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Toxic Effects of Nanographene Oxide on Testes of Rats

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ABSTRACT

The current study aimed to examine the effects of nanographene oxide on the testes. A total of 48 male albino rats were randomly divided into 6 groups. The first, second, third, fourth, and sixth groups were treated with graphene oxide nanopowder at 20, 30, 40, 50, and 60 mg/kg concentrations, respectively. The sixth group was considered the control group. The results indicated a significant decrease in the average testis weight of rats treated with different nanographene oxide dosages, compared to the control group. There was also a significant decrease in the level of FSH and testosterone of treated rats with nanographene oxide, while there was no significant difference in the level of LH hormone when compared to the control group. The histological examination of the testes in the treated rats indicated hemorrhage, decreased sperm count, decreased thickness of the tubular epithelium, dissociation of connective tissue between the seminiferous tubules, in addition to hematological congestion, necrosis of the tubular epithelium, divergence of the seminal tubules, absence of sperm, shattering of the seminal tubule wall and degeneration sperm-forming cells and edema formation. Using the transmission electron microscope, the findings revealed a range of cellular changes, such as the presence of two-headed spermatids, the destruction of the nucleus membrane, spermatoblasts, the destruction of the cell membrane, and the denting of the nucleus membrane. It can be concluded that the nanographene oxide at 20-60 mg/kg concentrations can have harmful effects on spermatogenesis and normal function testis in rats.

Keywords: Laboratory rat, Nanographene oxide, Testes, Toxic effect

INTRODUCTION

Graphene is the thinnest electronic material which possesses distinctive chemical and physical properties. Graphene has remarkable properties, such as high surface area, thermal conductivity, electrical conductivity, and mechanical strength. These unique properties have led to an explosion of recent research in its composition, characterization, and development of its applications, especially in electronic devices, transparent electrodes for solar cells, plasma screens, and energy storage devices (Dhiman and Dhamija, 2014). The two-dimensional allotropic structure and bio-inherent properties of graphene make it applicable for biomedical and therapeutic purposes (Priyadarsini et al., 2018). The derivatives of graphene, such as graphene oxide, have received great attention due to their excellent solubility in physiological media, their good biocompatibility at the level of human exposure, and their ability to combine with other nanomaterials (Markovic et al., 2011). Graphene oxide has been widely applied in cellular imaging, drug and gene delivery, tissue engineering, and antibacterial therapies. It is a strong antibacterial alternative since it has severe toxic effects on bacteria, fungi, and other pathogens (Wu et al., 2015). The multiple applications of nanographene oxide, especially in the biomedical fields are increasing concerns about the potentially toxic effects of this substance on health and tissue cells. Therefore, the current study aimed to investigate the toxic effects of nanographene oxide on the testes of laboratory rats.

MATERIALS AND METHODS

A total of 48 male albino rats with an age range of 10-12 weeks and a mean weight of 170 g were examined for clinical health in the present study. The graphene oxide nanopowder was purchased from Sky Spring Nanomaterials, Inc., USA, in the form of black powder with 97% purity, 2-nm thickness, and an average diameter of 3-10 nm. Different concentrations of graphene oxide nanopowder were prepared by dissolving it in a normal saline solution. The animals were then divided into six groups and each group has a replicate (4 rats in each replicate). The first, second, third, fourth, and fifth groups were respectively treated with graphene oxide nanopowder at concentrations of 20, 30, 40, 50, and 60 mg/kg. The sixth group was considered as the control group. The solution was administered orally to the rats using a f*eeding tube* about 2-3 inches in length to prevent wounding the animal. The volume of administration was 0.1 ml per

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each animal, for 30 days. At the end of the experiment, the blood samples were collected from the lateral tail vein of each rat into an EDTA tube and a silicon-coating tube. The samples of silicon coating tubes remained at room temperature for up to 30 minutes to enable clotting. The clot is removed by centrifugation at 2000 x g for 10 minutes and the resulting supernatant immediately transfer to a polypropylene tube using a Pasteur pipette. The serum levels of LH, FSH, and testosterone hormones were measured immediately after receiving the serum samples by commercial kits (Beijing Northern Biotechnology Research Institute, Beijing, China) in an automated chemistry analyzer (BS 200, Mindray, China). The light and electron microscope slides were prepared based on the method proposed by Luna (1968). The microscopical studies were carried out using a light microscope (Olympus, Japan) and a transmission electron microscope (TESCAN company, China) to see the histological and cellular changes resulting from the effect of this substance. The weights of the testes in the different groups were measured using a sensitive scale (PCE-LSZ 200C, PCE- Deutschland Gmbh & Co, Germany), and the lengths of these samples were also measured using the ruler.

Statistical analysis

The statistical analysis was carried out using one-way ANOVA followed by L.S.D. The probability level of ≤ 0.05 was considered statistically significant.

RESULTS

Effects of graphene oxide on blood parameters

The results of the current study showed that there was a significant decrease in the average number of red blood cells in group 5 when compared with other groups ($p \leq 0.05$, Table 1). The results of the current study showed a significant decrease in the hemoglobin level of the group treated with a concentration of 60 mg/kg when compared to other groups ($p \leq 0.05$). Moreover, there was a non-significant increase in the average number of white blood cells when comparing the treated groups with concentrations of 20, 30, 40, and 50 mg/kg and the control group ($p > 0.05$). There was a significant increase in the rate of white blood cells when comparing treated rats with 60 mg/kg with other groups except for the fifth group ($p \leq 0.05$). The current study showed a significant increase in the mean number of platelets when comparing the groups treated with 40, 50, and 60 mg/kg of graphene oxide with other groups (Table 1).

Effects of graphene oxide nanoparticles on weight and length of testes

Comparing the control group with the experimental groups showed a significant decrease in the mean testis weight of treated rats ($p \leq 0.05$, Table 2). There was a significant decrease in the mean testicular length of all treated rats, compared to the control rats ($p \leq 0.05$, Table 2).

Effects of graphene oxide on the levels of sex hormones

There was a significant decrease in the FSH level when comparing all the treated groups with the control group ($p \leq 0.05$, Table 3). The results of the current study also showed an insignificant difference in the rate of LH hormone when comparing all the treated groups with the control group ($p > 0.05$). However, there was a significant decrease in the rate of testosterone hormone in all experimental groups, compared with the control group ($p \leq 0.05$).

