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Research Article

Journal of Sexual Health and AIDS Research

Effect of Magnesium Oxide Nanoparticles on Human Sperm Parameters after Freezing

Sheida Mirgalooye Bayat[*1], Nasim Hayati Roodbari[2], Farahnaz Farzaneh[3], and Shahla Mirgaloybayat[4]

[1]*MSc of cell and developmental biology, Department of Biology, College of Basic Science, Tehran, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Department of Biology, Sciences and Research Branch, Islamic Azad University, Tehran, Iran*
[3]*Infectious Diseases and Tropical Medicine Research Center, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[4]*Endometriosis Research Center, Iran University of Medical Sciences, Tehran, Iran*

[*]**Corresponding author:** Sheida Mirgalooye Bayat, Department of Biology, College of Basic Science, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.

Submitted: 17 August 2024 **Accepted:** 23 August 2024 **Published:** 30 August 2024

Citation: *Sheida Mirgalooye Bayat, Nasim Hayati Roodbari, Farahnaz Farzaneh, Shahla Mirgaloybayat (2024) Effect of Magnesium Oxide Nanoparticles on Human Sperm Parameters after Freezing. Jour of Sexu Heal and AIDS Res 1(4), 01-08.*

Abstract

Background: *In reproductive science, freezing reduces the fertility potential of sperm due to the production of reactive oxygen species and physicochemical damage, which leads to a decrease in sperm viability and mobility. Adding antioxidants to freezing environments can protect sperm from cold damage and neutralize the harmful effects of ROS. This study aims to investigate the effect of magnesium in the form of magnesium oxide nanoparticles to examine sperm parameters and measure the quality of sperm cells caused by the effects of freezing.*

Materials and Methods: *Eight semen samples of normal humans referred to Noor Pathobiology Laboratory were evaluated in 8 Fresh and Freeze and control groups. Three fresh experimental groups were only exposed to MgO nanoparticles with concentrations of 5, 25, and 50 µg/ml and were analyzed half an hour later. Three freezing experimental groups were frozen after including concentrations of MgO nanoparticles and thawed and analyzed half an hour later. Two control groups including control 1: were analyzed half an hour after exposure to the environment and control 2: half an hour after freezing were thawed and analyzed. DNA fragmentation, morphology, motility, viability, and PH of sperm were evaluated. Data were analyzed using a one-way analysis of variance.*

Results: *Evaluations showed that magnesium oxide nanoparticles have a toxic effect on sperm cells, thereby causing a significant difference in motility and viability parameters between control group nanoparticles and both fresh and frozen groups at all concentrations. Also, the use of Mgo NPs in fresh and frozen processes effectively maintains the pH, morphology, and fragmentation of DNA in sperm cells.*

Conclusion: *MgO nanoparticles may negatively impact the movement and survival of sperm during both the fresh and freezing processes. The concentration and duration of exposure to these nanoparticles are crucial factors that determine their toxicity on the sperm cell.*

Keywords: MGO Nanoparticles, Sperm Parameters, Human Semen Fluid, Sperm Freezing.

Introduction

According to the World Health Organization, infertility is a disease in the reproductive system that occurs by failure to achieve clinical pregnancy after twelve months of regular and unprotected sexual activity, it should be noted that infertility does not mean the lack of gamete production, but the inability to give birth to a live baby is called infertility [1].

Infertility has various causes, and in many infertile couples, several factors may play a role in it at the same time. Infertility may be due to men's infertility problems, women's infertility problems, or without a specific reason and justification under the title of unexplained infertility. About 44 to 55 percent of infertility factors are due to male factors. From a diagnostic point of view, the concept of male fertility is measured by sperm count, motility, and morphology, which is calculated by semen analysis [2].

The sperm freezing technique is especially useful in preserving the fertility of men with various types of cancer before starting radiation or chemotherapy. Also, this technique is recommended in people who have non-malignant diseases such as diabetes and autoimmune diseases or high blood pressure that can lead to testicular damage, or who have a low number of sperm cells [3].

