JJBS

Volume 1, Number 4, December. 2008
ISSN 1995-6673
Pages 147- 152

Jordan Journal of Biological Sciences

Attempts for Detection of Nanoparticles-Nanobacteria and Distribution of their Antibodies in Jordanian Patients with Urolithiasis

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Abstract

In 1998, calcifying nanoparticles (CNPs) or nanobacteria was proposed as an explanation of certain kinds of pathologic calcification and stone formation. In the present study Enzyme Linked Immunosorbent Assay (ELISA), bacterial culturing, and staining techniques were used to investigate the incidence of calcifying nanoparticles IgG antibodies (anti-CNP Abs) in serum of Jordanian patients with urolithiasis and the living nature of these CNPs in the renal stones. Serum samples from 65 patients and related 20 healthy individuals were tested for anti-CNP Abs. Renal stones retrieved from the kidneys of 20 patients were processed and subjected to mammalian cell culture conditions, then bacterial growth and staining were observed from these cultures. Results revealed detection of anti-CNP Abs in 96% of patients and in 40% of healthy individuals. Although high anti-CNP Abs incidence were correlated strongly with the presence of CNPs and urolithiasis, no CNPs or bacterial growth was detected - following the applied staining and turbidity methods, which may reflect the non living nature of such particles. The findings of this study can be used as a tool for early prediction of kidney stone formation.

الملخص

في عام 1998 تم اكتشاف الجزيئات المتكلسه المتناهيه في الصغر أو الجراثيم المتناهية في الصغر(النانوبكتيريا). وارتبطت هذه الجزيئات المتكلسه ارتباطا مباشرا بتكوين حصى الكلى وحدوث الأمراض المزمنة ذات الطبيعة التكلسية. في هذا البحث تمت دراسة هذه الجزيئات باستخدام الطرق المناعية لتحديد الأجسام المضادة لها في عينات مصل المرضى كما تم زراعه عينات حصى الكلى في الأوساط الزراعية الخلوية لتحديد الطبيعة الحية لتلك الجزيئات المتكلسه. تم جمع 65 عينه دم من المرضى الأردنيين المصابين بحصى الكلى وكذالك 20 عينه دم من أناس أصحاء لاستخدامها كعينة سيطرة. تم فحص المصل لتلك العينات لتحديد الأجسام المضادة ضد تلك الجزيئات. كما تم جمع 20 عينه حصى كلى وتمت زراعتها في أوساط خلوية وتم مراقبه تلك الزروعات ومحاولة صباغتها لتحديد أي نمو بكتيري. تم تحديد الأجسام المضادة لتلك الجزيئات المتكلسه في 96% من عينات المرضى وفي 40% من عينات السيطرة. لم نتمكن من تكثير تلك الجزيئات المتكلسه في الأوساط الخلوية ولم تعطي محاولات الصباغة أي دليل لنمو بكتيري في تلك الزروعات. بالرغم من عدم زراعة تلك الجزيئات المتكلسه في الأوساط الخلويه إلا أن النسبه العاليه للأجسام المضاده المكتشفه لها في عينات المرضى وحتى في عينات الأصحاء تعكس الرابط القوي على وجود تلك الجزيئات والإصابه بالأمراض المختلفه ذات الطبيعه التكلسيه. ان نتائج هذه الدراسه تبين امكانية استخدام تحديد الأجسام المضاده في عينات المرضى لتلك الجزيئات كوسيله للتشخيص المبكر للأمراض المختلفه ذات الطبيعه التكلسيه مثل تكون حصى الكلى.

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***Keywords*:** Nanobacteria; Antibodies; Tissue Culture; Urolithiasis; Jordan

1. Introduction

Calcifying nanoparticles, or so called nanobacteria, were isolated and named by the Finnish researcher Olavi Kajander and the Turkish researcher Neva Ciftcioglu, working at the University of Kuopio in Finland (Kajander and Ciftcioglu, 1998). According to the researchers, the particles are self-replicated in microbiological culture, and the researchers further reported having identified DNA in these structures by staining (Carson, 1998). According to Kajander and Ciftcioglu, these are the smallest known self-replicating bacteria, and they are about 20–200 nm in length. CNPs are phylogenetically close relatives of mineral forming bacteria (Kajander et al., 1997; Kajander and Ciftcioglu, 1998). CNPs are thought to play an important role in extraskeletal calcifying diseases including periodontal stone formation, urolithiasis, atherosclerosis, chronic pancreatitis, rheumatoid arthritis, and various other tissues in the body (Carson, 1998). Apparently, these particles surround themselves with a mineral coating, and can serve as nidi for the genesis of renal calculi (Khullar et al., 2004; Ciftcioglu et al., 1999). Cuerpo (2000) showed that when these CNPs were injected intravenously, they accumulated in the kidney and produced apatite. The Finnish scientists

