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Zingiber officinale mitigates diazinon-induced testicular toxicity via suppression of oxidative stress and apoptosis in mice model

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ABSTRACT: Diazinon (DZN), as an organophosphate insecticide, induces testicular toxicity through oxidative stress. Ginger, a herbal medicine, has antioxidant and anti-inflammatory properties. This study was designed to investigate the effects of ginger against DZN-induced testicular toxicity. In this experimental study, thirty-two adult male mice were randomly divided into four groups. The control group; ginger group (100 mg/kg); DZN group (10 mg/kg); ginger + DZN group. Ginger and DZN received for 30 consecutive days by gavage, and DZN has been treated one hour after receiving ginger. Sperm parameters (including motility, sperm count, sperm viability rate and morphological sperm abnormalities), biochemical (MDA and GSH), testosterone levels, histopathological and immunohistochemical assays of testis were evaluated. The results revealed that treatment with DZN caused significant damage of sperm parameters (sperm motility, count, viability rate and abnormalities), increased oxidative stress (increased MDA and decreased GSH level), significant histopathological changes and decreased Johnsen's Score, testosterone level and increased caspase-3 immunoreactivity. Ginger preserved sperm parameters and mitigated the toxic effects of DZN. Also, treatment with ginger significantly reduced caspase-3 immunoreactivity. Our results concluded that the ginger with anti-apoptotic and antioxidant activity and with scavenging free radicals protect testis against DZN-induced toxicity.

KEYWORDS: Organophosphate insecticide; diazinon; ginger; oxidative stress; testicular toxicity; caspase-3.

1. INTRODUCTION

Diazinon (O, O-diethyl O - 2-isopropyl –6- methyl pyrimidinyl -4- g- 1- phosphorothioate, DZN , as one of organophosphate insecticides, is being used in different agricultural and gardening and could be highly toxic [1]. Contamination of food and water to DZN leads to abnormalities in the function of liver [2], kidney [3], brain [4], intestine [5], heart [6], ovary [7] and testis [8]. DZN decrease testosterone level, sperm count, motility and inhibit spermatogenesis and result in testicular atrophy [8-10]. Pathogenesis of tissue damage following DZN exposure is usually being attributed to oxidative injury [11]. Increasing oxidative stress through mitochondrial dysfunction and immunosuppression can lead to testicular injury and sperm abnormalities [12]. DZN with oxidative stress and generation of free radicals and increment of lipid peroxidation induce DNA fragmentation and apoptosis [5]. Also, Pesticides exposure decrease the endogenous antioxidant levels leading to cell death. So, use of exogenous antioxidants with scavenging ROS help in cell survival and longevity [13]. Organophosphate insecticides with phosphorylation of proteins induce toxicity. DZN, in the final stage of spermatogenesis with phosphorylation of sperm nuclear proteins (protamines), causes chromatin condensation and testicular toxicity [14, 15]. Previous researches demonstrated that administrations of antioxidants protect organs against oxidative stress-induced damage [10].

Ginger (Zingiber officinale Roscoe, *Zingiberaceae*), as a spice is being used all over the world. *Ginger* in traditional herbal medicine has been used for diseases such as common cold, digestive diseases, rheumatism,

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neuralgia, colic and motion sickness [16, 17]. Also, *ginger* is an attractive diet for the study of oxidative stress caused by exposure to insecticides [18]. The antioxidant property of *ginger* has been approved in *in vitro* [19] and *in vivo* [6] studies. Biological properties of *ginger* are anti-inflammatory and antioxidant properties [20]. Researchers recently reported that *ginger* protects hepatotoxicity [21], gastric ulcer [22] nephrotoxicity [23] and cardiotoxicity [24]. Studies have also shown that *ginger* improves testicular toxicity caused by cisplatin [25], cadmium [26], sodium arsenic [27] and gentamicin [28]. Protective effect of *ginger* on biochemical parameters has been confirmed against DZN-induced hepatotoxicity, testicular and heart toxicity [6, 29].

Hence, *ginger* has the potential that protects against DZN-induced testicular damage, and the best of our knowledge, no such study has been done on histopathology and immunoreactivity of caspase-3. This study aims to investigate the effects of *ginger* on testicular damage induced by DZN in mice by biochemical, histopathological and immunohistochemical assessment.

