

A potential cause for kidney stone formation during space flights: Enhanced growth of nanobacteria in microgravity

NEVA ÇİFTÇİOĞLU, RUWAIDA S. HADDAD, D.C. GOLDEN, DENNIS R. MORRISON,
and DAVID S. MCKAY

Universities Space Research Association, Houston, Texas; Hernandez Engineering, Inc., Houston, Texas; and National Aeronautics and Space Administration, Lyndon B. Johnson Space Center, Houston, Texas

A potential cause for kidney stone formation during space flights: Enhanced growth of nanobacteria in microgravity.

Background. Although some information is available regarding the cellular/molecular changes in immune system exposed to microgravity, little is known about the reasons of the increase in the kidney stone formation in astronauts during and/or after long duration missions at zero gravity (0g). In our earlier studies, we have assessed a unique agent, nanobacteria (NB), in kidney stones and hypothesized that NB have an active role in calcium phosphate-carbonate deposition in kidney. In this research we studied effect of microgravity on multiplication and calcification of NB in vitro.

Methods. We examined NB cultures in High Aspect Rotating Vessels (HARVs) designed at the NASA's Johnson Space Center, which are designed to simulate some aspects of microgravity. Multiplication rate and calcium phosphate composition of those NB were compared with NB cultured on stationary and shaker flasks. Collected aliquots of the cultures from different incubation periods were analyzed using spectrophotometer, SEM, TEM, EDX, and x-ray diffraction techniques.

Results. The results showed that NB multiplied 4.6× faster in HARVs compared to stationary cultures, and 3.2× faster than shaker flask conditions. X-ray diffraction and EDX analysis showed that the degree of apatite crystal formation and the properties of the apatite depend on the specific culture conditions used.

Conclusion. We now report an increased multiplication rate of NB in microgravity-simulated conditions. Thus, NB infection may have a potential role in kidney stone formation in crew members during space flights. For further proof to this hypothesis, screening of the NB antigen and antibody level in flight crew before and after flight would be necessary.

Future exploratory missions to the moon and Mars, including the establishment of a permanently crewed base on the lunar surface, will extend the distances traveled,

the exposure to radiation, the duration of the mission, and the levels of confinement and isolation to which the crews will be exposed. This will raise several health issues involving microgravity and various stressors that may be limiting factors during these missions. The transition through various levels of gravity, such as from 1g through hypergravity to microgravity during launch, long-term exposure to microgravity during interplanetary transfer, transition from microgravity to hypergravity during deorbiting, and stay at reduced gravity on the celestial body—to mention just one half of the round-trip to the moon or Mars—have major implications for astronaut health. Deconditioning symptoms of this flight profile may include loss of muscle and bone mass, reduced cardiovascular and physical capacity, and changes of motor skills [1]. Increased bone resorption and the attendant hypercalciuria and hyperphosphaturia contribute significantly to raising the urinary state of saturation with respect to the calcium salts, namely calcium oxalate and calcium phosphate [1]. We know that some proteins regulate bone calcification [2], but we still don't know all of the endocrinology and biochemical mechanisms that link bone decalcification to kidney stone formation. It has been previously suggested that space flight may increase the risk of kidney stone formation, and this increased risk may occur rapidly upon exposure to microgravity, and apparently continues throughout the space flight and following landing [3]. Dietary factors may also adversely affect urine composition and increase stone formation risk during space flight. Reductions in urinary volume, pH, and citrate contribute to increased risk of stone formation [3]. In addition, the real mechanism of crystal initiation and kidney stone formation remains speculative.

There exists a recently discovered agent (termed nanobacteria or NB) that behaves as a microbe and appears to show a correlation with such diverse conditions as arterial heart disease [4], Alzheimer's disease [5], kidney stone formation [6–8], polycystic kidney disease [9], and malignant tumors [10]. Furthermore, these NB have unique properties including extremely small size, very

Key words: kidney stones, nanobacteria, microgravity.

Received for publication May 14, 2004

and in revised form July 19, 2004

Accepted for publication August 16, 2004

© 2005 by the International Society of Nephrology

slow multiplication rate (about 3 days doubling rate), rapid in situ precipitation of calcium phosphate from blood and other body fluids under conditions not normally conducive to such precipitation, and the apparent ability to extract and concentrate phosphorous from dilute solutions [11]. The concept that NB are living organisms will remain controversial as long as their putative nucleic acid is not sequenced [12, 13]. We still do not know their location in the phylogenetic tree of systematic microbiology. Whether this agent is a true microbe or something else is still under investigation, but NB appears to play a role in several pathologic calcification-related diseases.

In a small study, Garcia-Cuerpo et al found that translumbar, percutaneous intrarenal injection of NB into rats resulted in kidney stone formation [14]. Despite the small number of experimental animals ($N = 4$), the result provides evidence that kidney stone formation can be caused by introducing NB into kidney. Whether NB themselves serve as the nucleus for crystal formation, or whether the NB are simply able to lower the activation energy barrier, thus allowing precipitation and growth of crystals under much lower supersaturation conditions, is yet to be determined. Interestingly, Evan et al has confirmed a long-standing hypothesis that kidney stones, while not usually calcium phosphate, develop from calcium phosphate crystals [15]. Recent studies on NB have produced findings suggesting that NB might be calcium phosphate nuclei for kidney stone formation [6–8]. A key question for space flight medical issues might be asked: What is the effect of microgravity on NB multiplication and calcification? Could this unique entity have a role in kidney stone formation that may increase the risk to the flight crew when they are exposed to microgravity?