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who first discovered these CNPs have suggested that they are the *Helicobacter pylori* of kidney stone disease (Kajander et al., 1998; Ciftcioglu, et al., 1998).

Epidemiological studies have shown that only 10–20% of patients with renal stones have predisposing factors such as anatomical defects, metabolic or genetic disorders, and bowel disease (Parks and Coe, 1996). Others who develop stones due to any unknown cause are referred to as idiopathic stone formers. The progression of events leading to stone formation begins with urine supersaturation, crystal nucleation and aggregation, bringing about retention of crystals (nidi) and continued growth on the retained crystals (Menon and Resnick, 2002). Although the stimuli for calcium salt deposition are not completely known, it has become clear that nidi are needed for precipitation, even under supersaturated conditions.

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

Urolithiasis in Jordanian population has increased in the last decade with high recurrence (Dajani et al. 1981; Rizvi et al. 2002). Local data indicates that urolithiasis in Jordanians may developed during their life without any known etiological factors (Freundlich et al. 1982; Dajani et al. 1988).

The present study is conducted to assess the incidence of the anti-CNP Abs in Jordanian patients, and to investigate the living nature of these CNPs by attempted isolation of them in cell co-culture from the collected renal stones.

2. Material and Methods

2.1. Patients and Samples Collection

Serum samples were collected from patients who were receiving medication at the Urology department, Islamic hospital, Amman, Jordan during the years 2006-2007. The healthy group included individuals undergoing routine checkup with no other known diseases. Individuals who were included in this study are from the same geographical area, all are resident in the central of Jordan, with close mean age and same risk and environmental factors (Table 1). Patients who had undergone operative procedures such as pyelolithotomy, extended pyelolithotomy and/or nephrolithotomy for the removal of renal stones were also included in the study. Surgically removed calculi from 20 patients with renal stones were collected. The stones were analyzed for their chemical composition by standard chemical analytical methods. (Abboud IA, 2008)

2.2. Enzyme Linked Immunosorbent Assay (ELISA)

Eighty five unrelated serum samples were collected, including 65 patients with urolithiasis [mean age (45±10)] and 20 healthy individuals [mean age (40±10) years] (Table 1). Diagnosis of urolithiasis was determined depending on the clinical presentation, physical examination, and also confirmed with radiological tests.

Duplicate of 100-µL diluted (1:500) serum samples were used to detect anti-CNP Abs with the Nano-Sero IgG ELISA kit (Nanobac OY, Finland). The absorbance was read at 450 nm, with the reference wavelength at 650 nm, using an ELISA reader (Sunrise Tecan, Austria). 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).

Table 1:Characteristics of the Study Population.

Variables	Study Participants	
	Patients Samples	Healthy Samples
Total number	65 (100.0)[a]	20 (100)
Gender		
Male	45 (69.2)	15 (75)
Female	20 (30.8)	5 (25)
Age (years)		
Mean	45±10	40±10
Range	20-75	20–70
Age groups (years)		
20–29	5 (7.7)	2 (10)
30–39	9 (13.8)	6 (30)
40–49	20 (30.8)	6 (30)
50-59	29 (44.6)	5 (25)
60 and over	2 (3.1)	1 (5)

[a] Numbers in parentheses are percentages.

