Zinc phosphate nanoparticles as an antimicrobial agent and their impact on rats microbiota

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Abstract: Nano minerals, especially trace minerals, are widely used in different fields, but mostly in animal systems. They can improve overall immunity and also a digestive efficacy in livestock. In this case, nanometals as nano mineral substances were synthetized, particular the zinc phosphate-based nanoparticles (ZnNPs). The antibacterial activity against three bacterial strains – *E. coli, S. aureus* and methicillin-resistant *S. aureus* was determined using different methods. After promising *in vitro* testing, the impact of these zinc nanoparticles on rats after oral exposure during 30 days of treatment was investigated. The antibacterial effects on rats gut microbiota were monitored, with the aim to reduce the number of pathogenic bacteria.

Key Words: antimicrobial activity, nano minerals, zinc, rats

INTRODUCTION

Nano minerals are broadly used in various sectors including agriculture, animals and food systems and also in fields like biology, biotechnology, and physiology (Sindhura et al. 2014, Swain et al. 2015). The natural properties of nanometals are mainly determined by its size, shape, composition, morphology and crystalline structure (Dickson and Lyon 2000). The particle size is the main attribute, which heavily influences the functional activities of nano minerals (Lewis and Klibanov 2005, Rosi and Mirkin 2005). Nano zinc and nano zinc oxide (ZnO) are the third most globally produced metal nanoparticles (NPs), annually by volume, after nano SiO_2 and nano TiO_2 (Piccinno et al. 2012). This sudden rise in the demand is mostly attributed to their better antibacterial effects than the conventional ZnO (Padmavathy and Vijayaraghavan 2008). Zn-based nanoparticles, including ZnO NPs like the most studied representative of this type of nanoparticles, showed bactericidal effects on Gram-positive and Gram-negative bacteria as well as the spores, which are resistant to high temperature and high pressure (Azam et al. 2012). From these pathogenic bacteria, there are dominant bacterial causes of severe secretory diarrhoea, which can be still a significant reason of death. A few studies have already proved that the dose of ZnO NPs have influenced growth performance on livestock and also they can be used as antimicrobial and immune agent to reduce the diarrhoea rate in piglets (Hongfu 2008, Mishra et al. 2014).

In particular, ZnO inhibits the bacterial viability, but the precise mechanism of its antibacterial activity has not been well understood so far. One of the proposed possibility is the generation of hydrogen peroxide as the major factor of the antibacterial effect. It is also supposed that, due to electrostatic forces between the particles and the bacteria surface could be another mechanism of antimicrobial effect of ZnO NPs. In addition, generation of reactive oxygen species (ROS), zinc ion release, membrane dysfunction and nanoparticle internalization could be possible reasons of cell damage (Hajipour et al. 2012, Zhang et al. 2008).

The main purpose of this work was to synthesize zinc-based nanoparticles, specifically four types of zinc-phosphate nanoparticles, with the antimicrobial activity. These zinc-phosphate NPs were prepared by synthesis of zinc nitrate with hydrogen phosphate (ZnA and ZnB), diphosphate (ZnC) or triphosphate (ZnD). The antibacterial activity of ZnNPs were tested *in vitro* and then also *in vivo*. *In vitro* antibacterial effect was investigated on three bacterial strains – *E. coli*, *S. aureus* and methicillin-resistant *S. aureus*. Further, zinc nanoparticles were tested on rats, and the effect of ZnNPs on total aerobic bacteria and coliforms in their feces during 30 days of treatment was studied.

MATERIAL AND METHODS

Determination of antibacterial activity *in vitro*

The antimicrobial effect of ZnO and Zn-phosphate nanoparticles were evaluated using three strains of bacteria. Bacterial cultures (*Staphylococcus aureus* NCTC 8511, *Escherichia coli* NCTC 13216, methicillin-resistant *S. aureus* (MRSA) CCM 7110) were cultivated in Müeller-Hinton broth (MHB, Oxoid, Hampshire, UK) overnight at 37 °C and subsequently diluted to a concentration $\sim 1\times10^6$ CFU/ml, where the concentrations are determined by optical density at 600 nm (OD_{600}).

Growth curves: The 100 µl of diluted bacterial suspensions was added into a 96-well microplate and mixed with individual ZnO or Zn-phosphate NPs in ratio 1:1, with total volume 200 µl. The growth of bacteria suspension with Zn NPs, with maximal concentration 5 mM, was detected by Multiskan EX (Thermo Fisher Scientific). The optical density measurements (OD_{620}) were carried out at zero time-point, and then each half-hour for 24 h at 37 °C. An equal volume of sterile water instead of Zn NPs was used as a control, marked as 0 mM.