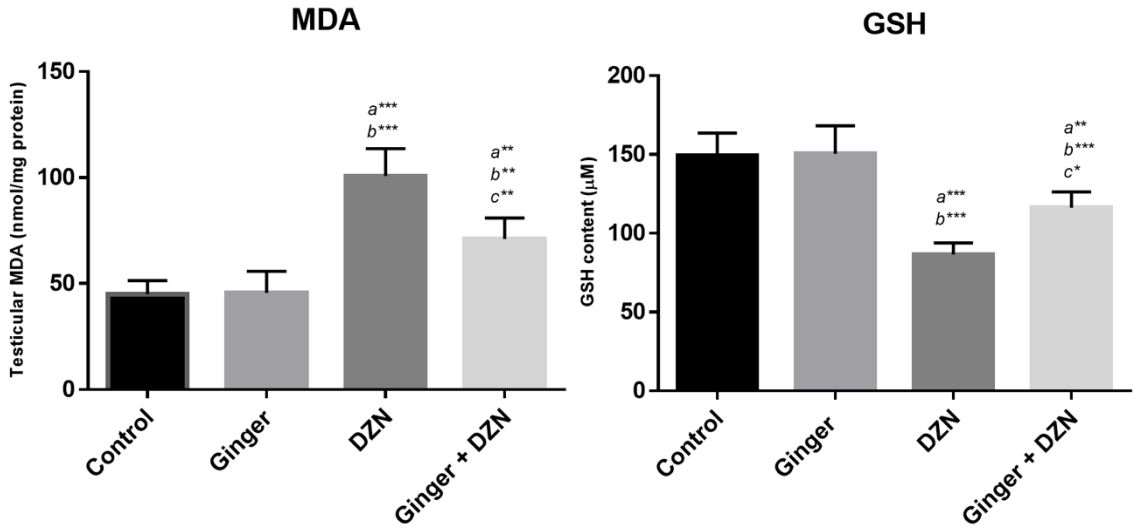
2. RESULTS

2.1. Total phenolic and total flavonoid contents

The total phenolic and total flavonoid contents of the extract were 237.9 ± 3 mg gallic acid equivalents and 101.33 ± 2.73 mg quercetin equivalents per gram dried extract, respectively.

2.2. Biochemical finding

The biochemical results are shown in Figure 1. The levels of MDA increased significantly in the DZN group that was comparable to control group ($p < 0.05$). Ginger treatment in DZN treated mice decreased significantly MDA compared with the DZN alone group ($p < 0.05$). GSH decreased significantly in the DZN group compared with the control group ($p < 0.05$). This antioxidant increased significantly in the ginger plus DZN group compared with the DZN alone group ($p < 0.05$). MDA and GSH levels were similar in control and ginger groups. Overall, DZN induced oxidative stress in testis and ginger decreased oxidative stress markers.



All values are expressed as mean ± SD. a significant vs. control, b significant vs. ginger and c significant vs. DZN groups. *; P<0.05, **; P<0.01, and ***; P<0.001. DZN; Diazinon, MDA; malondialdehyde, GSH; glutathione.

Figure 1. Histogram shows the levels of MDA and GSH.

2.3. Sperm parameters

The sperm parameters are presented in Table 1. In the ginger-treated group, sperm parameter findings were similar to control group. In the DZN-treated mice had significantly lower sperm counts, total epididymal sperm motility, cell viability rate and higher abnormal sperm morphology than the control group ($P < 0.05$; Table 1). The ginger plus DZN-treated mice had significantly higher total epididymal sperm motility and lower abnormal sperm morphology than the DZN-treated group ($P < 0.05$).

Table 1. Sperm characteristics, serum concentration of testosterone in different groups.

