













Effect of platinum nanoparticles on rabbit spermatozoa motility and viability

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Abstract: Male reproductive health is becoming a frequently asked question nowadays. Spermatozoa can be highly sensitive to various substances found in the environment. With the accretive usage of nanoparticles in technology and medicine, nanoparticles may also pose as potential environmental contaminants. Platinum nanoparticles are increasingly used in medicine, mainly for cancer treatment. The aim of this study was to investigate the effect of platinum nanoparticles on the motility and viability of rabbit spermatozoa *in-vitro*. Rabbit semen (n = 5) was cultured with solutions with different concentrations of platinum nanoparticles (control group or CON = only 0.9% NaCl; experimental groups D = 62.5 µg/ml, E = 31.25 µg/ml, F = 15.63 µg/ml, G = 7.81 µg/ml, H = 3.91 µg/ml, I = 1.95 µg/ml, J = 0.98 µg/ml, K = 0.49 µg/ml, and L = 0.24 µg/ml platinum nanoparticles) at time intervals of 0, 2, 4, 6, 9 and 24 hours. Selected parameters were analysed by computer-assisted sperm analyzer (CASA) technology – total motility (MOT, %), progressive motility (PRO, %), curvilinear velocity (VCL, µm/s) and viability (metabolic activity) of spermatozoa using the MTT test. Platinum nanoparticles negatively affected spermatozoa motility, progressive motility, and curvilinear velocity at almost all assessed time intervals, mainly at the highest used concentrations. The initial time interval shows a slight but statistically significant increase in MOT and PRO. Spermatozoa viability was not significantly affected, according to the results of this experiment. It was shown that the effect of platinum nanoparticles is time and dose-dependent. However, further analyses are needed to draw complete conclusions.

Introduction

Spermatozoa and the whole male reproductive system are highly sensitive to various substances present in the environment, naturally or due to human activity. With the rising technological progress, the number of toxic substances in soil, water, air, and food chains could be seen. Researchers have demonstrated a decreasing trend in male fertility during past decades due to environmental pollution (Ghosh et al., 2022; Massányi et al., 2020;

Tirpák et al., 2022; Madhu et al., 2022). Daily exposure to a broad spectrum of chemicals used in industry, agriculture, and medicine, such as heavy metals, bisphenols, dioxins, polychlorinated biphenyls, pharmaceutical residues, and many others, has adverse effects on the structure and function of male reproductive organs (Jambor et al., 2021; Ahmed and Soylak, 2023; Yilmaz et al., 2020; Massányi et al., 2020). Previous studies have reported deterioration in spermatozoa

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quality, such as reduced motility and sperm count, disruption of membrane and acrosome integrity, DNA fragmentation, and pathological changes in reproductive organs (Madhu et al., 2011; Sharma et al., 2020; Tirpák et al., 2021).

The progress of science and technology has brought increasingly frequent use of nanoparticles in many fields of industry, technology, and medicine. Nanoparticles are described as particles with a size of 1 – 100 nm in at least one of their dimensions (Medici et al., 2021; Ranjha et al., 2022; Halo Jr. et al., 2021). Their small size acquires unique abilities that can improve already existing processes. They are characterized by significantly different chemical and physical properties compared to larger particles of the same materials, such as larger surface area, high reactivity, strength, sensitivity, stability, magnetic, optical, thermal, mechanical, antimicrobial properties, etc. Currently, the use is spreading in medicine, electrical engineering, optics, construction, textile, chemical, food and automotive industries (Singh et al., 2022; Dianová et al., 2022).

