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Pathology

Evaluation of the Ameliorative Role of Naringin Against Di-N-Butylphthalate-Induced Testicular Injury in Male Rats

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ABSTRACT

The objective of this study was to assess the ameliorative effect of naringin (NG) against di-n-butyl phthalate (DBP)-induced testicular injury in rats. Six equal groups were treated orally, 3 times a week for six successive weeks: the control vehicle group received olive oil; the NG group received NG (80 mg/kg); the DBP 250 and DBP 500 groups received DBP 250 and 500 mg/kg, respectively; and the NG+DBP 250 and NG+DBP 500 groups received NG (80mg/kg), an hour before DBP administration at 250 and 500 mg/kg, respectively. Serum malondialdehyde (MDA) level and total antioxidant capacity (TAC), as well as the testicular pathological changes, were investigated after 2, 4, and 6 weeks of exposure. Administration of DBP at a dose level of 250 mg/kg caused insignificant differences in serum MDA level or TAC over the experimental period, whereas rats given DBP at a dose level of 500 mg/kg showed significant changes after 4 and 6 weeks of exposure. Meanwhile, various degrees of testicular degenerative changes were observed at both levels of DBP following 2, 4, and 6 weeks of exposure, where degeneration of different spermatogenic cells, decrease in the number or even complete absence of spermatozoa in the lumen of seminiferous tubules were the main observed alterations. The co-administration of NG to DBP-intoxicated rats improved, in a time-dependent manner, the serum oxidant/antioxidant status and the testicular morphology. Overall, these findings proved that oral administration of DBP induced changes in the serum oxidant/antioxidant status and testicular histoarchitecture in a dose- and time-dependent manner, as well as the protective and ameliorative role of NG.

Keywords: Di-n-butyl phthalate, Malondialdehyde, Naringin, Testicular injury and Total antioxidant capacity.

INTRODUCTION:

The indiscriminate pollutant discharges from various agricultural and industrial activities area global concern for animal and human health (Kumar *et al.*, 2020). Over the last decades, human exposure to environmental endocrine disrupting chemicals (EDCs) has gained exponential awareness, particularly because of their association with harmful

effects on reproductive health (Green *et al.*, 2021).

Phthalates are environmental pollutant identified as endocrine disrupting chemicals. Among them, di-n-butyl phthalate (DBP) is the most common phthalate that is widely used in the manufacture of many industrial and pharmaceutical products, resulting in a wide-spread animal and human exposure (Reddy and Giribabu, 2012 and Zeng *et al.*, 2013). Although DBP exposure can occur via food, water, air, or skin, the oral route is the main source for continuous and daily human exposure, because DBP is used as food packaging materials and is widely used as coating materials for many drugs (Schettler, 2006 and Zhou *et al.*, 2010).

The evidence from previous studies suggested oxidative stress as one of the substantial mechanisms of DBP-induced reproductive dysfunctions through different pathways (Aly *et al.*, 2016; Nelli and Pamanji, 2017; Zhang *et al.*, 2019 and Zhang *et al.*, 2021).This dedicates the indispensable ameliorative role of antioxidants against DBP-induced oxidative tissue damage.

Flavonoids, a well-known source of natural antioxidants, are widely found in a variety of vegetables, fruits, and grains. Nowadays, flavonoids are essential ingredients in various medicinal, pharmaceutical, and cosmetic products, particularly due to their antioxidant, anti-inflammatory, anti-apoptotic, and anti-carcinogenic activities. Therefore, current trends in research have focused on flavonoids, considering their potential roles in improving human health and preventing numerous diseases (Panche *et al.*, 2016).

Citrus peel is a citrus by-product that contains flavonoids, powerful bioactive compounds that possess a pivotal role in protection against oxidative tissue damages (Koolaji *et al.*, 2020). Naringin (NG) is a dietary bioflavonoid abundantly found in citrus fruit peel and degraded by intestinal bacteria into the highly absorbable form, naringenin (Chen *et al.*, 2014). Previous literatures have reported numerous biological and pharmacological properties of NG and naringinin, such as antibacterial, antioxidant, anti-inflammatory, and anti-carcinogenic properties (Chen *et al.*, 2016 and Salehi *et al.*, 2019). Additionally, mounting evidence has proven the therapeutic role of NG against a variety of metabolic, hepatorenal, cardiovascular, neurological, reproductive, and respiratory disorders (Alam *et al.*, 2013 and 2014; Adil *et al.*, 2014 and 2015; Gopinath and Sudhandiran, 2016 and Shi *et al.*, 2017).