The sperm freezing technique leads to damage in sperm cells due to temperature and destructive changes in sperm structure including cell membrane, mitochondria, and DNA, so adding estrogenic compounds or effective antioxidants to the freezing environment can play a role in reducing the risks caused by sperm freezing [4].

The common materials in sperm-freezing environments are albumin and glycerol. This group of anti-freezing materials is stronger than water at low temperatures and as a result, reduces the amount of ice formed inside the cell and reduces the amount of damage caused by physical changes in cells and membranes [3].

Magnesium oxide nanoparticles are widely used due to their unique properties, including easy synthesis, non-toxicity, and cheapness, as well as antibacterial and antimicrobial properties [5].

These nanoparticles are usually found in the size of 5 to 100 nm and a specific area between 25 and 50 square meters per gram. Although these metal oxide nanoparticles are very small in size, they have a very high melting point and boiling point that is several times that of the bulk material. These nanoparticles are widely used due to their new properties, such as simple stoichiometry, crystalline structure, and high ionic properties [6].

Through ongoing clinical research on magnesium nanoparticles, positive results have been achieved, for example, these nanoparticles offer precise drug delivery, improved permeability, and unique targeting to tumors with minimal or no side effects. Also, as drugs with characteristics such as molecular weight, pH, ionic strength, and particle size, they are simultaneously used in nano-cryosurgery, hyperthermia, tumor inhibition, and in brain tumor imaging [7].

A study has shown that magnesium oxide nanoparticles cause significant toxicity with genotoxic, biochemical, histopathological, and biodistribution parameters and also found high levels of malondialdehyde and reduced glutathione, catalase, and superoxide dismutase, which indicates the occurrence of oxidative stress. Therefore, genotoxicity may be caused by ROS [8].

The results obtained from the study show that exposure to high doses of magnesium oxide nanoparticles causes DNA damage and significant biochemical changes [9].

Therefore, this study aims to investigate the effect of magnesium oxide nanoparticles on sperm parameters after freezing.

Materials and Methods

Chemicals

Magnesium oxide nanoparticle powder was supplied by Nano-bazar Iran (CAS number 1309-48-4). magnesium oxide stock solution with concentrations of 5-25-50 µg/ml was prepared using deionized distilled water as a solvent.

Sample Collection and Analysis

Semen samples of 8 healthy men (n = 8) between 30 and 42 years old after 3 to 5 days of abstinence (normozoospermic men), from the Noor Pathobiology Laboratory in Tehran, Iran. Moreover, written informed consent was obtained from each subject before their inclusion in the study, and the approval of the ethics committee with code IR.IAU.SRB.REC was received from the biomedical research ethics committee. After initial evaluation, the samples were selected with a number of more than 10 million sperm per milliliter and a motility of more than 42%. Types of mobility including A, B, C, and D classifications were assessed by optical microscopy at 400× magnification and a light microscope equipped with the Computer-assisted sperm analysis (CASA) system and were classified according to WHO guidelines 2010 as progressive, nonprogressive, or immotile. Then, additional factors such as vitality were determined through the use of the trypan blue staining technique, pH measured using pH meter paper, DNA fragmentation assessed using the SCD method, and morphology analyzed through Diff-Quik staining.

Exposure Procedure

Each semen sample is divided into 8 parts as follows:

- **Control 1:** Seminal fluid is analyzed half an hour after being placed in the environment.
- **Control 2:** The semen is frozen for half an hour and then thawed and analyzed.
- **Fresh experimental group 3:** semen is affected by the concentration of 5 µg/ml of magnesium oxide nanoparticles and then analyzed.
- **Fresh experimental group 4:** Semen is affected by the concentration of 25 µg/ml of magnesium oxide nanoparticles and then analyzed.
- **Fresh experimental group 5:** Semen is affected by the concentration of 50 µg/ml of magnesium oxide nanoparticles and then analyzed.
- **Freezing experimental group 6:** Semen is affected by the concentration of 5 µg/ml of magnesium oxide nanoparticles and then it is frozen and analyzed.
- **Freezing experimental group 7:** Seminal fluid is affected by the concentration of 25 µg/ml of magnesium oxide nanoparticles and then it is frozen and analyzed.
- **Freezing experimental group 8:** Seminal fluid is affected by the concentration of 50 µg/ml of magnesium oxide nanoparticles and then it is frozen and analyzed.

