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Research Strategies for Treatment of Nanobacteria

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Abstract: Background: Modern medicine strives to get an efficient treatment for Nanobacteria (NB) as it highly treatment-resistant, persisters, dormant forms and biofilms containing hydroxyl apatite or carbonate. **The context and purpose:** To demonstrate the different steps for management of nanobacteria that plays an important role in extraskeletal calcifying diseases. As the treatment protocol started with the dissolution of calcified shells using substances like liquid zeolites and fulvic acid, which get in between the molecular bonds and thus compromise the shell's structure. This is followed up by sessions with chelating agents as Ethylene-Diamine-Tetra-Acetic acid (EDTA) and/or Dimethlyl Sulfoxide (DMSO) to further weaken that troublesome shell. **Conclusions:** we discuss diverse trials for inhibition and treating of NB *in vivo* and *in vitro* with the anti-nanobacterial agents. Furthermore, the treatment of extraskeletal calcifying diseases caused by NB with chemotherapeutic agents and natural herbs will be considered. Finally, we emphasize some new trends for suppression of NB as photomedicine (light and Laser), irradiation and optical nanoparticles.

Key words: Nanobacteria, anti-nanobacterial agents, herbal medicine, photomedicine

INTRODUCTION

Nanobacteria (NB) or so called calcifying nanoparticles, 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 Kajander and Ciftcioglu, the particles are self-replicating bacteria and the smallest described bacteria to date, with dimensions of 20-200 nm in length. Furthermore, these organisms were found to produce a biofilm containing hydroxyl apatite or carbonate, preventing their effective staining. NB 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, the biliary tract in patients with cholecystitis (Wen *et al*., 2005), inclusions of psammoma in ovarian cancers (Hudelist *et al*., 2004), peripheral blood from healthy subjects and atheromatous plaques (Bratos-Perez *et al*., 2008). NB are thought to play an important role in extraskeletal calcifying diseases including stones formation, urolithiasis and polycystic kidney disease (Kajander *et al*., 2003), atherosclerosis (Jelic *et al*., 2007), periodontal disease (Ciftcioglu *et al*., 2003), rheumatoid arthritis (Cassell, 1998) and prostatitis (Bock *et al*., 1989; Geramoutsos *et al* ., 2004). 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). Two strains, one of *Nanobacterium sanguineum* and the other of *Nanobacterium* sp., were isolated from kidney stones and human and bovine sera, respectively Kajander *et al* . (1997). NB are so hard to remove and treat because of their calcified shells, outer coverings made of heavy, resistant substances (protective shell) that keep the NB inside from the purging drugs and immune system. Nanobacteria cannot be killed by penicillin, cephalosporins, marolides and most other antibiotics, heat under 196 F, freezing, dehydration and gamma radiation under 150 Mrad (Demir, 2008). In this review, we discuss the different trials for inhibition and treating NB with various chemotherapeutic and naturally product agents.

DIFFERENT PHASES FOR GENERAL OF NANOBATERIAL THERAPY

Microbes, their toxins or their shed components may contribute to pathological calcifications in several ways. They may (1) damage cellular membranes, resulting in exposure of tissue components capable of forming

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crystallizing nidi (Wiessner *et al*., 2001) or (2) alter local levels of calcium and phosphate in tissue to saturating concentrations that in turn promote crystal formation on available nidi; (3) the microbe may be calcified directly (Streckfuss *et al*., 1974; Vogel and Smith, 1976; Van Dijk *et al*., 1998) or (4) microbial components may interact with tissue components to form complexes that are hybrid nidi. NB or its fragments may be nidi, but they are not necessarily the only nidi for the formation of pathological calcifications (Garcia Cuerpo *et al*., 2000). As microbial components are known to bind to apatite (Berry and Siragusa, 1997), NB may also contribute directly to the primary pathogenesis of disease by acting as a system for the delivery of microbial and other toxins to tissues (Akerman *et al*., 1997; Kajander *et al*., 2001), a process that would require endocytosis (Ciftcioglu and Kajander, 1998). Future research is required to determine the classical and potentially novel mechanism(s) by which drugs inhibit the growth of NB, alter the morphology of NB and affect the genesis of diverse types of microbial and tissue calcifications.

Dissolution of calcified shells of NB: The first step of the anti-nanobacteria phase is to weaken the calcified shells using substances like liquid zeolites and fulvic acid, which get in between the molecular bonds and thus compromise the shell's structure. This is followed up by sessions with Ethylene-Diamine-Tetra-Acetic Acid (EDTA) and/or Dimethlyl Sulfoxide (DMSO) to further weaken that troublesome shell.

