IN VIVO ANTIBACTERIAL STUDY OF ZINC PHOSPHATE-BASED NANOPARTICLES

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Abstract

In animal nutrition, the nanotechnology is mostly used for the preparation of nanominerals, especially trace minerals, due to their low bioavailability. These nanominerals also showed advantageous effects at lower doses compared to conventional minerals. In addition, nanominerals can improve digestive efficacy, immunity and overall performance in livestock. In that case, we suggested and synthesized four types of zinc phosphate-based nanoparticles (ZnNPs), which achieved a significant antibacterial activity. After successful *in vitro* testing, we realized also *in vivo* study. We investigated the effect of zinc nanoparticles on rats after oral administration. Among others, the influence on total aerobic bacteria and coliforms in rat feces at day 10 or day 30 of treatment was monitored. In general, it has been observed a decrease of total aerobic and coliform bacteria population compared to untreated control group.

Keywords: Antibiotics, antimicrobial activity, zinc nanoparticles, nanominerals, rats

1. INTRODUCTION

Nanotechnology is emerging technology which has massive potential to global revolutionize agriculture and livestock sectors [1]. With regard to animals, its major applications are in administration of drugs and probiotics, nutrients and supplements, diagnosis and treatment, and also they are useful in hormonal immune-sensors in the management of reproduction. In animal nutrition, the main use of nanotechnology is in preparations of nanominerals, particularly trace minerals, due to their low bioavailability [1,2]. Nanominerals are stable at higher temperature and pressure [3] and can be lightly taken up by gastrointestinal tract and consequently utilized in the animal system [4]. In addition, minerals as nanoparticles increase the absorption of trace minerals and reduce intestinal mineral antagonism, which consequently reduces minerals excretion and thus pollution of the environment. Nano-minerals can be used in animal and poultry feed for effective nutrient intake to make better use of feed and other dietary supplements. Studies also indicate that nutrition by nanoparticles also improved digestion, immunity and performance of livestock [1,5]

The third most globally produced metal nanomaterial is nano zinc, particularly zinc oxide, which is one of the most studied representative of this type of particles [6]. The uniqueness of zinc is precisely the fact that it is the second most numerous trace element in animal body, but it cannot be stored in the body, so its regular intake in food is needed [7]. The reason for this sudden increase in demand for this type of nanoparticles is due to their better immuno-modulatory effect, but mainly antibacterial effects as the conventional Zn, or more precisely ZnO. Zn-based nanoparticles (ZnNPs) exhibited bactericidal effects against both, gram-positive and gram-negative bacteria [8, 9]. Within pathogenic bacteria, the species, which are responsible for the bacterial causes of severe secretory diarrhoea, especially in pre- and post-weaned piglets, are still dominant and still can be a significant cause of death. There are many different pathogens and factors that can cause diarrhoea so outbreaks should be investigated thoroughly. However, it is important to keep in mind that the use of nano Zn as an antimicrobial and immune agent depends on its dosage [7, 10-12].

So far, the main source of zinc for feed supplementation is used in the form of its inorganic salts, such as zinc sulphate ($ZnSO_4$), zinc chloride ($ZnCl_2$) and zinc oxide (ZnO). However, bioavailability of zinc from inorganic sources has been found to be relatively low, and therefore it is important to looking for the other sources with higher bioavailability [13]. In this case, the major objective of this work was to synthetize zinc-based nanoparticles, specifically four types of zinc-phosphate nanoparticles, with the antimicrobial activity. These zinc-phosphate NPs (ZnNPs) were prepared by synthesis of zinc nitrate with hydrogen phosphate (ZnA and ZnB), diphosphate (ZnC) or triphosphate (ZnD). First of all, ZnNPs were charecterized by Dynamic light scattering (DLS) and Transmission electron microscopy (TEM). After that, these ZnNPs were examined for their antibacterial activity *in vitro*, using three different methods to determine their antibacterial effects. Because of the successful results from that part of the project, in next work we focused on the main purpose of the study - the *in vivo* experiments. The current study focuses on the effect of in-house prepared ZnNPs on rats after oral administration.

2. MATERIAL AND METHODS

2.1. Particle characterization

ZnNPs were prepared by reaction of zinc nitrate with hydrogen phosphate (ZnA and ZnB), diphosphate (ZnC) or triphosphate (ZnD). The particle size and distribution were measured by dynamic light scattering, DLS, on a Malvern Zetasizer (NANO-ZS, Malvern Instruments Ltd., Worcestershire, UK). For visualization of synthetized particles, the transmission electron microscopy, TEM, were used. The images were taken with Tecnai F20 microscope (FEI, Eindhoven, Netherlands) at appropriate magnifications.

