Effect of Graphene Nanoparticles on some Organs of Reproductive System in Male Albino Mice

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Abstract

Due to their exceptional physical properties, graphene nanomaterials have gained a great deal of interest in recent years for their application to state-of-the-art technology. On the other hand, nanotoxicity of graphene materials has also rapidly become a major concern, especially in the field of occupational health. There is a lack of evidence that such graphene nanomaterials influence the organs of the male reproductive system. Therefore, the goal of this study was to determine the effect of Graphene nanoparticles (GNPs) on mice body weight, reproductive organs weight and sperm quality, as well as certain reproductive organs injuries, after14 days of treatment with oral gavages of 0.1 ml of 10, 20, and 30 mg/kg of GNPs in male albino mice. The results showed a decrease in animal body weight and certain reproductive organs, as well as a decrease in the proportion of live sperm and the concentration of sperm in the tail of the epididymis and an increase in sperm abnormalities in all treatment relative to the control group. Injuries in the testes and epididymis (head and tail) were shown by histopathological analysis. This study concluded that oral GNPs gavages at various concentrations have a negative effect on the male reproductive system and can affect fertility.

Keywords: *graphene Nanoparticles, organs, Reproductive, male albino mice*

Introduction

A graphene (GNPs) is a mother of all atoms of carbon and is isolated from crystalline graphite. GNPs has a flat monolayer consisting of a single atomic thick, nanosized, two-dimensional sheets of hexagonally structure arranged as a honey comb lattice (1, 2) . Graphene derivatives vary in size from one to several hundred nanometers, and in thickness from 1 to 100 nanometers (3). In 2004, the significant attention was given to the discovery of these materials received considerable (4,5). Such materials were widely used to diagnosis, treatment and detection of genetic risk factors because of their extraordinary physical and chemical properties such as small size and large surface to- volume ratio(6-8). Moreover, GNPs are also used indifferent fields, including nano electronic equipment; energy storage; batteries(9-11); and biomedical applications such as antibacterials(12,13), biosensors(14-17), cell imaging(18,19), delivery of drug (20-22), and engineering of the tissue(23-25). However, increasing GNPs utilization and production may increase the risk of unintentional occupational or environmental exposure to these materials(26) .Recently, some research has been carried out on exposure to graphene materials in the workplace, and published data has shown that graphene materials occupational exposure has potential toxicity to workers and researchers (27,28). Intratracheal instillation(29), oral gavages(30), intraperitoneal and intravenous injection (31,32) ; as well as subcutaneous injection(33) can deliver graphene nanomaterials to bodies. GNPs can cause acute and chronic tissue injury by penetrating through blood-air barrier, blood-brain barrier, blood-testis barrier, and blood-placenta barrier,… etc., and then accumulate in different organs such as lungs, liver, and spleen ,….etc.

Su et al. (28) found that the majority of GNPs inhaled in the respiratory tract of human could easily pass through the head airways as well as the upper part of the tracheobronchial airways and then migrate to the lower airways, where undesirable biological reactions could be induced such as granuloma and lung fibrosis. Whereas, another study reported an inflammatory pulmonary response in rats following BSA-capped graphene (*bovine serum albumin-capped graphene*) administration[34].

Recently, the findings of study[35] have shown that oral gavage of 60 mg/kg GNPs in male albino mice for 7 and 9 days caused a decrease in the weight of animal body and liver weights, an increase in serum GOT enzyme levels as well as liver injury.

Many studies have shown that exposure to the graphene family may produce reactive oxygen species (ROS) depending on the concentration and duration of exposure, suggesting the occurrence of oxidative stress in many organs[36.37], resulting in histological injuries. Patlolla et al. [38] demonstrated that graphene oxide can be hepato toxic and that oxidative stress can mediate its toxicity.

Reproductive activity is usually an important factor when determining toxicology. In male animals, if environmental chemicals (e.g. GNPs) adversely influence the sperm quality , reproductive organs structure, the activities of certain main reproductive hormones, fertility could decrease and health offspring could be effected. In rats , the intraperitoneal injection of high nanoscale graphene oxide concentration (10.0 mg / kg BW) for 15 and 30 days resulted in decreased epidididymal sperm counts and motility, and increased sperm abnormalities, as well as histological damage to testicular tissue such as seminiferous tubules atrophy with reduced germ epithelium, loss of germ cell, and vacuole formation[39]. In fish, Dasmahatra et al. [40] found a connection between graphene oxide and gonad histpathology in fish, including granulosa and leydig cell changes.

Nevertheless, no information on the toxicity of GNPs to the male reproductive system has been given. Therefore, this research investigates the toxic effects of GNPs on animals body weights and some reproductive organs, sperm qualities, and reproductive organs injury (testes, head and tail of epididymis) in male of albino mice.