The Rotary Cell Culture System™, invented at the US National Aeronautical Space Administration (NASA), was introduced commercially by Houston-based Synthecon in 1990. This culture system uses a HARV attached to rotator base and rotated about its horizontal axis. The vessel is a disk-shaped container, 8.7 cm in diameter, 1.27 cm in height, and with 50 mL capacity. It has two luer lock ports attached to the front side enabling to load and sample the vessel [16, 17]. The rotating cell culture system has no impellers, airlifts, bubbles, or agitators; culture turbulence and damage is significantly decreased. Shear stress and damage is very low and is essentially insignificant. Under these conditions, cultures communicate and function as they would in vivo. HARV spins a fluid medium filled with cells to randomize most of gravity's effects and encourage cells to grow more like a 3D tissue construct. This microgravity simulation system has been also tested in some bacterial cultures [18, 19] where *Salmonella* virulence was increased [18]. These and other tests have shown that HARV is a good simulation system for re-

producing the effects of microgravity on cell growth and behavior.

We have examined the microgravity effect on multiplication and calcification rate of the unique self-replicating agent, NB, using this simulation system.

METHODS

We used one of the NASA-designed bioreactors, designated HARVs. It consists of a disk-shaped culture vessel with 50 mL capacity that rotates about a horizontal axis (Fig. 1). Adequate gas exchange is maintained by pumping air from the surrounding 5% CO₂ incubator through a membrane spanning the backside of the bioreactor. The vessel is filled with culture medium and NB are added. All air bubbles are removed from the culture vessel. This ensures that the fluid rotates without shear forces that would destroy the formed apatite coat on the surface of NB. The rotating vessel does not really cancel gravity, but ideally maintains NB in continual free-fall similar to that experienced by astronauts in the microgravity of space.

NB (DSM no.5819–5821; Braunschweig, Germany), available from NANOBAC OY (Kuopio, Finland), are cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) under mammalian cell culture conditions (37°C; 5–10%CO₂/90–95% air) for 27 days [20]. One-tenth dilution from the stock NB culture with turbidity equivalent to that of 0.5 McFarland standard (1.5×10^8 bacteria/mL, optical density at 650 nm, 0.020) was used as the starting concentration; this concentration was used in our earlier experiments [20]. The total culture volume was 50 mL, and all the preparations were made using strict aseptic techniques in a cell culture facility. Duplicate samples were incubated in three different physical conditions: (1) in HARVs; (2) within flasks on shakers; (3) within stationary flasks. As a control we incubated only DMEM containing 10% FBS in the same culture conditions for the same incubation periods. Control experiments were performed to determine whether spontaneous crystallization could occur in a culture medium with and without FBS. Five milliliter samples were collected for planned analysis, and 5 mL of fresh medium added to the culture vessels on 0, 3rd, 7th, 10th, 14th, 21st, and 27th days of the incubation period.

The collected samples were analyzed for positive identification of NB and sterility control; optical density measurement; transmission electron microscopic (TEM) analysis with and without negative staining; scanning electron microscopy (SEM) analysis; and x-ray diffraction and EDS analysis.

We checked NB culture quality by applying a double-staining method as previously described [6]. Double staining is the combination of immunofluorescence staining (IFS) and Hoechst #33258 fluorochrome (0.05 µg/mL)

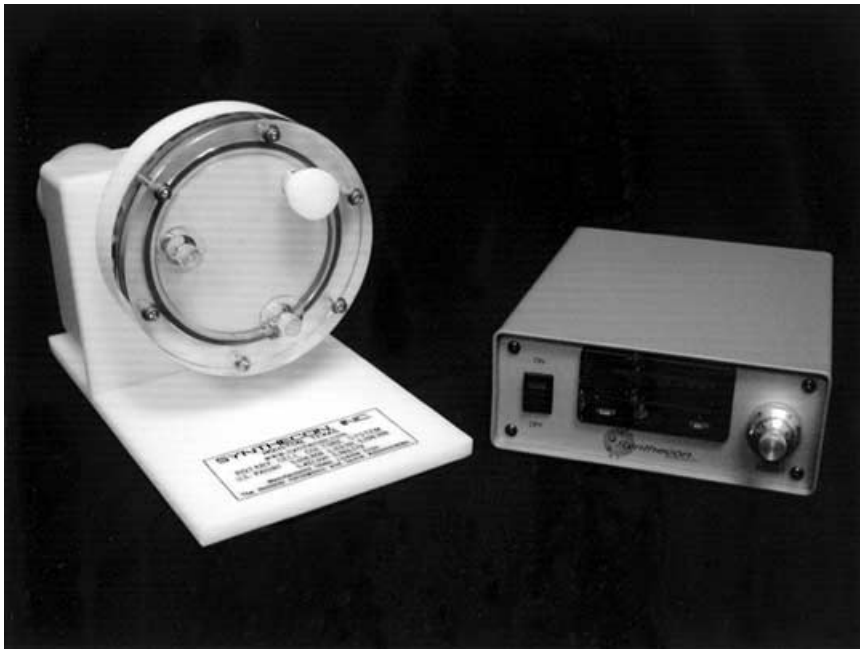


Fig. 1. Photograph of a High Aspect Ratio Vessel (HARV), rotary cell culture system™, designed by NASA, created by Synthecon™.

staining. We used anti-NB monoclonal antibody 8D10 (NANOBAOY) for IFS [21]. The DNA stain (Hoechst #33258) at the concentration used does not stain NB but does detect all known common bacteria [22]. Positive identification of NB also included typical growth in cell culture medium as monitored by optical density [23], with a doubling time of 1 to 3 days [24], and characteristic morphology by SEM or TEM [25]. Replication rate of NB can be measured by optical density at 650 nm as applied earlier [20]. The net absorbances are calculated by deducting the absorbance of the control cultures (cultures with no NB) from the absorbance of each set of the cultures in different culture conditions. For the statistical *P* values, regression analyses are used for each data set to determine the slopes. Then the slopes are used as the dependent response measure to compare vessels and the time points [26]. We calculated the ratio of the different slopes to find the amount of change in the multiplication rates of the cultures seeded in different vessels.