Despite the advantages that nanomaterials provide, e.g., in drug delivery, the food industry or cosmetics, their increasingly frequent inclusion in products raises concerns about the consequences and health threats they could bring (Ealia and Saravanakumar, 2017; Yılmaz and Soylak, 2016). Nanoparticles can not only circulate throughout the body, but also enter cells, and therefore it is especially necessary to investigate their effects on living organisms. They can have a cytotoxic and pro-inflammatory effect, mainly caused by the production of reactive oxygen species (ROS) (Wang and Tang, 2021). On the other hand, research also points to the antioxidant effect of platinum nanoparticles and their ability to act as a scavenger of free radicals in the cell. Platinum nanoparticles have a wide range of applications, which include the chemical industry, the automotive sector, biomedicine, and the therapeutic field. Due to their excellent biocompatibility, small size, antimicrobial, catalytic and other properties, they are destined for use in biomedicine. Nevertheless, they still can have significant cytotoxic effects (Bendale et al., 2017; Gholami-Shabani et al., 2023; Gutiérrez de la Rosa et al., 2022). This study, therefore, focuses on the possible effect of platinum nanoparticles on rabbit spermatozoa *in-vitro*.

Material and methods

Collection and preparation of experimental samples

Ejaculate samples were collected from adult New Zealand white rabbits aged 1 year \pm 2 months in a research breeding and production centre (Research

Institute for Animal Production Nitra, Slovak Republic). The rabbits were kept in individual cages and fed with a commercial feed mixture. The average air temperature was $20 \pm 2^{\circ}\text{C}$, and the relative humidity was $70 \pm 5\%$. Ejaculate samples were taken once a week, from 6 – 8 male rabbits, using an artificial vagina. Ejaculates were pooled to avoid inter-individual differences, and the final number of hetero-zoospermic samples was 5 ($n = 5$). Only ejaculates with a minimum motility of 80% were used for the experiments.

Spermatozoa were incubated at 4°C with different concentrations of platinum nanoparticles (3 nm particle size, 1000 ppm in H_2O obtained from Sigma Aldrich, 3050 Spruce Street, Saint Louis, MO, USA) dissolved in saline. Fresh ejaculates were diluted with saline (NaCl 0.9% Braun, B. Braun Melsungen AG, Germany) at a ratio of 1:9 for the control group (CON). Experimental samples were prepared by the same dilution with nine different concentrations of platinum nanoparticles ($\mu\text{g}/\text{ml}$) as shown in Figure 1.

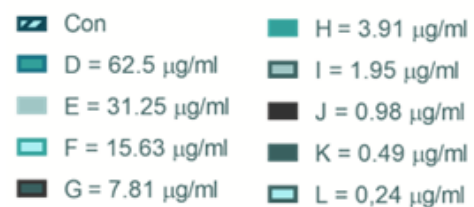


Figure 1. Concentrations of platinum nanoparticles used in the study

Spermatozoa motility analyses

Spermatozoa motility analyses were performed at time intervals of 0, 2, 4, 6, 9, and 24 hours, using computer assisted sperm analyzer (CASA) system with Sperm Vision software (Minitube, Tiefenbach, Germany) equipped with an Olympus BX 51 microscope (Olympus, Japan). The principle of the CASA system is to acquire a series of sequential microscopic images of motile spermatozoa in a static field of view (Massányi et al., 2008; Vincent et al., 2014). Diluted ejaculate samples were placed in a Makler counting chamber (Sefi-Medical Instruments, Germany) with a volume of 10 μl , preheated to 37°C for each analysis. Selected parameters were analysed - total motility (MOT; %), progressive motility (PRO; %), and curvilinear velocity (VCL; $\mu\text{m}/\text{s}$).

Spermatozoa viability

Spermatozoa viability and proliferation after 24 hours of exposure to platinum nanoparticles were determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Mosmann, 1983). This method is based on the conversion of the yellow tetrazolium salt MTT (Sigma Aldrich, St. Louis, USA) to blue formazan particles, by mitochondrial enzyme

(succinate dehydrogenase) of intact mitochondria inside living cells.