Table 1. Effects of different levels of nanographene oxide on some blood parameters of rats

Groups	RBC	Hb	WBC	Platelets
Treatment 1 (20 mg/kg)	8.11 ± 0.47^{a}	14.12 ± 0.77^{a}	5.06 ± 0.52^{a}	437.20 ± 11.67^{a}
Treatment 2 (30 mg/kg)	8.11 ± 0.46^{a}	14.08 ± 0.29^{a}	5.06 ± 1.18^{a}	490.00 ± 18.95^{ab}
Treatment 3 (40 mg/kg)	7.71 ± 0.45^{ab}	13.46 ± 0.61^{a}	5.28 ± 0.83^{a}	597.60 ± 64.36^{e}
Treatment 4 (50 mg/kg)	7.69 ± 0.26^{ab}	12.64 ± 0.11^{ab}	5.56 ± 1.11^{ab}	632.80 ± 44.48^{e}
Treatment 5 (60 mg/kg)	6.82 ± 0.53^{c}	10.76 ± 0.61^{c}	6.46 ± 0.49^{b}	640.60 ± 96.94^{e}
Treatment 6 (Control)	8.71 ± 0.53^{a}	14.26 ± 1.25^{a}	4.40 ± 0.40^{a}	368.80 ± 60.76^{a}

The numbers in the table represent mean values ± standard deviation. RBC: Red blood cells, Hb: Hemoglobin, WBC: White blood cells. The different superscript letters in the same column indicate a significant difference at the probability level of $p \leq 0.05$.

Table 2. Effects of different levels of graphene oxide nanoparticles on the average weight and length of testes in rats

Groups	Weight of testis	Length of testis
Treatment 1 (20 mg/kg)	0.71 ± 0.04^{b}	1.71 ± 0.09^{b}
Treatment 2 (30 mg/kg)	0.69 ± 0.04^{c}	1.63 ± 0.05^{c}
Treatment 3 (40 mg/kg)	0.68 ± 0.03^{c}	1.50 ± 0.12^{d}
Treatment 4 (50 mg/kg)	0.67 ± 0.03^{cd}	1.48 ± 0.07^{e}
Treatment 5 (60 mg/kg)	0.63 ± 0.04^{d}	1.35 ± 0.05^{f}
Treatment 6 (Control)	0.80 ± 0.04^{a}	1.85 ± 0.05^{a}

The numbers in the table represent mean values ± standard deviation. The different superscript letters in the same column indicate a significant difference at the probability level of $p \leq 0.05$.

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Table 3. Effects of different levels of graphene oxide nanoparticles on sex hormones of rats

Groups	FSH	LH	Testosterone
Treatment 1 (20 mg/kg)	0.52 ± 0.29^{b}	1.15 ± 0.25	2.12 ± 0.04^{b}
Treatment 2 (30 mg/kg)	0.42 ± 0.29^{b}	1.14 ± 0.19	2.02 ± 0.29^{b}
Treatment 3 (40 mg/kg)	0.30 ± 0.11^{b}	1.06 ± 0.18	2.02 ± 0.16^{b}
Treatment 4 (50 mg/kg)	0.25 ± 0.05^{bc}	1.02 ± 0.13	1.86 ± 0.18^{b}
Treatment 5 (60 mg/kg)	0.24 ± 0.05^{bc}	1.01 ± 0.21	1.42 ± 0.20^{bc}
Treatment 6 (Control)	0.92 ± 0.17^{a}	1.22 ± 0.08	2.72 ± 0.57^{a}

The numbers in the table represent mean values ± standard deviation. The different superscript letters in the same column indicate a significant difference at the probability level of $p \leq 0.05$.

Histological examinations

Histological examinations on the control rats showed the normal structure of the testis. As observed in the control group, the seminiferous tubules contain germ cells that represent the different stages of sperm formation. In addition to the mature sperms present in the lumen of the seminiferous tubule, the seminiferous tubules are separated from each other by connective tissue as they are the testicle surrounded by the capsule (Figure 1). The histological examinations of the testes in the rats of the group treated with a concentration of 20 mg/kg showed bleeding, while those treated with a concentration of 30 mg/kg indicated a decrease in the number of sperms and thickness of the tubular epithelium as well as the dissociation of the connective tissue between the seminiferous tubules (Figures 2 and 3). Necrosis of the tubular epithelium and severe bloody congestion as shown in figures 3 and 4. The histological examinations of the testes in the rats treated with a concentration of 40 mg/kg showed bleeding, a decrease in the number of sperms, dissociation of the connective tissue between the seminiferous tubules, and spacing of the seminiferous tubules (Figures 5 and 6). The tubular epithelium, lack of sperm, and the destruction of the seminal tubule wall, as well as the dissociation of the connective tissue between the seminiferous tubules, and a decrease in the thickness of the tubular epithelium, were seen in treated rats with a concentration of 50 mg/kg (Figures 7 and 8). Histological examinations of the testes in the experimental rats administered a concentration of 60 mg/kg showed a lack of connective tissue between the seminiferous tubules and necrosis of the tubular epithelium, as well as hematopoietic congestion, lack of sperm, degeneration of sperm-forming cells and edema formation (Figures 9 and 10).

Examinations by electron microscopy

Transmission electron microscopy images of the testis in the rats of the control group revealed a normal structure of spermatids, where the cell membrane, cytoplasm, vesicle, and nucleus could be observed (Figure 11). Moreover, transmission electron microscope images of the testis in the rats of the treated group at a concentration of 20 mg/kg of nanographene oxide showed the presence of a spermatid with two heads (Figure 12); however, the images of the testis in the rats treated with nanographene oxide at a concentration of 30 mg/kg indicated the destructions in the nucleus membrane (Figure 13). The nucleus membrane and the cell membrane of the testis were destroyed in the rats treated with nanographene oxide at concentrations of 40 and 50 mg/kg, respectively (Figures 14 and 15). The transmission electron microscopy images of the testis in the rats treated with nanographene oxide at a concentration of 60 mg/kg showed that the nucleus wall and the nucleus were dented in the sertoli cell (Figure 16).