For negative staining, NB samples were harvested by centrifugation at 40,000g for 1 hour, fixed with 3% glutaraldehyde-2% formaldehyde in PBS, maintained overnight at +4°C, and washed with PBS twice before the staining is applied. A drop of the suspension of NB in PBS was placed on a carbon-coated 400 mesh copper grid for 1 minute, washed with water, and stained on a drop of 5% uranyl acetate for a few seconds [24]. The grid was dried with the use of Whatman 1 paper and viewed directly by TEM (JEOL 2000FX; Tokyo, Japan) operated at 200 kV. Unstained and stained NB from all treatments were mounted on carbon substrates on Cu-grids for TEM analyses. Morphologic observations, EDX analyses, and selective area electron diffraction were per-

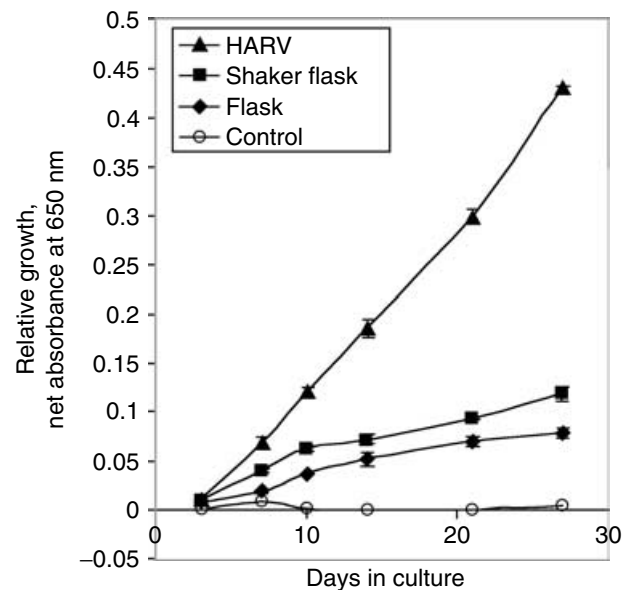


Fig. 2. Growth curve of nanobacteria in three different types of vessels during 27 days culture period. HARV, microgravity simulation system; shaker flasks, cultured on shakers in the incubator; flasks, stationary culture; control, culture medium with no nanobacteria addition.

formed on these samples. X-ray diffraction pattern analysis was performed as described before [27] on the dried slurries of unstained samples and compared to the ASTM database.

The samples were prepared for SEM analysis as described earlier [6] and analyzed both for morphology and chemical composition using JEOL 6340 Field Emission SEM with attached IXRF energy dispersive x-ray (EDX)

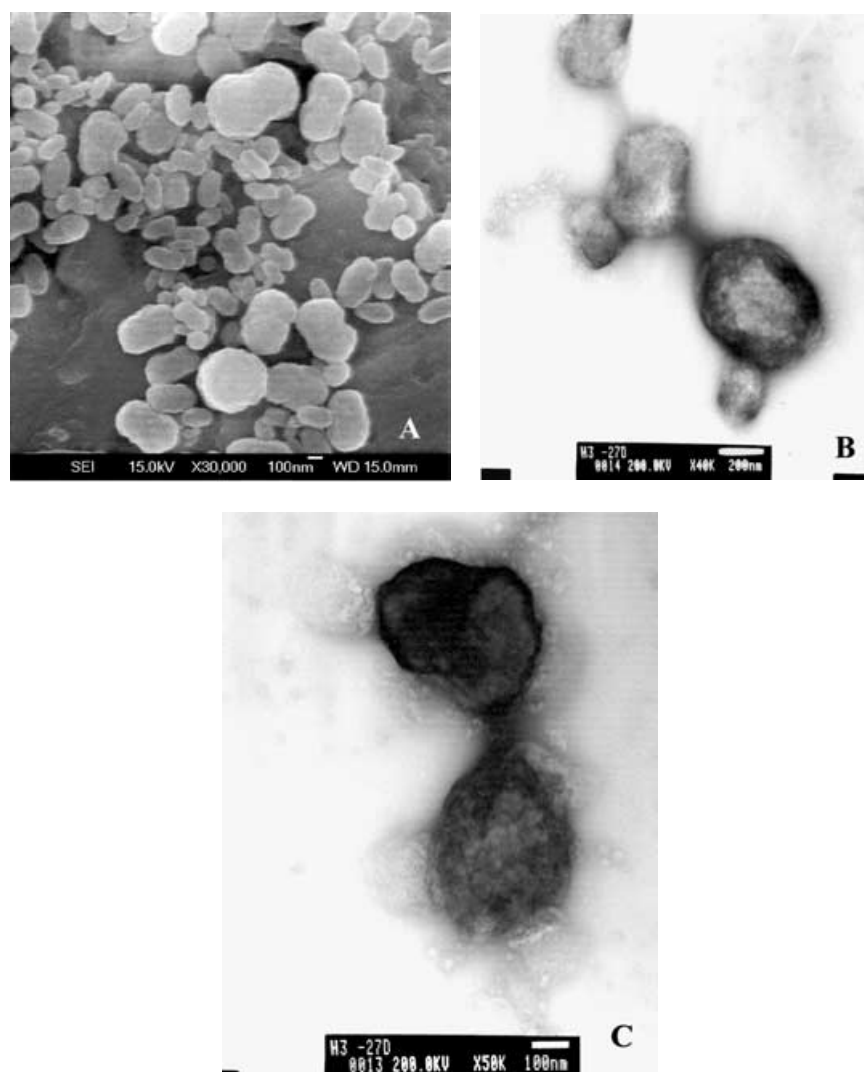


Fig. 3. Scanning (A) and transmission (B and C) electron microscopic images of nanobacteria. The samples shown in B and C were negative-stained with uranyl-acetate. Bars are (A) 100 nm, (B) 200 nm, and (C) 100 nm.

analyzer [28]. In EDX analyses, inorganic hydroxyapatite was used as a reference.