($P < 0.01$) and H ($P < 0.05$). as compared to control. Increasing motility was also observed after 2 hours of

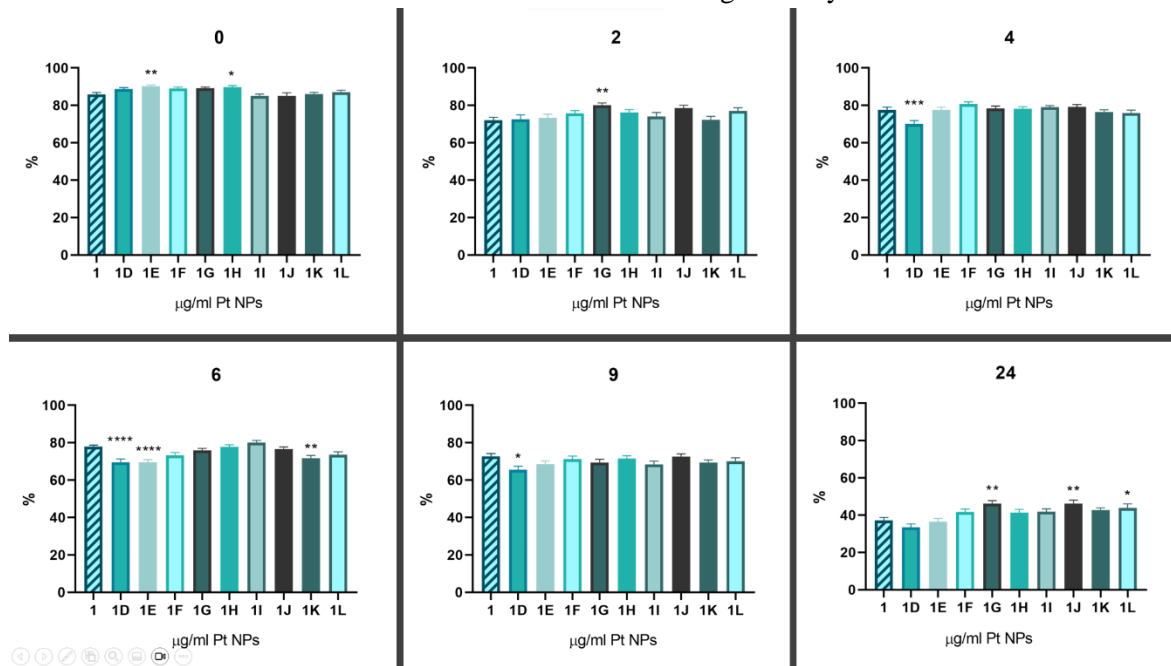


Figure 2. Motility (% MOT) of rabbit spermatozoa after 0, 2, 4, 6, 9 and 24 hours of exposure to platinum nanoparticles (Pt NPs) at various experimental concentrations (groups D = 62.5, E = 31.25, F = 15.63, G = 7.81, H = 3.91, I = 1.95; J = 0.98; K = 0.49, and L = 0.24 µg/ml)

Samples (100 µl) in all analysed concentrations were placed in a 96-well microplate. A tetrazolium salt solution dissolved in saline (NaCl 0.9% Braun, B. Braun Melsungen AG, Germany) was added in a volume of 20 µl to each chamber. Subsequently, culture took place for 1 hour at 37°C. Thereafter, the reaction was stopped by adding 40 µl of isopropanol (Centralchem, Bratislava, Slovakia). The intensity of formazan staining of the samples was quantified spectrophotometrically using an ELISA microplate reader (Multiscan FC, ThermoFisher Scientific, Finland) at a wavelength of 570/620 nm.

Statistical analyses

The values of the basic statistical characteristics were obtained by the GraphPad Prism 8 (GraphPad Software built, San Diego, USA) with the use of analysis of variance (one-way ANOVA) and subsequent comparative Dunnett's test, which compares the differences between the control group and the experimental groups. Significant differences were described at the levels of statistical significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Results

Motility

Platinum nanoparticles had a significant effect on spermatozoa motility at all assessed time intervals. Immediately after the addition of nanoparticles, at the time interval '0', there was a significant increase in spermatozoa motility in the experimental groups E

exposition to platinum nanoparticles in experimental group G ($P < 0.05$) in comparison to control. At the following time intervals of 4, 6, and 9 hours, a negative influence of nanoparticles could be seen. Motility was negatively affected in experimental group D with the highest addition of nanoparticles after 4 ($P < 0.001$), 6 ($P < 0.0001$) and 9 ($P < 0.05$) hours of exposure. Motility was found to decline also after 6 hours of incubation with platinum nanoparticles in experimental groups E ($P < 0.0001$) and K ($P < 0.05$). On the other hand, the final time interval of 24 hours showed a rising tendency of motility compared to the control group in experimental groups G ($P < 0.001$), J ($P < 0.001$) and L ($P < 0.05$) (Figure 2).