Spread-plate method: Bacterial cultures diluted at concentration $\sim 1\times10^8$ CFU/ml were further diluted in tenfold steps with MHB. The 900 µl of these bacterial suspensions were mixed with 100 µl of Zn-phosphate NPs or ZnO at concentration 5 mM. These mixtures were incubated for 2 hours at 37 °C. After incubation, 100 µl of inoculum from each sample was applied on MH agar plates by spreading and followed by incubation at 37 °C for 24 h.

Live/dead assay: The individual bacteria were incubated with ZnO and Zn-phosphate NPs and prepared and diluted as in the previous cases, then was the suspensions centrifuged and washed with 0.85% NaCl. The fluorescent dyes SYTO9 (Thermo Fisher Scientific, USA) and Propidium Iodid (PI; Sigma Aldrich, St. Louis, USA) were added into these prepared bacterial solutions. Further, 5 µl of each mixture was observed with an Olympus IX71 inverted fluorescence microscope (Olympus, Tokyo, Japan).

In vivo antibacterial testing

As a model animal for this experiment were selected male rats of the *Wistar albino* strain, which were divided into seven groups. The rats diet contained Zn NPs ZnO and also with commercial zinc nanoparticles ZnO-N, each of them in a dose of 2000 mg Zn/kg diet. Feed and water were available *ad libitum*. One group was served as a control (C) without zinc addition into the diet.

Total aerobic bacteria and coliforms counting: The samples of rat's feces were homogenized and diluted with sterile physiological solution 1:9 w/v. Then, the homogenate was furthermore diluted in tenfold steps. Further, 1 ml of each suspension was placed into empty Petri dishes, and then potting by Plate count agar (PCA) and MacConkey agar (MCA) in duplicate. After 24 h of incubation in 37 °C, total counts from PCA and counts of coliforms from MCA were determined.

RESULTS AND DISCUSSION

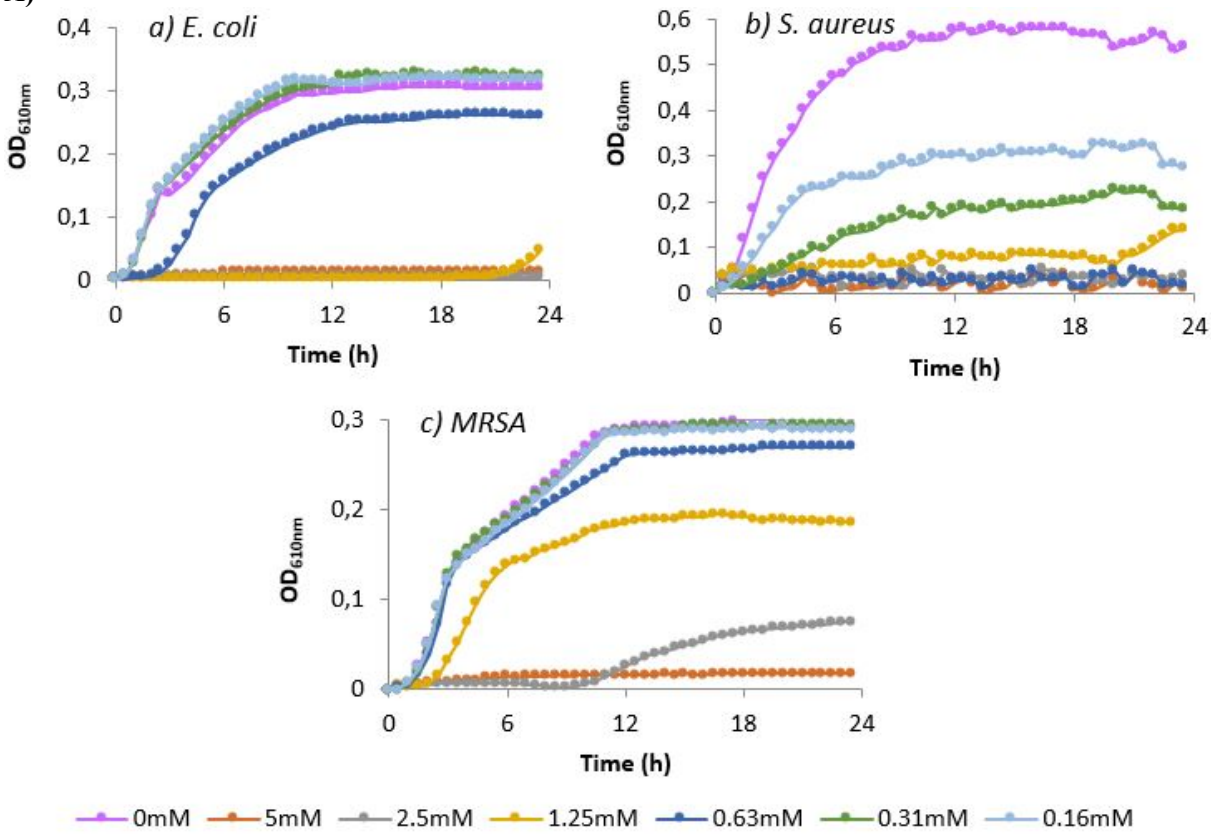
In vitro antibacterial activity characterization