Materials and Methods

The research was performed on 27 male albino mice, weighting between 26-34g. They were divided into four groups, each comprising of six animals. The first group was a control which was ingested with distilled water. The second, third and fourth groups were ingested for 14 days with 10, 20 and 30 mg/kg of GNPs, respectively. GNPs were made from the same powder that Aldakheely and Al-Bairuty used in their study[35], which was described in detail . Briefly, GNPs were prepared as a stored solution. The 10, 20, and 30 mg/kg GNPs concentrations were prepared by dissolving 37.5, 75, and 112.5 mg of GNPs in 15 ml of distilled water, respectively. After preparation of the concentrations, the mixture was placed in the ultrasonic bath for 30 minutes to mix and prevent the particles from agglomerating.

The powder was supplied by the company of Sky spring Nanomaterials (Inc., USA) with the following features: 99.5% purity, 6-8 nm sheet thickness of graphene, 15 nm average diameter, laminar form and black powder.

In study[35], the powder purity was tested by Energy Dispersive X-ray spectroscopy (EDX), which was 96.27% of carbon and 3.73% of oxygen (Fig. 1). The X-Ray Diffraction (XRD) was used to verify the 26 nm GNPs size and the Transmission Electron Microscopy (TEM) was used to identify the GNPs shape that had a laminar shape(Fig. 2).The results were almost identical to company descriptions .

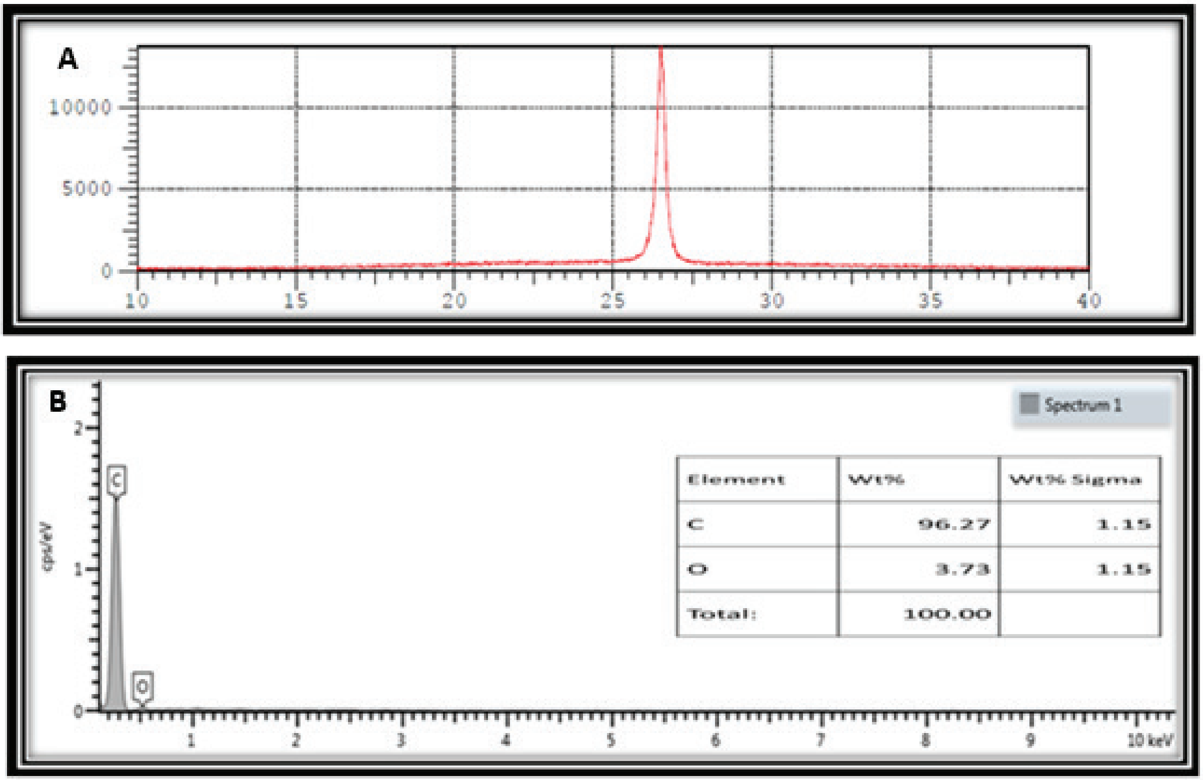

Figure (1) (A) The scale of GNPS powder using XRD, which was 26 nm. (B) The purity of GNPs powder using Energy Dispersive X-Ray Spectroscopy (EDS) was 96.27% of carbon and 3.73% of oxygen (impurities) [35].