RESULTS

Growth curves for the culture conditions of NB are depicted in Figure 2. NB cultured in HARVs showed 4.5 times faster multiplication rate compared to stationary culture conditions ($P < 0.005$), and 3.2 times faster replication compared to shaker flasks. Cultures on shaker showed only 1.4 times greater multiplication rate compared to stationary cultures (Fig. 2). The negative controls contained only culture medium, and FBS did not show significant changes in the absorbance during the 27 days total culture period.

The criteria for pure NB culture were retractile aggregates of typical coccobacillar shaped particles visible in SEM (Fig. 3A) and TEM (Fig. 3B and C) analysis, showing no Hoechst dye stainability at the concentration of 0.05 $\mu\text{L}/\text{mL}$ and IFS positivity with anti-NB mono-

clonal antibodies (results not shown). Negative Hoechst stainability results on all cultures shows that there were no conventional bacteria or any other possible contaminants in the cultures. Figure 3C shows a nanobacterium apparently in multiplication phase. The negative control contained only culture medium containing 10% FBS, and confirmed the absence of common bacteria and NB under the test conditions used in the study.

Negative staining for TEM imaging of all NB cultures revealed typical electron-dense coccobacillus-shaped NB with defined borders ranging in size from approximately 150 to 250 nm, but also revealed size differentiation depending on culture condition. In HARV and shaker cultures there were more of small-sized (about 100 nm) NB (Fig. 4B) with slimy connections with each other (Fig. 3B and 4C) compared to stationary cultures (Fig. 4C). X-ray diffraction of bulk samples and EDX analysis of selected cells clearly showed the existence of apatite crystal formation on the cell surface of all NB cultures (Fig. 5B). Control hydroxyapatite was correctly identified in the test

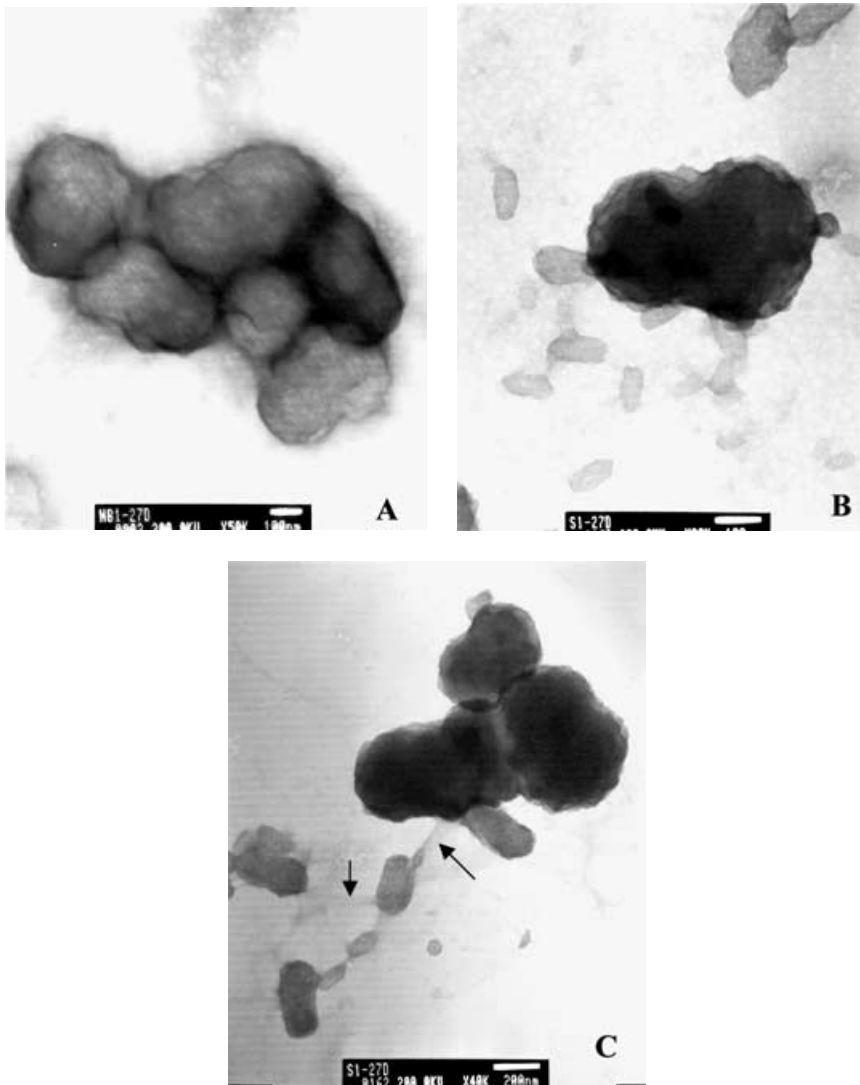


Fig. 4. TEM images of nanobacteria after cultured in HARVs (A), on shaker (B), and stationary (C) flasks. Arrows show the slimy connection among nanobacteria. Bars in (A and B) are 100 nm, and in (C) are 200 nm.

(Fig. 5A). The results observed in x-ray diffraction analysis confirmed the only mineral phase present is hydroxy apatite; this is in concurrence with the elemental analysis by EDX (Fig. 6). The selective area electron diffraction for the NB from stationary, shaker, and microgravity treatments are shown in Figure 6. This observation is consistent with our observation that the apatite layer on the NB cells cultured in HARVs was apparently thinner compared to shaker and stationary cultures (Fig. 7A and B). Because of the lower mineral density on the cell surface, NB cultures in bioreactors were stained lighter, with negative staining, compared to other NB cultures (Fig. 7). None of the NB cultures produce urease or alkaline phosphatase activity, and their culture medium remained at pH 7.4. All those observations related to chemical composition and calcification were the same in each time period of the incubation. However, the small sized NB 100 nm in size in HARV and shaker cultures were not observed before 14th day of the incubation period. Those

samples were filtered through the 100-nm sized pores. The filtrate is stained with IFS by using monoclonal anti-NB. All the samples gave positive signals showing the positive NB identity. However, the smaller-sized (<100 nm) NB appeared to be less mineralized (thinner or absent mineral coating) than the larger NB in negative staining TEM images (Fig. 4).

