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Clay Nanoparticles Ameliorate the Reproductive Toxicity Induced By Silver Nanoparticles in Adult Male Rats

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Abstract

Nano silver (Ag-NPs) is a commonly utilized type of nanoparticles in various fields. Meanwhile, nano clay (clay-NPs) possesses antioxidant properties. Our objective was to evaluate the reproductive toxicity of Ag-NPs (50 nm) at a dosage of 100 mg/kg on adult male albino rats and explore whether clay-NPs (100 nm) at a dosage of 40 mg/kg could mitigate the adverse effects of silver. We divided thirty-two adult male albino rats into four equal groups: the control group, the Ag-NPs group, the nano clay group, and the clay-NPs/Ag-NPs group (combination group). The rats were orally administered Ag-NPs (100 mg/kg) and clay-NPs (40 mg/kg) for a total of 60 days. The results of our study revealed that exposure to Ag-NPs had detrimental effects on sperm motility, morphology, viability, and concentration. Additionally, Ag-NPs induced oxidative stress, as evidenced by the comet assay, which indicated DNA damage in the rat sperm. Morphometric and histopathological examinations of the testes of rats exposed to Ag-NPs demonstrated various histological alterations. However, the co-administration of clay-NPs ameliorated most of these toxic effects of Ag-NPs, potentially through their antioxidative capacity

Keywords: clay nanoparticle silver nanoparticle antioxidants, DNA damage, sperm evaluation, testes histopathology

1. Introduction

The study of reproductive toxicity associated with nanomaterials (NMs) is a relatively new field of research, but it is increasingly recognized as an important aspect of risk assessment and hazard characterization of chemicals (Chou et al., 2018)(21).

Nanotechnology holds great potential across various industries, including agriculture, and offers possible solutions to current challenges. Nanoparticles have gained significant attention in commercial applications, but our understanding of their interactions with biological systems is still limited. The unique properties of nanoparticles at the nanoscale, different from those of the same material in bulk form, contribute to both their value and potential risks (Rameshaiah et al., 2015)(69).

Nanoclay, a type of nanomaterial, has attracted attention in agriculture as a potential nanofertilizer due to its unique properties. It refers to processed clay minerals, such as montmorillonite and halloysite, that are transformed into nanoparticles with high surface area and reactivity. These nanoparticles offer several advantages as nanofertilizers, including improved nutrient delivery, controlled release, increased water-holding capacity, and enhanced soil fertility (Zhang M. et al., 2017).(98)

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Nano clay has also garnered significant interest in the field of pesticide applications. The incorporation of nano clays into pesticide formulations provides several benefits, including enhanced efficacy, controlled release, reduced environmental impact, and improved safety profiles (Ghormade V. et al., 2015)(35).

Silver nanoparticles (AgNPs) are considered highly hazardous metal nanoparticles due to their widespread use and the inevitable exposure of humans. They have been extensively employed in the production of cosmetics, healthcare products, and wound dressings .

Nano silver has gained considerable attention in recent years due to its potential application as a nanofertilizer. Specifically, nano silver has shown promise in enhancing plant growth, disease resistance, and nutrient utilization (Zhou et al., 2017)(99).

Review of Literature

According to research, male factor infertility is responsible for about 40-50% of all infertility cases (Ethaeb et al. 2021).(32) This means that in roughly half of the cases where couples struggle to conceive, the issue can be attributed to problems with the male partner's reproductive system. It is important to note that female infertility factors and male infertility factors can coexist in some cases.

Despite a lot of information about individual nanomaterials are available, but the toxicity level of many NPs is still indefinable, thus the application of these materials is limited due to the lack of knowledge of risk assessments and effects on human health. (Prasadetal. 2017)(48).

The review by (Souza et al. 2021)(81) provides strong evidence from experimental in vitro and in vivo studies published in the last 20 years that NMs can damage the male reproductive system of mammals. Also, the male nanoreprotoxicity is dependent on the NMs' physicochemical properties, species, experimental design, exposure conditions, and exposure time. In this sense, it was observed that smaller NMs (≤50 nm), which have high surface area and reactivity, have a greater potential to cross biological barriers and cause toxicity Nanoparticles can penetrate the blood testes barrier (Lan et al. 2012)(50) and alter Leydig cell viability, proliferation, and gene expression to mediate reproductive toxicity (Komatsu et al. 2008).(47) These particles also reduce cellular proliferation in mammalian spermatogonial stem cells in vitro, which would be expected to decrease fertility in vivo (Braydich-Stolle et al. 2010)(16). In addition, several NMs demonstrated the ability to cross the BBB and BTB and accumulate in the brain (10%), testis (25%) and other organs of the reproductive system, such as the epididymis, prostate, and seminal vesicle (1% each), enhancing the risk of potential toxicity through direct action on reproductive tissues or indirectly through the hypothalamic pituitary testis (HPT) axis (Cheng et al. 2012; Kielbik et al. 2019).(20,45))

Nanotechnology poses some risks and problems for the health and environment. Nano materials have serious health hazards and show toxic effects when entered into the human body, causing tissue damage to all the vital organs. (Latha et al, 2016; Rameshaiah et al. 2015).(48,69)

In the field of agriculture and the food industry, nanotechnology is poised to bring about revolutionary advancements through innovative techniques. Its applications span the entire spectrum of agricultural production, processing, storage, packaging, and transportation. Nanotechnology facilitates improved nutrient absorption in plants, more efficient and targeted use of agricultural inputs, disease detection, and disease control (Saurabh Singh et al. 2015)(79). By harnessing the power of nanotechnology, the agricultural sector can achieve enhanced productivity, sustainability, and quality throughout the entire food supply chain.

Nano Fertilizers

Although fertilizers are very important for plant growth and development, it is necessary to minimize nutrient losses in fertilization, and increase the crop yield through the exploitation of new applications with the help of nanotechnology and nanomaterials. Nano fertilizers or Nano-encapsulated nutrients might have properties that are effective to crops, releasing the nutrients on-demand, controlled release of chemical fertilizers that regulate plant growth and enhanced target activity. In the recent decade Nano fertilizers are freely available in the market, but particularly the agricultural fertilizers are still not shaped by the major chemical companies. Nano fertilizers may contain Nano zinc, Nano silver, silica, iron and titanium dioxide, Nano clay, ZnCdSe/ZnS core shell QDs, InP/ZnS core shell QDs, Mn/ZnSe QDs, gold nanorods, core shell QDs, etc. (Dimkpa 2014; Zhang et al. 2016).(24)

Nano clay has gained significant attention in recent years as a potential nanomaterial for various applications, including its use as a Nano fertilizer. Nano fertilizers are nanoparticle-based formulations that can enhance nutrient availability and improve nutrient uptake by plants, leading to improved crop productivity. Nano clay, also known as layered silicates, is a type of clay mineral with a layered structure. Due to its unique properties, such as its large surface area, high cation exchange capacity, and excellent water-holding capacity, Nano clay has demonstrated promising potential as a Nano fertilizer (Wang P. et al)(89).

This review paper highlights the use of layered Nano silicates, including Nano clay, as Nano

fertilizers for sustainable plant nutrition and pest management. It discusses the mechanisms of nutrient delivery and the potential benefits for crop production (Pal S. et al).(68)

One of the key benefits of Nano clay as a Nano fertilizer is its ability to efficiently deliver nutrients to plants. The high surface area of Nano clay particles allows for better adsorption and retention of essential nutrients, such as nitrogen, phosphorus, and potassium. This improves nutrient availability to plants and reduces nutrient leaching, leading to better nutrient uptake and utilization. Nano clay also exhibits controlled-release properties, which means it can gradually release nutrients over an extended period. This controlled-release mechanism ensures a sustained nutrient supply to plants, minimizing nutrient losses and reducing the need for frequent applications. Thus, Nano clay-based Nano fertilizers can contribute to improving nutrient use efficiency and reducing environmental pollution. (Zhang M. et al.2017)(.98)

Nano silver particles have a high specific surface area and unique physicochemical properties compared to conventional silver compounds. These properties enable enhanced interaction and absorption of nutrients, improved water retention and controlled release of ions, resulting in improved plant nutrition and growth.