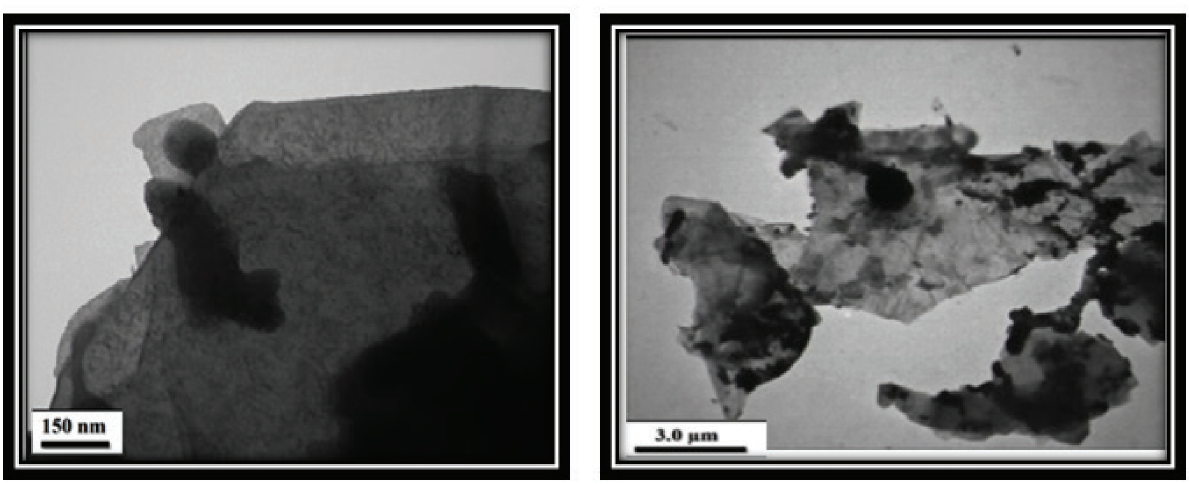

Figure (2) Transmission Electron Microscope (TEM) showing the laminar shape of GNPs.

Animals were obtained from the Ministry of Industry and Minerals, Industrial Research and Development Authority, the Al-Razi Center and then transferred to the house of animals in the department of Biology/College of Science for Pure Science/Ibn Al-Haitham. All animals were exposed to normal laboratory conditions of temperature, light, air, and were supplied with water and pelleted mice diet *ad libitum*.

Animals weights were recorded using the electrical balance before and after the treatment period with GNPs (14 days). After 14 days, the animals were sacrificed and the testes and epididymis in the left and right side were resected and removed fatty tissue adhered to them using a sensitive balance. The weights of the testes and epididymis (head and tail) were recorded. To study the sperm quality, the epididymis tail in the left side was resected, cut, crushed and mixed with 5 ml of saline solution. In a clean glass slide, a drop of tail solution was added and then a drop of eosin dye was placed and mixed with a drop of mash and made a smear [41]. The tail smear was left to dry and then fixed with DPX to study sperm abnormality (head, tail, middle piece of sperm and the cytoplasmic droplet site). Then, 100 sperm were extracted and abnormal ones examined by the Krazaanovskaya method[42] and all slides were examined with light microscopy with a magnification of 40x.The testes and tail in the right side were placed in formalin fixative for 48 hours, after which the organs were dehydrated in ascending concentration of alcohol, purified in xylene, and embedded in paraffin wax, then 4-5 μm thick sections were obtained by a rotary microtome and stained with hematoxyline Harris and Eosin[43]

Statistical Analysis

Statistical analysis was carried out by using SPSS, version 24. All data were presented as Means ± SE. *t*-test was used to identify the differences between initial animal body weight (before) and after the end of treatment. The other parameters were analysed by using One –Way Analysis of Variance (ANOVA) with least significant difference at a probability to investigate the difference between treatments. The value was considered statistically significant when the probability value of less than 0.05. The value were written as average mean ± standard error

Results

Body weightsandReproductive organs weights

As a result, the mean body weight of animals treated with 10, 20 and 30 mg / kg of GNPs for 14 days decreased significantly (P <0.05) compared to the animals themselves before treatment, with a percentage varying from 3-5% (Table 1). However, when comparing the mean body weights of the animals after oral gavages of all three GNPs concentrations with control groups, the mean body weights of the animals showed a significant decrease (P<0.05) (Table 1).

Table 1. Animals body weights before and after treated with different concentrations of GNPs for 14 days.

Treatments	Number of animals	duration of exposure (days)	Animal bodies weights(gm)	
			Before exposure	After exposure
Control	6	-	33.92±1.78 aA	35.63±1.67 aA
10 mg/kg GNPs	6	14	26.22±0.41 bA	22.88±0.79 bB
20 mg/kg GNPs	6	14	29.12±0.62 bA	24.02±1.36 bB
30 mg/kg GNPs	6	14	28.47±0.43 bA	25.07±0.44 bB

Values represent mean ± standard error

Similar horizontal and vertical capital letters indicate no significant change (P >0.05)

Different horizontal and vertical small letters indicate significant change between treatments (P ≤0.05)

In comparison with the control group, statistical analysis revealed a significant decrease (P≤0.05) in mean testes weight of mice treated with 10, 20, 30 mg/kg of GNPs with a concentration effect (Table 2). The mean epididymis head weights showed a significant decrease in mice treated with 10, 20, 30 mg/kg of GNPs compared to control animals. Whereas the mean epididymis tail weights in mice treated with all GNPs concentrations were not significant (P>0.05) in all treated animals relative to the control group (Table 2).

Table 2. Changes in the weight of testes, head and tail of epididymis after 14 days of exposure to 10, 20 and 30 mg/kg of GNPs .

