nanobacteria in the new millennium.

REFERENCES

Abraham, P.A. and C.L. Smith, 1987. Evaluation of factors involved in calcium stone formation. *Miner. Electrolyte Metab.*, 13: 201-208.

- Akerman, K.K., J.T. Kuikka, N. Ciftcioglu, J. Parkkinen, K.A. Bergstroem, I. Kuronen and E.O. Kajander, 1997. Radiolabeling and *in vivo* distribution of nanobacteria in rabbit. *Proc. SPIE Int. Soc. Opt. Eng.*, 3111: 436-442.
- Asplin, J., J. Parks, J. Lingeman, R. Kahnoski and H. Mardis *et al.*, 1998. Supersaturation and stone composition in a network of dispersed treatment sites. *J. Urol.*, 159: 1821-1825.
- Blijenberg, B.G., M. van Vliet and L. Zwang, 1997. Validation of a natural urinary stone data based infrared library searching system with artificial survey samples. *Eur. J. Clin. Chem. Clin. Biochem.*, 35: 625-630.
- Bradbury, J., 1998. Nanobacteria may lie at the heart of kidney stones. *Lancet*, 352: 121-121.
- Carson, D.A., 1998. An infectious origin of extraskeletal calcification. *Proc. Natl. Acad. Sci. USA.*, 95: 7846-7847.
- Ciftcioglu, N. and E.O. Kajander, 1998. Interaction of nanobacteria with cultured mammalian cells. *Pathophysiology*, 4: 259-270.
- Ciftcioglu, N., A. Pelttari and E.O. Kajander, 1997. Extraordinary growth phases of nanobacteria isolated from mammalian blood. *Proc. SPIE*, 3111: 429-435.
- Ciftcioglu, N., M. Bjorklund, K. Kuorikoski, K. Bergstrom and E.O. Kajander, 1999. Nanobacteria: An infectious cause for kidney stone formation. *Kidney Int.*, 56: 1893-1898.
- Folk, R.L., 1993. SEM imaging of bacteria and nanobacteria in carbonate sediments and rocks. *J. Sediment. Petrol.*, 63: 990-999.
- Grases, F., A. Costa-Bauza and L. Garcia-Ferragut, 1998. Biopathological crystallization: A general view about the mechanisms of renal stone formation. *Adv. Colloid Interface Sci.*, 74: 169-194.
- Hyvonen, P.M., H. Hanhijarvi and K. Aho-silta, 1986. The effect of clodronate (dichloromethylene diphosphonate) on the mineral concentration of dental enamel and bone of the miniature swine. *Acta Pharmacol. Toxicol.*, 59: 129-134.
- Kajander, E.O. and N. Ciftcioglu, 1998. Nanobacteria: An alternative mechanism for pathogenic intra and extra cellular calcification and stone formation. *Proc. Natl. Sci. USA.*, 95: 8274-8279.
- Kajander, E.O., I. Kuronen, K. Akerman, A. Pelttari and N. Ciftcioglu, 1997. Nanobacteria from blood, the smallest culturable autonomously replicating agent on earth. *Proc. SPIE*, 3111: 420-428.

- Kajander, E.O., N. Ciftcioglu, K. Aho and E. Garcia-Cuerpo, 2003. Characteristics of nanobacteria and their possible role in stone formation. *Urol. Res.*, 31: 47-54.
- Khan, S.R, 1997. Animal models of kidney stone formation: An analysis. *World J. Urol.*, 15: 236-243.
- Mandel, N., 1996. Mechanism of stone formation. *Semin. Nephrol.*, 16: 364-374.
- Nash, P. and M.M. Krenz, 1991. Culture Media. In: *Manual of Clinical Microbiology*, Balows, A., Jr. W.J. Hausler, K.L. Herrmann, H.D. Isenberg and H.J. Shadomy (Eds.). American Society for Microbiology, Washington, DC. USA., pp: 1226-1288.
- Suzuki, T., M. Yano, S. Sumi, M. Honda, Y. Hosoya and K.I. Yoshida, 1997. Study of the structure of struvite stones with scanning electron microscopy and energy dispersive X-ray microanalysis. *Urol. Int.*, 58: 88-92.
- Vogel, G., 1998. Bacteria to blame for kidney stones. *Science*, 281: 153-153.