Another emerging technique is utilizing silver nanoparticles for the delivery of fertilizers to plants because of their antimicrobial properties, but studies have found that it poses a serious threat to the ecosystem, causing membrane damage. Silver nanoparticles are usually difficult to recover. Some plant species tend to use this nanoparticle maximum and accumulate them in their tissue beyond the limit (Latha et al. 2016; Rameshaiah et al. 2015).(49,69).

Several studies have investigated the effectiveness of Nano silver as a Nano fertilizer. For instance, a study by (Zhou et al. 2017(99) demonstrated that application of Nano silver in rice cultivation significantly increased plant height, root length, and biomass. Additionally, Nano silver-treated plants exhibited improved nitrogen assimilation and higher concentrations of essential nutrients, such as potassium and magnesium, in their tissues. In another study, (Xu et al. 2016)(93) investigated the impact of Nano silver on tomato production. The results showed that Nano silver-treated tomato plants had higher yields, larger fruits, and improved resistance against fungal pathogens compared to the control group. This was attributed to the enhanced nutrient absorption and uptake efficiency facilitated by Nano silver.

Furthermore, Nano silver has also shown potential in reducing the dependence on chemical pesticides. It has been reported to possess antibacterial and antifungal properties, leading to decreased crop diseases and improved plant health. However, it is crucial to ensure the precise dosage and application methods to prevent any adverse effects on the environment or human health (Singh J. et al. 2019).(79)

It is important to note that while there are promising findings, further research is still required to optimize the application and dosage of Nano silver as a Nano fertilizer. Long-term studies are needed to assess its impact on soil health, microbial activity, and potential accumulation in the food chain. (Agronomy, 9dar, J. C., 2013).(4)

Nanopesticides

It is well known that insect pests are the predominant ones in the agricultural fields and also in its products, thus NPs may have a key role in the control of insect pests and host pathogens (Khota et al., 2012). So, development of non-toxic and promising pesticide delivery systems for increasing global food production while reducing the negative environmental impacts to the ecosystem;)

Nano clay The industrial and biomedical applications of Nano sized clay particles have been rapidly increasing. Nano clay–polymer composites have found important biomedical applications, such as antimicrobial coatings, Nano containers for drug delivery, and pesticide carriers (Kryuchkova et al. 2016).(49)

One of the main benefits of using Nano clay in pesticides is its ability to improve the delivery and release of active ingredients. Nano clay particles have a large surface area and can adsorb and encapsulate pesticide molecules, protecting them from degradation and facilitating controlled release. This controlled release mechanism allows for sustained and targeted delivery of pesticides to the intended pests or plants, improving their effectiveness while minimizing off-target effects (Ghormade V. et al. 2015).(35)

Nano clay-based pesticide formulations also offer improved stability and reduced volatility. The addition of Nano clays can enhance the stability of pesticide molecules, preventing their degradation due to environmental factors such as sunlight, moisture, and temperature fluctuations. This increased stability extends the shelf life of the pesticide product and ensures its efficacy over a longer period. Furthermore, Nano clay particles can act as carriers for different types of pesticides, including insecticides, fungicides, and herbicides. The adsorption and encapsulation properties of Nano clays enable the loading of multiple active ingredients, allowing for the development of broad-spectrum pesticide formulations. This can be particularly useful in integrated pest management strategies, where multiple pests or diseases need to be

controlled simultaneously (Khodakovskay M. V. et al. 2013).(44)

In terms of environmental impact, the use of Nano clay in pesticides can help reduce the amount of active ingredient required for effective pest control. The controlled release mechanism provided by Nano clays ensures that the pesticides are released gradually, reducing the overall amount of chemical needed and minimizing potential harm to non-target organisms and the environment (Gogos A.et al. 2012).(36).

It is important to note that the development and application of Nano clay-based pesticide formulations are still areas of active research. Several studies have investigated the effectiveness and safety of these formulations, considering factors such as pesticide release kinetics, environmental fate, and potential toxicity. The research aims to optimize the formulation parameters and ensure the safe and sustainable use of Nano clay-based pesticides (Singh R. et al., 2011).(79). **Nano silver** is the most studied and utilized Nano particle for bio-system. It eliminates unwanted microorganisms in planter soils and hydroponics systems. It is being used as foliar spray to stop fungi, molds, rot and several other plant diseases. Moreover, silver is an excellent plant-growth stimulator . Nano silver has been reported to exhibit strong antimicrobial activity against bacteria, fungi, and viruses. Its nanoscale size enhances its surface area, facilitating greater interaction with pathogens. For example, a study by (Kim et al. 2007)(46) showed the efficient antifungal activity of Nano silver against plant pathogens such as Botrytis cinerea and Alternaria solani. Nano silver has also demonstrated insecticidal properties against various pests (Subramanyam et al. 2016)(82) investigated the efficacy of nano silver against stored product pests such as Tribolium castaneum and Sitophilus spp. The study found that Nano silver significantly reduced pest populations and exerted long-term control.

While Nano silver shows promise as a Nano pesticide, it is important to consider the potential environmental impacts. Some concerns relate to the potential accumulation of AgNPs in soil and water systems, which may adversely affect non-target organisms. It is essential to assess the risk-benefit ratio and conduct further research on the environmental fate of Nano silver (Handy R. D. et al. 2008).(40)

Different types of NPs have different effects on sperm cell functions either upon direct exposure. Fewer reports considered utilizing the antimicrobial properties of NPs on sperm preparations or treatment of genital infections. Nevertheless, toxic effects of NPs on male reproductive performance are evident, which increase the environmental concerns about the dispersion of these NPs and their biological effects.

Review of publications on semen quality in men indicating an even more pronounced decrease in sperm production than expressed by the decline in sperm density. There has been a genuine decline in semen quality over the past 50 years. As male fertility is to some extent correlated with sperm count the results may reflect an overall reduction in male fertility.

When it comes to diagnosing male infertility, several tests and evaluations can be conducted. These may include a thorough medical history review, physical examination, semen analysis to assess sperm count, motility, and morphology, hormone level testing, genetic testing, and imaging studies such as ultrasound or testicular biopsy. (Kumar & Singh 2015).(49).

2. Materials and Methods

Tested Materials and Doses Determination

Nano- Clay (NC):

The clay we used is sourced from the land of Sinai, Egypt, and to enhance its properties, heating the clay to a temperature of 750 °C for one hour, we got reactive clay. To achieve a finer particle size, we employ high-efficiency grinding techniques, obtaining Nano-sized clay particles, which possess unique characteristics and applications.

Table (1): The chemical oxide composition of Nano clay

Type	Clay	Active clay
SiO_2	45.67	53.59
Al_2O_3	34.34	39.75
Fe_2O_3	0.33	0.38
CaO	0.34	0.42
MgO	0.04	0.04
SO_3	0.00	0.00
K_2O	0.05	0.07
Na_2O	0.01	0.01
Cl	0.00	0.00
LOI	12.95	0.35

Our Nano Clay had the Blaine surface area about_20000 cm2/g and its particle size dimensions were about 16-71 nm. The XRD, TEM for NC are given in figs. 1, and 2 respectively.

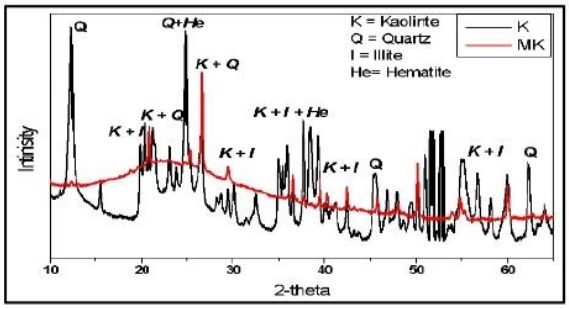


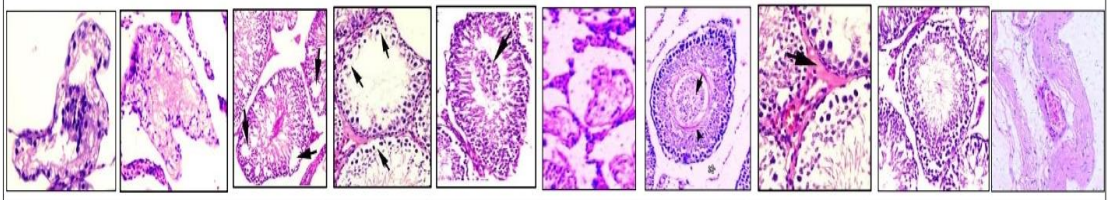
Fig. (1): XRD pattern of MK & NMK

Nano- Silver (Ag NPs):

Collection of Plant Stem:

The collection of plant stems was from agricultural lands. The stems undergo several cleansing steps. They are washed multiple times with tap water, followed by several rinses with distilled water to eliminate any traces of dust particles. Once cleansed, the stems are dried naturally at room temperature to remove any remaining moisture.