Treatments	Number of animals	duration of exposure (days)	Organs Weight (mg)		
			Testes	Epididymis	
				Head	Tail
Control	6	-	91.35±2.04a	38.91±2.93a	21.58±0.72a
10 mg/kg GNPs	6	14	83.5±1.23b	21.00±1.033b	20.92±1.00a
20 mg/kg GNPs	6	14	72.42±1.91b*	21.83±3.17b	18.67±0.45a
30 mg/kg GNPs	6	14	72.75±2.54b*	18.75±1.26b	18.17±1.03a*

Values represent mean ± standard error

Different vertical letters indicate significant change between treatment (P ≤0.05)

* significant change (P ≤0.05) between concentration.

Changes of sperm characteristics in the tail ofepididymis

The results of the statistical analysis showed that the percentages of live sperm and the concentration of sperm in the epididymis tails of mice treated with all three concentrations (10, 20 and 30 mg / kg) of GNPs decreased significantly (P <0.05) after 14 days compared to the control group (Table 3).

The percentage of sperm abnormalities in the epididymis tails of mice treated with all GNPs concentrations (10, 20 and 30 mg / kg) showed statistical increases (P≤0.05) after 14 days in contrast to the control group (Table 3). The percentage of sperm distortions has increased with increasing GNPs concentration (Table 3).

The sperm abnormalities involved sperm head absence , tail absence, tail warp , tail curvature , median piece convolution , and cytoplasmic droplets appearance in the median piece (Fig. 3)

Table 3. Changes in the percentage of sperm live , sperm abnormalities and concentrationof sperm in epididymis tail of albino mice treated with 10,20 and 30 mg/kg GNPs for 14 days

Treatments	Number of animals	duration of exposure (days)	(%) Live of sperm	Concentration of sperm* 103	(%) of spermatic abnormalities in epididymis tail
Control	6	-	75.17±3.36 a	13708.33±1250.28 a	6.33±1.28 a
10 mg/kg GNPs	6	14	13.50±0.62 b	8708.33±668.23 b	71.17±2.30 b
20 mg/kg GNPs	6	14	11.17±1.19 b	6270.83±1439.55 b	78.33±0.76 c
30 mg/kg GNPs	6	14	20.33±0.76 b*	6916.67± 722.16 b	85.67±1.17 d

Values represent average mean ± standard error

Differentvertical letters indicate significant change (P ≤0.05) between treatments