DISCUSSION

Although some information is available regarding the cellular and molecular changes in immune system exposed to microgravity, little is known about the reasons of the increase in the kidney stone formation in astronauts during/after long duration missions at 0g [3]. We now report a potential role of NB in that problem depending on our ground-based simulated microgravity model system.

In a successful space science biology and health maintenance program, the limits of established knowledge and

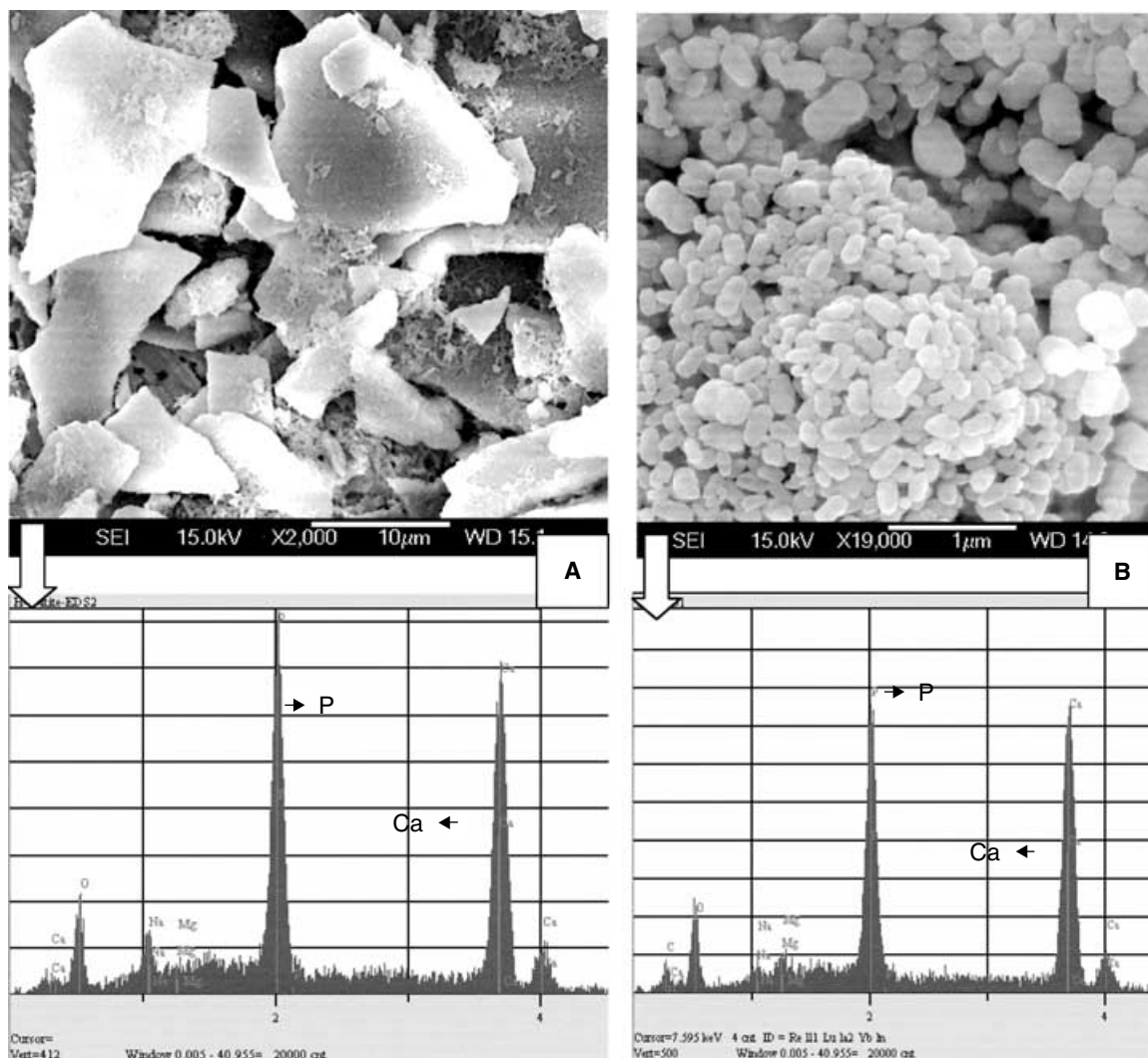


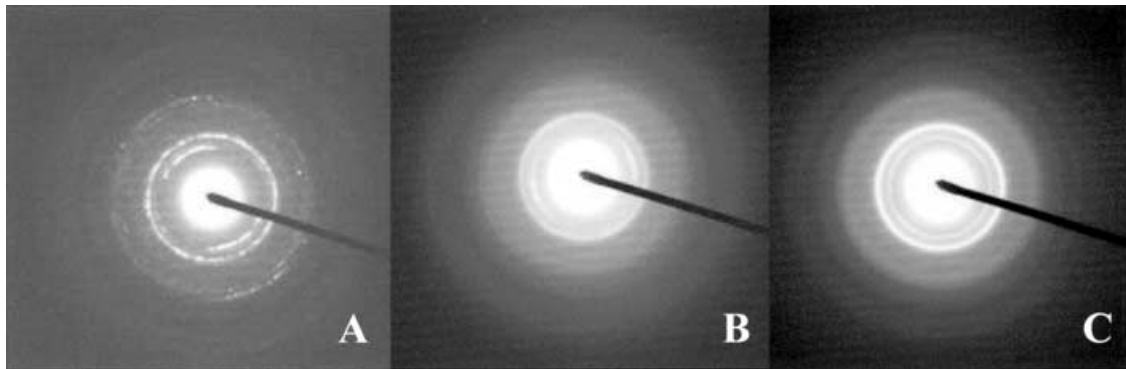
Fig. 5. Ca and P peaks detected by using EDX analyze of commercial hydroxyl apatite (A) and nanobacteria culture (B). At the top, SEM images of the samples are seen.

the implied uncertainties of the estimated effects on astronaut health have to be identified and quantified. In particular, our limited knowledge of the microgravity effect on human health creates the greatest uncertainty in planning the future space science operations and missions.