Tabulated figure 1: Showing the recorded histopathological lesions in the studied testis paraffin tissues section stained with haematoxylin and eosin.

Histopathological lesions	Irregular basement membrane	Detached sperm cells Aspermia	Vacuolation	Spermatogenic arrest	Sloughing necrotic spermatocytes	Edematous stroma	Hyaline material in tubular lumen	Interstitial bleeding	Detachment of spermatogenic cell	Thickness of tunica with dilated and congested of B.V
										
GP1: Control	-	-	-	-	-	-	-	-	-	-
GP2: Low dose	+	++	++	+	+	++	++	++	+	+++
GP3: high dose	++	+++	++	+++	+	++	+++	+++	+	+
GP4 Recovery of low dose	-	+	+	-	-	+	+	+	-	-

Severe: +++ Middle: ++ little: + No: -

Preparation of Stem Extract:

Once dried, the stems are carefully cut into small pieces. 10 grams is then placed in a 250ml Erlenmeyer conical flask, accompanied by 10ml of double-distilled water. The flask is heated, boiling the contents for a precise duration of 15 minutes at a temperature of 500 °C. After cooling, the resulting extract is filtered using Whatman No. 1 filter paper and stored at a temperature of 40 degrees Celsius for another step. The color of this extract presents itself as a delightful light brown.

Preparation of the $AgNO_3$ Solution:
To prepare a solution of nano silver, we combine 90ml of the stem extract with 10ml of a carefully prepared 1mm silver nitrate solution. This combination induces a transformation, as the color of the solution transitions from a light brown to a dark brown. This color change is attributed to the excitation of surface Plasmon, a phenomenon unique to the formation of nano silver.The dimensions of these silver nanoparticles are truly remarkable, ranging from 40 to 75 nanometers in size.

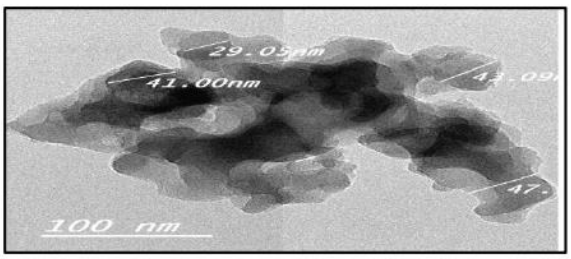


Fig. (2): TEM photographs of NMK

The chemicals used in this process were sourced from Sigma grade in the USA.

Animal Grouping:
Group I: control group orally administered saline.

Group II: Silver nanoparticle orally administered 100 mg / kg.

Group III: Nano Clay orally administered 40 mg / kg.

Group IV: Nano Clay and Nano Silver at the same dose orally administered 40 mg / kg and 100 mg / kg respectively.

Experimental Design

In this experiment, 32 adult male albino rats that were 16 weeks old and weighed between 150-180 grams. They were housed in the laboratory of the Zoology department at Fayoum University. The rats were kept in a stable environment with a temperature of 22 ±1ºC. After a period of 15 days to acclimate to their surroundings, the rats were randomly assigned to 4 groups, with 8 rats in each group.

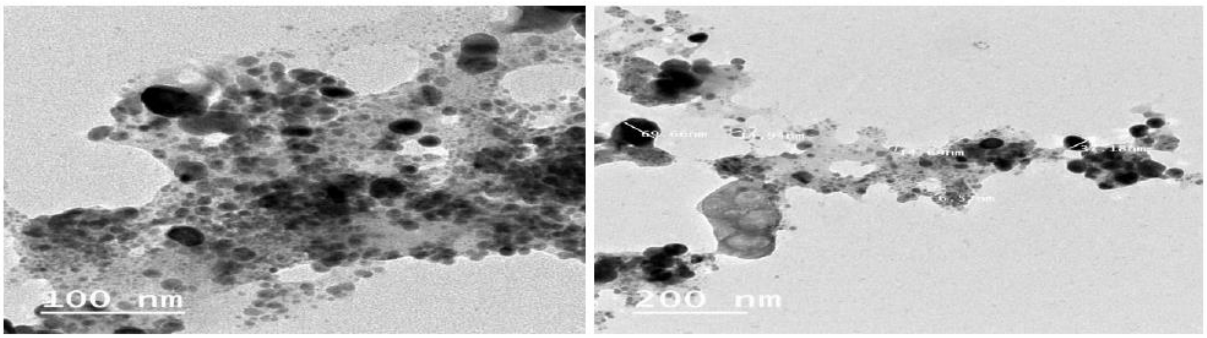


Fig. (3): TEM photographs of AgNP

The first group, known as the control group, received distilled water. The second group, called the Nano silver group, received 100 mg/kg BW of AgNPs. The dose of AgNPs was chosen based on the NOAEL (no observable adverse effect level) and the LOAEL (lowest observable adverse effect level) which were determined to be 30 mg/kg and 125 mg/kg respectively. The third group, known as the Nano clay group, received 40 mg/kg BW of clay nanoparticles. This dose was selected because it is within a range that is well-tolerated and does not cause significant adverse effects in rats. Lastly, the fourth group received both the previously mentioned doses of clay-NPs and Ag-NPs. Finally, after 60 days, the rats were weighed and sacrificed for sample collection.

Sampling:

Blood Sampling:

Blood samples were collected from the eye and centrifuged to obtain clear sera, which were then stored in a deep freezer for further analysis.

Specimen Collection:

The testes of the rats were dissected and divided into three pieces. One piece was used for

oxidative and antioxidant parameter evaluation, another piece was used for molecular parameters, and the third piece was fixed in formal saline for histological study.

Clinical Symptoms:

During the experimental period, the rats were observed daily for any signs of toxicity.

Measured Parameters:

Measurement of Body and Testicular Weight and Gonadosomatic Index(GSI):

The body weights were recorded at the beginning of the study and then at the end of the experiment, and the rats were euthanized and their testes were weighed. Measuring the gonadosomatic index (GSI) was calculated by dividing the testis weight by the body weight of each rat (Predes et al., 2007)(35).

Sperm Analysis:

The epididymis was excised and by placing in a pre-warmed petri dish containing 0.2 mL of calcium and magnesium-free Hank's solution at 37 ˚C. The tissue was minced with scalpels for approximately 1 min. and placed in a 37 ˚C incubator for 15 min to evaluate the sperm motility, viability, count, and abnormality.

Sperm motility:

Sperm motility was assessed by counting the number of progressively motile cells out of the total number of spermatozoa observed and then expressed in percentage according to the method .

Sperm viability

Sperm viability was determined using the Eosin-Nigrosin staining techniques, where live sperm cells appeared white and dead sperm cells appeared pink. The viability percent was calculated out of the number of live sperm cells from the total number of cells observed.

Sperm count

Sperm count was obtained using the hemocytometer using this equation for finding the sperm count (Ekaluo *et al*., 2008).(38).

$$\text{Sperm count} = \frac{\text{No. of spermatozo a counted} \times \text{Dilution factor} \times \text{Depth factor}}{\text{No. of area count}}$$

Sperm abnormality

sperm abnormalities were assessed by examining the head, mid-piece, and tail of the sperm cells. The percentage of sperm head abnormalities was calculated according to (Ekaluo et al., 2009).(39)

Comet Assay

We used The comet assay to determine DNA damage as designated by (Singh et al. 1988) with minor modifications. The sperm cells were processed and observed under a fluorescent microscope, and the tail length of DNA migration was measured to assess the severity of DNA damage.

Regular agarose (RA) and low melting point agarose (LMPA) were prepared at 0.75% and 0.5%, respectively, in Ca^{++} and Mg^{++} free PBS. 110 µL of RA were added to fully frosted microscope slides. 75 µL of LMPA containing 105 cells were added. At the end, a top layer of 75 µL LMPA was added. In lysis solution the slides were immersed for at least 1 h at 4˚C, and then left in an alkaline buffer for a quarter of an hour to allow the DNA unwinding and the expression of alkali-labile sites.