* significant change (P ≤0.05) between concentration

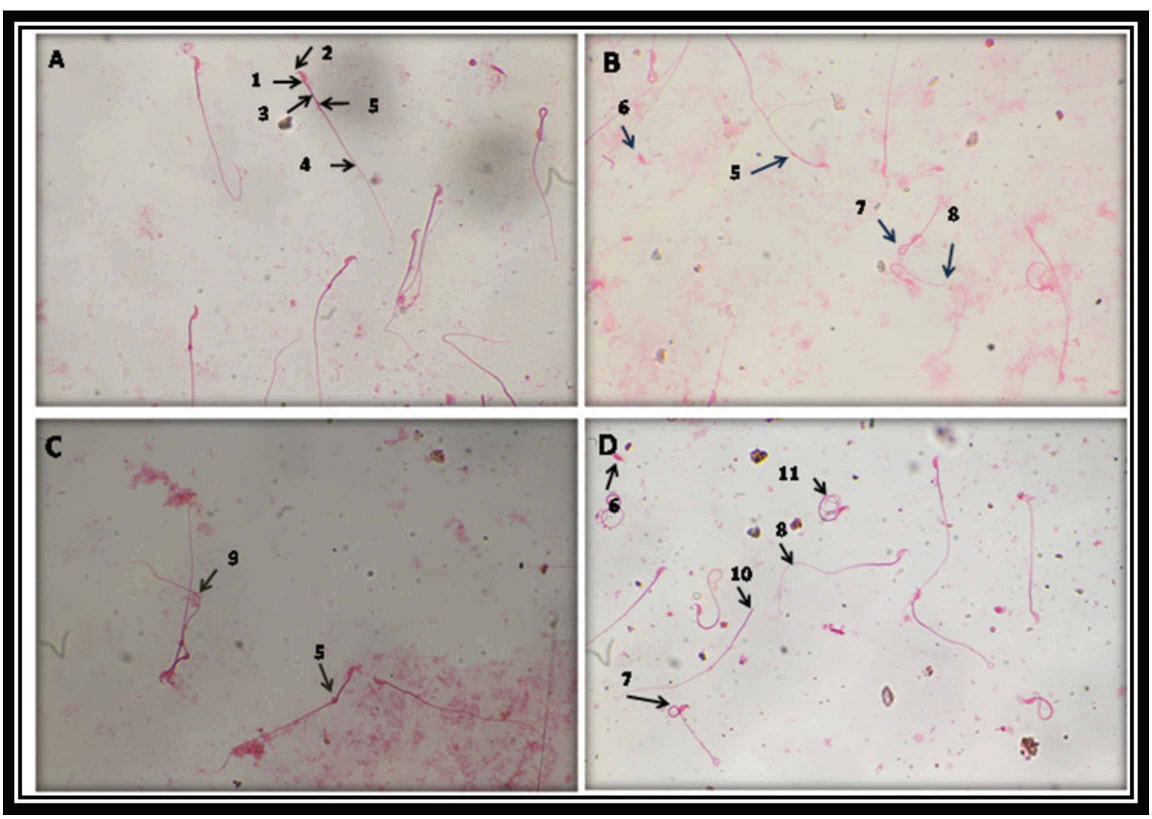

Figure 3(A) shows the normal sperm in albino mice of the control group (B) abnormal sperm in mice treated with 10 mg / kg of GNPs for 14 days (C) abnormal sperm in mice treated with 20 mg / kg of GNPs for 14 days. (D) abnormal sperm in mice treated with 30 mg / kg of GNPs for 14 days. The numbers refer to: 1. Sperm head , 2.Spine of the head; 3. Median piece; 4. Sperm tail ; 5. Cytoplasmic droplets.; 6. Loss of head and median piece; 7. Convolution of median piece. 8. Tail bending.; 9. Tail zigzag.; 10. Loss of head; 11.

Convolution of tail.

Changes in the structure of testes and epididymis

Histological results showed normal seminiferous tubule architecture in the control group testicles tissue (Fig.4a). Exposure to 10, 20 and 30 mg / kg of GNPs for 14 days resulted in changes in the structure of testes involving soci of cell necrosis, degeneration of seminiferous tubule cells, vacuolation, expansion of space between seminiferous tubules, low number of leydig cells (Fig.4 b,c and d). In mice treated with all three GNPs concentrations, the epididymis head showed changes involving degeneration of the epididymal tubule, hyperplasia, pyknotic nucleus cell, expansion of the space between tubules, epithelium lifting , vacuole formation, congestion of the blood vessels and low epithelium lining thickness compared to the normal epididymis head structure in the control group (Fig.5). The structure of the epididymis tail showed changes in mice treated with 10, 20 and 30 mg/kg of GNPs involving tubule degeneration, space expansion between tubules, epithelium lifting, low epithelium thickness with low sperm density in the epididymis tail lumen compared to the normal epididymal tail structure (Fig. 6).