Chelation therapy: EDTA is chelating calcium, copper and iron, high blood and tissue concentrations of which are suspected to promote atherogenesis through oxidative stress. EDTA chelates and removes via urine other poisonous heavy metals which may promote atherogenesis. Interestingly, very high-dose (3 g day^{-1}) oral EDTA or subcutaneous EDTA-magnesium therapy have been reported to reduce cholesterol content in hypercholesterolemic rabbits (Uhl *et al*., 1992; Evans *et al*., 2001). Lipid modulating effects of EDTA are also supported by the present findings: comet therapy improved blood lipid patterns in Coronary Artery Disease (CAD) patients even under statin therapy. Increased activity of matrix metalloproteinases has been implicated in atherosclerosis in several ways. Metalloproteinase activity is dependent on zinc and calcium ions (Sierevogel *et al*., 2003). Both tetracycline and EDTA inhibit matrix metalloproteinases. EDTA and tetracycline also inhibit oxidative enzymes and act as antioxidants, even reducing experimental ischemic and reperfusion lesion sizes. EDTA has strong inhibitory action on blood clotting. EDTA may inhibit many calcium-mediated signaling pathways directly or indirectly via changes in the concentration of extracellular ionized calcium affecting function of calcium channels in cell membrane. One such target is immunological activation, another is smooth muscle contraction, both of importance, e.g., in coronary angina. All these action mechanisms could be pharmacologically important in atherosclerosis. Novel rectal administration of EDTA has been shown to result in high blood EDTA levels sustained for a long time. Furthermore, the contributory effects of the oral powder component should be evaluated. It contained several antioxidants, vitamins, amino acids among other agents. Further studies are needed to delineate its actions and targets.

Drug therapy: Carson (1998) studied on the effects of drugs on the growth and morphology of NB within the larger context of microbes as provocateurs of soft tissue calcifications, lesions that occur in a surprisingly wide array of important diseases. He found that when drugs altered the morphology of NB, there was a loss of (1) electron density, (2) coccobacillary shape and (3) defined borders. Exceptions were enlarged NB observed with nitrofurantoin and the amorphous debris and paucity of residual NB observed with inhibitory concentrations of tetracycline and ampicillin. The relatedness, if any, of the findings for this test system for the detection of inhibition of NB to the reported drug-induced effects on the viability and morphology of classical bacteria and fungi is yet to be determined (Davis *et al*., 1997; Mintz and Fives-Taylor, 2000; Yokochi *et al*., 2000). Regarding the test system for detection of the inhibition of NB described here, our earlier investigations showed that NB reach the log period of multiplication within a month if the *A*650 of the initial inoculum density was lower than 20 (turbidity equivalent to that of a 0.5 McFarland standard). The inoculum density of the NB used in this inhibitory test allowed us to obtain logarithmic growth over the 14-day test period. Previous work also demonstrated that the absorbance of NB grown in the presence of Fetal Bovine Serum (FBS) is due to an increase in the number of NB and not an increase in the mass of each NB particle (Ciftcioglu and Kajander, 1999). In the present study, Transmission Electron Microscopy (TEM) of the negative control showed no evidence of protein precipitation or classical crystal formation (Miller, 1998), thus discounting these factors as being responsible for the changes in absorbance. Furthermore, if protein precipitation had occurred, an increase in absorbance of the negative control (Dulbecco's modified Eagle's medium (DMEM) plus 10% gamma-irradiated FBS) should have occurred.

Use of absorbance to monitor the growth of NB is preferred because NB can exhibit clumping, making particle counting by flow cytometry unreliable and scanning electron microscopy SEM laborious. In this study, NB was inhibited *in vitro* at clinically achievable levels in serum and urine (Garrison, 2000) by ampicillin, trimethoprim, trimethoprim-sulfamethoxazole, nitrofurantoin (a urinary antiseptic) and tetracycline HCl. It is commonly known that ampicillin inhibits bacterial cell wall synthesis, but like some other penicillins, it is also a calcium chelator (Crossland, 1970). The inhibition by ampicillin may also have been influenced by the lack of detectable β-lactamase in NB and the somewhat zwitterionic nature of ampicillin that enables it to penetrate the cell walls of gram-negative bacteria (Livermore and Williams, 1996). Trimethoprim, trimethoprim-sulfamethoxazole and nitrofurantoin are reported to inhibit protein and DNA syntheses; we did not find reports of calcium chelation activities for these drugs.