The slides were electrophoresed at 25V and 300 mA for 20 minutes, then washed with a neutralized buffer and stained with ethidium bromide (2 g / ml in distilled water). By using 400X magnification fluorescent microscope (Olympus) with an excitation filter of 515 - 560 nm and a barrier filter of 590 nm. From the trailing edge of the nucleus, the tail length was measured to the leading edge of the tail, using a calibrated scale in the ocular. Measuring The severity of DNA damage by comparing comet tail lengths with the diameter of the nucleus of undamaged cells observed in the same field. Two parameters were evaluated to determine the effect of malnutrition on the extent of DNA damage: the proportion of damaged cells and the mean tail length of DNA migration.

Oxidative/Antioxidant Parameters

Blood samples were also collected and Centrifugated at 3000 rpm for 30 min for sera collection for biochemical analysis including the measurement of total antioxidant capacity (TAC) by using ELISA assay. The TAC kit was obtained from (Biodiagnostic company) for diagnostic and research reagents, Dokki, Giza, Egypt).

Histopathological Examinations and Morphometric Study

Sections from The testes were processed for histological examination, and were stained with hematoxylin and eosin (Bancroft & Gamble, 2008), and were examined under a light microscope (Olympus, Warsaw, Poland). Haphazardly selections were made on seminiferous tubules from each testis and by using the Image j program, for measuring the transverse and the longitudinal tubular diameters various parameters, such as tubular diameter, germinal epithelium height, and blood vessel diameter, were measured by randomly chosen fields from each section. (Batra et al., 2001)(14).

PCNA Immunohistochemistry:

Immunohistochemistry was conducted to detect the presence of PCNA in the testes sections. The sections were incubated with specific primary antibodies against PCNA (GB13030; Wuhan

Saiweier Biological Technology, China). PCNA was detected in the nuclei of the mitotically divided spermatogonia or primary spermatocytes as a brown color.

Statistical analysis

We made Statistical analysis to find any significant differences among the treatment groups using the analysis of variance (ANOVA) and were run on the computer by using the SPSS program where level of $p<0.05$ and highly significant at $p<0.01$.

Results

Clinical signs and symptoms:

Many clinical signs and symptoms were observed during the experiment period and after administered with the studied NS dose (100 mg/kg) such as general aggressiveness, disgusting smell of urine, diarrhea, palpitation, decrease in the food and water consumption and hair fall but these symptoms not observed in all rats in Gp_{III} and with less degree only in Gp_{IV}. Nano silver group (Gp_{II}) appeared red in color and swollen as accumulation of fats and fluids (Fig. 5). Furthermore, the study showed that the administration of NS has a lethal effect on rats as shown in the table below.

3.2. Body, testicular weights and gonado-somatic index (GSI):

The weights of rats were recorded on the first and last day of the study. Animals treated with NS (100 mg/kg body wt) (Gp_{II}) appear to have a significant increase in their weight change in comparison with the control group. No significant change in body weights was reported in rats treated with NC Individually (40mg/kg) (GpIII) or in combination group NC/NS (40 mg NC /kg followed by 100 mg NS/kg) (GpIV). For testicular weight, test weights and percent of gonadosomatic index showed significant increase in (Gp_{II}) compared with control group (Gp_I). The final body weights in group (Gp_{III}) did not significantly change when compared with (Gp_I). Also, The testes weights and the percent of gonadosomatic index of nano clay treated group (Gp_{III}) showed no significant change when compared with (Gp_I) (Table 3).

Table (2): Mortality rate% in control and male albino rat treated with NC, NS and mixture (NC/NS) for 60 days.

Animal groups N=8	Period of the experiment								Mortality rate %
	1st week	2nd week	3rd Week	4th week	5th week	6th week	7th week	8th week	
Control group (GpI)	0	0	0	0	0	0	0	0	Zero
Nanosilver (NS) (GpII) 100 mg/kg bw	1	1	0	1	0	0	0	0	37.5
Nanoclay (NC) (GpIII) 40 mg/kg bw	0	0	0	0	0	0	0	0	Zero
Mix (NC/NS) (Gp_{IV}) 40 mg/kg bw/100 mg/kg bw	1	0	0	0	0	0	0	0	12.5

Table (3): Initial weight, body weight gain, final body weight change and the percent of body weight changes in male albino rat treated groups.

Animal groups N=8	Initial body Weight (g)	Final body Weight (g)	Body weight gain (g)	Total testes Weight (g)	Gonado somatic index %	Epididymis Weight (g)
Control group (Gp_I)	190	211.66 ± 2.2	20.0	2.17 ± 0.02	1.08 ± 0.01	0.31
Nanosilver (NS) (Gp_{II}) 100 mg/kg bw	200	273 ± 2.4	70.0	3.72 ± 0.07	1.46 ± 0.04	0.48
Nanoclay (NC) (Gp_{III}) 40 mg/kg bw	188	205 ± 1.9	17.25	1.9 ± 0.02	0.91 ± 0.03	0.22
Mix (NC/NS) (Gp_{IV}) 40 mg/kg &bw 100 mg/kg bw	190	201.5 ± 2.0	14.0	2.03 ± 0.03	1.12 ± 0.02	0.26

Table 4: Assessment of sperm count ($X10^6/mm^3$), motility (%) and viability (%) in male albino rats treated groups.

Groups / Parameters	Control	Nanosilver	Nanoclay	Mix
Count	59.98 ± 1.14	26.28 ± 0.77*	58.07 ± 1.08	43.82 ± 1.00*
Motility	80.12 ± 1.07	36 ± 1.22*	70.37 ± 1.01*	58.37 ± 0.98*
Viability	82.75 ± 1.30	39.62 ± 1.1*	74.62 ± 1.4*	61.37 ± 1.45*

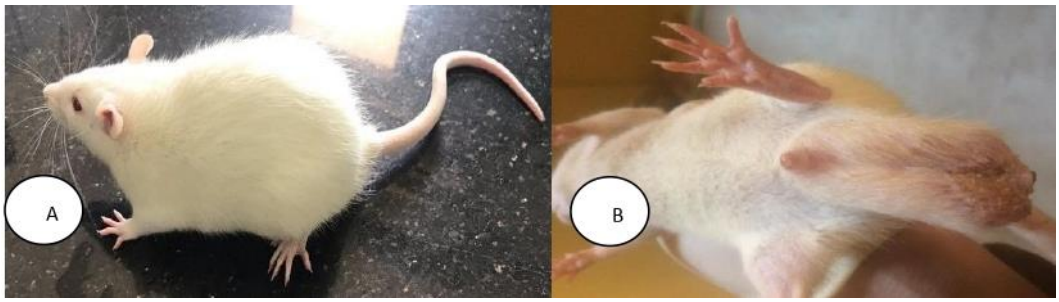


Fig.4: Photomicrograph showing normal scrotal sacs in control group (A) and reddish swollen ones in AgNPs treated groups (B).

Sperm Evaluation

Sperm count, Motility and Viability Sperm, count, motility and viability in Nano silver (Gp_{II}) showed a significant decrease in czomparring to control group (Gp_I). While exposure to nano clay (Gp_{III}) had no significant change in sperm count when also compared to the control group and there was a slight significant decrease in motility% and viability% compared with it.

On the other hand, (Gp_{IV}) appeared a significant increase in the count, motility, and viability of the sperms when compared to the Ag-NPs group.(Table 4)

Morphological Abnormalities

Sperm morphology in the Ag-NPs group showed a significant increase in sperm morphological abnormalities in comparison with the control group. While exposure to clay-NPs group appeared with no significant change in sperm morphological abnormalities compared to the control group. Moreover, concurrent administration of clay-NPs with Ag-NPs resulted in a slightly significant decrease in sperm morphological abnormalities when compared with the group that received Ag-NPs only (Gp_{II}) (Table 5) .