Changes in the diet, urinary volume, and urinary biochemistry of crew members during long duration spaceflight demonstrated increases in the supersaturation of the stone-forming salts. The effects of dietary intake, especially fluid intake, may have a significant impact on the potential for renal stone formation [29]. However, increasing urinary output during flight may not be entirely effective in minimizing the potential risk of renal stone formation [30]. There have been many studies on the possible mechanisms of crystal aggregate formation following the initial nucleation of crystals from supersaturated urine. Even supersaturated urine requires nidi (nucleus) for crystallization to appear. It is not known

how these nascent crystals are retained in the nephron to form calculi in certain individuals in normal (1g) or in microgravity conditions. In our early studies, we have proposed that a novel infectious agent, NB, may serve as nidi, that attach to, invade, and damage the urinary epithelium of collecting ducts and papilla forming the calcium phosphate centers found in most kidney stones [6–8].

If NB cause kidney stone formation, and are potential infectious agents, how might this affect the crew members during and after space flights? Up until now, all the studies performed during space flight have shown that the main sources of microbial contaminants are the crew members themselves [31]. The studies performed during long duration flights have revealed that the bacterial flora of the crew members were uniform, probably due to the close confinement [32, 33]. If any of crew members happened to have NB infection, it is very likely that



Electron diffraction analysis of crystals associated with NB			
Ring #	d-spacing Å	d-hyd.ap. Å	{hkl}
d1	3.44	3.45	[002]
d2	3.17	3.16	[102]
d3	2.77	2.77	[112]
d4	1.83	1.84	[213]
d5	1.72	1.73	[004]

Fig. 6. Electron diffraction patterns for the NB samples from stationary (A), shaker (B), and microgravity (C) experiments. Samples from stationary flasks had five prominent rings (see table), and the other two samples had 4 lines each. Shaker and microgravity products are less crystalline than the stationary samples. As seen in the table, electron diffraction patterns for NB from stationary, shaker, and microgravity experiments showed the diffraction lines characteristic of hydroxyl apatite. The spacing for d2 was observed only in the stationary sample.

the infection will spread. Additional to close-confinement reason, the following two major factors might play roles in spreading any kind of infection easier during the space flight compared to 1g conditions. Space flight and models that created conditions similar to those that occur during space flight have been shown to affect a variety of immunologic responses [34]. These changes might have adverse effect on potential infection. In early studies, it has been shown that some microorganisms change their multiplication rate [35], cell behavior [36], and even increase virulence [18] in microgravity. In this study we showed that unique, infectious calcifying agent, NB, multiplies about 5 times faster in microgravity-simulated culture conditions (Fig. 2). In our earlier studies, we have shown the cytotoxic effect of NB on mammalian

cell cultures [21]. Possible changes in cytotoxicity and virulence of NB in microgravity conditions should be evaluated.

We do not fully understand the control mechanism of biomineralization either in primitive or in developed organisms. Recent investigations in space science have demonstrated the ability of living organisms to control crystallization through a biomineralization process that involves application of specialized macromolecules which enable nucleation and growth of crystalline structures of carbonates, phosphates, oxides, oxalates, silicates, and other inorganic materials. This process has been demonstrated in both 1g and analog microgravity conditions. Although some researchers believe that microgravity does not affect crystal formation, bone loss

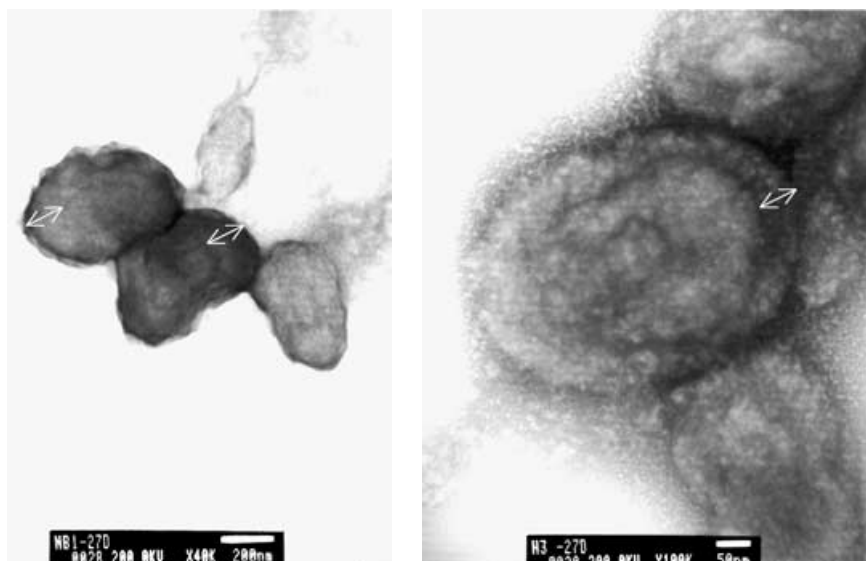


Fig. 7. TEM images of nanobacteria cultured in stationary flasks (A), and in HARVs (B). Arrows show the thickness of the apatite wall of nanobacteria. Bars in (A) are 200 nm, and in (B) are 50 nm.