First, we treated the bacteria with synthetized ZnNPs and also with ZnO and commercial ZnO-N. A substantial antibacterial activity trend was observed by measuring the optical density at 620 nm during 24 h. From growth curves, which are shown below, the minimal inhibition concentrations (MIC) were found out. The representative measurements with ZnA are shown in Figure 1A. In this case the MIC values ranged from 1.25 mM for *E. coli* and *S. aureus* to 2.5 mM for MRSA. In general, the substantial inhibition effect exhibited the Zn NPs against *S. aureus*. For instance, the 5 mM ZnC, as a maximal

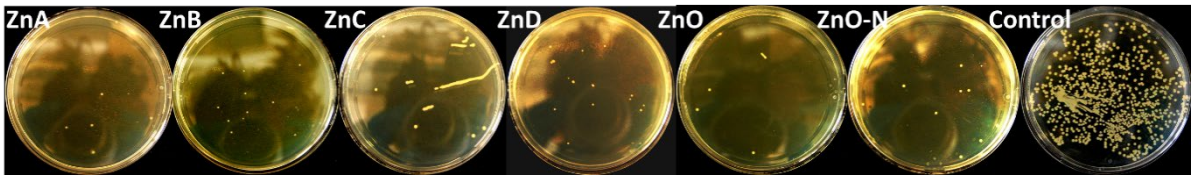
concentration, had no effect on *E. coli* bacterial population. Lower efficacy can be seen on MRSA experiment, where in MRSA phenotype, different SCCmec genotypes confer different resistance characteristics and virulence factors present in this SCCmec cassette (Kaito et al. 2011) or a putative metal resistance gene was also found in various MRSA isolates (Cavaco et al. 2010).

Figure 1 Characterization of antibacterial activity by A) growth curves; B) spread-plate method

A)



B)



The amount of viable bacteria after treatment with 5 mM Zn was found out by spread-plate technique. In Figure 1B is obvious decrease of *S. aureus* bacterial colonies compared to control. Based on the Table 1, the growth was significantly reduced compared to control. Almost all types of zinc compounds caused above 90% inhibition of bacterial growth, except lower inhibition effect of ZnC (no inhibition) and ZnD against *E. coli*. Another inhibition effects lower than 90% were observed against MRSA with ZnC, ZnO and ZnO-N, these outcomes correspond with growth curves results.