Tetracycline is reported to inhibit bacterial protein synthesis, chelate calcium and inhibit metalloproteinases, a property of potential use in the treatment of osteoarthritis, periodontitis and cancer (Hartzen *et al*., 1997). Tetracycline is already used in the treatment of some periodontal diseases and dental stone formation (Ryan *et al*., 1996).

NB have been isolated from human dental stones (Ciftcioglu *et al*., 1998). There was a difference in the *in vitro* activity of tetracycline HCl (MIC, 1.95 μg mL^{-1}) and that of doxycycline (MIC, 62.5 μg mL^{-1}) against NB. Although doxycycline is more highly protein bound and approximately 10 times more lipophilic than tetracycline HCl (Cunha *et al*., 1982), their activities against NB observed *in vitro* correlated with their comparative levels of calcium binding. The level of chelation of tetracycline to calcium (40%) is reported to be twice that for doxycycline (19%) (Von Wittenau, 1968). The aminoglycosides are primarily known as inhibitors of protein synthesis but more recently it has been recognized that they displace cell biofilm-associated calcium and magnesium that link polysaccharides of lipopolysaccharide molecules (Peterson *et al*., 1985). Gentamicin, kanamycin and neomycin did not block the multiplication of NB. However, gentamicin caused a reduction in the amount of putative biofilm surrounding NB.

NB are positive by the differential *Limulus* amoebocyte lysate assay (Hjelle *et al*., 2000) but the lipopolysaccharide of NB has not been sufficiently characterized to allow further speculation regarding the observed lack of activity of these antibiotics.

IN VITRO INHIBITION OF NB BY ANTIMICROBIAL DRUGS

Ciftcioglu *et al*. (2002) tested 16 classes of antimicrobial drugs for their abilities to inhibit the *in vitro* multiplication of NB, found in human kidney stones and kidney cyst fluids from patients with Polycystic Kidney Disease (PKD). They describe a modified microdilution inhibitory test that accommodates the unique growth conditions and long multiplication times of NB for 14 days in cell culture medium. Bactericidal or bacteriostatic drug effects were distinguished by subsequent subculture in drug-free media and monitoring for increasing absorbance. NB isolated from Fetal Bovine Serum (FBS) were inhibited by tetracycline HCl, nitrofurantoin, trimethoprim, trimethoprim-sulfamethoxazole and ampicillin at levels achievable in serum and urine; all drugs except ampicillin were cidal. Tetracycline also inhibited multiplication of isolates of NB from human kidney stones and kidney cyst fluids from patients with PKD. The other antibiotics tested against FBS-derived NB either had no effect or exhibited an inhibitory concentration above clinically achievable levels; the aminoglycosides and vancomycin were bacteriostatic. Antibiotic-induced morphological changes to NB were observed by electron microscopy. Bisphosphonates, aminocaproic acid, potassium citrate-citric acid solutions and 5-fluorouracil also inhibited the multiplication of NB in a cidal manner.

It is not clear that inhibition of the growth of NB requires chelation. Calcium binding may contribute to the effects against NB of the potassium citrate and citric acid mixture (Hjelle *et al*., 2000) and some of the antimicrobics and other drugs tested. However, the nonchelators nitrofurantoin, 5- fluorouracil and aminocaproic acid were active against NB. Conversely, ciprofloxacin, a known chelator, was not active. The classical mechanisms of action of these drugs against NB would be consistent with reports that NB contain protein, DNA, apatite and muramic acid (Kajander *et al*., 1994) and are positive for endotoxin by the differential *Limulus* amoebocyte lysate assay and by immunoblotting. Of course, drugs effective against NB *in vitro* may act via nonclassical mechanisms. New mechanisms of antibiotic action are increasingly appearing in the literature, such as (1) gentamicin's effect on ribosomes to correct the enzyme deficiency that causes Hurler's syndrome in cultured fibroblasts from patients (Kajander *et al*., 1994) and (2) the discovery that a genetically engineered live-attenuated human immunodeficiency virus will reproduce only in the presence of doxycycline (Verhoef *et al*., 2001).

NB are sensitive *in vitro* to tetracycline and its action is increased by EDTA dissolving NB apatitic protective coat (Ciftcioglu *et al*., 2002). Thus, combination of these drugs might offer a novel treatment for calcific atherosclerotic disease. The present trial treatment regimen also included oral powder containing EDTA plus amino acids, vitamins and proteins to support the EDTA-tetracycline therapy and to elicit beneficial effects on known risk factors for heart disease.

