ATTEMPTS FOR DETECTION OF NANOPARTICLES-NANOBACTERIA AND DISTRIBUTION OF THEIR ANTIBODIES IN SAUDI PATIENTS WITH UROLITHIASIS

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Abstract

Calcifying biologic nanoparticles (CNPs) have been identified from diverse tissue samples including kidney stones and calcified aneurysms. Whether or not they represent independent, biologic entities or a form of self-perpetuating biomineralization remains controversial.

In this study, 50 serum samples collected from Saudi patients with urolithiasis and 20 samples collected randomly from healthy individuals were tested for detecting anti- CNP IgG using commercially available ELISA kits. Seven renal stone samples were obtained from National Guard-Health Affairs, King Abdullaziz Medical City, KSA. Each stone sample is divided into two fragments; one fragment was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum under cell culture condition to investigate the living nature of CNPs and the other fragment was used for chemical analysis.

Results indicated that 98% of patients have anti-CNP Abs compared with 20% in healthy individuals. Light Microscopy with DIC optic revealed the absence of any microorganism in the tested culture media and no visible mineralized biofilm were observed in cell culture in any of the seven tested stones. Chemically, 71, 4% (5 out of 7) of tested stones were calcium oxalate and 28, 6% (2 out of 7) were urate.

Keywords: Nanobacteria, urolithiasis, renal stone, CNP, Saudi patients

Introduction

Nanobacteria’’ are mysterious particles that have spurred one of the biggest controversies in modern microbiology (Kajander and Ciftcioglu, 1998; Drancourt *et al*., 2003). First discovered by geologists as 100 nm coccoid particles present on mineral surfaces **(**Folk, 1993**),** such structures were later found in human and cow blood as well as in commercial cell culture serum (Akerman *et al*.,1993). The culturability of ‘‘nanobacteria’’ was then reported by Kajander *et al.,* (1997) who established a link between these particles and kidney stone formation **(**Ciftcioglu *et al*., 1999). In the last few years, these calcifying nanoparticles CNP have been associated with several human diseases including polycystic kidney disease, renal calculi, and chronic prostatitis **(**Kajander, 2006**).** However, despite the various pathological disorders they cause, whether nanobacteria are living or nonliving cells is still under debate (Miller *et al*., 2004).

Kajander and Ciftcioglu (1998) show that a new class of bacteria, designated nanobacteria because of their small size (0.05–0.5 mm in diameter), produce sufficient calcium apatite to initiate pathologic calcification and stone formation. The nanobacteria were discovered in white films sticking to the surfaces of tissue culture vessels containing mammalian cells and media supplemented with bovine serum (Kajander *et al*., 1997). A member of the Proteobacteria family, which includes *Bartonella* and *Brucella* species, the nanobacteria have distinctive properties, including heat resistance and the ability to pass through 0.1-mm sterilization filters. Their most remarkable characteristic is the formation of carbonate apatite crystals at neutral pH and at physiologic phosphate and calcium concentrations. The extracellular mineralization forms a hard protective shelter for these hardy microorganisms, and it enables them to survive conditions of physical stress that would be lethal to most other bacterial species. Although it is not clear exactly how the nanobacteria induce calcification, other bacteria in aqueous sediments have been demonstrated to release oligopeptides that nucleate calcium apatite (Mojzsis *et al*., 1996**).**

Proteobacterial infections are common in cows, and fetal bovine serum is the presumed origin of the tissue culture contaminants. Kajander and Ciftcioglu (1998) have found that more than 80% of fetal bovine serum batches, each pooled from several thousand animals, have nanobacteria, as determined by immunoassay with monoclonal antibodies and by direct culture.

Due to lack of their genomic evidence, CNP are controversial agents as prions were, and critics have proposed hypotheses explaining them as precipitates of proteins or crystals (Cisar *et al* .,2000). Although CNP cause specific infection (Ciftcioglu *et al*., 2007), and are detected in pathological calcification, general debate over their existence continues. The detection of CNP in human urinary stones (Shiekh *et al*., 2006) inspired the hypothesis that CNP might be the initiating agents in the formation of RP and subsequently renal stones.