Progressive motility

In the case of progressive motility of spermatozoa, a slight increase was noted in the initial time interval in experimental groups E, F, G, and H ($P < 0.01$). After 2 hours of exposure to platinum nanoparticles, there was no significant change in the experimental groups as compared to control. Subsequently, at subsequent time intervals, progressive motility declined mainly in the groups with the highest concentrations of platinum nanoparticles. In group D, a significant decrease was noted at 4 ($P < 0.01$), 6 ($P < 0.0001$), 9 ($P < 0.001$) and 24 ($P < 0.0001$) hours of exposure to nanoparticles. Progressive motility also notably decreased in experimental groups E and F ($P < 0.001$), too, after 6

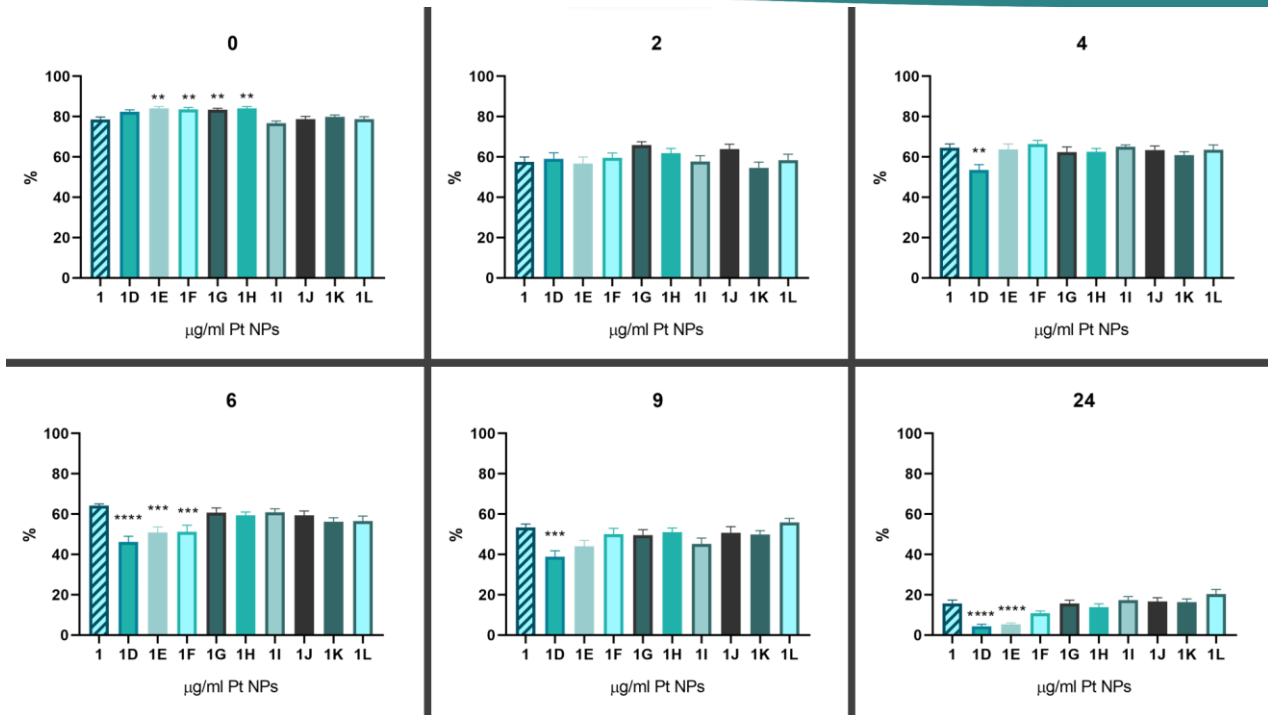


Figure 3. Progressive motility (% PRO) of rabbit spermatozoa after 0, 2, 4, 6, 9 and 24 hours of exposure to platinum nanoparticles (Pt NPs) at various experimental concentrations (groups D = 62.5, E = 31.25, F = 15.63, G = 7.81, H = 3.91, I = 1.95; J = 0.98; K = 0.49, and L = 0.24 µg/ml)