Groups	Control	Ginger (G)	Diazinon (DZN)	G + DZN
Sperm count (× 10^6)	4.66 ± 0.2	4.61 ± 0.37 $a^{***}b^{***}$	2.96 ± 0.27 c^{*}	3.96 ± 0.57
Sperm abnormality (%)	8.4 ± 2.51	8.83 ± 2.23 $a^{***}b^{***}$	30.86 ± 6.72 $a^{**}b^{**}c^{*}$	20.14 ± 1.68
Sperm viability (%)	90.2 ± 0.99	89.53 ± 1.3 $a^{***}b^{***}$	77.93 ± 2.98 c^{***}	86.12 ± 2.28
Sperm motility (%)	90.4 ± 5.27	89.43 ± 5.89 $a^{***}b^{***}$	54.71 ± 5.96 $a^{***}b^{***}c^{*}$	64.57 ± 6.6
Testosterone (nmol/L)	11.82 ± 1.51	11.98 ± 1.23 $a^{***}b^{***}$	4.17± 1.61 $a^{**}b^{**}$	7.44 ± 1.87

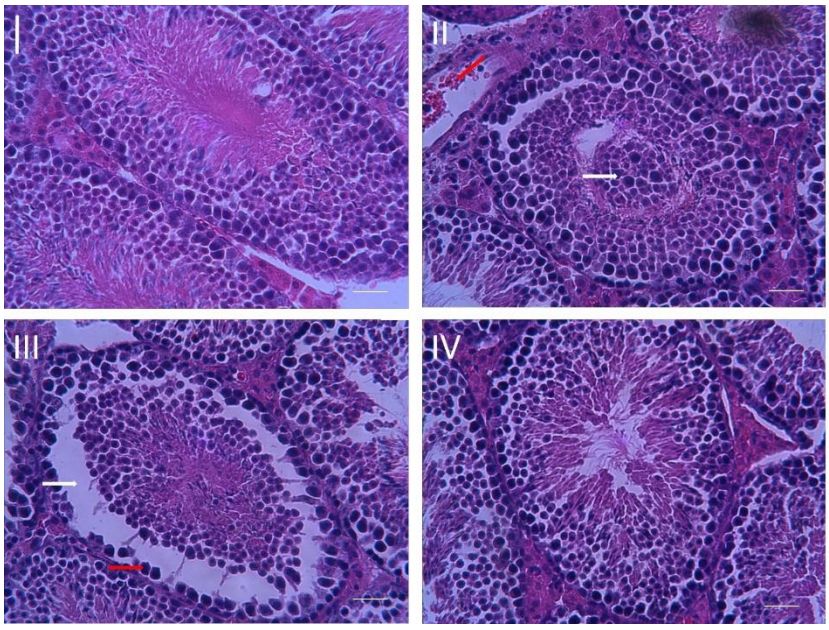
All values are expressed as mean ± SD. a significant vs. control, b significant vs. ginger and c significant vs. DZN groups. *; P<0.05, **; P<0.01 and ***; P<0.001.

2.4. Testosterone level

Serum testosterone levels are presented in Table 1. Serum testosterone levels were significantly decreased in DZN group compared with the control and ginger groups ($P < 0.05$). Ginger treatment in DZN treated mice increased testosterone levels compared to DZN group, but it was not significant.

