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ORIGINAL ARTICLE

Resistance of nanobacteria isolated from urinary and kidney stones to broad-spectrum antibiotics

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ABSTRACT

Background and Objective: Nanoscopic life forms called Nanobacteria or calcifying nanoparticles (CNP) are unconventional agents. These novel organisms are very small (0.1 to 0.5 microns) and possess unusual properties such as high resistance to heat and routine antimicrobial agents. Nanobacteria are 100 times smaller than bacteria and protected by a shell of apatite, so they could be as candidate for emerging and progress of *in vivo* pathological calcification. In this study, the inhibitory effect of broad-spectrum antibiotics on growth of these new forms of life has been investigated.
Material and Methods: Powdered urinary and kidney stones were demineralized with HCl and neutralized with appropriate buffers and became filtered. Finally suspension was incubated in DMEM medium with Fetal Bovine Serum (FBS) and broad-spectrum antibiotics (100U/ml for penicillin and 100µg/ml for streptomycin) for 60 days.
Results: In the presence of broad-spectrum antibiotics, Scanning Electron Micrographs (SEM) showed a spherical shape of these nanobacteria. Also, Energy Dispersive X-ray spectroscopy (EDS) showed a pick for calcium and phosphor. Transmission Electron Microscopy (TEM) results illustrated cover around the nanobacteria.
Conclusion: The growth of calcifying nanoparticles after adding the broad-spectrum antibiotics may be due to their apatite hard shells supporting them against penetration of the antibiotics.

Keywords: Broad-spectrum antibiotics, Nanobacteria, kidney stones

INTRODUCTION

One of the issues in recent years has attracted many urologists vision are Nanobacteria. Calcifying nanoparticles (CNP) or Nanobs (are the others name of nanobacteria) considered as potential nidi for human calcific disease. These agents could accumulate different minerals under*in vivo* as well as *in vitro* condition. On the other hands, biomineralization and calcification are the main properties of nanobacteria. However, in many studies it has been proven that presence of nanobacteria could start the biomineralization and calcification process that eventually will cause disease (1). These diseases include arterial heart disease (2), Alzheimer's disease (3), kidney stone formation (1), polycystic kidney disease (PKD) (4), gallstones and gallbladder inflammation, (5) prostatitis (6), calciphylaxis (7), and cancer (8). One reason seems to be due to the tenacious incredible cover of this organisms. Penicillinis and other β-lactam derived antibiotic are used in the treatment of bacterial infections caused by Gram-positive bacteria (9). Streptomycin is used to inhibit the growth of bacteria, fungi, and algae. Tetracycline, nitrofurantoin, trimethoprim, trimethoprim-sulfamethoxazole, and

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ampicillin are able to inhibit the NB growth at levels achievable in serum and urine. However, other antibiotics had no effect on nanobacteria growth (10). For prevention of bacterial growth in the human cell culture such as DMEM, researcher added various antibiotics. Penicillin and streptomycin are the most common antibiotics that used in medium. The purpose of this study was to evaluate the inhibitory effects of penicillin and streptomycin as broad-spectrum antibiotics on these novel nanoorganisms. We also intended to show whether these antibiotics used in the medium culture of human cells are able to inhibit the growth of nanobacteria.

MATERIALS AND METHODS

Fifteen urinary and fifteen kidney stones were collected from northeastern Iranian population. For CNP isolation, 0.05 g of stone fragments were manually grounded and demineralized by incubation with 1 N HCl for 10 min at room temperature, and then neutralized by the addition of Tris buffer (pH=10.5). After centrifugation at 20,000 g for 40 min, the precipitation was suspended in Dulbecco's Modified Eagle medium (DMEM). The suspension was filtered through a 0.22-µm membrane filter and cultured in six-well plates that each well containing 5 mL DMEM and 10% fetal bovine serum (FBS) gamma-irradiated at a dose of 30 kGy and antibiotics (100U/ml for penicillin and 100µg/ml for streptomycin). Culturing was carried out using strict aseptic techniques at 37°C in humidified 5% CO_2-95% air. Negative control was a medium containing 5 mL DMEM, 10% gamma-irradiated fetal bovine serum (FBS) and antibiotics but without stone. CNP growth was monitored by measuring them at 650 nm. After 60 days, the positive plates based on optical density were monitored and white-colored sediment on the bottom of the wells were used for SEM and TEM analysis. In the SEM study, floating and adherent materials in a culture plates were examined separately.

RESULTS

The nanobacteria growths which monitored by using of spectrophotometry at wavelength of 650 nm with an interval of 15 days, indicated that in two early weeks, nanobacteria growth was too low. pH variation was measured during the culture period, but the changes were in the range of 7 to 8. During the 3 days of incubation in nutrient media filtrate, no bacterial and fungal contamination was observed at 25, 30, 37 and 50°C .Inverted optical microscope micrograph showed nanobacteria start crystallization and attached to the bottom plate (Fig. 1). After 60 days of nanobacteria, sediment sticking to the bottom plate analyzed with scanning electron microscopy and showed nanobacterial spherical shapes. SEM study showed a spherical shape in size of 56.36 to 93.86 nm (Fig. 2). In culture without powdered stones positive CNP detection was zero. Microscopic methods such as SEM indicated that nanobacteria were grown in the medium include penicillin and streptomycin at 100U/ml and 100µg/ml concentration respectively. TEM results illustrated cover around the nanobacteria (Fig. 3). Energy-dispersive X-ray analysis in culture medium showed calcium and phosphate peaks rather than other minerals such as magnesium and sodium (Fig. 4)