In all groups, analysis is done half an hour after freezing or adding nanoparticles.

Sperm Freezing and Thawing

In this research, magnesium oxide nanoparticles with a particle size of 20 nm in the form of powder (Iran Nanobazar Company)

were used (Figure 1). For freezing, a freezing medium (origio, Denmark) consisting of glycerol, glucose, and HEPES (a buffering agent) was used in a ratio of 1:1, meaning that 1 cc of the freezing medium was added to 1 cc of sample fluid. A volume of 250 µl of semen was mixed with 250 µl of freezing medium. To start the freezing process, the sperm freezing medium was extracted from the refrigerator and the appropriate amount was carefully placed drop by drop on the sperm sample inside the cryotube. It was gently mixed with the sample and left at room temperature for 15 minutes. Then the cryotubes were exposed to nitrogen vapor for 15 minutes and then transferred to liquid nitrogen at -196 degrees Celsius for 30 minutes and as a result, the samples were frozen. Following a 30-minute period of cryostorage, the cryotubes were extracted from the nitrogen tank and left at ambient temperature for 15 minutes to facilitate the thawing of ice crystals. Subsequently, these cryotubes were immersed in standard tap water and subjected to an additional 15-minute incubation period. Following this sequence of events, the analysis was recommenced.

Sperm Viability and Motility

First, a volume of 50 microliters of semen sample was added to a test tube, followed by the addition of 50 microliters of trypan blue dye in a 1:1 ratio. The mixture was then thoroughly mixed in a shaker for a duration of 10 seconds. 10 microliters of the homogenous sample were placed on a glass slide and observed using a binocular optical microscope and a 40 lens, the count of sperm was meticulously recorded using a counter. Sperms that exhibited a blue color and had the dye penetrate their head were identified as dead sperms, while sperms that did not display dye penetration in their head were considered alive. A total of 100 sperms were counted in each sample, and the percentage of live and dead sperms was meticulously documented.

In addition, motility is used by the ocular method with a microscope and then by the CASA system. Firstly, a semen sample of 5 microliters was carefully placed onto the slide. Subsequently, the motion of the sperm cells was precisely distinguished and documented using a microscope that was linked to CASA software, all while utilizing a magnification of 20x. Based on the WHO guidelines, sperms can be either progressive motile, move linearly or in a large circle, or be non-progressive, move on a side or slowly without any forward movements, including beating flagellum, moving in small circles, or being immotile [10]. Also, in the optical method, 5 microliters of the semen sample was placed on a slide and re-examined with a microscope and 40x magnification at different angles.

Sperm DFI Detection

DNA fragmentation was determined by utilizing the SDFA kit (Dain Bioassay, Iran) in accordance with the guidelines provided by the manufacturer. In brief, 50 µL of semen was diluted in Ham's F10 medium, and a semen aliquot was combined with 50 µL of 6.5% agarose. Subsequently, 20 µL of the resulting mixture was applied onto a glass slide that had been pre-treated and then placed on a chilled surface at a temperature of 4°C for a duration of 5 minutes. The slides were then subjected to a denaturizing solution for a period of 7 minutes, followed by treatment with a lysing solution for 15 minutes. Subsequent to this step, the slides underwent a washing process with distilled water lasting for 5 minutes. Dehydration was achieved through the utilization of ethanol at increasing concentrations of 70%, 90%, and 100%. Finally, the air-dried slide was subjected to staining. A minimum of 200 spermatozoa were observed and evaluated under a microscope with a magnification of 100x. Spermatozoa demonstrating a large or medium halo were categorized as having intact chromatin, whereas those possessing either no halo or a small halo were classified as spermatozoa with fragmented DNA. The results were presented as sperm DNA fragmentation index (DFI) [10].