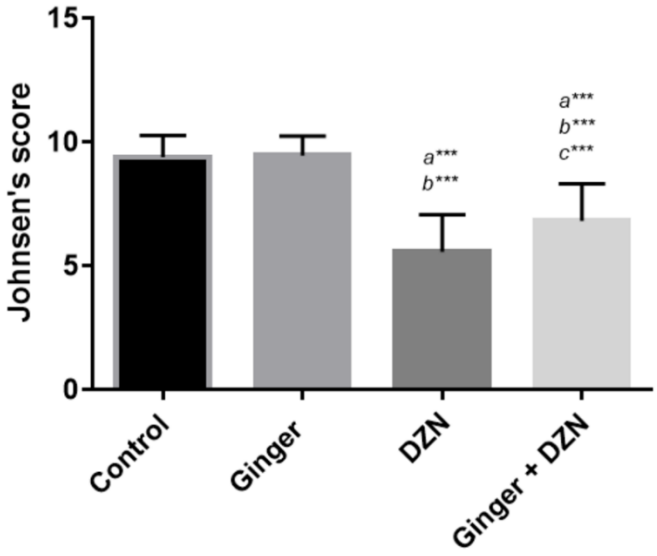
2.5. Histopathological findings

The photomicrographs of testes are shown in Figure 2. Normal spermatogenic cells, Sertoli and Leydig cells and precise spermatogenesis with abundant spermatids can be seen in the control group (Fig 2-I). Testis in ginger treated mice was similar to control group. DZN induced the impairment in the testes. In the DZN group, seminiferous tubules (ST) showed disruption of spermatogenesis with disorganization in the germinal epithelium layer and atrophy of ST, desquamations, necrosis and degeneration, diffuse interstitial oedema, desquamation and formation of giant cells by spermatocyte and spermatids and congestion (Fig 2-II and III). Ginger administration in DZN treated mice mitigated histopathological findings compared to DZN group (Fig 2-IV). Johnsen's mean scores of all groups were presented in Figure 3. DZN decreased testicular injury score. Johnsen's scores in DZN plus ginger group was higher compared to DZN alone ($P < 0.05$).



Control (I), DZN (II and III) and G + DZN (IV) groups. Normal structure in control group, disorganization, detachment (red asterisk-III), degenerative cells, giant cells formation (white arrow-II) condensation nuclei (red arrow-III), oedema (white asterisk-III), congestion (red arrow-II) and vacuolization (white arrow-III) in the seminiferous tubules in DZN group. Treatment with *ginger* ameliorates these changes. (H & E. Mag; ×40. Scale bar = 100 µm. G; *ginger*, DZN; Diazinon.

Figure 2. Photomicrographs showed histological structure of testis in the groups.



Data are presented as Mean ± SD. *a* significant vs. control, *b* significant vs. *ginger* and c significant vs. DZN groups. ***; *P*<0.001. DZN; Diazinon.

Figure 3. The histogram shows Johnsen's score of testicular tissue in the all groups.

Morphometric results in DZN treated group showed decreased seminiferous epithelial thickness and seminiferous tubules diameter in the majority of tubules as compared to control (P < 0.05). Ginger treatment increased the mean germinal epithelium thickness and tubular diameter in DZN treated mice. Reduction in thickness of the epithelium and diameter of seminiferous tubules were statistically significant compared to the DZN alone (P<0.05) (Table 2).

Table 2. Epithelial thickness (ET) and seminiferous tubules diameter (SD) in groups.

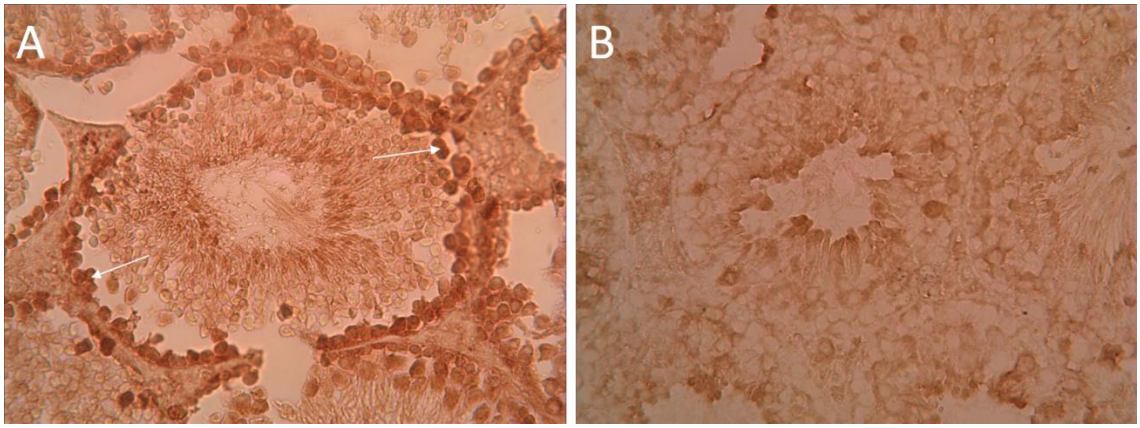
Groups	Control	Ginger	DZN	CP+NC
ET (µm)	65.49 ± 7.03	66.53 ± 6.58	30.52 ± 5.58 ^{a***b***}	59.51 ± 6.92 ^{a***b***c***}
SD (µm)	198.1 ± 14.82	195.4 ± 12.93	160.7 ± 13 ^{a***b***}	188.3 ± 10.99 ^{a***b*c***}

All values are expressed as mean ± SD. *a* significant vs. control, *b* significant vs. *ginger* and c significant vs. DZN groups. *; *P*<0.05, ***; *P*<0.001. ET; epithelium thickness, SD; seminiferous tubules diameter, DZN; Diazinon.