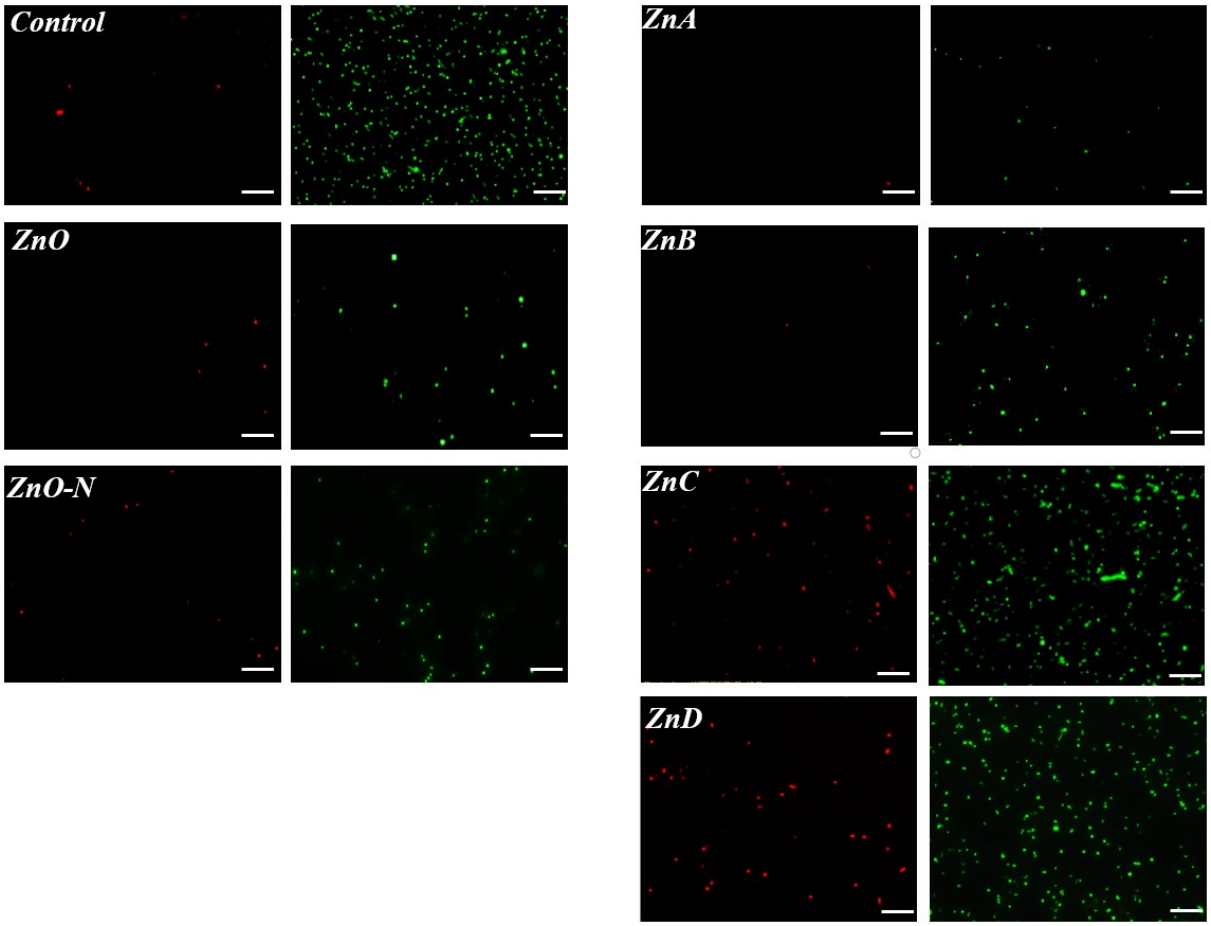
Table 1 Overview of % inhibition effects of Zn NPs and ZnO on using Spread-plate method

	ZnA	ZnB	ZnC	ZnD	ZnO	ZnO-N
E. coli	99.57	100.00	0	71.75	98.54	96.61
S. aureus	98.03	99.31	98.61	99.50	98.49	97.63
MRSA	93.56	92.14	84.81	92.37	87.58	88.02

In order to verify previous results and test the viability of bacterial cells, Live/dead assay was performed. Using this method with a fluorescence microscope we could track the ratio of living cells to

the dead cells, if some populations were present. As examples there are pictures of MRSA after interaction with fluorescence dyes (Figure 2). The micrographs show that despite lower inhibitory effects in previous assays, in this case it can be seen that ZnNPs and ZnO have good inhibition effect, except ZnC and ZnD, although there is no significant reduction of bacterial growth, but the number of dead cells has increased markedly.

Figure 2 Fluorescent micrographs showing live and dead (SYTO9, green) and dead (PI, red) bacterial cells from Live/dead assay; the scale bar – 20μm



Impact of ZnNPs on rats – counts of bacteria in feces

The synthetized Zn NPs exhibited great antimicrobial properties against bacterial strains *in vitro*. Thus their activity was further tested in whole animal organism. The total number of bacteria and coliforms in rats feces decreased.The rats were treated with ZnNPs and ZnO during 30 days. After first 10 days, there were not significant changes in amount of bacteria against control group, whereas after 30 days there were considerable decrease of bacteria populations. Especially in the case of ZnA and ZnC were significant decrease of coliforms, which are mostly the main cause of digestive problems, immunity and overall performance.

CONCLUSIONS

This work dealt with different types of Zn phosphate-based nanoparticles and their antibacterial effects characterization. In the first part, the *in vitro* antimicrobial activity against bacterial strains using three techniques was investigated. ZnNPs have shown sufficient antimicrobial activity, so we tested them in whole animal organism, rats. They were orally treated by zinc for 30 days, when they had uncontrolled access to feed containing ZnNPs and also ZnO. After this time, we evaluated their effect on bacterial population in rats.

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