Diff-Quik Staining

Morphology detection was performed using a Diff-Quik kit (Ideh varzan farad, Iran). Firstly, a volume of 10 microliters of specimen was carefully placed onto the slide and the surrounding area was made ready. Subsequently, the slide was transferred to the incubator and left there until the semen had completely dried. Following the preparation of the slides, the staining process was carried out in accordance with the guidelines provided by the manufacturer. The slides were then placed in the environment or incubator to dry, and the morphology was meticulously examined using a magnification of 100x.

pH

In this research to evaluate pH, before adding nanoparticles to semen liquid, put a drop of seminal fluid on the pH meter paper, alkaline or Its acidity was observed and pH was recorded. Also, in the next step Adding nanoparticles to the semen and after the freezing process, the pH was recorded in the same way.

Statistical Analyses

The data were presented as average values with the addition or subtraction of the standard error. The outcomes were subjected to statistical analysis through the utilization of a one-way analysis of variance. In order to analyze the data and generate visual representations, SPSS (Version 25, Armonk, New York, USA) was applied to analyze the data. A $P < 0.05$ was statistically considered significant.

Results

In the comparison of the fresh experimental groups with the frozen groups, no significant changes in pH were observed, and there was no significant difference between the groups receiving nanoparticles and the control groups 1 and 2 (Figure 1).

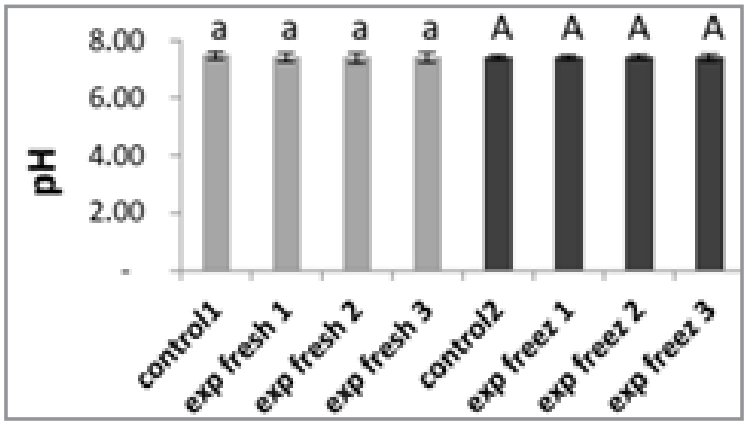


Figure 1: The trend of changes in pH parameters in different groups after 30 minutes in the environment (a) and 30 minutes after freezing (A). Small letters (a) represent fresh groups and capital letters represent frozen groups (A).

Regarding the viability parameter, the results indicated a decrease in the viability percentage in the groups receiving Mgo nanoparticles compared to both control groups. A significant difference was observed in the freezing experimental group receiving 50 µg/ml compared to the control group of the same group. In control group 1, compared to control group 2, the percentage of vitality decreased, but it was not significant (Figure 2).

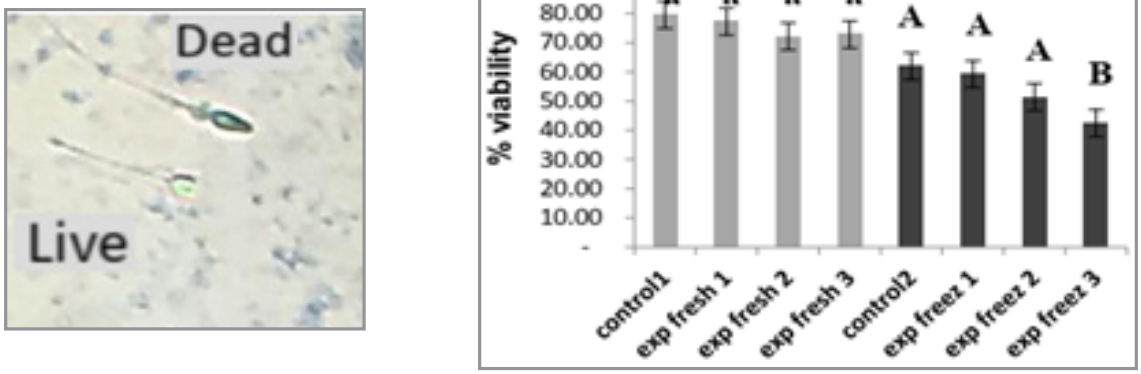


Figure 2: Evaluation of the sperm viability.A. trypan blue staining showing the heads of viable (white) and dead (blue) sperms respectively (400x eyepiece magnifications), and B. The trend of changes in sperm viability in different groups after 30 minutes in the environment (a) and 30 minutes after freezing (A). The non-uniformity of capital letters (A/B) indicates a significant difference between the frozen group compared to the control of the same group (p <0/05).