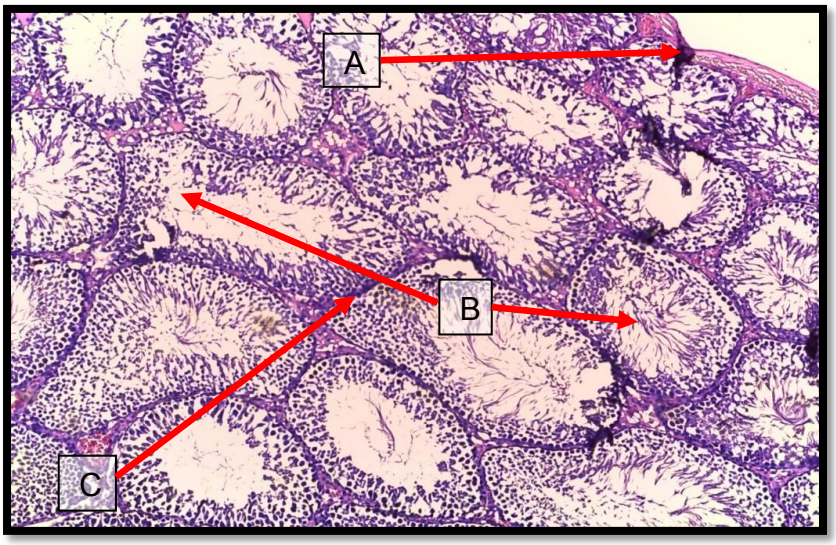

Figure 1. Normal testicular tissue of rats showing tunica albuginea (A), seminiferous tubules (B), the connective tissue between seminiferous tubules (C), H&E, 100X.

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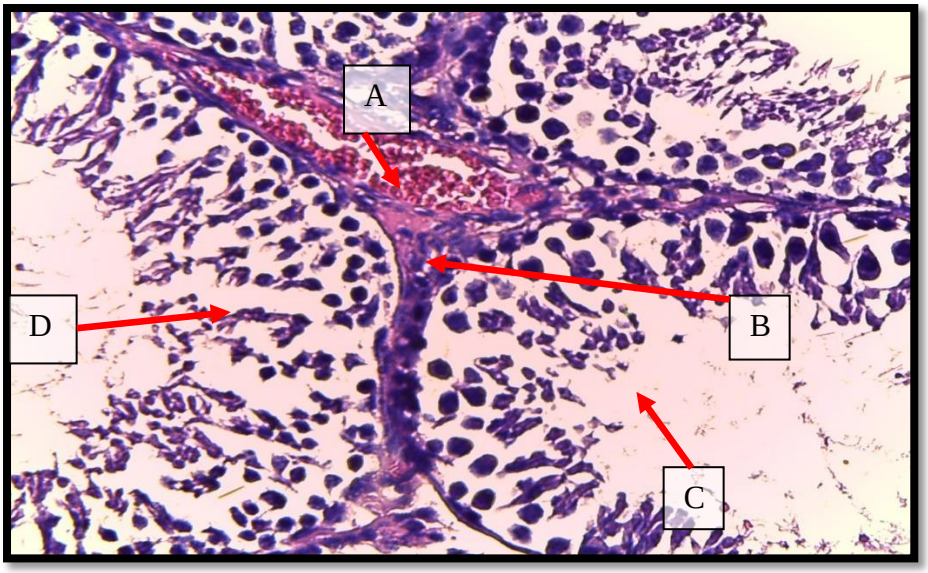

Figure 2. Testicular tissue of the rats treated with nanographene oxide at a concentration of 20 mg/kg indicates hemorrhagic bleed (A), progenitor sperm (B), the lumen of seminiferous tubule (C), spermatocytes in different stages of formation (D), H&E, 400X

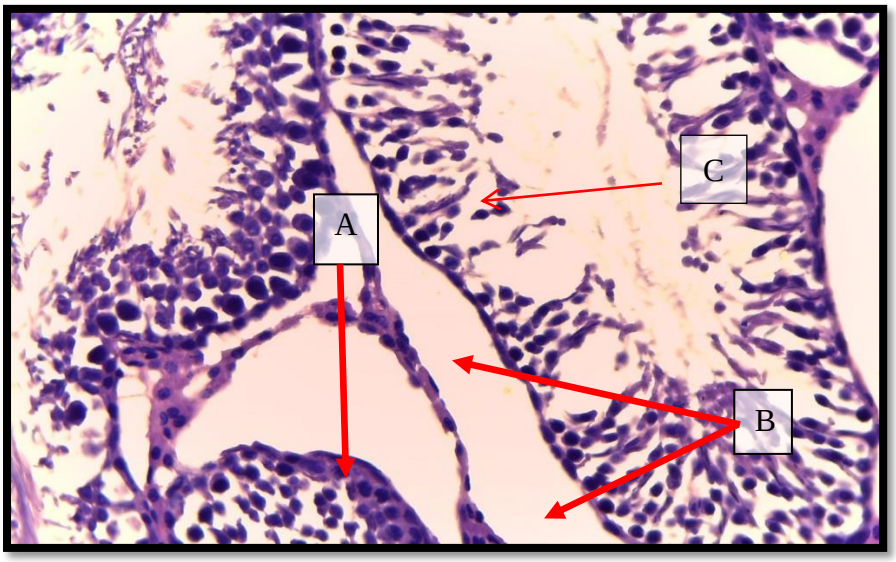

Figure 3. The testis tissue of the rats treated with nanographene oxide at a concentration of 30 mg/kg showis the dissociation of connective tissue between seminiferous tubules (A), necrosis of the tubular epithelium (B), decreased sperm count (C), H&E, 400X