2.2. Analyses of *in vitro* antibacterial activity

The *in vitro* antimicrobial effect of synthetized ZnNPs and also with ZnO and commercial ZnO based NPs (ZnO-N) was determined and evaluated on three bacterial cultures – *Staphylococcus aureus*, *Escherichia coli*, methicillin-resistant *S. aureus* (MRSA).

For counting of bacterial colonies, bacteria were diluted in Muller-Hinton broth to a concentration $\sim 1\times10^8$ CFU/ml, and further diluted in tenfold steps. The cultures were mixed with samples to a final concentration 5 mM, in ratio 9:1. After 2 h of incubation at 37 °C, 100 µl of each suspension was applied on agar plates. After 24 h of incubation, the colonies were counted.

Bacterial cultures at concentration $\sim 1\times10^6$ CFU/ml were used for the growth curves method. In 96-well microplate, these diluted cultures were mixed with samples in maximal concentration 5 mM in ratio 1:1, where total volume in one single well was 200 µl. The growth curves were carried out by measuring the optical density at 600 nm during 24 h (Multiskan EX; Thermo Fisher 144 Scientific, Bremen, Germany).

For Live/dead assay, the MRSA culture ($\sim 1\times10^8$ CFU/ml) was incubated with samples in ration 9:1 for 24 h, and the individual suspensions were then centrifuged and washed by 0.85 % NaCl solution. Fluorescent dyes, SYTO9 and propidium iodide (PI), were used for observation under fluorescence microscope (Olympus IX71 inverted fluorescence microscope – Olympus, Tokyo, Japan).

2.3. *In vivo* antibacterial testing

For this experiment, a male rat of the *Wistar albino* strain was selected as a model animal. The rats were divided into 7 groups - four groups of rats were fed by Zn-phosphate NPs (ZnA, ZnB, ZnC and ZnD), the fifth was fed by ZnO, the sixth by commercial zinc nanoparticles (ZnO-N). The last group served as a control group (C) without a zinc supplementation in the diet. Each of them were administrated in a dose of 2000 mg Zn/kg diet. Feed and water were available *ad libidum*. The rats were treated with ZnNPs and ZnO during 30 days.

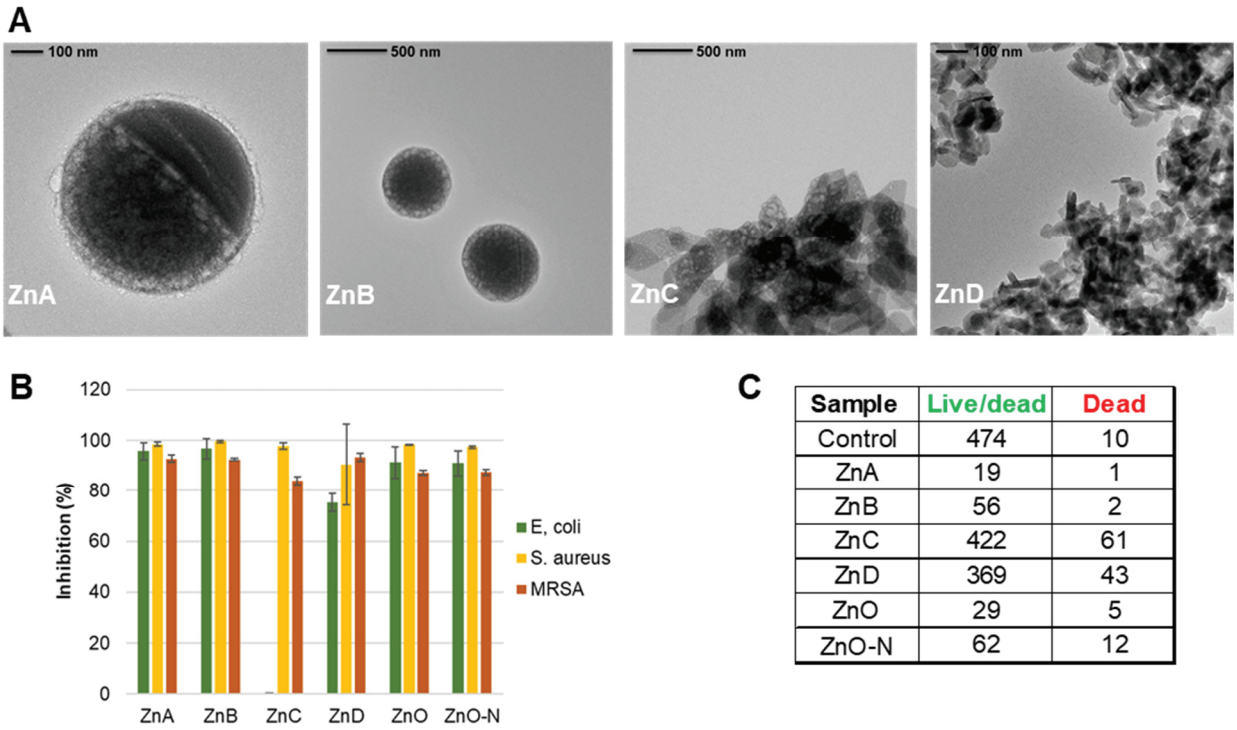
The experiment was carried out in the facility of the Institute of Animal Nutrition and Forage Production at Mendel University in Brno. Throughout the experiment, the microclimatic conditions in the laboratory were observed and controlled.

