The Role of Zinc Oxide Nanoparticles in the Modulation of Movement Characteristics of Bull Spermatozoa

Michal Lenický [1], Marek Helczman[1], Denis Bažány[1], Peter Ivanić[2], Marko Halo Jr.[1]

[1]*Slovak University of Agriculture, Faculty of Biotechnology and Food Science, Institute of Applied Biology, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovak Republic*
[2]*Slovak Biological Services Ltd, Kremnička 2, Banská Bystica 97405, Slovak Republic*

Abstract
Zinc oxide (ZnO) nanoparticles have gained significant attention in reproductive biology due to their unique physicochemical properties and potential applications in enhancing gamete functionality. This study investigates the effect of ZnO nanoparticles in modulating the motility characteristics of bull spermatozoa, which are critical for successful fertilization. Given the importance of spermatozoa motility in agricultural and veterinary practice, understanding the interactions between ZnO nanoparticles and spermatozoa could provide insight into new strategies to improve reproductive efficiency in cattle. We used computer-assisted sperm analysis (CASA system) for accurate determination of motility, to evaluate the effect of different concentrations (1000; 500; 250; 125; 62.5; 31.2 and 15.6 µg/ml) of zinc oxide nanoparticles (ZnO NP) on parameters such as total motility (MOT, %), progressive motility (PRO, %) and velocity curved line (VCL, µm/s) of bull spermatozoa at 37°C in time 0h, 3h and 5h. Pure saline served as a control, and the experimental concentrations of ZnO NPs were diluted also in saline. The results of our experiments show that the addition of 31.2 and 62.5 µg/ml ZnO NPs has a beneficial effect on the motility characteristics of bull spermatozoa. On the other hand, concentrations of 1000 and 500 µg/ml ZnO NPs were cytotoxic. Our findings suggest that it is necessary to elucidate the pathways by which ZnO nanoparticles affect spermatozoa motility, thus contributing to the broader discourse on nanotechnology in the field of reproductive health.

Keywords: nanoparticles ZnO, bull spermatozoa, CASA system, motility

1. Introduction

In the past, artificial insemination was introduced as a tool against the spread of venereal diseases in cattle. Over time, artificial insemination techniques have gradually improved with the introduction of new approaches and methods such as cryopreservation or the use of semen extenders have contributed to better results [1]. One of the main factors affecting efficient reproduction in cattle is impaired semen quality. Even with the use of effective artificial insemination techniques and the use of sufficient spermatozoa in the insemination ration, there are still differences in fertility between bulls [2]. One possible solution is the application of new technologies in the form of nanoparticles. The fundamental property of nanoparticles is the fact that they can exhibit unique properties that are not similar to their chemical equivalent on a larger scale. And it is precisely the properties of nanoparticles such as high chemical bioactivity of substances, their reactivity, cellular as well as tissue and organ penetration ability that are characteristics that facilitate their wide application in various industries. However, the aforementioned advantages can also be pathways for potential toxicity [3]. Zinc plays a key role in the body as a cofactor for many metalloenzymes, has an effect

* Corresponding author: Michal Lenický, +421 949 475 470, xlenicky@uniag.sk

on spermiogenesis, increases the activity of antioxidant enzymes, and participates in proper motility, capacitation, and the acrosome reaction [4]. It is for these reasons that we decided to apply zinc oxide nanoparticles to bull spermatozoa and observe the effect on the motility characteristics of bull spermatozoa at different concentrations.