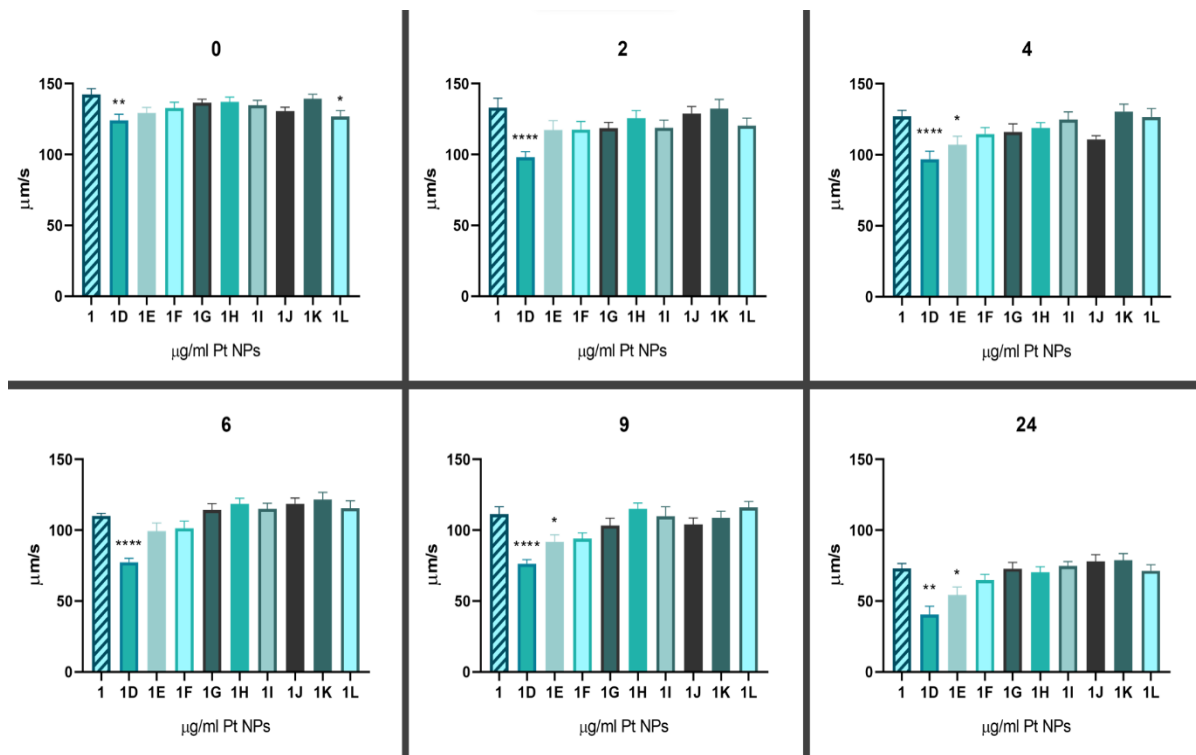


Figure 4. Curvilinear motility velocity (µm/s VCL) of rabbit spermatozoa after 0, 2, 4, 6, 9 and 24 hours of exposure to platinum nanoparticles (Pt NPs) at various experimental concentrations (groups D = 62.5, E = 31.25, F = 15.63, G = 7.81, H = 3.91, I = 1.95; J = 0.98; K = 0.49, and L = 0.24 µg/ml)

hours. At 24 hours, platinum nanoparticles initiated a reduction of progressive motility in experimental group E ($P < 0.0001$), in a similar fashion to the group D (Figure 3).