Regarding the types of mobility, there was a decrease in the movement of the progressive type A-B class in all groups receiving Mgo nanoparticles compared to both control groups. A significant difference was observed in the movement of class A in all concentrations of fresh test groups compared to control group 1. Also, a significant difference was seen in both fresh and frozen control groups (P<0.05) (Figure 3). In class B movement in the fresh and frozen test groups receiving 50 micrograms/ml of nanoparticles compared to the control group of the same group (1 and 2), and also between both fresh and frozen control groups, a significant difference was observed (Figure 3). In class C movement, there was a decrease in fresh and frozen groups receiving Mgo nanoparticles compared to both control groups. A significant difference was observed in all the received concentrations of nanoparticles in the fresh test groups compared to the control group 1 and in the received concentrations of 25 and 50 µg/ml in the frozen test groups compared to the control group 2. Also, the difference between both fresh and frozen control groups was significant for P < 0.05 (Figure 3). Also, a significant increase in all concentrations was observed in the number of non-motile sperm in the groups receiving nanoparticles compared to the control groups. Also, the difference between both the fresh and frozen control groups was significant, and a significant difference was observed in the received concentration of 50 µg/ml in frozen test group 3 compared to control group 2 (Figure 3).

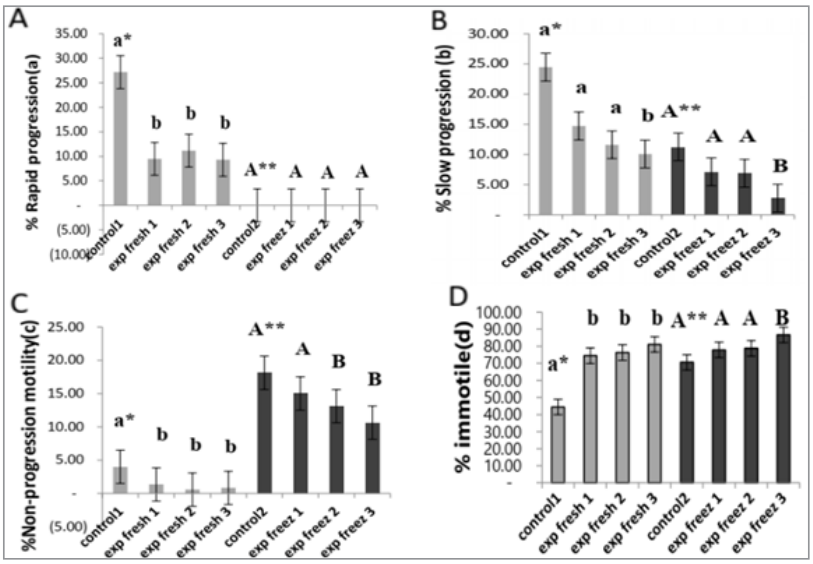


Figure 3: Evaluation of the sperm motility. A: The trend of changes in the percentage of rapid progression (a) in different groups after 30 minutes in the environment (a) and 30 minutes after freezing (A). B: The trend of changes in the percentage of slow progression (b). C: The trend of changes in the percentage of non-progression (c). D: The trend of changes of the immotile sperm percentage. The non-uniformity of small and capital letters (a/b) (A/B) indicates a significant difference between the fresh and frozen groups and the same control groups ($p<0.05$). The non-identity of the * indicates a significant difference in the fresh and frozen control groups ($p<0.05$).

In the present study, the morphological problems of the sperms treated with magnesium oxide nanoparticles were also observed before sperm freezing. The current images are related to sperms that were fresh and frozen-thawed and show that abnormal sperms still exist and magnesium oxide nanoparticles had no positive or negative effect on sperm morphology compared to the control group (Figure 4).