In the light of this background, this study aimed to evaluate the dose- and time-dependent testicular injury induced by DBP in male rats and the prospective mitigating and ameliorative role of NG.

MATERIALS AND METHODS

Materials:

Di-n-butyl phthalate, DBP (CAS No.84-74-2; purity of 99%) and Naringin, NG (CAS No. 10236-47-2; purity of ≥ 90%) were obtained from Sigma–Aldrich Company. Diagnostic kits for assessment of serum level of malondialdehyde (MDA) and the total antioxidant capacity (TAC) were obtained from Biodiagnostic Company, Dokki, Giza, Egypt. The Other chemicals were of analytical grades and commercially available.

Animals and experimental design:

The animals' care and handling followed the ethical guidelines of the International Animal Care and Use Committee, IACUC, Faculty of Veterinary Medicine, University

of Sadat City (Approval No. VUSC-014-1-19). Generally, ninety healthy male albino rats (180-200 g) of three months old were obtained from Alzyade Experimental Animals Production Center, Giza, Egypt. Rats were housed in the polypropylene cages at a naturally ventilated room under a standard laboratory condition (28±2 °C, 50-65 % relative humidity, and natural daily dark/light cycle) and were provided with a standard commercial diet and clean tap water *adlibitum* during the acclimatization period and all over the experiment.

Rats were assigned into six groups (n= 15), and treated orally, three times per week for sixsuccessive weeks. Control vehicle group: Rats were given olive oil (DBP vehicle). NG group: Rats were administered 80 mg/kg NG dissolved in distilled water. DBP 250 and DBP 500 groups: Rats were administered 250 mg/kg and 500 mg/kg DBP dissolved in olive oil, respectively. NG+DBP 250 and NG+DBP 500 groups: Rats received NG (80 mg/kg), an hour before DBP (250 mg/kg) and (500 mg/kg) administration, respectively. The doses of both NG and DBP were based on earlier studies by Arumugam *et al.* (2016) and Yin *et al.* (2016), respectively.

Sample collections:

Every 2 weeks along the experimental period, 5 rats from each group were fasted, weighed, anesthetized by inhalation of isoflurane and sacrificed for blood and tissue samples collection. Blood samples were obtained from the inner eye canthus in dry, clean, and labelled glass centrifuge tubes without anticoagulant, left for clotting at room temperature, and then centrifuged in cooling centrifuge at 3000 rpm for 15 min for sera collection, which were stored at -20°C for further biochemical analysis. The collected testicles from each rat were fixed in neutral-buffered formalin 10% for histopathological examination.

Serum testosterone was determined using direct, competitive immunoassay kit (Feldman *et al.,* 2002).

Serum testosterone was determined using direct, competitive immunoassay kit (Feldman *et al.,* 2002).

Assessment of serum oxidant/antioxidant biomarkers:

Serum level of MDA and TAC were estimated according to the procedure adopted by Ohkawa *et al.* (1979) and Koracevic *et al.* (2001), respectively.

Histopathological Examination:

Following the method adopted by Bancroft and Layton (2013), the formalin-preserved testes were routinely processed, embedded in paraffin wax, cut into sections (3-5 µm thicknesses) using a microtome, and finally stained with hematoxylin and eosin, before being examined by Lieca DMLB microscopes and photographed Leica EC3 digital camera.

Statistical analysis:

Values are displayed as mean ± standard error (SE). Significant($P<0.05$) differences among the mean values were detected by one way ANOVA (Analysis of Variance) followed by Duncan's multiple range test for post hoc analysis using SPSS (Statistical Package for Social Sciences) Version 16, 2007.