Approximately 7% of adult men develop renal or bladder stones containing calcium mineral salts (Saklayen, 1997). Life-threatening calcification may occur after hemodialysis, in scleroderma, and in patients with sclerotic aortic valves. The stimuli for the calcium salt deposition in these conditions are unclear, but nidi for precipitation and crystallization are needed even under supersaturation conditions.

CNPs antigens have been reported in 97% of human kidney stones (Ciftcioglu *et al*., 1999; Kajander *et al*., 1997). Apparently, these CNPs surround themselves with a mineral coating, and can serve as nidi for the genesis of renal calculi (Cuerpo *et al*., 2000).

Biochemical and spectral analysis shows that some NPs are composed of proteins, carbohydrates, lipids and nucleic acids (Benzerara *et al*., 2006; Cisar *et al*., 2000; Raoult *et al*., 2008). However, whether NPs contain a unique collection of biomolecules, and whether they possess specific biomolecular and biologic activity, is unknown.

Urolithiasis is the presence of stones in the urinary system, and remains a common public health issue as well as a preventable cause of morbidity. The estimated global prevalence of urolithiasis in developed countries is between 5% and 13%, and, in developing countries, it is 0.5-1%. (Kim *et al*., 2002; Lee *et al*., 2002; Menon, 1992; Ramello *et al*., 2000).

The overall probability of forming stones differs in various parts of the world: 1-5% in Asia, 5-9% in Europe, 13% in North America and 20% in Saudi Arabia (Kim *et al*., 2002; Lee *et al*., 2002; *Ramello et al*., 2000). Sub-tropical and tropical climate and better socio-economic standards of living may predispose to the increased occurrence of urinary stone disease.

The high incidence of urolithiasis in the Gulf is due to an adverse combination of dietary and environmental factors. The highest recorded incidence of upper urinary-tract stones appears to be in the oil-rich states of the Arabian or Persian Gulf, such as the United Arab Emirates (UAE) (Robertson and Hughes., 1994) , Kuwait (Barkworth SA *et al*., 1989) and Saudi Arabia (KSA) **(** Abomelha *et al*., 1990**;** Freeg *et al*., 2012**)**, where the main types of stones consist of calcium oxalate (CaOx) and/or uric acid (UA) **(**Abomelha *et al*, 1990; Barkworth *et al*., 1989**;** William, 2012**)** . On the other hand, the populations of the countries in this region have fewer calcium phosphate (CaP)-containing stones and fewer infection stones than are reported from most Western countries.

Aqel (2008) reported the presence of high incidence of Anti-CNP in Jordanian Patients with urolithiasis with absence of any signs of live microorganism. He also recommended that further studies are required to validate the living nature of CNPs, to establish the exact mechanism by which CNPs are involved in the causation of renal stones, and to assess the role of the anti-CNP Abs distribution as a prediction of any extraskeletal calcification. These recommendations inspired us to conduct similar study among Saudi patients with urolithiasis.

Materials and Methods:

1-Samples:

1.1. Kidney Stones samples

Seven kidney stones obtained from National Guard-Health Affairs, King Abdullaziz Medical City, KSA. One fragments of each stone was used for nanobacterial culture and the other fragment was used to determine its chemical composition.

1.2 Serum samples:

Fifty serum samples (35 from male and 15 from female) were collected from urolithiasis patients with different ages and 20 serum samples were randomly collected from healthy individuals.

2. Serological Assay:

The commercially available Nano-Sero IgG ELISA kits Nanobac Oy, Finland) were used for detecting anti- CNP IgG in serum samples collected from the patients and healthy individuals. All measurements were run in duplicates. The absorbance was read at 450 nm, with the reference wavelength at 650 nm, using an ELISA reader. Anti- CNP Abs units were calculated from the standard curves using the kit standards via a linear equation. The assays were controlled using negative and positive controls. Anti- CNP Abs were classified as negative [unit value ≤2 × (mean of negative control - standard deviation)], borderline positive [unit value >2 × (mean of negative control - standard deviation), but <2 × (mean of negative control)] and positive [unit value ≥2 × (mean of negative control)] (Pretorius *et al.*, 2004).

3. Chemical Analysis of Renal Stones:

Chemical compositions of renal stones were analyzed by standard chemical analytical methods as described by Abboud (2008).