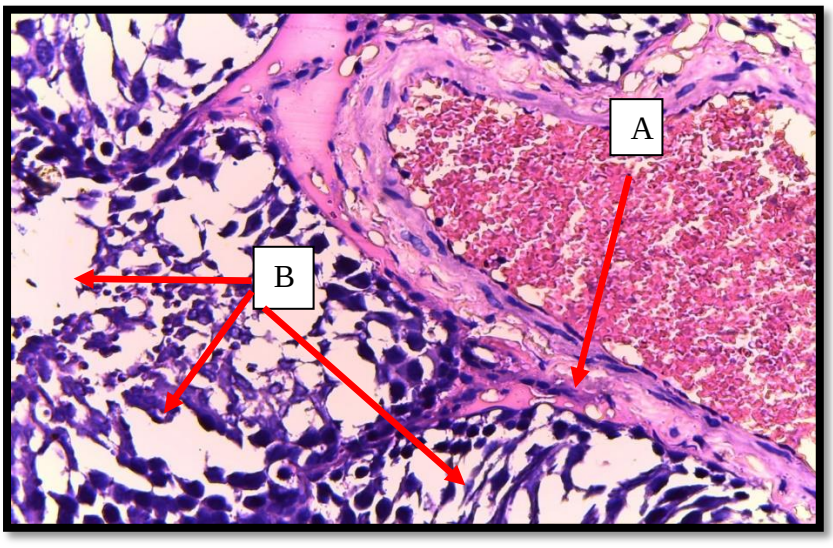

Figure 4. The testis tissue of the rats treated with nanographene oxide at a concentration of 30 mg/kg shows severe blood congestion (A) tubular epithelium necrosis (B) H&E, 400X

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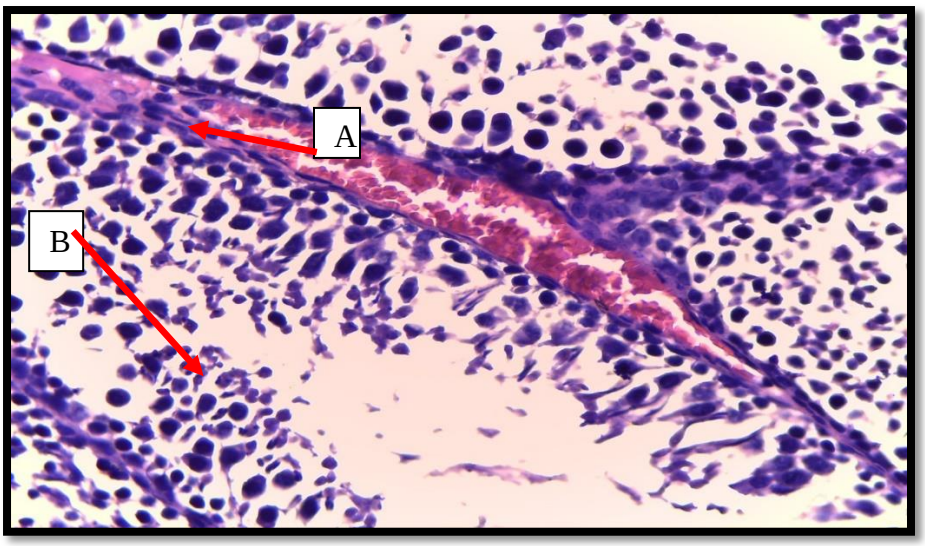

Figure 5. A cross-section of the testicular tissue of the rats treated with nanographene oxide at a concentration of 40 mg/kg indicates hemorrhage (A), decrease in sperm count (B) H&E, 400X

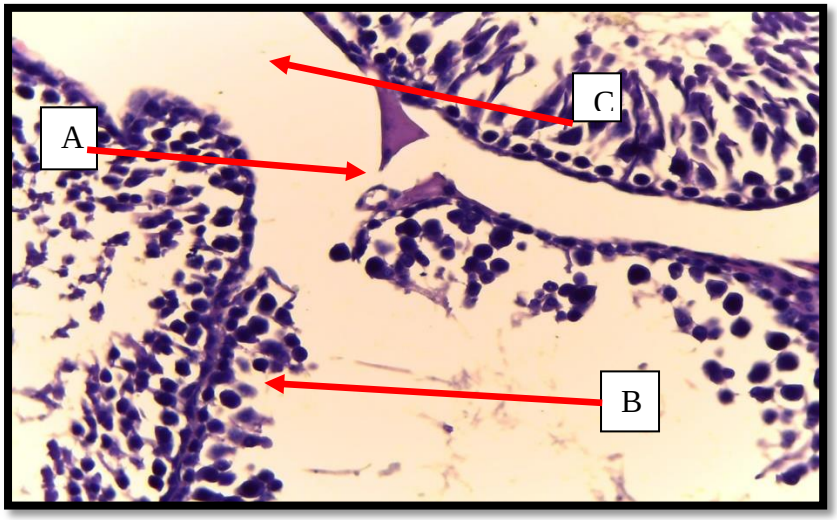

Figure 6. Testicular tissue of the rats treated with nanographene oxide at a concentration of 40 mg/kg reveals the dissociation of connective tissue between the seminiferous tubules (A), decrease in the number of sperm (B), spacing between the seminiferous tubules (C) H&E, 400X

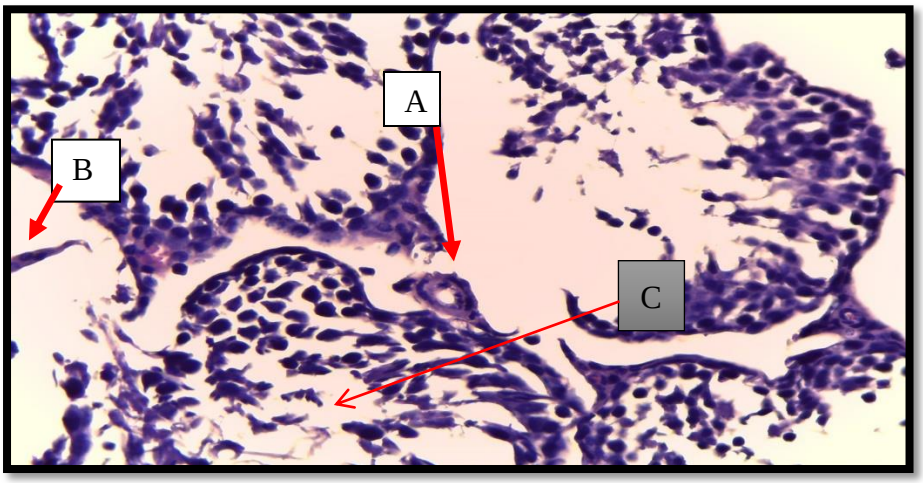

Figure 7. The testis tissue of the rats treated with nanographene oxide at a concentration of 50 mg/kg indicates the rupture of the wall of the seminiferous tubule (A), the dissociation of the connective tissue between the seminiferous tubules (B), Necrosis of the tubular epithelium (C), H&E, 400X