RESULTS

Naringin alleviated the di-n-butyl phthalate-induced disturbances in the serum oxidant/antioxidant status:

The general oxidant/antioxidant status was evaluated via estimating the changes in the serum MDA level and TAC (Figure1),

following the exposure to DBP and/or NG for 2, 4, and 6 weeks.

Rats administrated NG and/or DBP for 2 weeks did not exhibit any significant (*P<0.05*) variations in the serum MDA level and TAC, compared with the corresponding control values.

Concerning the normal control values, no significant (*P<0.05*) variations in the serum MDA level and TAC were observed in the NG and DBP 250 groups after 4 weeks of exposure. Otherwise, significant increases in the serum MDA level concomitantly with a significant decrease in TAC were recorded in the DBP 500 group, compared to those of the control group. Administration of NG one hour before DBP at dose levels of 250 or 500 did not induce any significant changes in serum MDA level and TAC, compared to the corresponding values of DBP 250 and 500, respectively; however, these values were around the normal control values in the NG+DBP 250 group.

Following six weeks of exposure, there were no significant (*P*<0.05) variations in the mean values of serum MDA level or TAC between the control and NG-treated groups. Furthermore, insignificant variations were observed in the mean values of serum MDA level and TAC between the control and DBP 250 groups and between the NG+DBP 250 and DBP 250 groups. Nevertheless, significant elevations in the mean values of serum MDA level along with significant reductions in serum TAC were recorded in the DBP 500-intoxicated group when compared with those of the control group, while co-administration of NG with DBP 500 induced significant improvements in serum MDA level and TAC, compared to the corresponding values of the DBP 500 group. Interestingly, co-administration of NG either with DBP 250 or DBP 500 restored the normal control values of the serum levels of MDA and TAC.

In terms of the aforementioned findings, the NG-treated group did not cause any significant (P<0.05) changes in the mean values of serum MDA level and TAC over the experimental period, when compared to the corresponding control values. Conversely, the oral administration of DBP induced changes in the serum MDA level and TAC, in a dose- and time-dependent manner, whereas significant changes were recorded only in the DBP 500 group at 4 and 6 weeks of exposure. The co-administration of NG to DBP-intoxicated rats improved, in a time-dependent manner, the serum MDA level and TAC to be within the normal control values at 6 weeks of exposure.