2. Materials and methods

Fresh ejaculate samples were collected from sexually mature, healthy Holstein bulls (n=7) (Slovak Biological Services, a.s., Nitra). The ejaculate was obtained using an artificial vagina and immediately subjected to a basic motility analysis (minimum 80% motile spermatozoa). Subsequently, the samples were promptly transported to the laboratory in a thermodynamically sealed container. In the laboratory, the spermatozoa were diluted at a ratio of 1:50 with pre-prepared zinc oxide nanoparticle solutions and incubated in a thermostat at 37.5°C.
For the preparation of experimental solutions with varying nanoparticle concentrations, we used an water dispersion of zinc oxide (ZnO, particle size < 100 nm (TEM), average particle size ≤ 40 nm (APS), 20 wt.% in H_2O) (Sigma-Aldrich, Saint Louis, MO 63103, USA), which was diluted in physiological saline (0.9% NaCl, Braun, B. Braun Melsungen AG, Germany). The control group (Con) consisted of pure physiological saline (0.9% NaCl, Braun, B. Braun Melsungen AG, Germany). The experimental groups had the following ZnO nanoparticle concentrations: 15.6, 31.2, 62.5, 125, 250, 500, 1000 µg/ml.

Computer-assisted sperm analysis (CASA)
Spermatozoa motility characteristics such as total motility (MOT), progressive motility (PRO) and velocity curved line (VCL) were evaluated using a computer-assisted semen analysis system called CASA system. The system combines an optical mircroscope Olympus BX 51 (Olympus, Japan) with a camera and a computer with a software Andro-Vision (Minitube, Tiefenbach, Nemecko). The system is species whispering and was set up to analyze bull spermatozoa, while dilution and total ejaculate volume were also entered. For the application of the sample to the microscope, a Makler counting chamber (Sefi-Medical Instruments, Nemecko) was used, which must be preheated to 37.5°C. A 10-µl spermatozoa sample with ZnO NP was placed on the heating stage of the microscope. CASA indexes each single spermatozoa in the field of view of the microscope and from a short recording calculates the percentage of total motile spermatozoa (MOT), progressively motile (PRO) and at what speed they traverse the recorded path (VCL) [5]. CASA was assessed at 0, 3 and 5 hours of incubation with ZnO NPs at 37.5°C

Statistical processing of results
The results were processed with the computer program GraphPad Prism 9.0.0 (GraphPad Software Inc., San Diego CA, USA) using One-Way ANOVA (Dunett's test). Data are expressed as mean values ± standard error of the mean. Significance of differences between control and experimental groups was set at *P<0.05; **P<0.01; ***P<0.001, **** P<0.0001.

3. Results and discussion

According to results listed in Figure 1a we can conclude that after adding spermatozoa to the experimental solutions at time 0, we did not notice any significant differences (P>0.05) compared to the control. After 3 hours (Figure 1b), we observed a significant decrease (P<0.0001) in total motility at concentrations of 500 (51.84 ± 10.50%) and 1000 (38.36 ± 12.81%) µg/ml ZnO NPs compared to the control (63.15 ± 5.31%). Such high doses of ZnO NPs have been shown to be cytotoxic. Barkhordari et al. [6] confirmed that concentrations of 500 and 1000 µg/ml ZnO NP after 180 minutes of incubation caused a significantly higher percentage of cell death compared to the control. They also confirmed that with increasing exposure time, the toxicity to sperm increases, which clearly indicates a time-dependent process of toxicity at high doses of ZnO NP. Conversely, lower doses of ZnO NP 10 µg/ml were associated with the lowest % of dead sperm. After 5 hours of incubation, we can observe (Figure 1c) a significant increase (P<0.05) in total motility at 62.5 (75.41 ± 16.17%) µg/ml ZnO NP compared to the control (53.88 ± 22.40%). Concentrations of 500 (20.42 ± 14.22%) (P<0.001) and 1000 (14.61 ± 4.83%) µg/ml (P<0.0001) ZnO NP after 5 hours of incubation caused a significant decrease in total motility.