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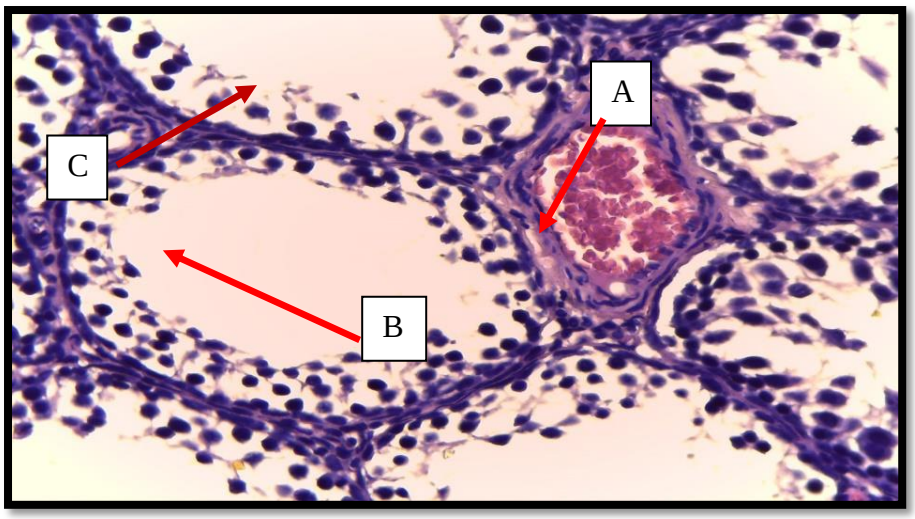

Figure 8. The testis tissue of the rats treated with nanographene oxide at a concentration of 50 mg/kg presents hemocongestion (A), lack of sperm (B), decrease in the tubular epithelium (C), H&E, 400X

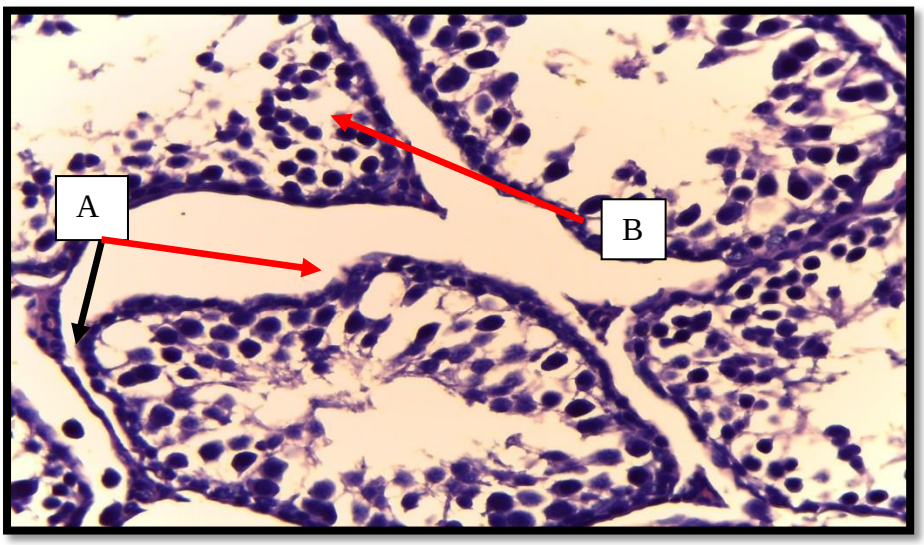

Figure 9. The testis tissue of the rats treated with nanographene oxide at a concentration of 60 mg/kg demonstrates a lack of connective tissue between the seminiferous tubules (A) necrosis of the tubular epithelium (B), H&E, 400X

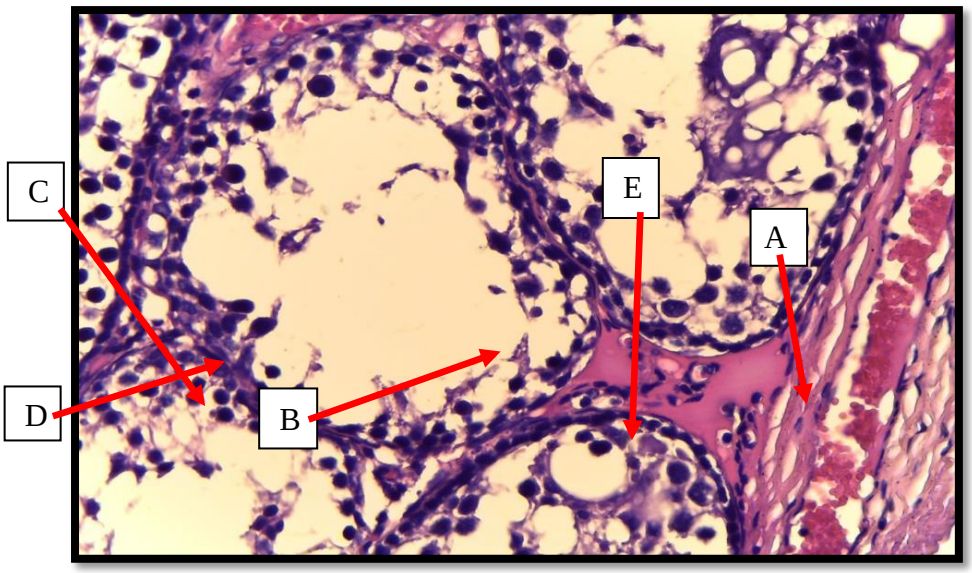

Figure 10. The testis tissue of the rats treated with nanographene oxide at a concentration of 60 mg/kg indicates blood congestion (A), necrosis of the tubular epithelium (B), lack of sperm (C), degeneration of sperm-forming cells (D), occurrence of edema (E), H&E, 400X