Comet assay:

The results represented a highly significant increase in the DNA % of damage from 7.4±0.29 in the control (Gp_{II}) to 12.90 ±0.18 in the AgNPs groups (Gp_{II}). However, (Gp_{III}) showed no significance (6.80±0.23) when compared to control, but there was a slightly significant decrease in (Gp_{IV}) (9.5 ±0.19) compared with (Gp_{II}) (12.90±0.18). The tail length appeared a significant increase in (Gp_{II}) (8.37±0.48), also there was no significant change in (Gp_{III}) (4.81±0.15) and in (Gp_{IV}) (5.9±0.17) all in comparison with control one (5.3±0.29).

As well the DNA % in the comet tail represented a significant increase in (Gp_{II}) (8.74±0.53), while (Gp_{III}) showed no significant changes (4.83±0.51) when compared with the control group (5.94±0.31). Also, (Gp_{IV}) (6.58±0.26) appeared slightly significant decrease compared to (Gp_{II}) and the tail moment

results showed a highly significant increase in Gp_{II} (0.78± 0.03) when compared with the control group (0.62±0.06). However, (Gp_{IV}) appeared a slightly significant decrease (0.42±0.04) in comparing with the (Gp_{II}) (0.78± 0.03).

Showing typical sperm nuclei of undamaged sperm cells of control. DNA damage observed as comets that were seen in the AgNPs group and with less degree in Gp_{VI} (NC/ AgNPs) group.

3.6.2. Total antioxidant capacity:

There was a highly significant decrease in the level of total antioxidant capacity in the AgNPs group (Gp_{II}) (0.87± 0.04) compared with the control group (1.48 ± 0.09) and Nano clay group (1.39 ±0.14). while a marked significant increasing in the level of total antioxidant capacity (1.14 ±0.09) was recorded in (Gp_{IV}).

Table 5: Effects of exposure to Ag-NPs and/or Nanoclay on sperm morphological abnormalities

Animal groups N=8	Normal Sperm %	Abnormal Sperm %	Abnormalities				
			Head			Tail	
			Amorphous	Without Hook	Banana Shape	Short Tail	Coiled Tail
Control group(Gp_I)	**75**	**25**	**15**	**4**	**3**	**1**	**2**
Nano silver (NS) (Gp_{III})	**56**	**44**	**25**	**6**	**7**	**2**	**4**
Nano clay (NC) (Gp_{II})	**80**	**20**	**12**	**3**	**2**	**1**	**2**
Mix (NC/NS) (Gp_{IV})	**72**	**28**	**18**	**5**	**3**	**1**	**1**

Table 6: Comet assay in the sperm of control adult male rat and other treated groups for 60 days.

Animal groups	% of damage	Tail length (PX)	%DNA in tail	Tail moment
Group l Control	7.4 ± 0.29	5.3±0.29	5.94 ± 0.31	0.34 ± 0.03
Group III nanosilver (100mg/kg/d)	12.9 ± 0.18	8.37 ± 0.48	8.74 ± 0.53	0.78 ± 0.03
Group II nanoclay (40mg/kg/d)	6.8 ± 0.23	4.81 ± 0.15	4.83 ± 0.51	0.33 ± 0.03
Group IV Mix Nanoclay/Nanosilver	9.5 ± 0.19	5.9 ± 0.17	6.58 ± 0.26	0.42 ± 0.04
F	170.7	27.003	15.41	39.43

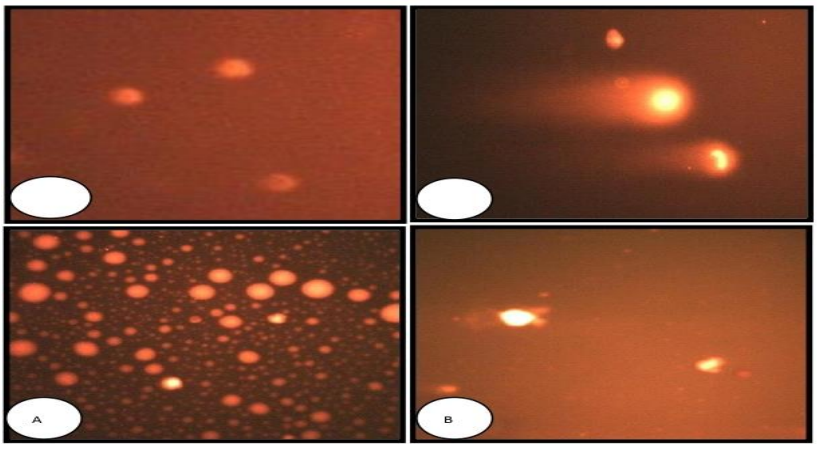


Fig. 6 Fluorescent microphotographs represent DNA damage, using comet assay in male rat sperm as determined by single cell gel electrophoresis with acridine orange staining. A. Showing typical sperm nuclei of undamaged sperm cells of control. B. DNA damage observed as comets that were seen in the NS group and with less degree in Gp_{IV} (NC/NS) group

Morphometric and Histopathologic Observations :Morphometric Analysis

The results represented a highly significant decrease in the diameter (81.51±2.91µm) and in the germinal epithelial height of the spermatogenic cells (7.29±0.54 µm) also in tubular lumen (66.93±2.6) of the seminiferous tubules of AgNPs group (Gp_{II}) all when compared with control group and any other ones. Also, a highly significant increase in the thickness of the tunica albuginea (239.32±7.99µm) and the diameter of the blood vessels (113.45±3.19µm) of the testicular tissue in (Gp_{II}) was also observed when compared with control group. All measured parameters in the mix group (Gp_{IV}) were significantly increased comparing with (Gp_{II}) and became more or less similar to control and Nano clay groups. **Histological examination of the testis:**

However, microscopic examination of transverse sections from the testes of rats in AgNPs (100 mg/kg) revealed disruptions in the germinal epithelium. The epithelium appeared irregularly placed on the basement membrane and, in certain areas, detached from it towards the lumen of the seminiferous tubules. Vacuoles were observed in the spermatogenic tissue, and hyaline material was present in the tubule lumen. Several tubules exhibited spermatogenic arrest, wherein sperm formation was hindered. Additionally, some seminiferous tubules were filled with only primary**.** spermatogonial cells, lacking sperm formation due to the sloughing of spermatogenic cells with pyknotic nuclei. Accumulation of sloughed cells was detected in the center of the seminiferous tubules. Furthermore, giant multinuclear spermatocytes were observed in the lumina of several seminiferous tubules. The interstitial tissue displayed acidophilic hyaline material,an edematous stroma containing small groups of Leydig cells, and dilated and congested blood vessels. Conversely, the testes of rats administered both Nano clay and Nano silver exhibited a more or less normal architecture with improvements in most of lesions, including the germinal epithelium of the seminiferous tubules and the interstitial tissue. The majority of seminiferous tubules appeared nearly normal, lined with a normal arrangement of spermatogenic cells and Sertoli cells. Additionally, their lumina were filled with mature spermatozoa. These findings indicate that the reparative effects of Nano clay against the reproductive toxicity induced by Nano silver were more pronounced, although a few seminiferous tubules still exhibited vacuolated and detached cells from the basement membrane.

Table (10): shows morphometeric analysis in testes of male albino mice in different studied groups.

group	Diameters	Lumen	Germinal height	Thickness of Tunica albugenia	Diameters of blood vessels
Control	384.22 ± 9.81	284.50 ±14.6	49.86 ±2.64	22.714 ±1.09	17.19 ±0.78
NS	81.51 ±2.91*	66.93 ±2.6*	7.29 ±0.54*	239.32 ±7.99*	113.45 ±3.19*
NC	331.91 ±12.58*	238.51 ±12.52*	46.699 ±1.825	29.01 ± 0.58	21.56 ±0.90
MIX	258.32 ±4.73*	174.85 ±7.46*	40.36 ±2.02*	69.085 ±1.53*	28.21 ±0.82*