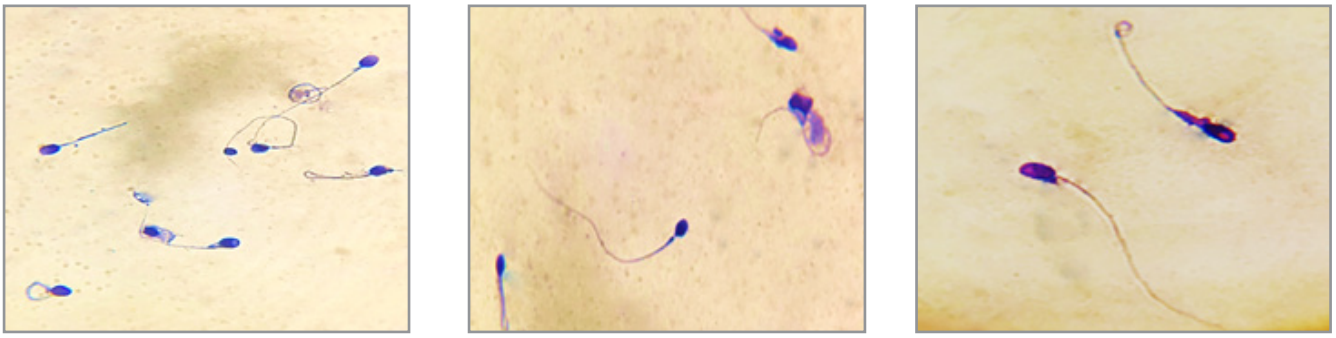
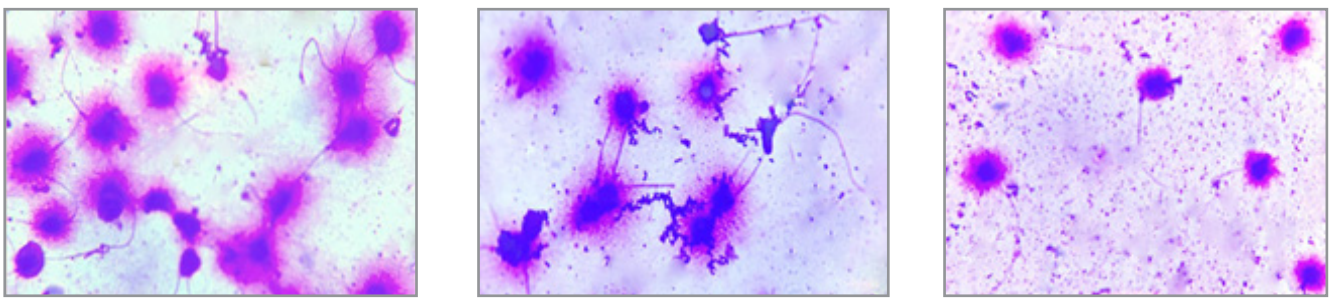


Figure 4: morphology detection of sperm using Diff Quick staining in different groups (1000x eyepiece magnifications) A: fresh control group B: freezing control group C: freezing + MgO NPs group



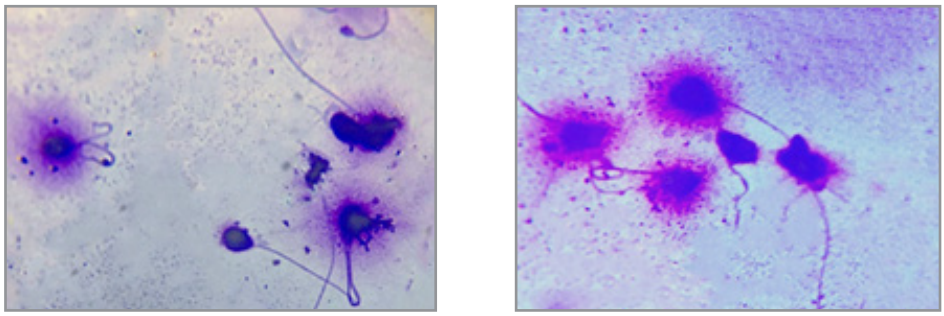


Figure 5: Human sperm DNA fragmentation as analyzed by an SCD assay in the different treatment groups: (1000x eyepiece magnifications) A: fresh control group B: fresh + 5 µg/ml MgO NPs C: freezing + 5 µg/ml MgO NPs D: freezing + 25 µg/ml MgO NPs E: freezing + 50 µg/ml