4. Culture Methods for Nanobacteria:

The cultures were prepared using strict aseptic techniques in a cell culture facility. Collected stones were washed with double-distilled water, dried, pulverized using a mortar and pestle, and stored at 4°C in plastic vials. To extract NPs, pulverized stones were demineralized using 1N HCl for 10 minutes with constant stirring, neutralized with 1N NaOH, and centrifuged at 14,000 x *g* for 15 min. The pellet was suspended in serum free DMEM (GIBCO), filtered through a Whatman No. 42 filter, sterile-filtered through a 0.2 μm Millipore filter (Sigma). One ml of suspended pellet inoculated into 25 ml sterile tissue culture flasks (Corning; Corning, NY) containing 10 ml of standard DMEM supplemented with with 10% heat-decomplemented fetal calf serum (Sera-Lab, Crawley Down, Sussex, U.K) under nanobacterial cell culture conditions (37°C; 5–10% CO_2/90–95% air) for 4 weeks. Subculture was done every 14 day by doing a 1:10 dilution in this culture medium.

Controls containing only culture media without sample filtrates were incubated in parallel with the test cultures to determine whether spontaneous precipitation can occur. Every week, cultures were assessed qualitatively by light microscopy with Differential Interference Contrast (DIC) Optics and quantitatively by turbidimetry in Nephelometric Turbidity Units (NTU).

Results

ELISA results for the presence of anti-CNP Abs in the patients serum samples and in healthy individuals are shown in table (1) and table (2) respectively. Neither mineralization nor white biofilm or floccules to the culture flasks was observed during the 6-weeks follow-up after incubation in any of the seven stone samples. In addition, Light Microscopy with DIC optic revealed the absence of any other microorganism in the tested culture media.

Chemical analysis of the renal stones examined indicated that 71, 4% (5 out of 7) stone examined were calcium oxalate and 28, 6% (2 out of 7) were urate.

Table.1. Detection of Anti-NCP antibody in Saudi patients with urolithiasis using Enzyme Linked Immunoassy (ELISA) test.

Sex	Age range /years	Total number	Positive number	Positive %
Male	20- 30	5	5	100%
Male	31- 40	12	12	100%
Male	41-50	15	15	100%
Male	51- over 60	3	3	100%
Female	31- 40	3	2	66.6%
Female	41-50	10	10	100%
Female	51- over 60	2	2	100%
Total number		50	49	(98%)

Table.2. Detection of Anti-NCP antibody in healthy individuals using Enzyme Linked Immunoassy (ELISA) test.

Sex	Age range /years	Total number	Positive number	Positive %
Male	20- 30	2	0	0 %
Male	31- 40	6	1	16.6%
Male	41-50	5	2	40%
Male	51- over 60	2	1	50%
Female	31- 40	3	0	0%
Female	41-50	2	0	0%
Total number		20	4	(20%)

Discussion

Urolithiasis is a multifactorial recurrent disease of world-wide distribution in rural, urban, industrial and non-industrial regions. Changes in urinary pH is a risk factor especially with hyperuricosuria, hypercalciuria or hyperoxaluria. With recurrence, hypercalcuria and higher urinary oxalate levels are more frequent. Hypercalciuria and hyperuricosuria showed correlation with family history of stones (Rabie, 2005).

Several hypotheses regarding the pathogenesis of calcification in soft tissues have been proposed, including: (i) calcium deposit is formed on degrading cells and/or apoptotic bodies (VU *et al*., 1998); (ii) nanobacterial4ilce particles (PLP) surround themselves with sphere-like structures from deposited calcium (Kajander and Ciftcioglu, 1998; Miller *et al*., 2004); (iii) induction of supersaturated calcium liquid is used as a building block for passive calcium sedimentation with or without participation of phospholipids or proteoglycans (Kim, 1997; Poggi *et al*., 2001) and (iv) smooth muscle cells undergo bone-like differentiation and hyper phosphonemia is stimulated by vascular calcification (Giachelli *et al*., 2005).