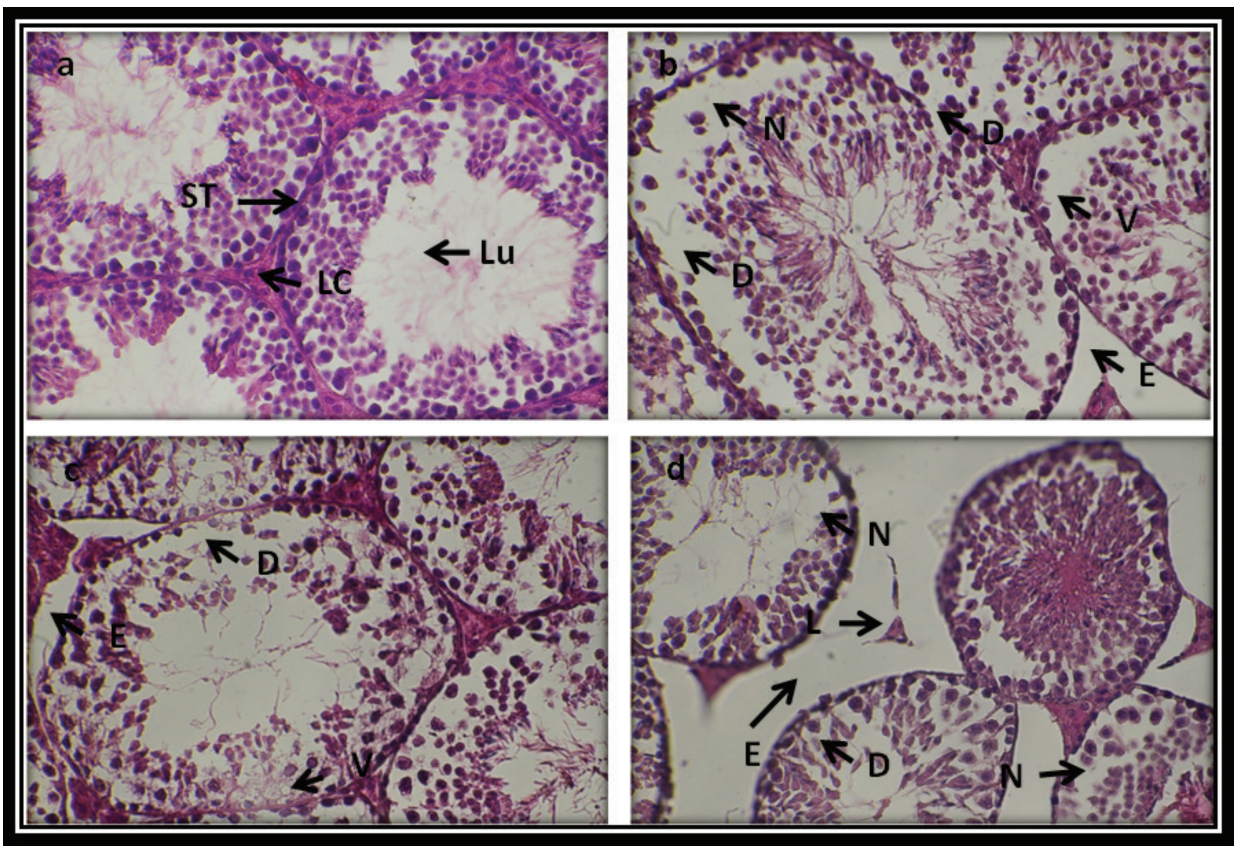

Figure 4 showed transvers sections of male albino mice testis in (a) control group (b) mice treated with 10 mg / kg of GNPs for 14 days (c) mice treated with 20 mg / kg of GNPs for 14 days (d) mice treated with 30 mg / kg of GNPs for 14 days. The testis of control group showed normal structure of seminiferous tubule (ST), lumen (Lu) and the presence of leydig cell between tubules. All treatment showed lesions that involved cell necrosis (N), degeneration of seminiferous tubule cells (D), vacuolation (V), enlargement of space between seminiferous tubule (E) , low number of leydig cells (L). Hematoxyline- eosin, 40X.

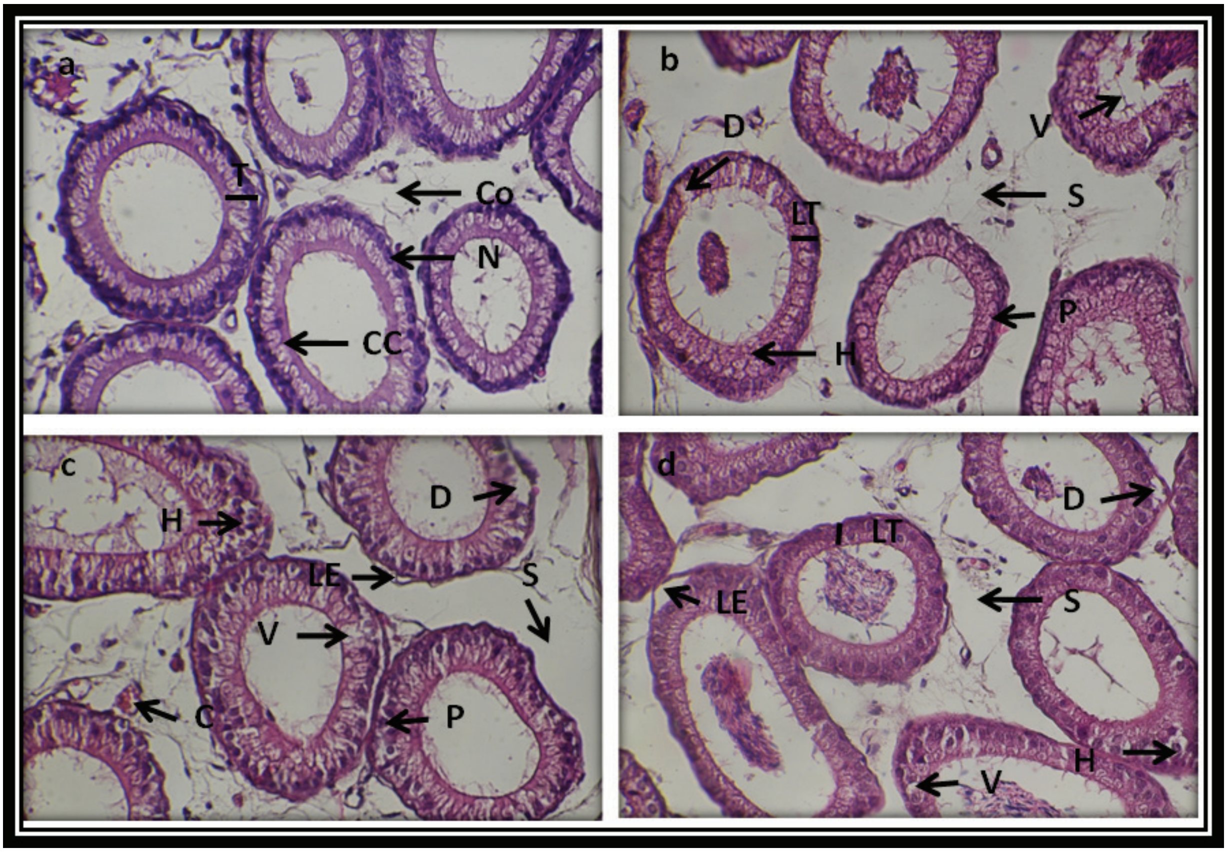

Figure 5 (a) showed transvers sections of epididymis head in male albino mice of (a) control group (b) mice treated with 10 mg / kg of GNPs for 14 days (c) mice treated with 20 mg / kg of GNPs for 14 days (d) mice treated with 30 mg / kg of GNPs for 14 days. The head of control group showed normal structure and thickness (T) of epididymis tubule that lined by ciliated columnar cell (CC) with normal nuclei (N) and normal presence of connective tissue in space between tubules (Co). All treatment showed lesions that involved tubule degeneration (D), hyperplasia (H), pyknotic nuclei (P), enlargement of space between tubule (S) , lifting epithelium (LE), vacuole formation (V), blood vessel congestion (C) and low thickness of epithelium (LT). Hematoxyline- eosin, 40X.