2.4. Counting of total aerobic bacteria and coliforms

The samples of rat's feces were collected at day 10, 20 and 30 of treatment. They were homogenized with sterile physiological solution 1:9 w/v. Then, the homogenate was furthermore serially diluted in tenfold steps. Subsequently, 1 ml of selected diluted suspension was places into empty Petri dishes and then potting by Plate count agar (PCA) and MacConkey agar (MCA) in duplicate. Total counts of aerobic bacteria from PCA and counts of coliforms from MCA were determined after 24 h of incubation in 37 °C. The results are expressed as CFU/g feces.

3. RESULTS AND DISCUSION

Four different precursors were used for ZnNPs synthesis, hydrogen phosphate for ZnA and ZnB, diphosphate for ZnC and triphosphate for ZnD, and the characterization of these particles was determined by DLS and TEM. By these two analyses, we find out that the ZnA and ZnB particles were in spherical shape with average diameter about 470 and 525 nm, respectively. Whereas ZnC and ZnD had irregular shape, and created small aggregates. The average diameter was then determined as 448 (ZnC) and 1030 (ZnD) nm. The different shapes are shown in **Figure 1A**. After the characterization of in-house prepared ZnNPs, the antibacterial activity against three bacterial species was investigated, and also the difference between individual particles connected with their structure was found out.



Sample	Live/dead	Dead
Control	474	10
ZnA	19	1
ZnB	56	2
ZnC	422	61
ZnD	369	43
ZnO	29	5
ZnO-N	62	12

Figure 1 (A) TEM images of synthetized ZnNPs, (B) inhibition effect of particles on three bacterial strains by spread-plate method, (C) the average amount of live and dead bacteria (SYTO9) and dead bacteria (PI) during microscope assay

Three methods - counting of bacterial colonies, growth curves and Live/dead assay - have been employed to determine their antimicrobial effects, and their antibacterial activity was assessed. These analyses were

performed with synthetized ZnNPs and also with ZnO and commercial ZnO based NPs (ZnO-N) for comparison. By spread-plate method, the amount of viable bacteria after 5 mM Zn treatment and 24 h incubation was found out. Overview of % inhibition effects of ZnNPs and ZnO are shown in **Figure 1B**, where almost all types of Zn caused more than 90 % inhibition of bacterial growth.

A significant trend of antibacterial activity was found out by measuring the optical density during 24 h. From these growth curves the minimal inhibition concentrations (MIC) were determined, and ranged from 1.25 mM for *S. aureus* and *E.coli* to 2.5 mM for MRSA type. It is known, that MRSA phenotype confer different resistance characteristics present in SCCmec genotype cassette, which are probably responsible for this lower efficacy [14]. In various MRSA isolates, a metal resistance genes were also found [15].

The bacteria viability was analyzed by the fluorescence microscopy. The individual images were taken on three fields of sight with a 20 μm approximation. From the resulting bacterial population, the average was calculated and the results are shown in the table in **Figure 1C**. From the given values, similar differences can be observed as are seen, in the physical characterization of the particles. While nanoparticles ZnA and ZnB treatment led to inhibition of bacterial growth, ZnC and ZnD did not reduce the bacterial growth, but they caused the increase of the dead cells.