DISCUSSION

CNPs have a major controversy in modern microbiology. So far, several papers have accepted the viability evidence of these new forms. This evidence includes the study of culture turbidity, using of monoclonal antibodies, and examination by SEM and TEM. Our study focuses on the broad-spectrum antibiotics inhibitory effects on nanobacteria growth. It is important to assure that in the each analysis there is no contamination. To achieve this reality, it is necessary to apply three approaches in the experiments. In the first step, all components should be sterilized. Using 0.2 micron filters most pathogen entrance into culture can be prohibited. At the end, if any contamination has been exist, microscopy methods could display them but in our study any of microscopy and staining methods did not exhibit any contamination. Based on the results of this study and reports of other investigators, nanobacteria are covered via a hard shield (3). As nanobacteria age rises, the coating thickness also increases. Their cover is so stronghold that is not readily dissolved in hydrochloric acid. Because of too strong cover in nanobacteria, penetration of any pernicious agents exposed with problem. Hence, the penetration of broad-spectrum antibiotics into their hard shell will be impossible. In this study, the main purpose for using of broad-spectrum antibiotics was

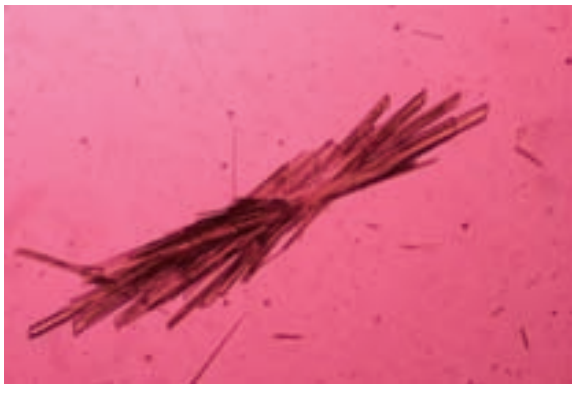

Fig.1. Cultures of CNPs observed by invert optical microscope (200× magnification).

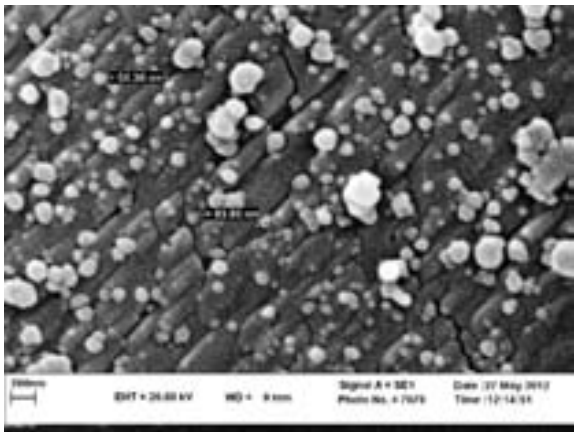

Fig.2. SEM micrograph of Nanobacteria: nanobacteria showing a small size (scale bar 200 nm).

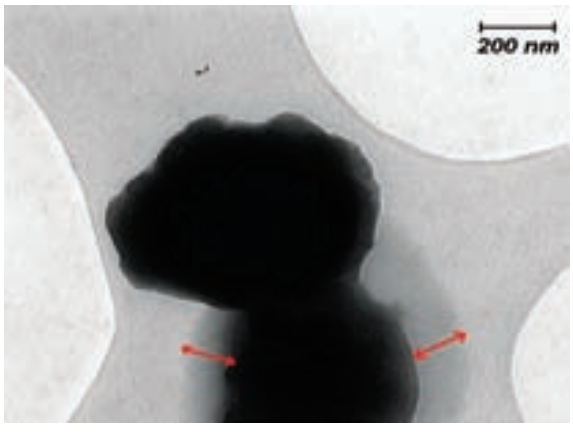

Fig.3. TEM micrograph of Nanobacteria: Wrap around the nanbacteria (scale bar 200 nm). This cover is a reseon that why nanobaceria have impenetrable propeties.

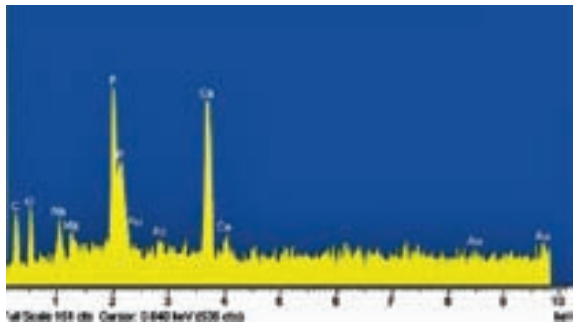

Fig. 4. Energy Disspersive X-ray analysis of nanobacteria: nanobacteria via accumulation of minerals, specially calcium and phosphate cause *in vitro* biomineralization.

many methods for nanobacteria grouth inhibition are exposed with defects. Streptomycin inhibits the protein synthesis via binding to the small 16S rRNA of the 30S subunit of the bacterial ribosome (11). β-Lactam antibiotics such as penicillin can inhibit the formation of peptidoglycan cross-links in the bacterial cell wall. Hence, reduce the cell wall strength of the bacteria that eventually raise the osmotic pressure causing cell death. However, as nanobacteria covered with an apatite hard shell which is impenetrable to many inhibitory material it may be the main reason why nanobacteria grow, in exposure to broad-spectrum antibiotics such as penicillin and streptomycin.

This study has revealed the CNPs resistance to broad-spectrum antibiotics. Nanobacteria buildup different mineral around themselves leading to inhibiting the penetration of lethal agents. The growth of calcifying nanoparticles after adding the broad-spectrum antibiotics may be due to their apatite hard shells that can support them from the antibiotics penetration to their cells. As nanobacteria are different in every area in the world, further studies are required to test CNPs resistance to specific antibiotics. These investigations might lead to novel approaches for preventing nanobacteria growth and consequentially facilitating pathological therapies which promote or started by nanobacteria.

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