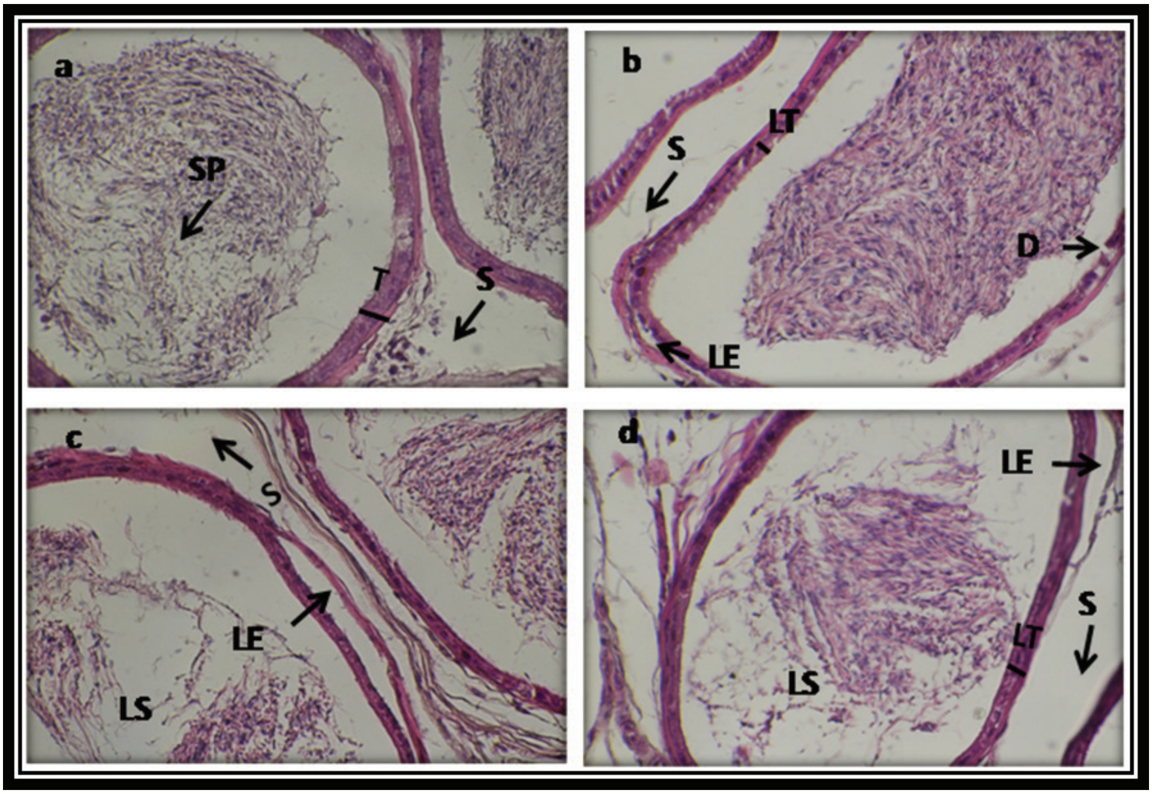

Figure 6 (a) showed transvers sections of epididymis tailin male albino mice of (a) control group (b) mice treated with 10 mg / kg of GNPs for 14 days (c) mice treated with 20 mg / kg of GNPs for 14 days (d) mice treated with 30 mg / kg of GNPs for 14 days. The tail of control group showed normal structure and thickness (T) of epididymis tubule that contain sperm (SP) and normal presence of connective tissue in space between tubules (S). All treatment showed lesions that involved tubule degeneration (D), enlargement of space between tubule (S) , lifting epithelium (LE), low thickness of epithelium (LT) with low density of sperm (LS). Hematoxyline- eosin, 40X

Discussion

There is a lack of studies on the toxicity of graphene nanoparticles in the male reproductive system. In this regard, further animal studies are urgently needed to understand the systemic behavior and potential systemic toxicity of these nanomaterials. Current findings indicate a reduction in the overall body weight of animals treated with all three GNPs concentrations relative to the control group.

These findings are in agreement with the [35] study, which showed that oral administration of 60 mg/kg of GNPs to male albino mice resulted in a decrease in average body weight. A return to the accumulated GNPs in the stomach or intestines may be the reason for this result,, leading to disruption of the absorption process due to the impact of GNPs on the mucous membrane of the gastrointestinal tract or loss of appetite in animals.

The toxic effect of various GNPs concentrations on the body weights of animals before and after exposure was evident, which showed a decrease in the weights of animals treated with GNPs for 14 days relative to the control group, suggesting the negative effect of GNPs on the body weights of the animal, which reflects the critical function of its internal organs and the lack of appetite due to its accumulation in the digestive system and prevents the absorption process. This interpretation is consistent with the [44] study in which it was shown that the decrease in animal body weights after treatment with nanoparticles induces loss of appetite due to the accumulation of nanoparticles in the stomach and intestines, thus affecting the digestive system's absorption process .