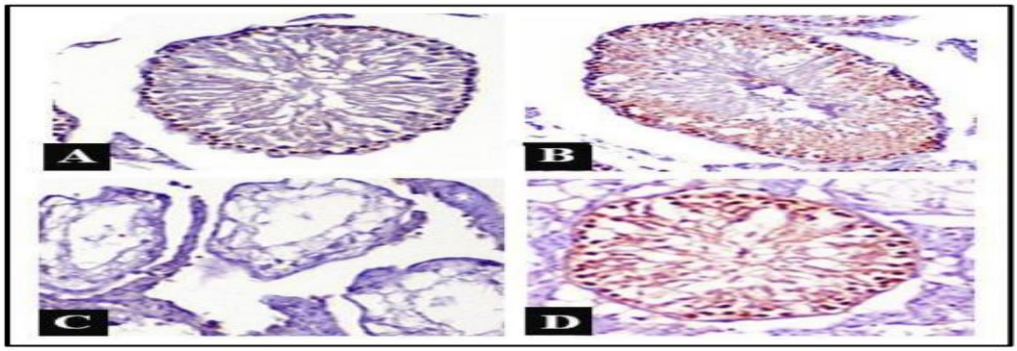
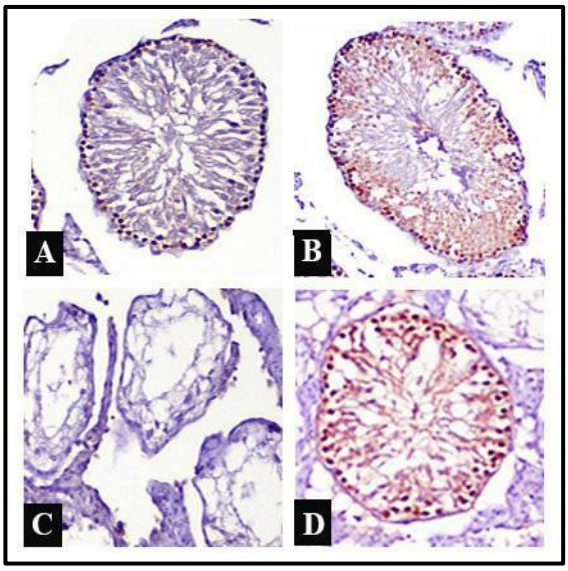


Fig. 7 : Photomicrographs showing immunohistochemical demonstration of PCNA in transverse sections of rats testes in different studied groups. (A) Control group (B) Nanoclay (40 mg/kg/d). (C) Nanosilver (100 mg/kg/d) D) Nanoclaytherafternanosilver group (40 mg/kg/d co- administered 100 mg/kg nanosilver. Note: nuclei of spermatogonic cell exhibited brown colour indicate positive reactivity (arrow) others give violet colour indicating weak or negative reaction (arrowhead). X400.

The tubular diameter, the height of the germinal epithelium and the luminal diameter of the seminiferous tubules, the thickness of the tunica albugina and the blood vessel diameter which all measured in the morphometric analysis were adversely affected in nano silver (Gp_{II}) and showed moderate to severe significant changes, While in nano clay (Gp_{III}) there were little and sometimes no significant changes in comparing with control (Gp_{I}) and mix group(Gp_{IV}) was showed ameliorative effects in most of lesions mentioned before as shown in the tabulated figure and table (1&8)**.**

Immunohistochemically Studies:

Proliferating Cell Nuclear Antigen (PCNA) Expression:



Appearance of brown granules in the nuclei of spermatogonia and some of spermatocytes is an indication of the positive PCNA reaction, which is a marker for deoxyribonucleic protein, that is necessary for deoxyribonucleic acid (DNA) synthesis in a mammalian cell.

control(Gp_{I}) and Nano clay (Gp_{III}) groups, the nuclei of spermatogonic cells and primary spermatocytes in the seminiferous tubule epithelium expressed strong immunopositive PCNA reaction (Fig. 7A, B). The nuclei of spermatogonic cells and primary spermatocytes in the seminiferous tubules in Nano silver(Gp_{II}) group showed a marked decrease in PCNA expression as a violet color (Fig. 7 C). While, sections with Nano clay and thereafter Nano silver observed an elevation in PCNA expression (Fig. 7 D).

Fig. 7: Immunohistochemically expression of PCNA in transverse testes sections of control and all groups. A: Control group, (B) Nano clay (40 mg/kg), (C) Nano silver (100 mg/kg) and (D) Mix (40 mg/kg Nano clay &100 mg/kg Nano silver).

Discussion

In recent years, the use of Nano clay minerals in biomedical applications has gained significant attention. These silicate-based materials possess excellent physicochemical properties, biocompatibility, and low toxicity, making them ideal for use in pharmaceuticals and other medical fields. Nano clay minerals have a broad surface area, diverse morphology, and high ion exchange capacity, making them effective drug delivery vehicles (Dong et al., 2021)(26).

Environmental factors and exposure to toxicants can have detrimental effects on gametogenesis, impacting fertility and reproductive health (Schagdarsurengin et al., 2016).(74)

The field of nanotechnology has introduced nanoparticles (NPs) to the male reproductive system, raising concerns about potential risks. To ensure the safe use of nanomaterials in consumer products and pharmaceuticals, it is crucial to assess their impact on reproduction and fertility (Elsharkawy et al., 2019)(30). NPs can have adverse effects on both germ and somatic cells, potentially affecting fertility and the transmission of genetic information (Habas et al., 2021).(39)

While the use of nanoscale materials in biomedical applications has expanded, their toxic effects on human health have become a subject of concern. However, the specific molecular interactions between NPs and male testicular cells are still not fully understood, making it essential to study their effects on reproductive medicine (Dantas et al., 2022). Other studies mentioned that the effect of nanoparticles on the cell cycle or release of spermatozoa to the mid duct of seminiferous tubules led to a significant decrease in sperm stem cells (Miresmaeili, et al., 2013).(56)

These NPs can also cause mitochondrial dysfunction and the generation of reactive oxygen species (ROS), resulting in protein, nucleic acid, and cell proliferation impairment (Brohi et al., 201; McShan et al., 2014).(17,58).

The reproductive and developmental toxicity of nanomaterials is a crucial aspect of nanotoxicology (Ema et al., 2017)(31). Various NPs have shown toxic effects on reproductive parameters, but only limited research has focused on the effects of silver nanoparticles (AgNPs) on the male and female reproductive systems (Lafuente et al., 2016). (49)Mitochondrial abnormalities have been observed in sperm exposed to AgNPs, as these nanoparticles can disrupt the mitochondrial respiratory chain and

reduce mitochondrial activity AgNPs may also induce the production of reactive oxygen species (ROS) in the cytoplasm, leading to toxic effects on spermatic cell. AgNPs have gained significant attention due to their unique properties and wide range of applications. However, even small amounts of AgNPs can exhibit toxicity.

This cytotoxicity is primarily caused by oxidative stress and the release of silver ions (Ag+), with the toxicity being dependent on exposure time and nanoparticle concentration (Zhang et al., 2014; Asare et al., 2012). (98,10)

Oral administration Prepubertal Wistar rats exposed to non-coated AgNPs (60-nm) from PND23 to PND53 presented delayed puberty, altered reproductive development, and compromised spermatogenesis. In the 50 µg/kg dosage, there was a numerical reduction in daily sperm production, disorganization of seminiferous epithelium, and sloughing of the germ cells. The authors suggest through evidence that the observed effects were not reversed after the end of treatment (Sleiman, et al., 2013).(80) AgNPs can alter reproductive parameters in male rats, resulting in reduced sperm production and compromised testicular structure (Mathias, et al., 2015).(56)

The LD50 is the dose that causes death in 50% of research subjects. The dose of Nano clay used in our study was much lower than the LD50 of calcium bentonite, indicating its safety (Audiyananda et al., 2021). (12)

Nanoclay didn't seem to have apparent acute toxicity where its LD50 was > 5,000 mg/kg No weight loss occurred due to the administration of calcium bentonite and a significant increase in body weight was even observed in the male rat group. Interestingly, a significant decrease was found in the female rats group when compared to the control group (Audiyananda *et al.*, 2021).(12)

There were no deaths in both male and female rats up to 7 days after the administration up to the highest dose of 5,000 mg/kgBW at a single dose of oral calcium bentonite, suggesting that the median lethal dose value of calcium bentonite was >5,000 mg/kg BW (Audiyananda *et al*., 2021). (12)

Furthermore, it is apparent that, calcium bentonite practically non-toxic materials according to Loomis Criteria where substances with LD50 values higher than 5,000 mg/kgBW by the oral route are regarded as being safe or practically non-toxic (Loomis & Hayes, 2019). (53)

Also, these findings were consistent with the safety data sheet from ThermoFisher m Scientific and the Carl Roth Company, in which the LD_{50} value of oral bentonite was greater than 5700 mg/ kg BW or more than 5000 mg/kg BW (Audiyananda *et al*., 2021). (12)

Clinical signs

Throughout the 60-day experiment, we diligently monitored the overall condition and clinical symptoms of the animals exposed to AgNPs orally. During the second month specially, we observed several stress symptoms, including icreased appetite and aggression, and nervousness. About 37.5% of male rats died in the nanosilver treated group after 60 days of treatment, compared with 12.5% in the mix groups and no mortality recorded in the control or nanoclay treated groups.