Discussion

It is important to note that similar results were obtained in both the fresh and frozen experimental groups, suggesting that the magnesium oxide nanoparticle functions under the same principles at room temperature and freezing conditions. However, it is worth mentioning that the frozen samples exhibited a greater decrease in the measured parameters compared to the samples at ambient temperature.

The extensive utilization of magnesium oxide nanoparticles can be attributed to their distinctive characteristics, including their ease of synthesis, non-toxicity, affordability, and possession of antibacterial and antimicrobial properties [5].

One possible approach to address the issue of oxidative erosion when freezing sperm cells is the introduction of various antioxidants into the preparation and freezing environment. This is because antioxidants serve as the only moderating factor between the physiological and pathological functions of reactive oxygen species (ROS). In essence, antioxidants function to counteract reactive oxygen species (ROS) and mask its active and aggressive nature, thus inhibiting its participation in the process of oxidative erosion [11].

The mitochondrial pathway of apoptosis plays a crucial role in cell death caused by metal oxide nanoparticles among various pathways leading to cell death. This is primarily because mitochondria serve as one of the main organelles targeted by the oxidative stress induced by nanoparticles [12]

The findings of the study suggest that exposure to significant quantities of magnesium oxide nanoparticles can result in both DNA damage and notable changes in biochemical functions [9].

The primary cause of reproductive toxicity induced by nanoparticles is oxidative stress [13]. Infertility issues in men are primarily caused by reactive oxygen species, as the heightened production of ROS results in cell apoptosis and impaired spermatogenesis [14].

When nanoparticles are discharged into the surroundings, they have the potential to easily penetrate cells through receptor-mediated endocytosis or passive diffusion, thereby engaging with cellular proteins, lipids, and genomic DNA [15].

Certain nanoparticles possess the capacity to influence the reproductive system by disrupting protective tissues within the system, including epithelial, placental, and reproductive cells such as germ cells, Leydig cells, and Sertoli cells. Furthermore, these nanoparticles possess the capability to generate reactive oxygen species, which function as molecular mediators in the transmission pathways of spermatogenesis, steroidogenesis, and the regulation of the hypothalamus-pituitary-gonadal axis. As a result, this interference has implications for the maturation of sperm, as well as the occurrence of toxic effects such as the condensation of DNA and flagellum [5].

In this study, the impact of magnesium oxide nanoparticles at concentrations ranging from 50-25-50 µg/ml, compared to the control group, on the progressive type of movement both in its fresh state and after undergoing freezing, demonstrated a substantial decrease. Additionally, a noteworthy reduction in movement was observed in the non-progressive type or class C, specifically within the frozen groups that were subjected to the concentrations of 50 and 25 µg/ml of nanoparticles, in comparison to the frozen control group. This decrease was similarly maintained in the fresh test samples. In contrast, a significant increase was observed in all concentrations of Mgo nanoparticles received in the non-moving type or class D, when compared to their respective control groups. Moreover, a significant increase was observed in the group exposed to the concentration of 50 g/ml of this nanoparticle during the freezing process, in contrast to the control group.

In this investigation, the impact of magnesium oxide nanoparticles at concentrations of 50-25-50 µg/ml was observed in relation to the control group in terms of progressive movement (grades A and B), which demonstrated a significant decrease subsequent to the freezing process.

In addition, a noteworthy reduction was observed in the locomotion of the non-progressive type or class C within the freezing cohorts that were exposed to concentrations of 50 and 25 µg/ml of the nanoparticle. This decline exhibited significant deviation when compared to the control group, and furthermore, this decline persisted in the subsequent experimental samples.