In this study, a significant decrease in testes and epididymis weights was observed in GNPs- treated animals at concentrations of 10, 20 and 30 mg / kg and after 14 days of exposure . Decreased weights of organs (testes and epididymis) can be returned to organ tissue damage. The study of Attia showed that exposure of male mice to silver nanoparticles causes testicular tissue damage and programed cell death, thus reducing testicular weights compared to the control group(45).

In a study of Olugbodi et al. (46), rats were subcutaneously exposed to Ag-NPs at doses of 10 and 50 mg/kg body weight (bw) for 7 days, resulting in a decrease in the relative weight of the epididymis and testes, suggesting that Ag-NP induced epididymis and testes atrophy in male rats. Previous research has shown that when nanomaterials are given to animals as a subacute intravenous injection, they circulates in the blood predominantly as particulate forms, interacting with blood components and cells to cause coagulation (47,48).

In the current analysis, the percentages of live sperm and the concentration of sperm in the epididymis tails of mice treated with all three concentrations of GNPs were found to be lower. This may be due to a reduction in testosterone levels (data not shown), which would cause spermatogenesis to be impaired or cause cell cycle arrest and death of intermediate stages of sperm formation. Research has shown that low testosterone levels in animals have affected spermatogenesis by reducing sperm count and motility, increasing the percentage of defective sperm cells, and modifying the histomorphology of the testis and/or epididymis(49-50). Nirmal et al. study demonstrated that after 15 and 30 days of exposure to nano graphene oxide, total sperm count decreased with dose effects(39). Whereas, Yoshida et al. (51) found that intratracheal administration of carbon nanoparticles to male mice resulted in decreased sperm production and tissue changes in the testes.

Tabish et al. (52) showed that injecting of 5 and 15 mg / kg of graphene nanopores intraperitoneally into rat for 27 days caused an imbalance in the spermatogenesis process, the appearance of vacuoles, and the expansion of the germ layer, as well as the breakdown of secondary sperm cells, damage to the germ layer of the seminiferous tubules and the appearance of gaps.

Morphological abnormalities in epididymis tail sperm have increased significantly in all three concentration of GNPs, indicating that oxidative stress is being produced. According to the results of Nirmal et al (39) study, nanographene oxide induce oxidative stress in testicular tissue. Oxidative stress triggers the oxidation of cell membrane that rich in polyunsaturated fatty acids. As a consequence, sperms rapidly lose intracellular adenosine triphosphate (ATP), causing axonemal injury. Elevated morphological abnormalities and sperm motility are closely correlated related with this effect(53). A study of Pinho et al.(54) showed that exposing the testes of mice to different doses of ZnO NPs (1, 5, 10, and 20 μg) for 6 and 12 h led to changes in the cytoskeleton and nuclear structure in sperm cells, as well as an increase in the levels of reactive oxygen species (ROS) and DNA damage and thus cell death, indicating a toxic effect on sperm in a time and dose dependent.

Oral administration of GNPs to mice at 10, 20 and 30 mg/kg bw resulted in degenerative changes in the cellular architecture of mice testes and epididymis relative to control. These degenerations in mice testes and epididymis are lines of evidence supporting the GNPs ability to cause cellular and oxidative injury to mice testes and epididymis. The study(39) found that high dose of nanographene oxide (10 mg/kg BW) caused substantial histological injury to testicular tissue, including seminiferous tubule atrophy, decreased germinal epithelium , germ cell destruction, and vacuolization. Kong et al. (55) found that the oral gavages of 15 and 45 mg/kg nickel nanoparticles to rats caused epithelial shedding of the seminiferous tubules, disordered arrangement of cell in the tubules, as well as apoptosis and cell death. Whereas another study reported disorganization of germ cells layers, detachment and sloughing of immature germ cells, and vacuolization of the epithelium of seminiferous tubules after exposure to a high concentrations of ZnO NPs(57)

Degeneration of the epididymal tubule, hyperplasia, pyknotic nucleus cell, expansion of the space between tubules, lifting epithelium, vacuole formation, congestion of the blood vessels, and low epithelium lining thickness were also seen in the head and tail of epididymis after 14 days of exposure to 10, 20, and 30 mg/kg of GNPs in the current study. These histological changes in the testes and epididymis are indicative of functional damage to

Sertoli cells, which are responsible for supporting and protecting germ cells during spermatogenesis [56] or due to oxidative damages. Olugbodi et al. [46] found that the administration of Ag-NPs to rats at 50 mg/kg BW induced degenerative changes in the cellular architecture of the testes and epididymis. These degenerations are lines of evidence supporting the NPs ability to cause cellular and oxidative damage to rats testes and epididymis.

As a result, the present study findings revealed that GNPs could significantly alter the fertility potential of male mice.

Conclusion

The current research concluded that graphene nanoparticles have a toxic effect on the male reproductive system that could contribute to functional disorder. However, further studies were required to investigate the toxicity of these materials to offspring and fertility.

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

Ethical Clearance: "All experimental protocols were approved under the Department of Biology and all experiments were carried out in accordance with approved guidelines".

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