Aqel (2008) reported the presence of high incidence of Anti-CNP in Jordanian Patients with urolithiasis with absence of any signs of live microorganism. He also recommended that further studies are required to validate the living nature of CNPs and to assess the role of the anti-CNP Abs distribution as a prediction of any extraskeletal calcification. These recommendations inspired us to conduct similar study among Saudi patients with urolithiasis.

In our study, the chemical analysis of the examined stone indicated that the majority of examined renal stones were calcium oxalate (71, 4%) and 28, 6% (2 out of 7) were urate. These results confirmed the findings of previous retrospective study conducted by Freeg *et al*., (2012) on 760 Saudi patients with urolithiasis. They found that the male to female ratio was 5:1; 87 percent of the patients were aged thirty to sixty years and 11 patients were under age fourteen. Seventy-six percent of stones analyzed (239) were calcium oxalate, 20.5 percent urate, and 3.3 percent phosphate.

Consistent with data published by other studies (Aqel, 2008**;** Holmberg, 2001**),** we detected high incidence of anti-CPNs antibody was detected among Saudi patients with urolithiasis (98%) compared with healthy individuals (20%). High anti-NB Abs distribution in both patients and healthy study groups proved high rate of CNPs exposure. CNPs may found in different samples like environmental and animal samples, and may be transmitted directly to human beings (Kajander and Ciftcioglu, 1998; Travis, 1998). Other studies suggest that transplacental or perinatal transmission of CNPs and anti-NB Abs from infected mothers to their babies could be possible (Pretorius *et al*., 2004).

Neither mineralization nor white biofilm or floccules to the culture flasks was observed in our study during the 6-weeks follow-up after incubation in any of the seven stone samples. In addition, Light Microscopy with DIC optic revealed the absence of any other microorganism in the tested culture media and this may indicate the absence of living nature of CPNs. A significant controversy has erupted over the existence and significance of CNPs as living or non living particles (Abbott, 199; Abbott, 2000; Ciftcioglu, *et al*., 1997; Ciftcioglu *et al*., 2006; Drancourt *et al*., 2003).

The culturability of ‘‘nanobacteria’’ was then reported by Kajander’s team (1997) who established a link between these particles and kidney stone formation (Ciftcioglu *et al*., 1999). The data described by Cisar’s group reached completely opposite conclusions as Kajander’s original assertion considering nanobacteria as living microorganisms (Cisar *et al*., 2000). In contrast to what would be expected from growth of a living entity, Cisar *et al*., (2000) failed to detect nucleic acids and suggested that observed biomineralization may be initiated by non living macromolecules generating self propagating microcrystalline apatite.

Consistent with data published by Cisar *et al*., (2000) and Raoult *et al*., (2008) failed to clearly demonstrate the presence of nucleic acids in nanons. Indeed, they observed discrepant results using various nucleic acid stains, such as nanons being easily stained by orange acridine but poorly stained by DAPI and Hoechst 33342. Also, the growth of nanons was not altered in presence of either DNAse or RNAse. Finally, 16S rRNA gene amplification and sequencing most often identified aproteobacteria and c-proteobacteria, both known to be waterborne contaminants in PCR-based experiments (Borst *et al*., 2004). It is thought that previously reported 16S DNA amplifications by PCR using ‘‘nanobacteria’’ as template result from PCR artifacts (Cisar *et al*., 2000; Pitcher and Fry, 2000). These data led us to hypothesize that nanons might have the ability to trap any contaminant 16S rDNA fragment present in the medium or environment rather than displaying original sequences from an emerging microorganism. All together, the data suggest that the nanon is a nucleic-acid free, transferable biological entity.

More conflicting results have been reported concerning the bacterial culture succeed, Khullar *et al*., (2004) successfully cultured 40 different renal stones from patients with nephrolithiasis. In contrast, Drancourt *et al*., (2003) failed to culture CNPs from 10 upper urinary tract stones.

In a recent study conducted by our colleague in Egypt **(**K. Abo-El-Sooud *et al*., 2001**),** they detected nanoparticles in four of eight stone examined using scanning Electron microscope and transmission electron microscope.

Conclusion

Our studies indictaed that the majority of renal stone Ca oxalate with the presence of high incidence of Anti-CNP antibodies in Saudi patients with urolithiasis. However, no CNPs or bacterial growth was detected.

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