and enhanced kidney stone formation-like disorders in astronauts in space flights are examples for modified crystal formation in vivo. If the observed increased risk of kidney stone development in flight crews was caused only on body chemistry changes resulting from bone loss, nucleation rates for kidney stone formation should also depend only on chemical changes. Here we show that nucleation and growth of apatite crystal, a likely precursor nuclei for kidney stone development, depends on reduced simulated gravity effects, even though the systems and controls all have the same basic chemical composition. Nucleation and mineralization processes may provide a unifying theme connecting such human health problems encountered in human space flight as bone loss and kidney stone formation. Other pathologic calcification formation and growth within the body may also occur. The reasons are probably multifactorial. To unravel the individual factors causing these problems, the mechanism of biomineralization must be better understood. Up until now, calcification-related problems in microgravity were blamed on cell-level abnormalities. However, studies in microgravity-simulated conditions noted no remarkable differences in the cell cycle, ultrastructure, and metabolism of individual cells [37]. It has been shown that the results of molecular mechanisms of some cellular signaling and growth in bioreactors are more similar to results obtained in vivo research when compared to other cell culture conditions [38, 39].

We propose a model system for the molecular analysis of biomineralization processes in simulated microgravity conditions by using rotating bioreactors and NB cultures. NB cultures are the only known systems that produce apatite-coated particles in identical size (about 150 nm) and morphology (coccobacillar) under physiologic conditions [6]. To our knowledge there is no other known, ei-

ther biogenic or nonbiogenic, in vitro model for studying apatite mineral formation in neutral pH and physiologic conditions.

This model (NB culture in bioreactors) could also be adopted as a drug-screening program for discovery of novel therapeutic agents affecting the biomineralization process in microgravity and similar mechanisms in patients with hypercalcemic conditions. Earlier we have shown that NB was inhibited in vitro at clinically achievable levels in serum and urine by ampicillin, trimethoprim, trimethoprim-sulfomethaxazole, nitrofurantion (a urinary antiseptic), and tetracycline HCl [20]. Etidronate and clodronate (bisphosphonates that lack N moieties), 5-FU, citrate, and aminocaproic acid completely inhibited the growth of NB [20]. Effects of these chemotherapeutics on NB multiplication and their calcification in microgravity conditions are not known. Concerning antibiotic resistance, some bacteria (*Escherichia coli* and *Staphylococcus aureus*) have been found to be more resistant to antibiotics in space than on Earth [40]. This finding may indicate a need for a higher antibiotic dosage in space than on the ground. Previously, it has been thought that pharmacologic intervention should only be directed at raising urinary volumes, diminishing bone losses, and preventing reductions in urinary pH and citrate.

CONCLUSION

New and rapid testing for the presence of NB in human bodies must be developed. Success in reducing the risk for stone formation in astronauts by using unique approaches and techniques would also be of potential major benefit to the estimated 20 million Americans with nephrolithiasis. [41].

ACKNOWLEDGMENTS

We appreciate Dr. Daniel L. Feeback for allowing us to use his molecular biology research facilities. We thank Craig S. Schwandt and Georg Ann Robinson for their expertise and help in electron microscopy.

Reprint requests to Dr. Neva Çiftçioğlu, USRA/NASA JSC, Mail code SA13, 2101 Nasa Road 1, Houston, TX 77058.
E-mail: nciftcio@ems.jsc.nasa.gov