Shoskes *et al*. (2005) developed a treatment to eradicate calcification formed by NB in the etiology and symptoms of Category III chronic prostatitis/Chronic Pelvic Pain Syndrome (CPPS). It consists of an antibiotic (tetracycline), a nutraceutical that purportedly allows the antibiotic to penetrate the stone and a suppository containing EDTA to dissolve the stone. Although, this therapy warrants further study for the placebo effect and to explore the role of nanobacterial infection as a cause of prostatic stones and the role of prostatic stones.

HERBAL MEDICINE IN TREATMENT OF NB

Hadjzadeh *et al*. (2007) stated that ethanolic extract of *Nigella sativa* seeds had a preventive effect on (calcium oxalate) CaOx calculus formation in the kidney of rats due to ethylene glycol consumption. The ethanolic extract also decreased the number of CaOx calculi in the treated group by 57%. Aglichon and flavonoids (qurecetin and kaempferol) which are present in black seeds have strong antioxidant and scavenging effects; thus, it may be suggested that the preventive and disruptive effects of black seeds on CaOx calculi are attributed to these mechanisms (Comalada *et al*., 2006). It has been reported that CaOx calculi such as struvite calculi may have a bacterial origin such as NB (Kramer *et al*., 2000). Black seeds also have antibacterial effects and therefore, may be effective in this mechanism of CaOx calculus formation (Hanafy and Hatem, 1991).

Various researchers have shown that garlic extracts exhibit a wide spectrum of antibacterial activity against gram-negative and gram-positive bacteria including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus* and *Clostridium*. Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic. Research shows that garlic extracts are effective against *Helicobacter pylori*, the cause of gastric ulcers. Garlic extracts can also prevent the formation of *Staphylococcus enterotoxins A*, *B* and C1 and also thermonuclease. Researchers Cavallito and Bailey (1944) were the first to demonstrate that the antibacterial action of garlic is mainly due to allicin. Interestingly, allicin has also been proven to be effective against various bacterial strains resistant to antibiotics such as Methicillin Resistant Staphylococcus Aureus (MRSA) as well as other multi-drug-resistant enterotoxicogenic strains of *Escherichia coli*, *Enterococcus*, *Shigella dysenteriae*, *S. flexneni and S. sonnei* cells (Hughes and Lawson, 1991; Yamada and Azuma, 1997).

TREATMENT OF UROLITHIASIS (KIDNEY STONES)

The relationship between urinary infections and stone formation has been recognized since ancient times and it has been over a century since bacterial degradation of urea was postulated to cause stones. Specific therapy for urease-producing bacteria, such as urease-inhibitors and antibiotics, has allowed for treatment for this subset of urinary stones. Future directions for research include development of novel urease-inhibitors and chemicals to enhance the protective glycosaminoglycan layer. An improved understanding of the pathogenesis of calcium-based stones has led to the discovery of potential roles for nanobacteria and *Oxalobacter formingenes* (Rahman *et al*., 2003).

The use of catheters, both urethral and ureteral, is common in the urinary tract and is associated with significant morbidity, primarily from associated infections. Catheters to prevent bacterial colonization and formation of biofilms have been created using various coatings, including ciprofloxacin, hydrogel and silver. Use of these types of catheters may minimize infections and encrustation inherent with their placement in the urinary tract.

TREATMENT OF PROSTATITIS

The standard treatment for chronic bacterial prostatitis involves a 1-3 months course of prostate-penetrating antibiotics such as fluoroquinolones, trimethoprim-sulfamethoxazole (Septra), or trimethoprim (Protoprim). The cure rate is 60-80% with fluoroquinolones and about 30-50% with Septra and Protoprim. Overall, it is estimated that about one-third of category II patients have recurrences after a seemingly successful first treatment. It is not clear why this is but there is some speculation that stones (calculi) and other debris lodged in the ducts of the prostate may prevent the antibiotics from reaching and completely eliminating the infectious bacteria (Nickel *et al*., 1999). It is also recommended that additional tests (ultrasound, CT scans, MRI) be performed in order to determine if an underlying cause can be found and eliminated (Habermacher, 2006).

It is very important to supplement with live probiotics (*L. acidophilus*, *L. bifidus*, *L. casei*, etc.) during and for a couple of months after treatment with antibiotics, especially the broad-spectrum ones like fluoroquinolones. These antibiotics destroy the normal flora in the gut (large intestine) and their use can result in a nasty case of candidiasis which can be very difficult to eradicate (Murray and Pizzorno, 1998).