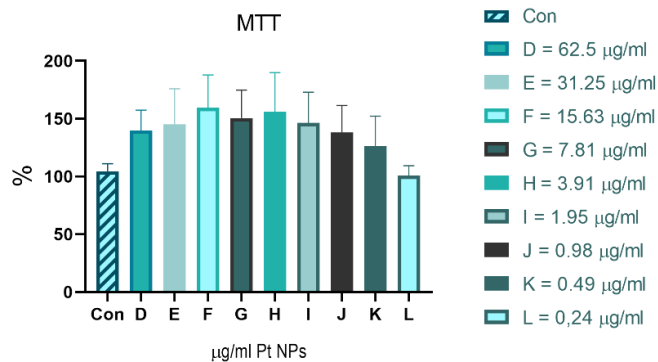


Figure 5. Metabolic activity (% control) of rabbit spermatozoa after 0, 2, 4, 6, 9 and 24 hours of exposure to platinum nanoparticles (Pt NPs) at various experimental concentrations (groups D = 62.5, E = 31.25, F = 15.63, G = 7.81, H = 3.91, I = 1.95; J = 0.98; K = 0.49, and L = 0.24 µg/ml)

Curvilinear velocity

VCL, as a parameter that shows the speed and passed distance of spermatozoa was significantly affected by platinum nanoparticles exposure, especially in the case of the highest added concentration. Experimental group D exhibited notably lower VCL at all measured time intervals compared to the control sample (at time 0 – $P < 0.01$; after 2, 4, 6, 9 hours – $P < 0.0001$; at 24 hours – $P < 0.01$). Reduced value of VCL was also observed at time 0 with the lowest concentration of platinum nanoparticles, i.e., group L as compared to control. Experimental group E also noted a sharp decrease in VCL ($P < 0.05$) after 4, 9 and 24 hours of exposure in comparison to control (Figure 4).

Metabolic activity

MTT assay did not reveal any significant difference in the viability of rabbit spermatozoa after experimental exposure to platinum nanoparticles, in comparison to control. Graphical representation of the results in Figure 5 indicates towards an increasing trend initially until and thereafter a declining trend as the concentration of nanoparticles increased gradually.

Discussion

Due to the lack of published studies on the effect of platinum nanoparticles on spermatozoa motility, the results were compared with scientific publications in which the effect of other metal nanoparticles on sperm quality parameters in different species of animals and humans were analysed.

After 90 minutes of exposure to zinc oxide (ZnO) nanoparticles at 5°C, Halo et al. (2022) reported lower percentage of motile stallion spermatozoa in samples treated with a concentration of 12 and 24 mg/ml ZnO nanoparticles. Progressive motility was reduced at the same concentrations at 60 and 90 minutes compared to control. However, Moretti et al. (2013) pointed out in their work the adverse effects of gold and silver nanoparticles on the motility of human spermatozoa *in-vitro*. In their experiment, ejaculate samples were obtained from 10 donors. Subsequently, different concentrations of gold and silver nanoparticles ranging from 30 to 500 µM were added to the samples for analyses after 60 and 120 minutes of culture at 37°C. The results confirmed that high concentrations of nanoparticles (above 125 µM) had a demonstrably adverse effect on human sperm motility, which is also confirmed by our results in the case of platinum nanoparticles on rabbit spermatozoa.

On the contrary, Lafuente et al. (2016) reported no significant change in rat epididymal spermatozoa motility after oral exposure to 0, 50, 100 and 200 mg/kg silver nanoparticles for 90 days. However, Halo Jr. et al. (2021) recently found a significant decrease in motility and progressive motility of rabbit spermatozoa after 2 and 3 hours of culture at ZnO concentrations above 24 mg/ml.

In contrast, VCL in this case was not significantly affected by the addition of ZnO nanoparticles. Castellini et al. (2014), in their experiment, dealt with the long-term effects of silver nanoparticles on the reproductive activity of rabbits. They reported a significant reduction in the percentage of motile spermatozoa when rabbits were intravenously administered 0.6 mg/kg silver nanoparticles for 126 days. However, the negative effect of silver nanoparticles on rabbit spermatozoa motility reduced slightly after 42 days of the experiment. Moreover, Miura et al. (2019) cultured spermatozoa obtained from the epididymis tail of mice with titanium nanoparticles at concentrations of 2.19, 4.38 and 8.76 ng/µl for 3 hours. There was a demonstrable decrease in motility at concentrations of 4.38 and 8.76 ng/µl. They concluded that titanium nanoparticles can have a direct impact on the physiological functions of spermatozoa in a dose-dependent manner. Recently, adverse impacts of zinc and manganese nanoparticles were also reported on the quality parameters of turkey spermatozoa. After 24 and 48 hours of culture in sperm extender with the addition of 25 and 50 µM zinc or manganese nanoparticles, a significant reduction of almost all path velocity and speed parameters of spermatozoa, including motility, progressive motility, VCL, amplitude of lateral head