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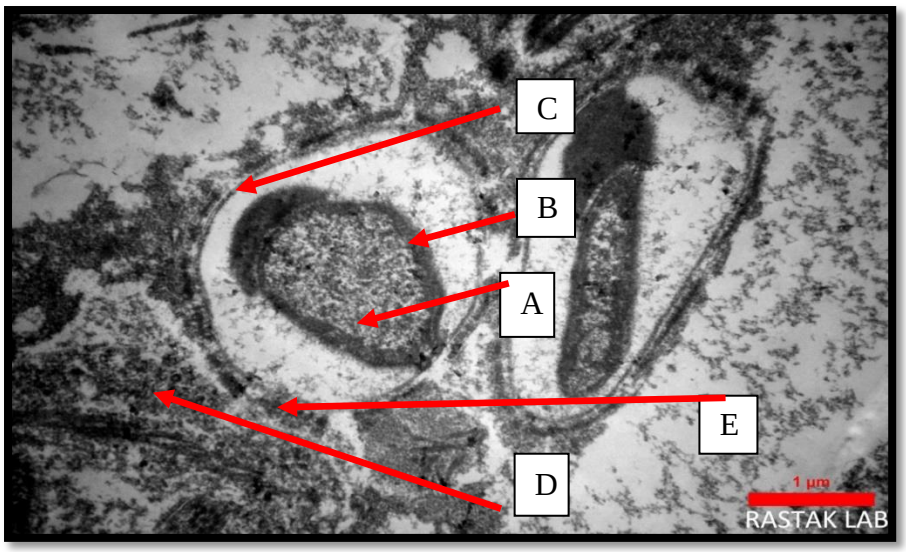

Figure 11. The testis tissue in control group rats showing the structure of the spermatid (A), cell membrane (B), cytoplasm (C), vesicle (D), centrioles (E), nucleus, transmission electron microscopy

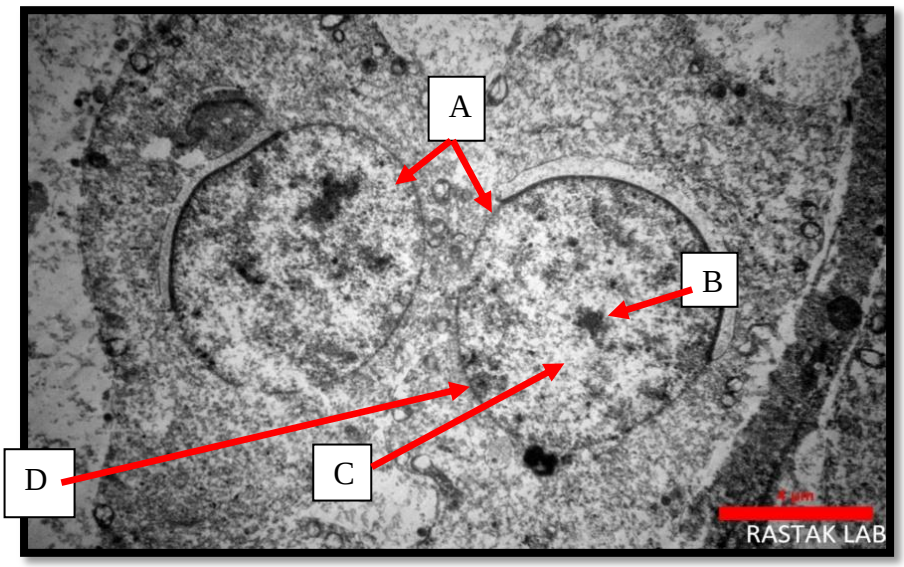

Figure 12. The testicular tissue of the rats treated with nanographene oxide at a concentration of 20 mg/kg indicates the structure of a double-headed spermatid (A), nucleus (B), cytoplasm (C), centriole (D), cell membrane, transmission electron microscopy

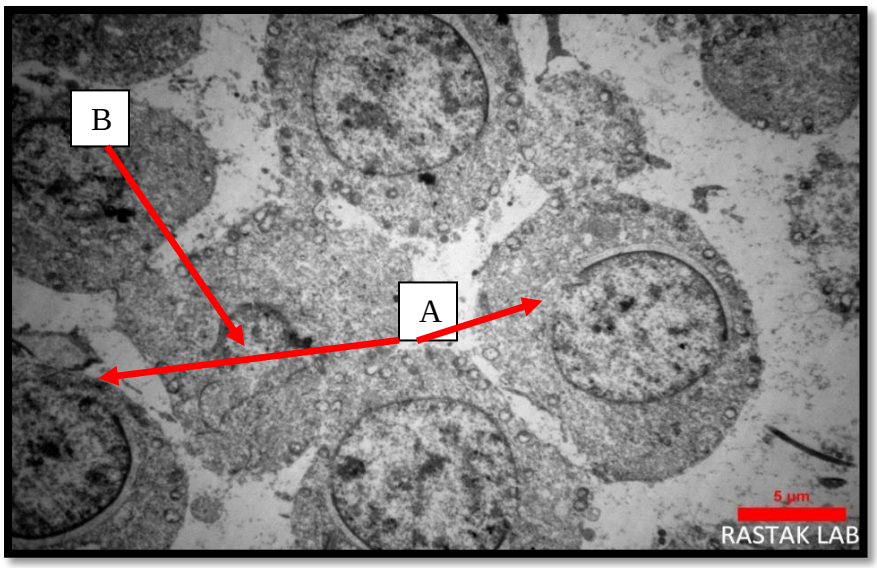

Figure 13. The testicular tissue of the treated rats with nanographene oxide at a concentration of 30 mg/kg. A nucleophilic breakdown in the cytoplasm (A, B), transmission electron microscopy

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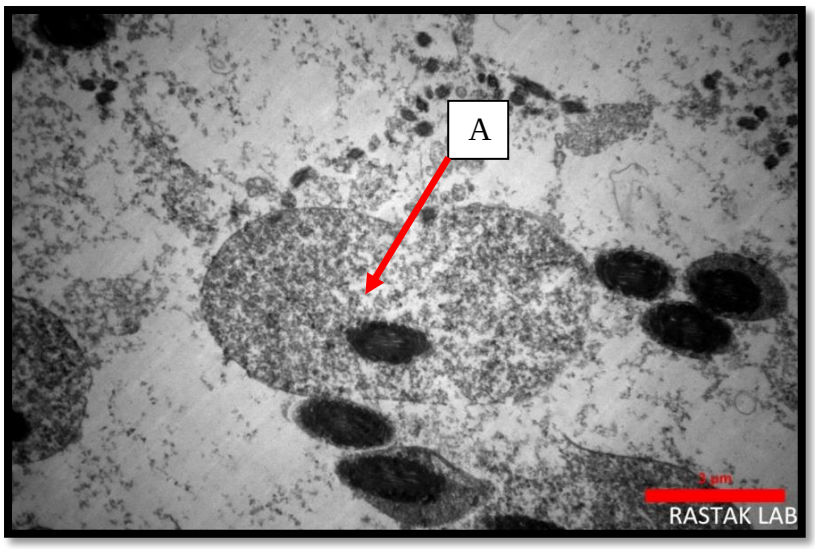