Farhadi et al [7] found that the addition of 0.1 or 1 µg/ml ZnO NP significantly increased the percentage of total and progressive motility, and also found that higher concentrations of ZnO NP in bull spermatozoa caused higher levels of malondialdehyde (MDA), which indicates oxidative damage to spermatozoa membranes and thus loss of their motility. As with total motility, the addition of ZnO nanoparticles at the initial time point 0h did not cause significant differences (P>0.05) compared to the control or in progressive motility (Figure 2a). After 3 hours (Figure 2b) we recorded a significant decrease (P<0.01) in progressive motility at a dose of 500 (44.55 ± 18.25%) and 1000 (41.89 ± 17.92%) µg/ml ZnO nanoparticles compared to the control (64.20 ± 9.43%). Progressive motility of bull spermatozoa, characterized by their ability to move straight forward, is essential for successful fertilization, as it determines the efficiency of spermatozoa movement through the female reproductive tract and their ability to reach and fertilize the oocyte [8]. A significant increase in progressive motility (P<0.05; P<0.01) was observed (Figure 3c) after 5 hours of incubation at doses of 15.6 (60.93 ± 17.18%), 31.2 (65.16 ± 41.64%) and 62.5 (67.87 ± 21.23%) µg/ml ZnO nanoparticles compared to the control group (35.96 ± 6.71%). The highest concentration of 1000 (13.23 ± 3.66%) µg/ml ZnO nanoparticles caused a significant decrease (P<0.01) in progressive motility.

A study investigating the effect of different concentrations of ZnO NPs (20, 40 and 60 µg/ml) on the quality of porcine semen during liquid preservation at 18°C found that all used concentrations of ZnO NPs significantly improved spermatozoa motility, acrosome and plasma membrane integrity, and reduced lipid peroxidation compared to the control group, with the best results achieved at a concentration of 20 µg/ml [9]. Storage of bull spermatozoa in a solution containing ZnO NPs did not cause a significant increase (P>0.05) in the velocity curve compared to the control group. Only the highest concentration of 1000 (32.40 ± 16.58 µm/s) µg/ml showed a toxic effect. After 5 hours (Figure 3c) there was a significant decrease (P<0.01) in the curvilinear sperm motility compared to the control (103.4 ± 56.11 µm/s). The toxic effects of nanoparticles may be due to the antimicrobial activity of nanoparticles when they can generate ROS, however, high doses can cause an imbalance in ROS levels and the emergence of oxidative stress, which can lead to lipid peroxidation and subsequent damage to spermatozoa structures, including the spermatid, which leads to reduced motility and velocity curved line [10, 11].

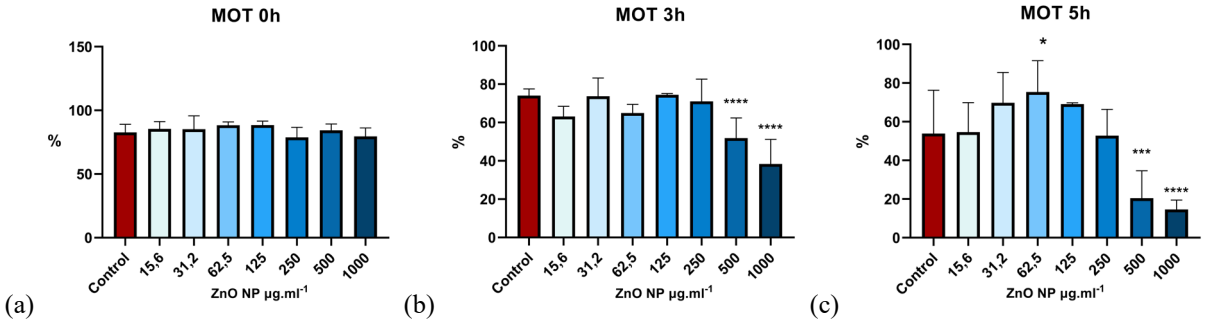


Figure 1. Effect of ZnO NP on total spermatozoa motility (%) at 0 (a), 3 (b) and 5 (c) h of incubation at 37.5°C. The significance level was set at**** (P<0.0001) *** (P<0.001), ** (P<0.01) and * (P<0.05).