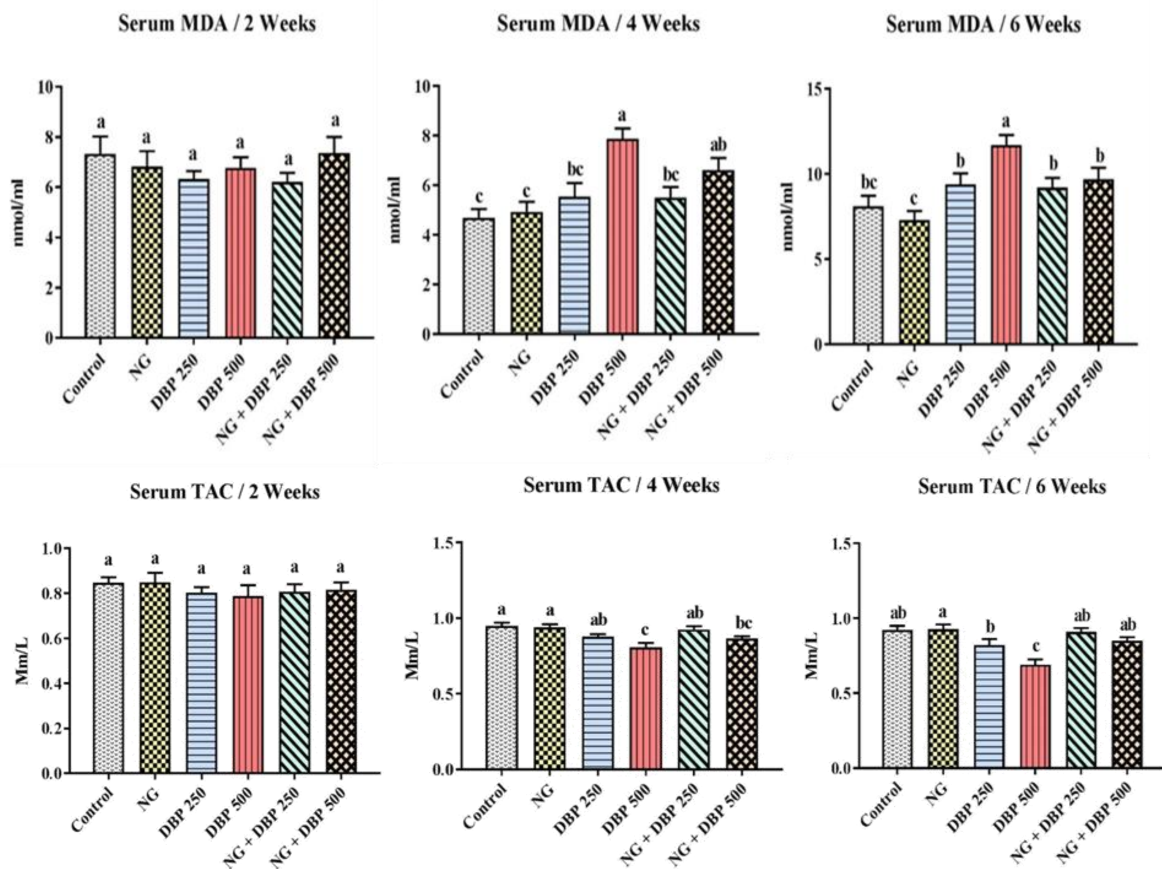

Figure 1: Serum malondialdehyde level and total antioxidant capacity of the different treated groups. Different letters mean significant changes at *P< 0.05*. MDA: malondialdehyde, TAC: total antioxidant capacity, NG: naringin, DBP 250: di-n-butylphthalate (250 mg/kg), DBP 500: di-n-butylphthalate (500 mg/kg).

Naringin improved the di-n-butyl phthalate-induced changes in the testicular histoarchitectures:

Testicular histopathological alterations of the different treated groups after 2, 4, and 6 weeks of exposure were presented in Table 1 and figures 2, 3, and 4.

Table 1: Histopathological scoring of the testicular alterations of the different treated groups:

Lesions / Group	Degeneration of the spermatogenic cells of the seminiferous tubules			Depletion of the spermatozoa inside the lumen of the seminiferous tubules		
	2 weeks	**4 weeks**	**6 weeks**	**2 weeks**	**4 weeks**	**6 weeks**
Control vehicle	-	-	-	-	-	-
NG	-	-	-	-	-	-
DBP 250	-	++	+++	-	++++	++++
DBP 500	-	+++	++++	+++	++++	++++
NG+DBP 250	-	-	-	-	++	++
NG+DBP 500	-	-	-	-	++	++

The histopathological changes are graded as follows: (−) indicates normal appearance, (+) indicates slight changes, (++) indicates mild changes, (+++) indicates moderate changes, and (++++) indicates severe changes. NG: naringin, DBP 250: di-n-butylphthalate (250 mg/kg), DBP 500: di-n-butylphthalate (500 mg/kg).

Normal histological architectures were observed in the testicular tissues of all the different treated groups after two weeks of exposure, except a marked decrease of the spermatozoa was observed in the lumen of the seminiferous tubules of the DBP 500 group (Figure 2).