2.6. Immunohistochemical findings

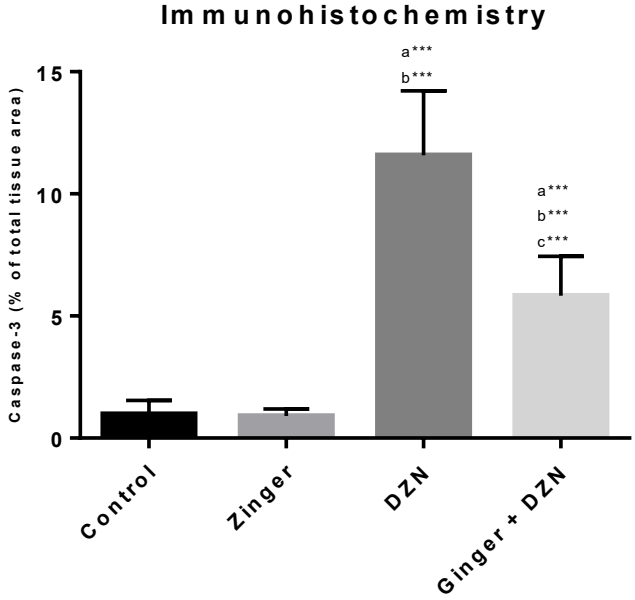
Immunohistochemical photomicrographs of the testes are shown in Figure 4. Section of testes in the control group showed no caspase-3 immunoreactivity. Expression of caspase-3 was similar in the ginger and control group. Increase immunoreactivity level of caspase-3 displayed in DZN-treated mice. Immunoreactivity staining mainly was localized in the spermatogonia cells (Fig 4.A) of the testis. Mild immunoreactivity staining of caspase-3 was shown in the spermatogonia cells (Fig 4.II) in ginger + DZN group compared to DZN alone group.

The histograms of the semi-quantitative analysis of caspase-3 staining in all groups are shown in Figure 5. The most immunoreactivity of caspase-3 was confirmed by semi-quantitative analysis in DZN treated mice compared with the other groups (p < 0.05). DZN administration decreased the severity of immunoreactivity of caspase-3. Level of caspase-3 in the control group was similar to the ginger group.



(A) Immunohistochemical staining showed the caspase-3 immunoreactivity in the DZN group that were remarkable in spermatogonia cells (arrow) and very weaker in spermatocyte cells. (B) *Ginger* treatment mitigated caspase-3 immunoreactivity in DZN-treated mice. DZN; Diazinon.

Figure 4. Immunohistochemical staining of caspase-3 in the groups.



Data are presented as Mean ± SD. *a* significant vs. control, *b* significant vs. *ginger* and c significant vs. DZN groups. ***; *P*<0.001. DZN; Diazinon.

Figure 5. The histogram shows densitometry analysis of immunohistochemical staining for caspase-3.

3. DISCUSSION

DZN, as an organophosphorus (OPs) insecticide, is one of the most widely used pesticides that can affect various organs [30]. In the present study, we showed pathological changes in mice testis that were exposed to DZN. These changes include decreasing of spermatogenesis, testosterone, apoptosis, sperm counts, motility, sperm viability rate and increased abnormal sperm morphology and apoptosis. Ginger improved the reduction in serum testosterone level, apoptosis, sperm count and abnormal sperm in DZN-treated animals. It also decreased lipid peroxidation and increased GSH level. Its protective mechanisms may be related to its anti-oxidative and anti-apoptotic properties of ginger.

OPs insecticides such as DZN induce cytotoxicity due to the generation of reactive oxygen species (ROS). ROS interrupts intracellular homeostasis. Glutathione (GSH), as an important antioxidant, is able to moderate ROS [31]. In this study, the biochemical assay confirmed the decrease in antioxidant enzyme like GSH and increase in MDA level in the testis, that showed oxidative stress which is consistent with previous study [11]. Antioxidants in the most studies are being used as pre-treatment, post-treatment or combined

treatment. We administrated ginger during the course of treatment, 1 hour before receiving of DZN. Ginger significantly decreased lipid peroxidation as a marker of oxidative stress and was comparable with DZN alone group. Previous studies showed that ginger efficiently scavenges ROS and inhibit the generation of oxidative injury in testis induced by malathion. This study is consistent with another study that showed ginger with free radicals scavenging can alleviate lipid peroxidation [32].