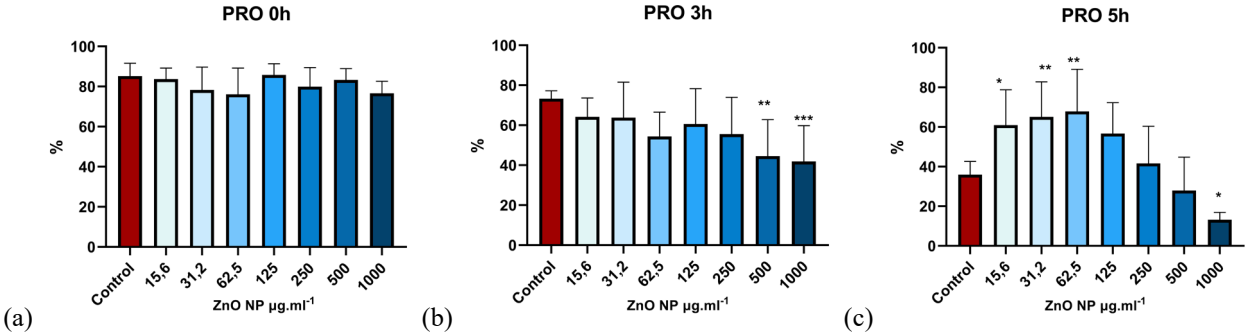
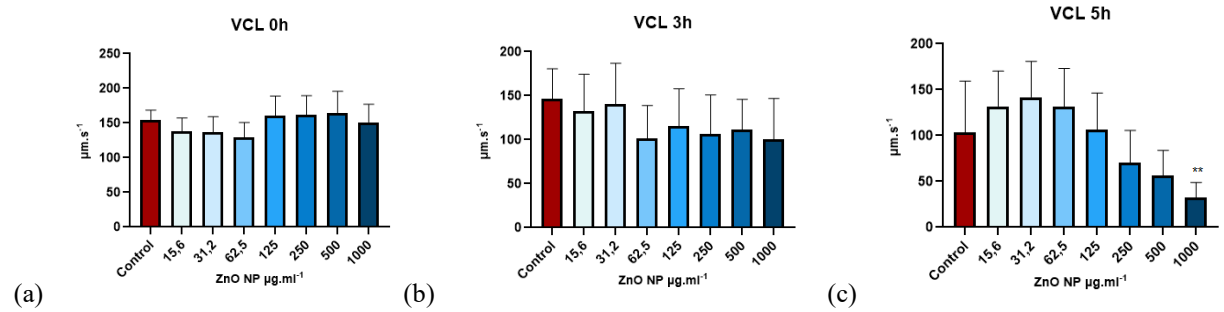


Figure 2. Effect of ZnO NP mixture on progressive motility (%) at 0 (a), 3 (b) and 5 (c) h of incubation at 37.5°C. The significance level was set at**** (P<0.0001) *** (P<0.001), ** (P<0.01) and * (P<0.05).



(a) (b) (c)

Figure 3. Effect of ZnO NP mixture on velocity curved line (µm/s) at 0 (a), 3 (b) and 5 (c) h of incubation at 37.5°C. The significance level was set at**** (P<0.0001) *** (P<0.001), ** (P<0.01) and * (P<0.05).

4. Conclusions

The results of our experiments show that the addition of 31.2 and 62.5 µg/ml ZnO NPs has a beneficial effect on the motility characteristics of bull spermatozoa. On the other hand, concentrations of 1000 and 500 µg/ml ZnO NPs were cytotoxic. Our results indicate that the effect of ZnO NPs is dose and exposure time dependent, with lower concentrations able to achieve the desired beneficial effect, but at high doses of ZnO NPs toxic aging of the nanoparticles occurs. Our findings suggest that it is necessary to elucidate the mechanistic pathways by which ZnO nanoparticles affect spermatozoa motility, thus contributing to the broader discourse on nanotechnology in the field of reproductive health.

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