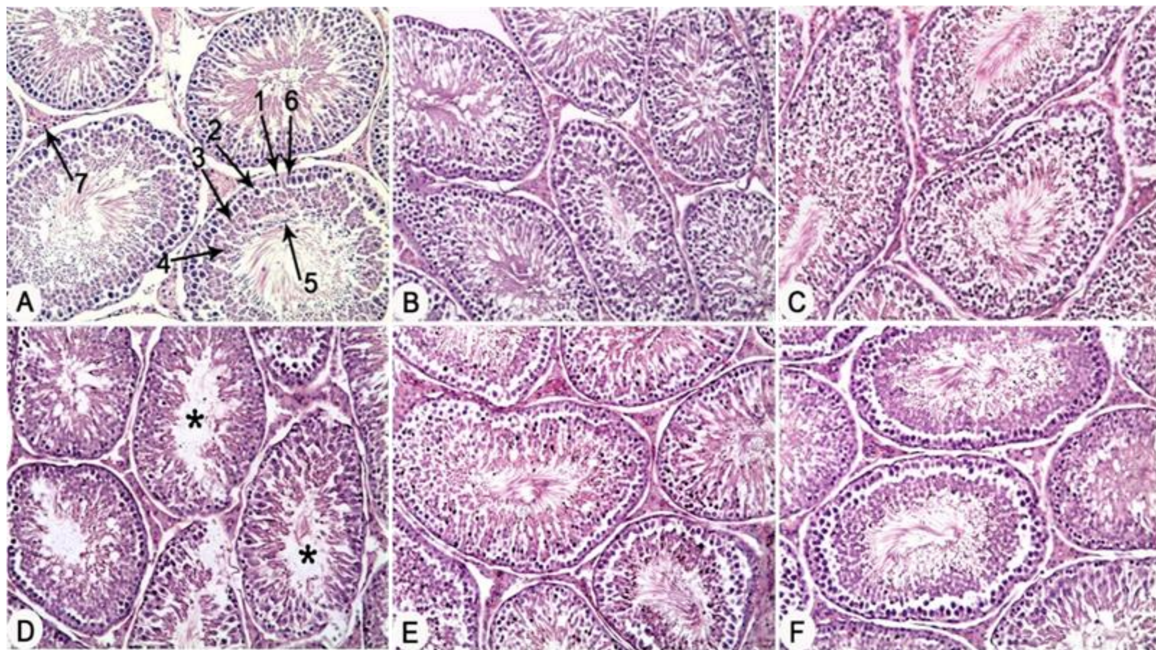

Figure 2. Photomicrographs of histopathological changes in testes of different treated groups, after two weeks of exposure (H&E stain ×10).1, spermatogonia; 2, primary spermatocytes; 3, secondary spermatocytes; 4, spermatids; 5, spermatozoa; 6, Sertoli cells; 7, Leydig cells. (A) Control vehicle, (b) NG and (C) DBP 250 groups showing normal histological architectures. (D) DBP 500 group showing marked decrease of the spermatozoa in the lumen of seminiferous tubules (asterisk). (E) NG+DBP 250 and (F) NG+DBP 500 groups showing normal histological architectures.

Following four weeks of treatments, the testicular tissues of the control and NG groups showed normal architectures. The absence of spermatozoa from the lumen of seminiferous tubules and degenerative changes in the spermatid cell layer were observed in the DBP 250-intoxicated group. Besides, degenerative changes in the secondary spermatocyte were observed in the DBP 500-intoxicated group. On the other hand, administration of NG one hour before DBP 250 and DBP 500 intoxication showed only decrease in the spermatozoa in the lumen of seminiferous tubules (Figure 3).