In another study by (Elsharkawy et al., 2019),(30) rats orally exposed to 5.36 mg/kg of AgNPs for six months displayed behaviour similar to the control group. However, rats administered 13.4 mg/kg of AgNPs showed stress symptoms, including poor appetite, increased aggression, and hair loss in different body parts during the 5th and 6th months of exposure.

The duration of 60 days for our reproductive toxicity study aligns with the development of spermatogonia into spermatozoa in rats, which takes approximately 48-52 days. Oral administration of AgNPs was chosen as it reflects a relevant route of exposure and allows for a better assessment of human exposure to nanoparticles (Yoisungnern et al., 2015).(95)

Body and testes weights

It is worth noting that AgNPs can accumulate and redistribute within various organs, potentially leading to decreased body weight (Sung et al., 2009; Kim et al., 2010; . (78)

However, in a study by Ćurlin et al. (2021),(23) there were no changes in body weight, hair condition, breathing, behavior, and movement in AgNP-treated Wistar rats compared to the control group. These rats were orally administered low doses of polyvinylpyrrolidone-coated small-sized AgNPs.

significant growth retardation in rats after subacute intravenous injection of Ag-NPs over a 28-day period. However, (Lee et al. 2018) reported no significant dose-related changes in body weight during or after administration of Ag-NPs, Au-NPs, or a combination of both in rats.

Another study conducted found a significant decrease in the body weight of mice treated orally with AgNPs of different sizes (ranging from 3 to 20 nm) at doses of 5, 10, 15, and 20 mg/kg body weight for a duration of 21 days. Similarly, (Mahmoudian et al. 2016) (55) reported that rats fed 300 mg/kg of AgNPs showed significantly lower body weight gain compared to the control group during a 28-day experiment.

No significant effects of AgNPs were observed in epididymis or testes. This is also in good agreement with the results of previous studies, although the route of administration and the doses were different (intravenous exposure of 1, 5 and 10

mg/kg of AgNPs). There was a significant decrease ($P < 0.05$) in the body weight of male rats after 4 weeks of exposure, although there were no significant changes in food or water consumption during the study period.

Changes in relative organ and body weight can serve as sensitive indicators of adverse effects caused by drugs, chemicals, or toxicants (Berinyuy et al., 2015).(15) The significant reduction in the relative weight of the epididymis and testes in rats treated with Ag-NP for 7 days suggests that Ag-NP induced atrophy in these organs.

In the case of orally ingested Sil-matrix 29% and Silica nanoparticles (SiO2-NPs) at various doses. reported no significant changes in body weight or organ weight in rats over a period of 28 or 45 days. The rats continued to mature without any significant toxic effects.

Our results revealed a significant increase in body weight and body weight change percent in AgNPs (Gp_{II}) 100 mg/kg compared to the control group. The increase also in testes weight was in the AgNP-treated group compared to control group. Interestingly, when AgNPs were mixed with nano clay NC/AgNPs (40 mg/kg NC: 100 mg/kg NS), there was no significant change in body weight percent or in testes weight too.

It is clear that the effects of AgNPs on body weight can vary depending on the administration method, dosage, and other factors. Further research is necessary to fully understand the mechanisms behind these effects and their implications.

Previous studies have presented conflicting findings regarding the effects of silver nanoparticles on body weight. The increase in body weight observed in our study could be attributed to physiological changes that affect the animals' appetite and feed consumption, ultimately impacting their body weight.

Sperm count, motility % and viability %

An apparent decrease in sperm count was observed in this study in the nano silver treated group, while in the nano clay treated group there was any decrease in sperm count compared with the control group. The abnormalities were more and clearly observed within nano silver treated groups especially in sperm heads that mostly appeared amorphous in shape and abnormalities in sperm tails were also found. All these abnormalities appeared in the mixed group but not as severe as in the nano silver group.

The rats intravenously administered a single bolus dose (5 and 10 mg/kg body mass) of 20nm silver nanoparticles (AgNPs) showed a clear decrease in sperm count. Folded, amorphous spermatozoa, cells lacking or showing a small hook, and cells with undulating or elongated heads were the most frequent abnormal forms found. In addition, in all groups examined at 1 and 4 weeks, elongated heads and two or three headed forms appeared more often than in groups examined at 24 h after injection (Gromadzka-Ostrowska *et al*., 2012).(37).

Treatment with AgNPs has resulted in dose-dependent decreases in sperm velocity parameters, total motility, and progressive motility, along with increases in non-progressive motility and immobility (Abu et al., 2013; Madan, 2013; Reuben et al., 2016; Obinna and Agu, 2018).(70).

Sperm abnormalities

The results of various studies have consistently shown that exposure to silver nanoparticles (AgNPs) can have detrimental effects on sperm morphology and reproductive parameters. (Baki et al. 2014)(13) found a significant reduction in the percentage of normal spermatozoa in experimental groups exposed to different concentrations of AgNPs compared to control groups. Similarly, observed sperm abnormalities and reduced sperm production in male rats exposed to AgNPs during prepubertal development.

(Yoisungnern et al., 2015)(95) reported that exposure to AgNPs led to abnormal morphological changes in spermatozoa, such as coiled, rolled, and bent tails, as well as an increase in dead spermatozoa and a decrease in live spermatozoa. (Lafuente et al., 2016)(74) demonstrated that sub-chronic oral exposure to PVP-coated AgNPs resulted in higher levels of sperm morphology abnormalities, although other sperm parameters were not significantly affected.

AgNPs have also been found to induce toxicity in different tissues and cause significant changes in sperm quality and quantity

(Sleiman et al. 2013)(80) reported that AgNPs could adversely affect reproductive development in prepubertal male rats, leading to changes in sperm production and seminiferous epithelium morphology.further highlighted the defects in spermatogenesis caused by AgNPs, resulting in morphological insult and impaired sperm function.

Furthermore, AgNPs have been shown to accumulate in the sperm tails and head, causing sperm immobility (Wiwanitkit et al., 2009).(91) These effects may be attributed to oxidative stress induced by AgNPs in the testis and epididymis, leading to impairments in sperm parameters .

Comet

In the context of DNA damage, (Gromadzka-Ostrowska et al.2012)(35) found that the Ag I and Ag II groups showed a significant increase in DNA damage (% DNA in tail) in germ cells at 24 hours after injection, which then decreased at later

time points (7 and 28 days after treatment). However, no significant difference in the extent of DNA damage was observed between the AgNPs treated groups and the control groups at day 7 and 28 after injection.

Similarly, (Asare et al. 2012)(90) discovered that silver nanoparticles with a size of 200 nm caused DNA strand breaks in NT2 cells. Many scientific reports suggest that the mechanism of silver nanoparticle toxicity is primarily related to the induction of oxidative stress and lipid peroxidation, leading to cellular changes such as DNA damage, apoptosis, and cytokine production. Several studies have also concluded that AgNPs exhibit cytotoxicity by affecting mitochondrial activity, with the severity increasing with higher doses and concentrations of AgNPs. Studies have shown that AgNPs can enter the nucleus, leading to changes in DNA integrity, affecting its synthesis process and resulting in cell mutation (Butler, et al., 2015; Asare, et al., 2016). This can result in a significant reduction in mitochondrial function, increased membrane leakage, necrosis, and the stimulation of apoptosis (Yang et al., 2017; Al-Bishri, 2018).(94,6)

Antioxidant

AgNPs produce significantly high concentrations of reactive oxygen species that lead to a decline of the testicular antioxidant enzymes, induce the oxidative damage of testicular cellular membranes, and finally induce a harmful effect on spermatogenesis (Attia, 2014; Mohamed, 2016; Nahla El-Eraky, et al., 2019). (11,63)

Smaller AgNPs, with their larger surface area and increased Ag+ release, have enhanced cytotoxicity. The interaction of Ag+ with antioxidants and induction of oxidative stress can lead to DNA damage and cell death (Arora et al., 2008). The large surface area of AgNPs also contributes to their cytotoxic activity by increasing their bioavailability and distribution.After oral intake, nano silver can be absorbed and distributed to various organs (Ferdous & Nemmar, 2020)(34).