The herbal medicine due to being non-toxic and without serious side effects are an excellent candidate for reducing oxidative stress [33]. Ginger, because of its flavonoids compounds, such as gingerols, shogaols, some phenolic ketone derivatives, volatile oils, alkaloids, saponins, scavenge all kinds of free radicals and have antioxidant properties [33]. So, ginger inhibits oxidation process and prevents from free radicals formation. Previous studies have shown that this medicinal herb significantly mitigated malathion-induced lipid peroxidation and oxidative stress. Ginger with free radicals scavenging protects toxicity of organs induced by organophosphate toxins [13]. Hepatoprotective effect of ginger against DZN was confirmed in the previous study [2]. In this study, ginger extract in dose of the 100 mg/kg b.w. improved some biochemical and histological changes induced organophosphorus pesticide DZN. These findings conform to antioxidant property of ginger and are consistent with other studies [34]. The antioxidant activity of ginger is related to gingerol and other compounds [35]. This medicinal herb with maintaining the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, reduces lipid peroxidation [32]. Ginger with biological components efficiently protects lipid peroxidation [32]

Our histopathological findings revealed that sub-acute exposure to DZN induce histopathological changes in the seminiferous tubules, such as disorganization in the germinal epithelium layer of seminiferous tubules (ST), desquamations, vacuolization, and exfoliation of tubular epithelium, arrest of spermatogenesis activity, giant cells formation in the central lumen of the seminiferous tubules, necrosis and degeneration, diffuse interstitial oedema, congestion and destruction in Leydig cell. These may be because of DZN induces pathological changes in the Leydig cells and subsequent changes in the spermatogenic cell line. This study is in line with another study [36] and ginger was able to significantly mitigate DZN-induced testicular damage in mice. Previous studies proved significant protection of ginger against Cisplatin-induced hepatotoxicity and cardiotoxicity [24], diabetic-induced nephrotoxicity [37], against acetic acid-induced colitis [38] and cisplatin-induced nephrotoxicity [23].

DZN exposure also reduces sperm counts has been shown previously [39]. In this study, mice treated intraperitoneally for 30 days with DZN, exhibited a decrease in sperm counts. Similarly, these mice exhibited a significant decrease in sperm motility and increased abnormal morphology rates. It is likely that these effects of DZN and other Ops, cadmium, are related to their ability to cross the blood–testis barrier [39]. OPs by inducing oxidative stress, effect on activities of mitochondrial enzymes and disrupt the microtubules structure in the sperm. Impairment of mitochondrial function decreases sperm motility [40]. As well, OPs with DNA damage affect male reproductive function [41, 42]. DZN, induces oxidative stress and lipid peroxidation that damages the biological membranes in the testes [5]. In the present study, DZN caused reduction of diameter and weight of testes, sperm counts, degeneration of the spermatogenic and Leydig cells, Sertoli cells and interrupts spermatogenesis [9]. Also, sperm parameter examination of testes showed that DZN significantly decreases sperm counts and sperm motility, sperm viability rate and increased abnormal sperm morphology. These changes are related to oxidative stress and DNA damage that can alter sperm parameter [41].

OPs like DZN exert their detrimental effects through oxidative stress, oxidation of lipids, proteins and DNA in the testis [43]. Ahmed and et al reported that administration of ginger with biological activities improves sperm parameters [32]. In the present study, co-treatment of DZN with ginger mitigated the effects of DZN on sperm counts, motility and morphology.

OPs may also affect male reproductive function by blocking steroid hormone biosynthesis in the Leydig cells [44]. Testosterone is a key hormone that regulates spermatogenesis. Exposure to DZN significantly reduces LH, FSH and testosterone levels [9]. However, other researchers have found that DZN increase FSH and LH levels [45]. In our study, the testosterone level in the DZN-treated mice was significantly lower compared with control group. Sub-acute DZN suppressed testosterone secretion. However notably co-treatment of DZN with ginger had a protective effect on testosterone level.