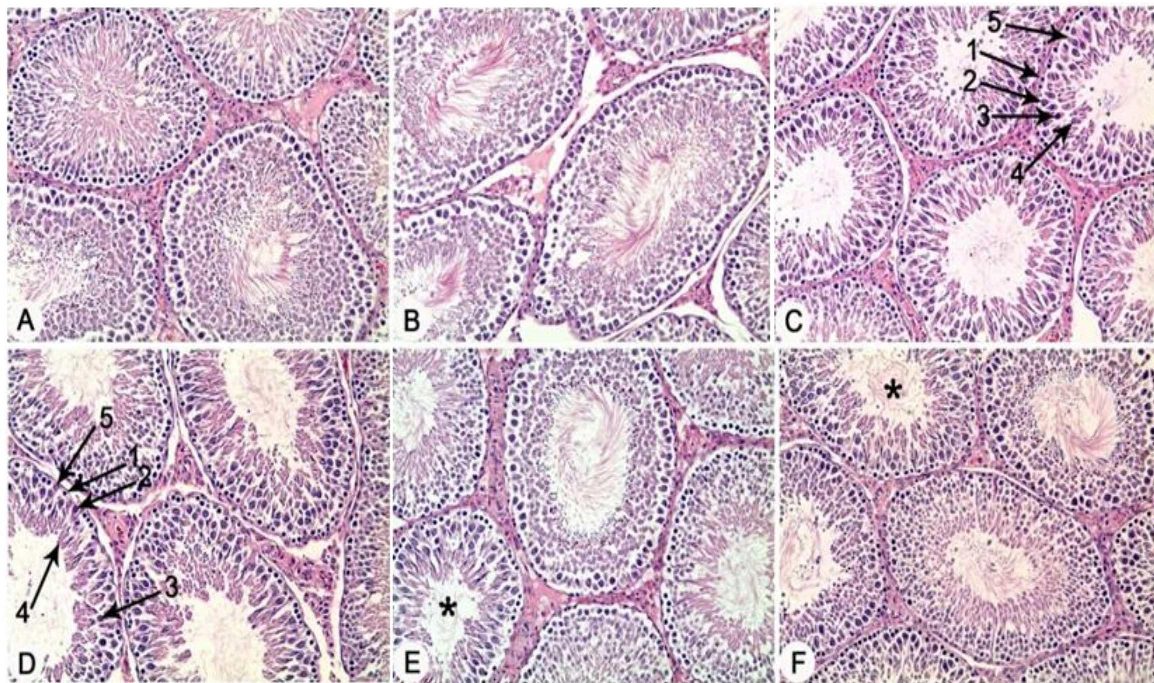

Figure 3. Photomicrographs of histopathological changes in testes of different treated groups, after four weeks of exposure (H&E stain ×10).1, spermatogonia; 2, primary spermatocytes; 3, secondary spermatocytes; 4, spermatids; 5, Sertoli cells. (A) Control vehicle and (b) NG groups showing normal histological architectures. (C) DBP 250 group showing absence of the spermatozoa from the lumen of seminiferous tubules and degenerative changes in the spermatid cell layer. (D) DBP 500 group showing absence of the spermatozoa from the lumen of seminiferous tubules and degenerative changes in the secondary spermatocyte and spermatid cell layers. (E) NG+DBP 250 and (F) NG+DBP 500 groups showing decrease of spermatozoa (asterisk).

Testicular tissues of the control and NG-treated groups showed normal histological architectures after a period of six weeks of exposure. However, marked pathological alterations were recorded in the DBP 250-intoxicated group, as evidenced by the absence of spermatozoa from the lumen of seminiferous tubules and the degenerative changes in the secondary spermatocyte and spermatid cell layers. In addition, degenerative changes in the primary spermatocytes were also observed in the DBP 500-intoxicated group. Although co-administration of NG with either DBP 250 or DBP 500 improved these degenerative changes; however seminiferous tubules of both NG+DBP 250 and NG+DBP 500 groups showed only decrease of spermatozoa (Figure 4).