Our results agreed with those of prior research which indicate that oral treatment of silver nanoparticles in groups $_{II}$ and $_{IV}$ causes a significant reduction in the activity of antioxidant enzymes compared to the control group, which is considered a state of oxidative stress in testicular tissue. Studies have indicated that the immobilization of AgNPs on Nano clay minerals reduces their cellular uptake and associated risks. This immobilization results in mild inflammatory responses and retains the antibacterial activity of AgNPs, making them safer for clinical use (Chang et al., 2021). (20)

Testicular histomorphology

The testis of rats orally treated daily with 100 mg /kg/day AgNPs for 60 days showed many and sever shrunken, disorganised seminiferous tubules with irregular basement membrane and a marked reduction in the thickness of their lining germinal epithelium with deeply stained nuclei. Other tubules reveal sloughing or detachment of germinal cells with a marked reduction in the number of spermatozoa. Separation and spaces between vacuolated germinal epithelial cells are also noticed. The interstitium in between the seminiferous tubules is edematous, wide, and contains vacuolated homogenous eosinophilic material and congested blood vessels. Similar results were reported by (El-Mesalmy et al. 2021) in the testis of rats intraperitoneally treated daily with 50 µg /kg/day AgNPs for 35 days from 23 postnatal days to 58 postnatal days. Vacuolization may be an effect of disturbances in membrane function, which result in an influx of water and sodium. Also, cellular swelling may be accompanied by cytoplasmic degeneration due to leakage of lysosomal hydrolytic enzymes (Abdelhalim, 2011)(2).

(Asare et al. 2012) found that nano silver appeared strong cytotoxicity and cytostatic properties, causing apoptosis, necrosis, and reduction of proliferation in a concentration and time dependent manner. Several scientists mentioned that AgNPs could cross the blood-testis barrier (BTB) and accumulate in the testes after intraperitoneal or intravenous injection (Wang, et al., 2013)(89). Therefore, AgNPs produced a reduction in the number of different germinal cells and led to injury and disorganisation of the seminiferous tubules (Thakur, et al., 2014; Ahmed, et al., 2017).(85,5)

Silver nanoparticles have been shown in the literature to induce pyknosis of germ cell nuclei due to DNA breakdown and damage (Al-naqeeb et al., 2017; Fathi et al., 2019; Nahla El-Eraky et al., 2019)(7,63). Some reporters attributed the vacuolation to the premature exfoliation of the cells of the seminiferous tubules.

Other investigators showed that the detachment of germ cells may be owing to the marked disruption of the Sertoli cell interaction, followed by the sloughing of the germ cells from the seminiferous epithelium (Thakur, et al., 2014; Ahmed, et al., 2017).(5) Subdermally, administration of AgNP to rats at 50 mg/kg bw for 28 days caused degenerative alterations to the cellular architecture of rat testes relative to the control. The lumen was devoid of spermatozoa with interstitial congestion, maturation arrest of tubules, and seminiferous tubules with atrophy exhibiting thick double cell layers' indicative of cessation of spermatogenesis (Olugbodi, et al., 2020).(65)

(Elsharkawy and collaborators 2019) (30) investigated the potential testicular toxicity induced by non-coated AgNPs (8.93–33.4 nm) by giving them to Sprague Dawley rats twice a week for six months. The results showed that AgNPs caused more

cytotoxicity in a high dose (13.4 mg/ kg), promoting vacuolization in Sertoli cells, inactivating Leydig cells, decreasing sperm counts and changing sperm parameters.

Sub-chronic exposure (once a day for 90 days) of Wistar rats to non-coated AgNPs (5–20 nm/UV–vis: 397 nm) also showed significant effects on spermatogenesis. Atrophy of the seminiferous tubules, germ cell sloughing (mainly spermatocytes and spermatids), reduced tubular lumen, and sperm cell necrosis were observed

In AgNPs treated rats, shrunken and disorganized seminiferous tubules were explained by some authors as the cytotoxicity of AgNPs is related to the increased formation of reactive oxygen species (ROS), which could induce apoptosis (Zhang, et al., 2015). (98)

Furthermore, the irregularities in the basal lamina could be secondary to myoid cell contraction or distorted seminiferous tubules (Mohamed, et al., 2014). Also, a marked separation and spaces between germinal cells with apparent diminished layers of germinal epithelium were seen. The latter was confirmed statistically by the highly significant decrease of the mean epithelial height in the AgNPs subgroup in comparison with the control group. Similar results were stated by another study that used nanoparticles orally for 90 days and confirmed that degenerative alterations in seminiferous tubules showed that nanoparticles could directly inhibit spermatogenesis . A few spermatozoa in tubules lumina were also seen in our study. This could be explained by the fact that a decrease in germinal stem cell number may have badly affected sperm production and led to a weakness in male fertility (Ong, et al., 2016)(66).

Higher doses of AgNPs, explained that undue accumulation of ROS might be a triggering agent for apoptosis and autophagy of the germ cells (Tan, et al., 2010; Ahmed, et al., 2017; El-Mesalmy et al., 2021).

Histological changes found in the interstitium were in agreement with other studies (Amin et al., 2015; Ahmed, et al., 2017)(8,5). The latter declared that interstitial tissue damage may be associated with AgNP deposition in the tissue. It was also observed that changes in the composition or volume of the interstitial fluid could also significantly affect the testicular function [Lirdi, et al., 2008].(52)

Proliferating Cell Nuclear Antigen (PCNA)

The expression of Proliferating Cell Nuclear Antigen (PCNA) serves as a reliable marker for evaluating cell proliferation. PCNA is involved in various cellular processes, including DNA replication, chromosome recombination, DNA methylation, nucleic acid metabolism, and RNA transcription. In the context of the testis, PCNA immunostaining is utilized as a proliferative marker to assess spermatogenesis. It offers a quick, sensitive, and quantitative method to detect early signs of testicular toxicity Reda et al., 2017).(70)

Our findings support the observations of other scientists who documented a significant decrease in PCNA immunostaining following exposure to AgNPs when compared to the control group. This decrease in PCNA expression is closely**.**

associated with increased apoptosis and a reduction in active DNA content within dividing spermatogenic cells (Nahla El-Eraky, et al., 2019)(63). Apoptosis is a complex biological process crucial for regulating cell survival and eliminating diseased or dead cells, (Iyiola, et al., 2018)(42). Exposure to AgNPs leads to the production of reactive oxygen species (ROS) accompanied by oxidative, cytotoxic, and genotoxic events. ROS accumulation and oxidising enzyme activation cause cell membrane, mitochondrial, and germ cell apoptosis (Ahmed et al., 2017)(5). multiple studies have demonstrated that exposure to AgNPs can cause significant alterations in sperm morphology, reproductive parameters, and mitochondrial function. These findings emphasize the potential reproductive toxicity of AgNPs and highlight the need for further research to fully understand the underlying mechanisms and potential implications for human health.

Conclusion

Here, AgNPs showed a potential reprotoxic effect on adult male rats exposed to oral administration of 100 mg/kg/d for 60 days. The histopathological and PCNA immune-staining changes that occurred in the testis tissue of AgNPs treated rats as well as the disturbance of the studied biochemical parameters and the results obtained by the comet assay are in agreement with the disturbance in sperm parameters observed in all AgNPs treated rats. On the other hand, Nano clay may be useful in ameliorating the biochemical and histopathological changes caused by AgNPs toxicity as a result of enhancing the antioxidant defense system where rats in GP_{IV} (mix group) showed a notable improvement compared to the other treated groups where the testicular histology, PCNA reactivity, biochemical parameters, and comet assay results became more or less similar to normal. Further investigation is necessary to clarify the mechanisms of AgNPs induced male reproductive dysfunction. Given these findings, we can conclude that AgNPs pose a potential risk to male fertility. So, contact with AgNPs is best avoided. Further clinical studies with human populations for long periods are still needed

to emphasize the results obtained from animal studies.

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