The organophosphorus pesticides are highly toxic and alter sperm DNA structure and induce testicular toxicity [41]. DZN decreases the plasma acetylcholinesterase activity [46]. Also, elevated concentration of free oxygen radicals modify DNA and promote local apoptosis [10]. In this study, DZN exposure for 30 days was associated with increase in immunoreactivity level of caspase-3 compared with the control group. Ginger treatment decreased caspase-3 immunoreactivity level. In a previous study, it was shown that 6-gingerol and

6-paradol are the main polyphenol compounds of ginger, that have anti-tumoral and anti-proliferative effects [47].

4. CONCLUSION

Thus, in summary, our study concludes that sub-acute DZN exposure could induce histopathological changes, oxidative stress in the testis. Ginger extract could improve testicular toxicity with anti-oxidative activity and through free radicals scavenging. In addition, protective effect may be attributed to its anti-apoptotic property, that is mediated through mitigating immunoreactivity of caspase-3. The most important limitation of this study was an assessment of the anti-inflammatory property of this extract.

5. MATERIALS AND METHODS

5.1. Plant material and extraction

The roots of the ginger plant were prepared from the local market. The herbarium specimen of this plant is stored in the herbarium of the Faculty of Biology, Ghaemshahr Azad University. The roots were dried in shadows, then homogenous powder was macerated in 70% ethanol for 72 hours (1:5 w/v), after which the hydroalcoholic extract of dried ginger was processed by removing the solvent using a Rotary (Heidolph, Germany). For this purpose, 10 g of dry plant roots were mixed with 50 ml of solvent. After 24 hours, methanol was removed and the new solvent was added again. This action was repeated for three times till complete extraction. After which the hydroalcoholic extract of dried ginger was processed by removing the solvent using a Rotary evaporator (Heidolph, Germany). The concentrated extract was kept at 4° used for in vivo study.

5.2. Determination of total phenolic and flavonoid contents

The total phenolic content of the hydroalcoholic extract was determined by Folin Ciocalteu method [48]. The calibration curve was plotted using various concentrations of gallic acid. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of extract. The total flavonoid content was estimated using aluminium chloride colourimetric assay [49]. Quercetin was used to make calibration curve and flavonoid content was expressed as milligrams of quercetin equivalents per gram of extract.

5.3. Animals

Thirty-two adult male BALB/c mice (weighing approximately 25-30 g) obtained from the Animal Research Center of Mazandaran University of Medical Sciences, Sari, Iran. The animals were fed a standard laboratory diet and water ad libitum, kept in 12 h light/dark cycle, laboratory temperature of 20 ± 2 C. They were allowed one week to acclimate to the Experiment environment. All the experimental methods were manipulated by the Institutional Animal Ethics Committee of the Mazandaran University Medical Sciences ID: IR.MAZUMS.REC.1395.S222.

5.4. Study design

In the experiment group, the mice were randomly divided to 4 groups (8/group): the control group (C), the *ginger* group (G), the DZN-treated group (DZN), and the *ginger* plus DZN-treated group (G + DZN). *Ginger* was administered at a dose of 100 mg/kg per day via gavage. In DZN group, were given DZN at a dose of 10 mg/kg b.w. per day via gavage. In *ginger* plus DZN-treated group, the mice were received *ginger*, and 1 hour later, were received DZN. At the end of the 4th week (30 days), the mice were anaesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). Blood samples were collected for testosterone assay. Then, the mice were sacrificed and dissected, testis and epididymis samples were taken to assess the biochemical, histological and immunohistochemical evaluations of testis and epididymal parameter analysis.

5.5. Evaluation of sperm parameters

One epididymis of each animal immediately after dissection was placed in 1 ml of Dulbecco's Modified Eagle's medium (DMEM) pre-warmed With 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a petri dish and minced by anatomical scissors. Afterwards, the pieces of epididymis were incubated for 15 min to allow the migration of all spermatozoa into DMEM. Then epididymis tissues were removed and cell suspension obtained was used to assess sperm parameters.

For sperm count, One drop 20 µL of the sperm suspension was placed on Neubauer hemocytometer and was allowed to stand for 5 min then at a magnification of × 40 was counted and expressed as million sperm cells per ml of suspension.