Figure 14. The testicular tissue of rats treated with nanographene oxide at a concentration of 40 mg/kg shows the breakdown of nucleus membrane (A), transmission electron microscopy

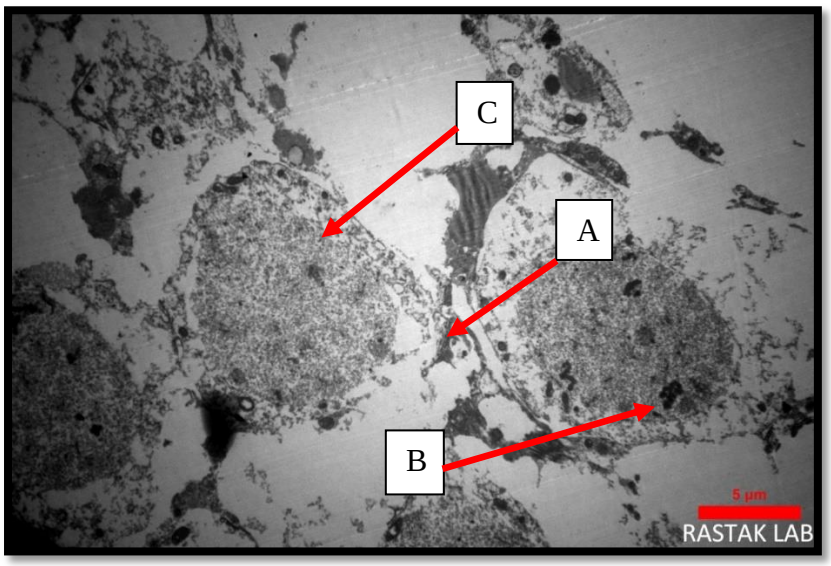

Figure 15. The testicular tissue of rats treated with nanographene oxide at a concentration of 50 mg/kg indicates cell membrane breakdown (A), nucleus (B), cytoplasm (C), transmission electron microscopy

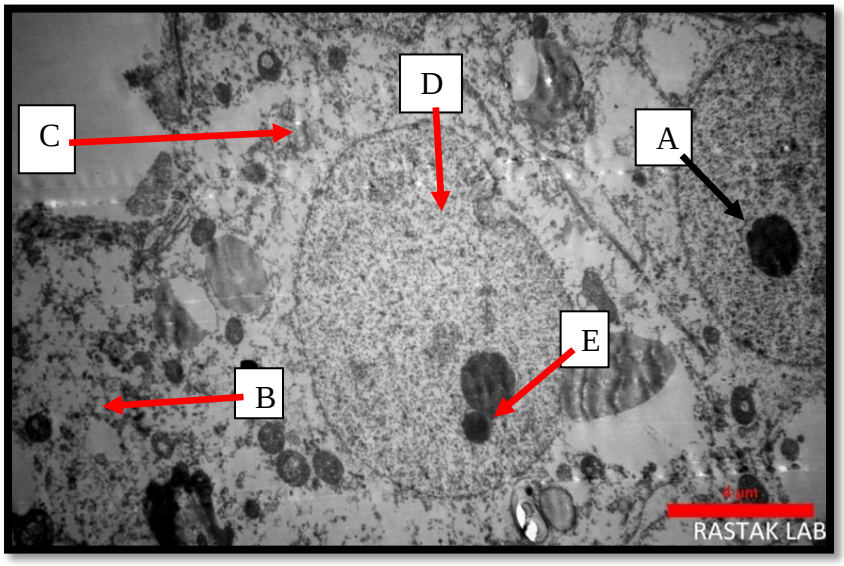

Figure 16. The testicular tissue of rats treated with nanographene oxide at a concentration of 60 mg/kg demonstrates buckling membrane of the nucleus (A), Golgi (B), Golgi apparatus (C), cytoplasm (D), nucleus (E), mitochondria in sertoli cell, transmission electron microscopy

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DISCUSSION

Effect of graphene oxide on the blood

The current study indicated a decrease in the number of red blood cells in all treated rats, especially in rats treated with 60 mg/kg of graphene oxide. The oxidative stress or pathological damage caused by the sharp edges of this material indicated that graphene oxide causes toxicity to the size and oxygen content of red blood cells leading to the dissolution of red blood cells (Liao et al. 2011). Moreover, this result agrees with a study of Puzyr et al. (2007), which showed the presence of toxic effects on blood parameters after intravenous injection, as graphene oxide interacts with the outer membrane of red blood cells through electrostatic interactions. This causes a disturbance in the polarity or permeability of the membrane and promotes hemolysis. These results are not in line with the obtained result of Qu et al. (2013), revealing that carbon nanoparticles do not cause any side effects to blood cells because they do not exert any toxic effects on blood cells. The results of the current study also revealed a decrease in the percentage of hemoglobin in the groups treated with nanographene oxide, compared to the control group. Hemolysis can be caused by the sharp edges of these nanomaterials that damage cell membranes, which was reported by Feng et al. (2015). The morphological changes, aggregation, and hemolytic effects on red blood cells when treated with graphene oxide are also indicated by Sasidharan et al. (2012). The reason behind the aggregation and hemolysis is the interaction between the hydrophobic surface of graphene and the lipid bilayer of the red blood cell membrane, or other interactions, such as hydroxyl and carboxylic groups in graphene oxide. These results also agree with those obtained by Stone et al. (2017), where a significant decrease was found in the hemoglobin percentage of groups treated with graphene oxide at concentrations of 80, 120, and 200 mg/kg, compared to the control group. In contrast, Escudero et al. (2019) reported that treating rats with graphene and graphene oxide could lead to smaller-sized red blood cells containing a high percentage of hemoglobin, justifying the reason for the high hemoglobin to compensate for the small size of red cells. The results of the current study also showed an increase in the numbers of white blood cells and platelets in the groups treated with graphene oxide, compared to the control group. There was an increase in the number of white blood cells although it decreased over time meaning that this effect was temporary. These results are consistent with a study by Vuppaladadium et al. (2020), where it was found that the number of white blood cells increased in mice treated with graphene oxide. This enhancement can be related to the foreign body, as it occurs after consuming the drug or graphene oxide. On the contrary, Rathnam et al. (2020) found that the hemoglobin content and the number of red blood cells were close to the control group. They also reported a slight increase in leukocytes, perhaps due to the response to nanomaterials during treatment. Pinto et al. (2013) and Zainab et al. (2021) also found that graphene-based nanomaterials were compatible with blood and did not cause hemolysis, platelet activation, changes in coagulation, or abnormalities in blood parameters. However, Lindstrom et al. (2015) reported that the number of white blood cells did not change in the animals treated with reduced graphene oxide.