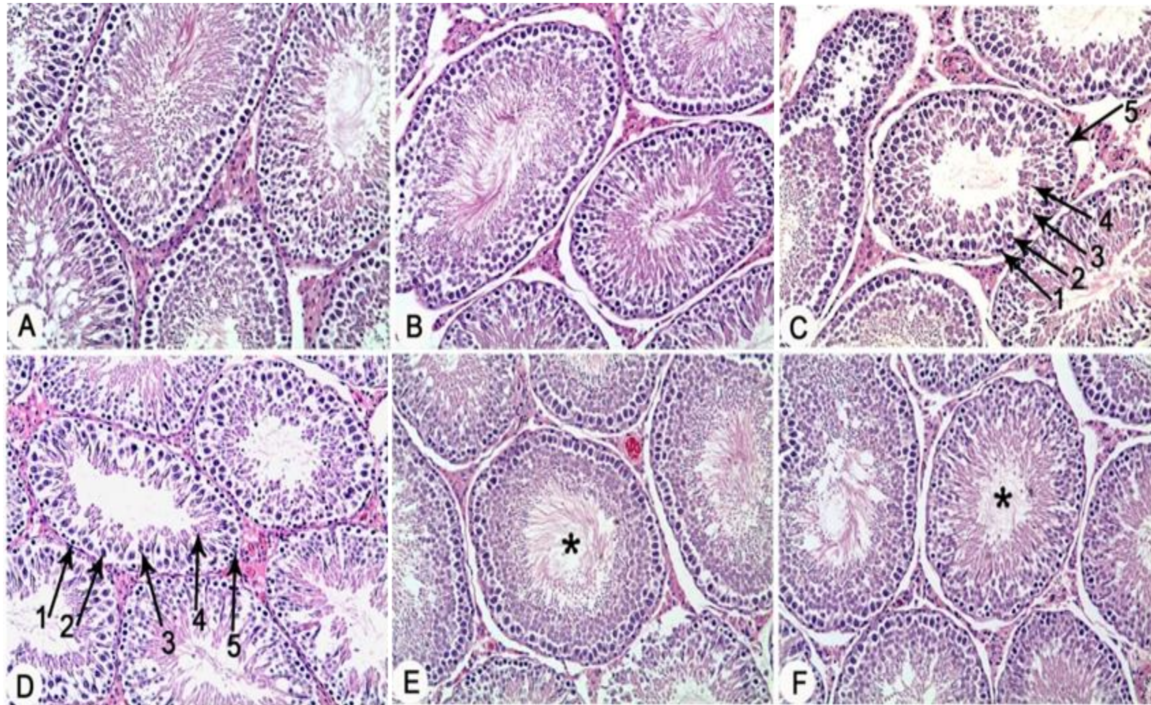

Figure 4. Photomicrographs of histopathological changes in testes of different treated groups, after six weeks of exposure (H&E stain ×10).1, spermatogonia; 2, primary spermatocytes; 3, secondary spermatocytes; 4, spermatids; 5, Sertoli cells. (A) Control vehicle and (b) NG groups showing normal histological architectures. (C) DBP 250 group showing absence of the spermatozoa from the lumen of seminiferous tubules and degenerative changes in the secondary spermatocyte and spermatid cell layers. (D) DBP 500 group showing absence of the spermatozoa from the lumen of seminiferous tubules and degenerative changes in the primary spermatocyte, secondary spermatocyte and spermatid cell layers. (E) NG+DBP 250 group and (F) NG+DBP 500 group showing decrease of spermatozoa (asterisk).

DISCUSSION

Male reproductive disorders are a well-known public health issue that has gained attention worldwide (Akinola *et al.*, 2010). The indiscriminate exposure to various environmental pollutants, particularly the endocrine disrupting chemicals, is among the main causes of the exponential growth of male reproductive disorders (Green *et al.*, 2021). Consequently, there are growing scientific attempts to investigate natural remedies for mitigating such disorders. Hence, this study aimed to investigate the dose- and time-dependent testicular injury induced by DBP in male rats and the prospective mitigating and ameliorative role of NG.

The current findings demonstrated that oral administration on DBP at dose levels of 250 and 500 mg/kg for 2, 4, and 6 weeks induced changes in the serum level of MDA and TAC, in a dose and time-dependent manner, inflecting the general oxidant/antioxidant imbalance induced by exposure to DBP. Further, the recorded disturbance in the general oxidant/antioxidant status were associated with a dose and time-dependent histopathological alterations in the

testicular tissue of the exposed rats. Absence of spermatozoa and the degenerative changes in the different spermatogenic cell layers were the remarkable observed histopathological alterations.

Following oral administration of DBP at dose levels of 200, 400, or 600 mg/kg/day for 15 consecutive days, Aly *et al.* (2016) also observed a dose-dependent reduction in serum TAC, testicular antioxidant enzymes, and elevation of testicular MDA, as well as necrosis of seminiferous tubules and degeneration with absence of sperms. Moreover, rats orally treated with DBP at a dose of 500 mg/kg for 4 weeks induced oxidative testicular damage via increasing the MDA level and decreasing the antioxidant markers with various histopathological alterations (Başak Türkmen *et al.*, 2022).