HERBAL TREATMENT FOR PROSTATITIS

Quercetin is a naturally occurring bioflavonoid found in green tea, onions and red wine. It has documented anti-inflammatory, antioxidant and nitric oxide-inhibiting properties. Several studies have shown it to be effective in the treatment of chronic prostatitis. Quercetin was particularly effective in reducing pain and improving quality-of-life score (Shoskes *et al*., 1999).

Cernilton or cernitin is bee pollen gathered from the rye flower. At least two clinical trials have found it to be effective in alleviating CPPS symptoms. Buck *et al.* (1989) reported that patients with CPPS experienced complete and lasting relief or a marked improvement after supplementing with cernilton. Rugendorff *et al.* (1993) reported that cernilton also was effective in alleviating symptoms of BPH like urethral strictures, prostatic calculi (stones) or bladder neck sclerosis.

Saw palmetto is being used successfully in the treatment of Benign Prostatic Hyperplasia (BPH) (Gordon and Shaughnessy, 2003). However, there are no clinical trials indicating that it is effective in the treatment of CPPS. As a matter of fact, one trial comparing finasteride and saw palmetto in the treatment of CPPS found no beneficial effect of saw palmetto (Yang and Te, 2005).

Small-flowered willow herb (*Epilobium parviflorum*) is a well known folk remedy for the treatment of prostate problems, including BPH and prostatitis. Steenkamp *et al.* (2006) found that *Epilobium*, both as a tea and ethanol extract was highly effective in inhibiting the growth of *E. coli* in culture; the ethanol extract was substantially more effective than the water extract (tea). The ethanol extract of *Epilobium* was also very effective as both a COX-1 and COX-2 inhibitor in culture experiments and showed significant antioxidant activity.

Waterhouse (2007) suggested triple protocol for treatment-resistant cell wall deficient bacteria (NB) involves immune modulation, which consists of vitamin D reduction and higher than usual dosages of the angiotensin II receptor blocker, olmesartan. These two components of the protocol enable the immune system to kill the NB weakened by the third component is very low dosages of certain antibiotics.

NEW TRENDS FOR ERADICATION OF NANOBACTERIA WITH PHOTOMEDICINE

Sommer *et al.* (2003) evaluated the effect of various wavelengths of light on Nanobacteria (NB). The results indicated that suitable wavelengths of light could be instrumental in elevating the vitality level of NB, preventing the production of NB-mediated slime and simultaneously increasing the vitality level of mitochondria. This finding could stimulate the design of cooperative therapy concepts that could reduce death caused by myocardial infarcts.

Sommer (2007) identified the synergistic effects in the interaction of light with biosystems in the presence of chemical agents. Their systematic analysis promises therapeutic strategies. He concluded that Low-Level Light (LLL) therapy is compatible with antiinfectives and even capable of enhancing effects of superficially applied and/or absorbed antiinfectives. Temporal coordination between light treatment and drug administration maximizes drug effects and minimizes possible adverse effects. Furthermore, irradiation should start when the drug concentration has reached its maximum in the desired field of action. Light-induced flow in nanoscale cavities could represent one mechanism of LLL therapy.

PROMISING OPTICAL NANOPARTICLES THERAPY

Sereemaspun *et al.* (2008) determined the *in vitro* effect of gold and silver nanoparticles as the two most frequently used metallic nanomaterials for therapeutics and diagnostic on the microsomes containing wild-type cDNA expressed human CYP450 enzymes CYP1A2, 2C9, 2C19 and 3A4. Results demonstrated that all of the CYP450s activities were down-regulated by metallic nanoparticles.

Lukhele *et al.* (2010) explored the use of nano-sized materials for the removal of bacteria in water using silver nanoparticles immobilized onto carbon nanotube and cyclodextrin polymers.

Sap-Iam *et al.* (2010) suggested that the silver nanoparticles synthesized by UV-irradiation can be employed in biocontrol of pest.

Gilaki (2010) stimulated investigational progressions for biosynthesis of silver nanometals using plant leaf extracts.

Semwal *et al.* (2010) developed novel and efficacious nanoparticles for drug delivery as a promising progress in cancer nanotechnology. The release characteristic of drugs from these polymeric systems is dependent on the drug loading contents and chain length of the hydrophobic/hydrophilic part of the copolymers.

Warisnoicharoen *et al.* (2011) used silver nanoparticles have been recently for a wide range of applications including health and household products even though an understanding of their mechanistic action in human.

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