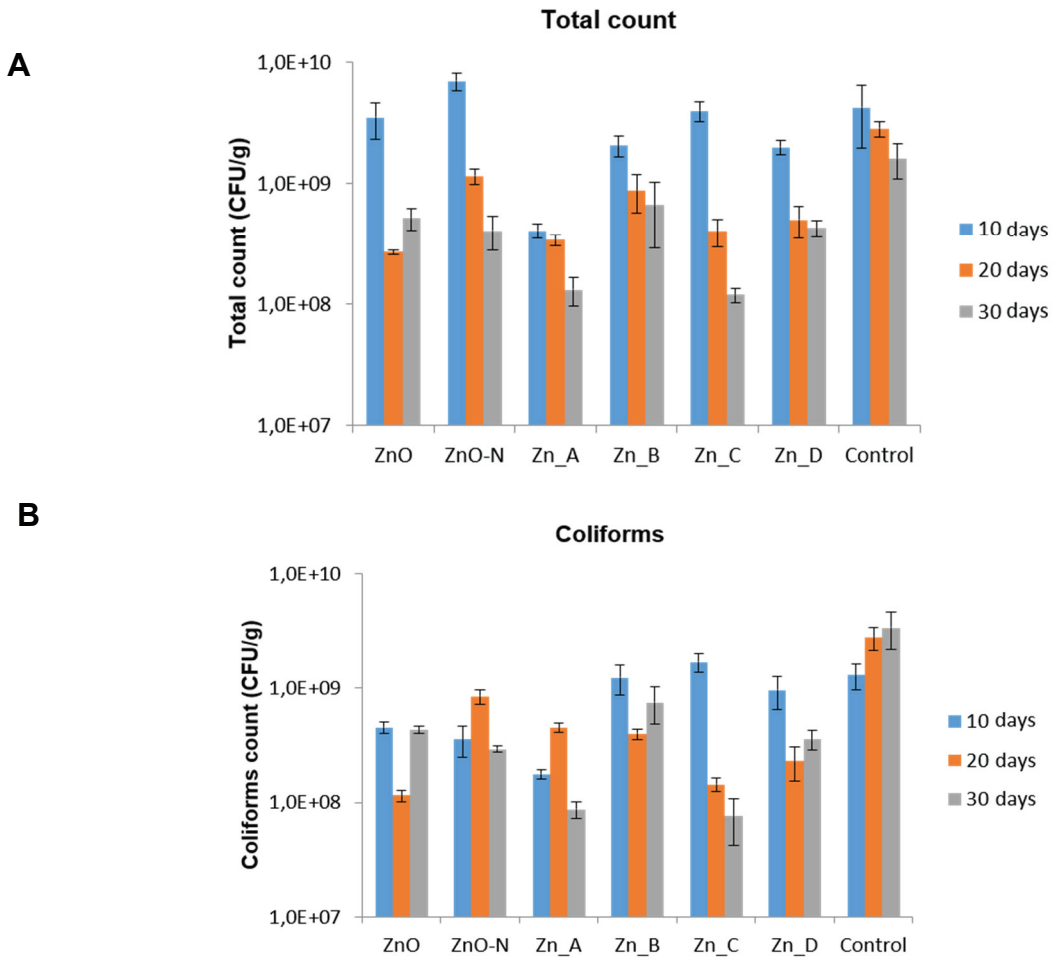


Figure 2 The ZnNPs effect on (A) total bacterial count and (B) count of coliform bacteria in rat feces

After promising and successful tests, the effects of these ZnNPs were subsequently monitored in rats, specifically their effect on rats gut microbiota. There were investigated the effect of zinc nanoparticles on rats after oral administration (**Figure 2**). In general, a decrease in the bacterial population was observed compared to the untreated control group with the duration of the experiment.

The decrease in both, the total aerobic bacteria and coliforms in rats feces was monitored, especially at day 30 of the treatment. After 10 and 20 days, few changes can be observed, but after 30 days there were apparent decrease of bacterial populations. Especially in case of nanoparticles types ZnA and ZnC the decrease of coliforms were significant (**Figure 2B**). Currently, these bacterial populations can cause digestive problems, if they are pathogens and their abundance is high in the intestines. They also cause problems with immunity and overall performance.

4. CONCLUSION

This work was concerned with testing the antimicrobial activity of zinc nanoparticles. At the beginning, their antibacterial effects were characterized *in vitro* by three testing methods against three bacterial strains. Zn-based nanoparticles have shown sufficient antimicrobial activity, which was the key point for their testing throughout the whole animal organism, the rats. The zinc nanoparticles effect on intestinal microbiota has been evaluated during the 30 day period.

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