2.3. Bacterial Cell Co-Culture

The stone samples were processed for the culture of CNPs according to Drancourt (2003), Khullar (2004) and Miller (2004). The stones were demineralised by incubation in 1 M HCl and neutralized with 1 M NaoH, (pH 10.5, Sigma) and the solutions were centrifuged at 20,000 *g* for 30 min at 4°C in a Sorvall RC5B centrifuge (GMI, Inc. USA). The pellet was suspended in serum free RPMI 1640 (Euroclone, Milan), filter sterilized through 0.2 µm Millipore filters (Sigma) and 1 ml of the filtrate were inoculated in screw-cap flasks containing 10ml of RPMI 1640 with 10% fetal bovine serum (FBS) (Gibco corp, USA). Cultures were incubated in CO_2 incubator (Heraeus, Germany) at 37°C, 5% CO_2 and 95% air for four weeks. Subcultures were carried out after 4 weeks of initial inoculation and subsequently after every 15 days. The cultures were aseptically precipitated by centrifugation at 20,000 g for 45 min at 4°C, washed with sterile phosphate buffered saline (PBS, pH 7.2), and then examined for bacterial growth by measuring the turbidity at 520 nm and 700 nm, using spectrophotometer (Hinotek, China) over a period of 6 weeks. RPMI with 10% FBS inoculated with no stone filtrate was used as a negative control. (Khullar, et al., 2004)

2.4. Bacterial Staining

Gram staining for the culture precipitant was done with a commercially available kit for any visible microorganisms (Wescor Inc., USA). Stained bacteria were observed under the oil immersion lens using the compound light microscope (Volker's optical, Germany).

Table 2. Anti CNP Antibodies in 65 Patients Samples.

Code	Antibody	Age	Sex	Code	Antibody	Age	Sex
1	0.36†	46	F	33	0.36†	55	M
2	0.56†	39	M	34	0.22*	56	M
3	1.37†	35	M	35	0.52†	56	F
4	0.80†	32	M	36	0.40†	51	M
5	0.75†	52	M	37	0.46†	52	M
6	0.59†	47	F	38	0.31†	50	M
7	0.38†	33	F	39	0.72†	52	M
8	0.29*	37	M	40	0.86†	53	M
9	0.64†	39	M	41	0.85†	55	M
10	3.38†	75	M	42	0.49†	51	M
11	0.66†	52	M	43	0.22*	53	M
12	0.29*	34	M	44	0.66†	54	M
13	0.62†	52	M	45	0.48†	59	M
14	0.73†	20	F	46	0.40†	56	M
15	0.36†	30	M	47	0.53†	53	M
16	0.49†	35	F	48	0.43†	22	M
17	0.40†	40	F	49	0.35†	46	M
18	0.41†	62	F	50	0.36†	45	M
19	0.42†	48	F	51	0.32†	47	M
20	0.15	23	F	52	0.50†	40	M
21	0.28*	53	F	53	0.56†	49	M
22	0.62†	55	M	54	0.85†	27	F
23	0.78†	40	M	55	0.82†	24	F
24	0.75†	52	M	56	0.33†	44	M
25	0.53†	58	M	57	0.56†	44	F
26	0.57†	51	M	58	0.19	45	M
27	0.83†	50	M	59	0.26*	58	F
28	0.82	43	M	60	0.27*	50	M
29	0.46†	46	F	61	0.85†	55	F
30	0.25*	47	M	62	0.32†	44	F
31	1.11†	55	M	63	0.25*	48	M
32	0.98†	47	F	64	0.69†	55	F
				65	0.24*	45	M

Antibodies were detected using enzyme-linked immunoassay (ELISA) kits and units were calculated from duplicate determinations. No symbol, negative results; †, positive results;*, borderline positive results; M, male; F, female.

Table 3. Anti CNP Antibodies in 20 Healthy Samples.

Code	Antibody	Age	Sex
1	0.13	21	M
2	0.40†	39	M
3	0.29*	22	M
4	1.42†	42	M
5	0.22*	40	M
6	0.16	52	M
7	0.38†	57	M
8	0.32†	36	M
9	0.13	43	M
10	0.08	51	M
11	0.11	33	M
12	0.12	40	M
13	0.15	34	M
14	0.18	42	M
15	0.12	38	M
16	0.32†	42	F
17	0.12	36	F
18	0.41†	55	F
19	0.16	51	F
20	0.19	70	F

Antibodies were detected using enzyme-linked immunoassay (ELISA) kits and units were calculated from duplicate determinations. No symbol, negative results; †, positive results;*, borderline positive results; M, male; F, female.