Effect of graphene oxide on the testes

The results of the current study showed a decrease in the rate of testes' weight in the groups treated with graphene oxide. Compared with the control group, the histological examinations of the testes in rats treated with graphene oxide showed a group of changes represented by hemorrhage, a decrease in the number of sperms, a decrease in the thickness of the tubular epithelium, in addition to the dissociation of the connective tissue between the seminiferous tubules, necrosis of the tubular epithelium and blood congestion. The reason for the decrease in the weight of the testicles in the treated groups may be attributed to the lack of sperm and the suspension of the cell cycle, which resulted from a defect in the process of sperm formation in addition to the necrosis of the tubular epithelium leading to the loss of spermatozoa. These results were supported by Nirmal et al. (2017), indicating a decrease in the number of sperm in that rats exposed to graphene oxide nanoparticles for 15 and 30 days.

Histopathological changes in the testes, such as necrosis and a decrease in the thickness of the tubular epithelium, may be due to oxidative stress, as oxidative stress leads to the oxidation of cell membrane lipids, and consequently cell death. Adenosine triphosphate (ATP) is rapidly removed from the sperm, causing axonal damage and increasing sperm morphological abnormalities. These results were confirmed in a study by Mathur and Dcruz (2011), where there was a decrease in the number of sperms, their movement, and deformation due to a defect in the process of sperm formation as a result of the imbalance between oxidizing factors and antioxidant factors. Similarly, Cherian et al. (2014) found that oxidative stress and reactive oxygen species decreased cell proliferation, reduced steroid hormones, loss of germ cells, and cell death in the germinal epithelium. These results were also consistent with a study by Thakur et al. (2014), where nanomaterials cause the irregular appearance of the testis with atrophy of seminiferous tubules, loss of sperm-generating germ cells, necrosis of germ cells, and decrease or disappearance of sperms. In the same line, Hafsan et al. (2022), Huldani et al. (2022), and Li et al. (2016) found that Zno nanoparticles could cause reactive oxygen species (ROS) generation and DNA damage to germ cells and downregulation of the expression of proteins in sertoli cells, which may cause damage to the blood barrier in the testis. However, Liang et al. (2015) reported that the level of testosterone

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hormone in the serum did not change as it was shown that these nanomaterials did not stimulate the tissue damage of the testes and epididymis. These results do not agree with a study by Nirmal et al. (2017), indicating the concentrations of sex hormones in the serum of rats treated with graphene oxide did not change, compared to the control group after intravenous injection and the sexual behavior in male rats as reproductive structures were normal.

Results of transmission electron microscope

The findings of the transmission electron microscope showed changes at the cellular level of the testis, represented by the appearance of bi-headed and smashed-headed spermatids, in addition to the presence of sperms with broken membranes and other shrunken flagella walls in sertoli cells. This may be attributed to the ability of graphene oxide sheets to penetrate the cell membrane due to their nanoscale, causing damage to cell components through several mechanisms, including direct interaction with large biomolecules present in the cell or through oxidative stress that causes cell damage. The interaction of graphene oxide with large biological molecules, such as proteins, can result from functional groups with electrical charges on the surface of graphene oxide. Consequently, abnormalities occur in the intracellular signaling pathways that regulate cell growth, proliferation, differentiation, or survival. Similarly, Shi et al. (2012) reported that graphene oxide can weaken membrane proteins that act as carriers of nutrients or essential biomolecules, thus leading to a decrease in metabolic activity in cells treated with graphene derivatives. The mechanisms of action of graphene nanomaterials on living organisms include oxidative stress, inflammatory response, apoptosis, autophagy, and necrosis (Wu et al., 2021). Another study showed that treatment of some *in vitro* cultured cells, such as THP-1 and BEAS-2B cells, with graphene oxide, causes cytotoxicity by oxidizing lipids in the membrane and causing damage to the cell membrane (Li et al., 2018; Ansari et al., 2022). Another study showed that small parts of nanosheets of graphene derivatives could enter the nucleus, interact directly with DNA, and cause damage (Xu et al., 2018; Abolhasani Zadeh et al., 2022). In contrast to these results, some researchers stated that the graphene family of nanomaterials does not negatively affect the nucleus and DNA (Jarosz et al., 2016; Bokov et al., 2022).

CONCLUSION

It can be concluded that the nanographene oxide at 20-60 mg/kg concentrations is a hazardous material for testis heath in rats which can have dangerous effects on spermatogenesis, testosterone, and FSH levels and can destroy the testicular structure and impairment the testicular function in rats. The examined results of the transmission electron microscope confirmed the negative effects of this material in investigated dosages. The authors of the present study suggest that lower doses of nanographene oxide will consider for future histopathological studies in laboratory rats.

DECLARATIONS

Authors' contributions

The authors contributed equally to the study design, collecting the samples, statistical analysis, writing the first draft of the manuscript, and following the revisions of the last draft of the article for submission to the journal.

Conflicts of interests

The authors of the present study have no conflicts of interest to declare.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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Availability of data and materials

The authors declare that they can prepare datasets for this study upon reasonable request.

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