However, several mechanisms have been linked to DBP-induced reproductive disorders, including male reproductive system deformities, Leydig and Sertoli cell dysfunctions, spermatogenesis impairment, and apoptosis of spermatogenic cells (Bielanowicz*et al.*, 2016; Liu *et al.*, 2016; Aly *et al.*, 2016 and Alam and Hoque, 2018), oxidative stress remains the primary mechanism through which DBP induces impairment of male reproductive performance and testicular injury (Nelli and Pamanji, 2017).

Oxidative stress occurs due to the shift in balance between the production and scavenging of ROS, which is associated with many toxicological and pathological conditions. Mammalian cells naturally generate ROS during regular cellular respiration (Small *et al.*, 2012); however, excessive production results in lipid peroxidation, and ultimately cell disruption and damage (Gaschlera and Stockwell, 2017).Due to the high amount of polyunsaturated fatty acids, testes are extremely susceptible to oxidative damage (Asadi *et al.*, 2017).

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids into lipid peroxides. Malondialdehyde is the main end product of this reaction that is a pivotal indicator of LPO (Zhang *et al.*, 2004). Accumulating evidence has suggested the ability of DBP to increase intracellular ROS production (Cheng *et al.*, 2019; Wang *et al.*, 2020 and Liang *et al.*, 2021). Increased levels of serum MDA in DBP-intoxicated groups reflects the overproduction of ROS and alterations in the antioxidant defence system, as evidenced by the concomitant reduction of serum TAC in DBP-intoxicated groups. Meanwhile, ROS have been proven to be implicated in signal transduction pathways and may induce necrosis and/or apoptosis (Redza-Dutordoir and Averill-Bates, 2016), which may illustrate the observed pathological changes in the testicular architecture.

Interestingly, the concurrent administration of NG at a dose level of 80 mg/kg, one hour prior to the administration of DBP at dose levels of 250 or 500 mg/kg, mitigated, in a time-dependent manner, the adverse effect induced by DBP and returned the serum levels of MDA and TAC to being within the normal control levels along with a remarkable improvement of the testicular histoarchitecture, reflecting its protective and ameliorative effect against DBP toxicity even at high dose.

Similarly, previous studies have reported the protective antioxidant role of NG, particularly against testicular injury (Akondi *et al.*, 2011 and Adil *et al.*, 2014). In parallel, oral administration of NG (50 mg/kg) for 6 weeks reversed the testicular damage induced by permethrin toxicity and improved the testicular histological structure (Mostafa *et al.*, 2016). Furthermore, administration of NG at dose levels of 40, 80, and 160 mg/kg for 30 days improved testicular function and structure in BPA-intoxicated rats, as evidenced by a decrease in MDA level concurrently with an increase in testicular antioxidants and an improvement in testicular morphology (Alboghobeish *et al.*, 2019).

The recorded improvement observed in DBP groups co-treated with NG may be attributed to the antioxidant properties of NG. NG inactivates ROS-forming enzymes like NADPH oxidase and plays an essential role in the regulation of antioxidant capacity by stimulating the expression of various antioxidant-related genes, and consequently increasing the intracellular antioxidants (Ciz *et al.*, 2012). Also, NG inhibits the release of oxidative and inflammatory mediators (Adil *et al.*, 2014). In the same context, Ahmed *et al.* (2019) attributed the protective effect of NG to the improvement of antioxidant defence system and the suppression of inflammation and apoptosis pathways. These studies clearly hypothesized the mechanistic role of the antioxidant effect of NG in enhancing the testicular function and structure.

CONCLUSION

The findings obtained in this study revealed that oxidative stress induced by DBP, as reflected by disturbances in serum oxidant-antioxidant balance, may be a primary cause of the recorded pathological alterations and testicular damage. Hence, NG could mitigate such adverse effects, seemly via its antioxidant properties.

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STATEMENTS & DECLARATIONS

Ethics approval and consent to participate:

Ethics approval and consent to participate this study was approved by the International Animal Care and Use Committee IACUC, Faculty of Veterinary Medicine, University of Sadat City (Approval No. VUSC-014-1-19).

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Consent to Publish:

All the authors have given their consent to publish this manuscript.

Competing Interest:

The authors declare no competing of interest.

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