There was also a notable augmentation in the density of 50 µg/ml of this nanoparticle within the freezing group in comparison

to its control group. The findings of the investigation indicated that the nanoparticle induces a cytotoxic impact on sperm cells, and the introduction of the magnesium oxide nanoparticle in concentrations of 5-25-50 µg/ml in contrast to the control group in progressive-type mobility was drastically diminished fresh and subsequent to freezing, as well as in non-progressive-type movement or Class C in freezing groups that exhibited a density of of 50 and 25 µg/ml of the nanoparticle we had witnessed a significant decline in freezing control groups, and the same reduction persisted in the new experimental samples. In the case of non-moving motion or class D, there was a significant increase in the concentrations of Mgo nanoparticles compared to their control groups that were observed.

There was also a notable increase in the concentration of 50 g/ml of this nanoparticle in the freezing group compared to its control group. This nanoparticle exhibits a cytotoxic influence on spermatozoa, and its exposure disrupts the motility of spermatozoa. The results of this investigation are consistent with the findings that demonstrated the ability of this nanoparticle to induce dysfunction in the mitochondria and the generation of reactive oxygen species by altering the potential of the mitochondrial membrane, ultimately leading to apoptosis and cell death [16].

In this investigation, there were no discernible adverse impacts on the structure, quantity, and pH characteristics subsequent to the freeze-thaw process, neither in the fresh experimental cohorts nor in any of the cohorts that were administered magnesium oxide nanoparticles. It is possible, however, that increasing the concentration of nanoparticles and prolonging the duration of exposure, both in vitro and in vivo, could potentially enhance their toxicity and result in more significant impairment in these parameters.

The process of freezing and thawing intensifies the harm to overall health, as demonstrated by the increase in DNA damage and breakage in the frozen test groups compared to the fresh test groups. This aligns with the discovery that DNA causes irreversible changes in the well-being of the sperm's genetic material. Additionally, in both the fresh and frozen experimental groups that were exposed to a concentration of 25 g/ml of magnesium oxide, the DNA breakage was higher than that of other doses derived from nanoparticles. However, this difference did not reach statistical significance.

In the context of DNA breakage, the quantity of DNA damage and breakage in the frozen test groups exhibited an escalation when compared to the fresh test groups. This discovery is in line with the findings of a study carried out by Fraser et al., which provided evidence that the freeze-thaw process exacerbates the negative impact on one's well-being. DNA induces irreversible alterations in the well-being of the sperm genome. Furthermore, in both the fresh and frozen experimental groups that received a concentration of 25 g/ml of magnesium oxide compared to the fresh and frozen control groups, the magnitude of DNA breakage exceeded that caused by other doses of nanoparticles. However, it is noteworthy that this disparity did not attain statistical significance.

Magnesium oxide nanoparticles are a category of metallic oxides that have a significant role in materials science and biomedical diagnostics due to their importance and extensive use. However, the potential toxicity of MgO nanoparticles towards bacterial and human cells as well as organs has not been thoroughly investigated. So far, the effect of magnesium oxide nanoparticles on semen parameters in an artificial environment, as well as the consequences after semen freezing, have not been studied in this way.

Several investigations have been conducted to examine the impact of MgO nanoparticles on different cell lineages. Lung cancer cells, which induce toxicity in lung cancer cells, may be responsible for the increase in levels of reactive oxygen species (ROS), as the potential of the mitochondrial membrane undergoes changes and triggers the phenomenon of apoptosis, resulting in cellular death [16].

The findings derived from the research indicate that exposure to elevated levels of magnesium oxide nanoparticles leads to DNA harm and notable alterations in the cellular biochemical profile [9].

Conclusion

In general, in the current research, it was found that adding magnesium oxide nanoparticles to human sperm, both fresh and frozen, can cause toxic effects on parameters such as sperm viability and motility, but in vitro, it is capable of morphological changes., the number and pH will not be broken, although the concentration and time of exposure to this nanoparticle are important and influencing factors on the toxicity of this nanoparticle on sperm cells.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This article, extracted from the Master's thesis, was supported by the Tehran University of Research Sciences. The authors wish to thank the compassionate cooperation of all personnel in the Noor Pathobiology Laboratory and the Andrology Laboratory of Avicenna Fertility Center.

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