3. Results

3.1. Chemical Analysis of The Renal Stones

Renal stones that were included in this study were analyzed chemically by standard chemical analytical

methods. 12 stone samples out of 20 (60%) were calcium oxalate stones, and they formed the majority,6 (30%) were uric acid/urate, and only 2 (10%) were phosphatic.

3.2. CNP Antibodies

ELISA readings for the presence of anti-CNP Abs in the patients serum samples indicated that 53 out of 65 samples (81%) were positive, and 10 (15%) were border line, and 2 (3%) were negative (Table 2). For the healthy individuals 6 out of 20 samples (30%) had detectable anti-CNP Abs, 2 border line (10%), and 7 were negative (60%). (Table 3)

3.3. Bacterial Culture and Staining

Cell co-culture and Gram staining of inoculated RPMI 1640 medium showed no growth and detection of CNPs in any of the 20 stone samples and after one week of incubation. In addition, spectrophotometric of turbidity measurements for bacterial growth revealed the absence of any other microorganism in the tested culture media.

4. Discussion

Urolithiasis is a common disorder responsible for serious human suffering and economic cost to society. Approximately 16% of men and 7% of women in Jordan will be diagnosed with urolithiasis at some time in their life with a recurrence rate of more than 30% in 5 years (Personal communications and Bani Hani et al., 1998), this is compatible with other international data (Lloyd et al., 1996).

Even if some risk factors are defined for stone formation, none of them can fully explain the etiopathogenesis. In this study (data not shown), kidney stones were removed from patient less than 10 years of age, also other cases with urolithiasis form Jordanian children and teenagers are diagnosed and treated by surgical removal in spite of the ambiguous cause of such stone formation in their urinary tract (Dajani et al., 1988). Urolithiasis in young patients may be considered as a strong evidence of biological cause of stones formation.

The incidence of anti-CNP Abs was examined, and an attempt to culture these CNPs from Jordanian patients with urolithiasis was also done. Despite the fact of strict application of the methods described previously (Drancourt et al., 2003; Khullar et al., 2004; Miller et. al., 2004), cultures of CNPs were not obtained from kidney stones. The study therefore wonders if there is a culture parameters not mentioned in publications that could explain discrepancy between our results and those previously reported. However, a significant controversy has erupted over the existence and significance of CNPs as living or non living particles (Abbott, 1999; Abbott, 2000; Drancourt et al., 2003).

It has been suggested that biomineralization attributed to CNPs may be initiated by nonliving macromolecules like phospholipids and by self-propagating microcrystalline apatite (Cisar et al., 2000). Alternatively, the formal possibility exists that our specimens simply did not contain any living substances, and so the living nature of the CNPs is remain unresolved. Conflicting results have been reported concerning the bacterial culture succeed, Khullar (2004) successfully cultured 40 different renal stones from patients with nephrolithiasis. In contrast, Drancourt (2003) failed to culture CNPs from 10 upper urinary tract stones.

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).

High detection of anti-CNP Abs in Jordanian population (96% of patients and in 40% of healthy individuals) was correlated positively with urolithiasis. In addition, anti-CNP Abs level was inversely correlated with the severity and the recurrence of many other extraskeletal diseases (Kajander and Ciftcioglu, 1998). CNPs as a cause of urolithiasis are not assessed in Jordanian hospitals. To the best knowledge of the researcher, this is the first observational clinical study to demonstrate the incidence and to distribute anti-CNPs Abs in Jordanian population with urolithiasis.

The scale of the urolithiasis epidemic in Jordan provides many opportunities to study the impact of the

etiology. The source of CNPs acquisition and the mode of transmission of such particles are still unknown. The environmental source is the most possible rout of obtaining CNPs in Jordanian patients, and must be studied further.

Further tests like electronic microscopy, histochemistry staining, and PCR analysis must be performed for CNPs co-culture plates in order to confirm the presence or absence of any bacterial growth.

Results presented in this pilot study could have therapeutic relevance and links between CNPs and urolithiasis, especially in individuals with family history for kidney stones formation.

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.

Acknowledgments

This research was supported by a grant (1/1/9/331/2005) from the Deanship of Research and Graduate Studies, Zarqa Private University, Zarqa, Jordan. The technical work assistance by Heba Alakhras and Sinan Nassar and editing of the manuscript by Dr. Wafa Abu Hattab is highly appreciated.

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