displacement (ALH) and beat cross frequency (BCF) was noted (Orzolek et al., 2021). These findings also support our results in the case of platinum nanoparticles.

Mitochondrial energy metabolism is a crucial factor affecting several sperm functions. These organelles are responsible for the function of metabolic pathways during germ cell development and fertilization. In addition, spermatozoa can use different substrates and thus activate various metabolic pathways depending on the available substrates and the physicochemical conditions under which they operate. These functions are critical to ensure fertilization success. However, the most valuable aspect of the function of mitochondria in all types of cells is the production of chemical energy in the form of ATP, which, in the case of spermatozoon, is necessary to ensure motility. Mitochondrial activity is one of the main determinants of male fertility (Piomboni et al., 2012).

In their study on the exposure of different ZnO nanoparticles to cultured stallion spermatozoa at 5°C for 0, 30, 60 and 90 minutes, Halo et al. (2022) showed that the higher concentrations of nanoparticles used (12 and 24 mg/ml) caused a significant decrease in spermatozoa mitochondrial activity after 90 minutes of exposure. However, platinum nanoparticles biosynthesized from pomegranate showed an effect on the viability of human breast adenocarcinoma cells MCF-7. Using MTT assay, platinum nanoparticles were demonstrated to cause a dose-dependent decline in the viability of MCF-7 cells at a concentration range of 2.5 to 50 µg/ml. A decrease in cell viability of almost 80% was observed for 2.5 µg/ml to 50 µg/ml concentrations after 72 hours of exposure to nanoparticles. The results indicate that the MCF-7 breast adenocarcinoma cell line is highly sensitive to biosynthesized platinum nanoparticles (Şahin et al., 2018). Results similar to this study were also reported by Konieczny et al. (2013) wherein the effect of platinum nanoparticles on normal human epidermal keratinocytes (NHEK) cells was evaluated using MTT assay. After 24 and 48 hours of culture with the addition of 6.25, 12.5 and 25 µg/ml platinum nanoparticles at a temperature of 37°C, no significant effect could be seen on the mitochondrial activity of NHEK cells (although a slightly decreasing trend in cell viability was observed although statistically insignificant as compared to control). However, Bendale et al. (2017) used cancer cell lines to assess the *in-vitro* cytotoxic activity of platinum nanoparticles. Lung adenocarcinoma cells A549, ovarian teratocarcinoma cells PA-1, and pancreatic cancer cells Mia-Pa-Ca-2 when cultured with platinum nanoparticles for 48 hours, the highest growth inhibitory effects were noted on PA-1 cells. A specific cytotoxic effect was

observed at a dose of 50 g/ml1 platinum nanoparticles, while the maximum effect was achieved at a concentration of 200 g/ml. Gholami-Shabani et al. (2023) also reported the cytotoxic properties of platinum nanoparticles upto a concentration of 400 µg/ml after 24 hours of culture with nanoparticles human liver cancer cells HepG-2. Viability of HepG-2 cells was found to decrease with increasing concentrations of platinum nanoparticles (from 25 to 400 µg/ml).

Conclusions

The result of the present study indicates towards an adverse impact of platinum nanoparticles on rabbit spermatozoa motility *in-vitro*. However, the viability as measured by the metabolic activity of spermatozoa remained unaffected even after the addition of platinum nanoparticles. Mechanisms of the action of metal nanoparticles, including platinum nanoparticles, on living organisms are still insufficiently investigated. Further research in this field is required in the future as the usage of such nanoparticles rises in consumer products.

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Conflicts of interest

The authors have confirmed that there are no conflicts of interest to report.

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