REFERENCES

- HEER MN, KAMPS C, BIENER C, et al: Nutrient supply during recent European missions. *Pflugers Archive* 441 (Suppl 2-3):R8-R14, 2000
- HUMPHREY MB, OGASAWARA K, YAO W, et al: The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. *J Bone Miner Res* 19:224-234, 2004
- WHITSON PA, PIETRZYK RA, SAMS CF: Space flight and the risk of renal stones. *J Gravit Physiol* 6:87-88, 1999
- JELIC TM, MALAS AM, GROVES SS, et al: Nanobacteria-caused mitral valve calciphylaxis in a man with diabetic renal failure. *South Med J* 97:194-198, 2004
- KAJANDER EO, LIESI P, ÇİFTÇİOĞLU N: Do autonomously replicating sterile-filterable particles have an association with amyloid accumulation? Viruses and virus-like agents in disease, *2nd Karger Symposium*, Basel, Switzerland, 1993, pp 41
- KAJANDER EO, ÇİFTÇİOĞLU N: Nanobacteria: An alternative mechanism for pathogenic intra- and extra-cellular calcification and stone formation. *Proc Natl Acad Sci USA* 95:8274-8279, 1998
- ÇİFTÇİOĞLU N, BJÖRKLUND M, WILLMAN K, et al: Nanobacteria: An infectious cause for kidney stone formation. *Kidney Int* 56:1893-1898, 1999
- KHULLAR M, SHARMA SK, SINGH SK, et al: Morphological and immunological characteristics of nanobacteria from human renal stones of a north Indian population. *Urol Res* 32:190-195, 2004
- HJELLE JT, MILLER-HJELLE MA, POXTON IR, et al: Endotoxin and nanobacteria in polycystic kidney disease. *Kidney Int* 57:2360-2374, 2000
- SEDIVY R, BATTISTUTTI WB: Nanobacteria promote crystallization of psammoma bodies in ovarian cancer. *APMIS* 111:951-954, 2003
- KAJANDER EO, ÇİFTÇİOĞLU N, AHO K, et al: Characteristics of nanobacteria and their possible role in stone formation. *Urol Res* 31:47-54, 2003
- BARR SC, LINKE RA, JANSSEN D, et al: Detection of biofilm formation and nanobacteria under long-term cell culture conditions in serum samples of cattle, goats, cats, and dogs. *Am J Vet Res* 64:176-182, 2003
- CISAR JO, XU DQ, THOMPSON J, et al: An alternative interpretation of nanobacteria-induced biomineralization. *Proc Natl Acad Sci USA* 97:11511-11515, 2000
- GARCÍA CUERPO E, KAJANDER EO, ÇİFTÇİOĞLU N, et al: Nanobacteria: Un modelo de neo-litogenesis experimental. *Arc Esp de Urol* 53:291-303, 2000
- EVAN AP, LINGEMAN JE, COE FL, et al: Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. *J Clin Invest* 111:602-605, 2003
- WANG SS, GOOD TA: Effect of culture in a rotating wall bioreactor on the physiology of differentiated neuron-like PC12 and SH-SY5Y cells. *J Cell Biochem* 83:574-584, 2001
- CLEJAN S, O'CONNOR K, ROSENSWEIG N: Tri-dimensional prostate cell cultures in simulated microgravity and induced changes in lipid second messengers and signal transduction. *J Cell Mol Med* 5:60-73, 2001
- NICKERSON CA, OTT CM, MISTER SJ, et al: Microgravity as a novel environmental signal affecting *Salmonella enterica* serovar Typhimurium virulence. *Infect Immun* 68:3147-3152, 2000
- ENGLAND LS, GORZELAK M, TREVORS JT: Growth and membrane polarization in *Pseudomonas aeruginosa* UG2 grown in randomized microgravity in a high aspect ratio vessel. *Biochim Biophys Acta* 1624:76-80, 2003
- ÇİFTÇİOĞLU N, MILLER-HJELLE MA, HJELLE JT, KAJANDER EO: Inhibition of nanobacteria by antimicrobial drugs as measured by modified microdilution method. *Antimicrob Agents Chemother* 46:2077-2086, 2002
- ÇİFTÇİOĞLU N, KAJANDER EO: Interaction of nanobacteria with cultured mammalian cells. *Pathophysiology* 4:259-270, 1998
- BATTAGLIA M, POZZI D, GRIMALDI S, PARASASSI T: Hoechst 33258 staining for detecting mycoplasma contamination in cell cultures: A method for reducing fluorescence photobleaching. *Biotech Histochem* 69:152-156, 1994
- KAJANDER EO, TAHVANEN E, KURONEN I, ÇİFTÇİOĞLU N: Comparison of Staphylococci and novel bacteria-like particles from blood. *Zentralbl Bakteriol (Suppl 26)*:147-149, 1994.
- MILLER-HJELLE MA, HJELLE JT, ÇİFTÇİOĞLU N, KAJANDER EO: Nanobacteria: Methods for growth and identification of this recently discovered calciferous agent, in *Rapid Analytical Microbiology, The Chemistry and Physics of Microbial Identification*, edited by Olson WP, Godalming, Surrey, UK, Davis Horwood International Publishing, Ltd., 2003, pp 297-312
- ÇİFTÇİOĞLU N, PELTTARI A, KAJANDER EO: Extraordinary growth phases of nanobacteria isolated from mammalian blood, in *Instruments, Methods, and Missions for Astrobiology*, edited by Richard B. Hoover, The International Society for Optical Engineering, Proceedings of SPIE (3111) 1997, pp 429-435
- SNEDECOR GW: *Statistical Methods*, 7th ed., Ames, IA, The Iowa State University Press, 1965
- GOLDEN DC, MING DW: Nutrient-substituted hydroxyapatite: Synthesis and characterization. *Soil Sci Soc of Amer J* 63:657-664, 1999
- ZHANG Y, FU T, HAN Y, et al: In vitro and in vivo tests of hydrothermally synthesised hydroxyapatite coating. *Biomol Eng* 19:57-61, 2002
- WHITSON PA, PIETRZYK RA, MORUKOV BV, SAMS CF: The risk of renal stone formation during and after long duration space flight. *Nephron* 89:264-270, 2001
- WHITSON PA, PIETRZYK RA, SAMS CF: Urine volume and its effects on renal stone risk in astronauts. *Aviat Space Environ Med* 72:368-372, 2001
- PIERSON DL: Microbiology, in *Space Physiology and Medicine*, 3rd ed., edited by Nicogossian A, Hontoon C, Pool S, Philadelphia, Lea&Febiger, 1994, pp 157-166
- PIERSON DL, VIKTOROV A: Microbiological investigations of the Mir Space Station and flight crew, in *Phase 1 Research Program Quarterly Research Report*, Houston, NASA JSC, 1998, pp 4/111-4/193
- PIERSON DL, VIKTOROV A: Microbiological investigations of the Mir Space Station and flight crew, in *Phase 1 Research Program Quarterly Research Report*, Houston, NASA JSC, 1999, pp 4/75-4/83
- COGOLI A: The effect of space flight on human cellular immunity. *Environ Med* 37:107-116, 1993
- WOLF L: Bioregeneration in space. *Adv Space Biol Med* 5:341-356, 1996
- WALTHER I, BECHER B, MULLER O, et al: Cultivation of *Saccharomyces cerevisiae* in a bioreactor in microgravity. *J Biotechnol* 47:113-127, 1996
- AKINS RE, SCHROEDL NA, GONDA SR, HARTZELL CR: Neonatal rat heart cells cultured in simulated microgravity. *In Vitro Cell Dev Biol Anim* 33:337-343, 1997
- ZHAU HE, GOODWIN TJ, CHANG SM, et al: Establishment of a three-dimensional human prostate organoid coculture under microgravity-simulated conditions: Evaluation of androgen-induced growth and PSA expression. *In Vitro Cell Dev Biol Anim* 33:375-380, 1997
- FREED LE, VUNJAK-NOVAKOVIC G: Microgravity tissue engineering. *In Vitro Cell Dev Biol Anim* 33:381-385, 1997
- TIXADO R, RICHOLLEY G, GASSET G, et al: Study of minimal inhibitory concentration of antibiotics on bacteria cultivated in vitro in space (Cytos 2 experiment). *Aviat Space Environ Med* 56:748-751, 1985
- ZERWEKH JE: Nutrition and renal stone disease in space. *Nutrition* 18:857-863, 2002