For evaluation of the sperm motility, one drop of cell suspension was placed on a slide, covered by a coverslip and the percentage of sperm motility according to the type move was assessed routinely by counting 200 cells/slide [50], using light microscope at 200 magnification. The motility estimations were performed in three different fields for each sample, and average numbers were considered as the final motility score.

To determine the percentage of sperm morphology, 10 µL of the sperm suspension with 10 µL of Eosin mixed. After 1 min incubation, smear prepared with a drop of 12 µL onto a glass slide. After drying, sperm morphology checked. Abnormal shape of the head and tail of sperm analyzed in the prepared slides and mean data were recorded.

To assess sperm viability 20 µL of sperm suspensions with 20 µL of 1 % Eosin-Y were mixed and after 3 to 4 min, 200 sperm of stained and unstained cells per slide [51] were counted for viability by using haemocytometer with ×40 magnification inverted microscope. Each sample was measured at least three times.

5.6. Biochemical analysis

5.6.1. Evaluation of lipid peroxidation

The testicular lipid peroxidation was measured by MDA level using the thiobarbituric acid with a spectrophotometric assay. To begin the analysis, 0.25 mL phosphoric acid (0.05 M) was mixed with the 0.2 mL of sample and then 0.3 mL of 0.2% thiobarbituric acid (TBA) was added. Samples were kept in a boiling water bath for 30 min. The sample tubes were placed to the ice-bath and then 0.4 mL of n-butanol was added to each sample. Then were centrifuged (3500 rpm) for 10 min and MDA was measured based on reacts with thiobarbituric acid (an MDA-TBA complex). Created MDA in each sample was calculated in the supernatant at 532 nm with ELISA reader (Tecan, Rainbow Thermo, Austria). MDA content was expressed as nmol/mg protein. Tetramethoxypropane (TEP) was used in this experiment as standard [52].

5.6.2. Measurement of glutathione (GSH) content

The content of the glutathione in the testis tissue was measured by a spectrophotometer (UV-1601 PC, Shimadzu, Japan). In brief, 0.5 ml sample or standard solution with 0.25 ml of 1 M sodium phosphate buffer (pH 6.8) and 0.5 ml 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as an indicator was mixed. After 5 min, the absorbance was measured at 412 nm and expressed as µM [53].

5.7. Testesterone analysis

Levels of testosterone in the serum were determined according to manufacturer's instructions (Mouse Testosterone ELISA Kit, Bioassay, Cat. No. E0260MO). Testosterone amount in the samples was calculated from testosterone standard curves using a spectrophotometer and expressed as nmol/L. All samples were carried in duplicate.

5.8. Histopathological examinations

For histopathological examination and to determine the effect of DZN exposure on the testis, samples were fixed in 10% buffer formalin, processed and embedded in paraffin. Sections of 5 µM thickness stained with hematoxylin and eosin (H & E) and were investigated for light microscopic examination (Olympus, Japan). Histopathological findings were investigated with Johnsen scoring system [54]. Five sections per animal and 10 seminiferous tubules per section were assessed using a score of 1–10 under ×40 magnification.

For quantitative evaluation, the average diameter of seminiferous tubule (ST) and thickness of the germinal epithelium of the ST (from the basement membrane to lumen) in 10 tubule per testicular section and 10 section per groups were measured at ×40 magnifications by using calibrated OLYSIA Soft Imaging System GmbH, version 3.2 (Japan) [55]. All specimens were evaluated by a histologist as blind.

5.9. Immunohistochemical assay

The immunohistochemical technique was performed according to the instructions kit manufacturer (Abcam Company, USA). After deparaffinization and rehydration, endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol for 15 minutes. Then, tissue sections were incubated at 4°C overnight with primary antibodies (anti-caspase 3 rabbit polyclonal antibody, 1:100 in PBS, v/v, Abcam, Lat: GR224831-2). After incubated with secondary antibody conjugated with horseradish peroxidase (Mouse and Rabbits

Specific HRP/DAB, Abcam, Lat: GR2623314-4) for 10 minutes, sections were incubated with diaminobenzidine tetrahydrochloride for 5 minutes [56]. Then, the samples were dehydrated and mounted. The primary antibody was omitted for negative controls. For the quantitative analysis, immunohistochemical photomicrographs were assessed using MacBiophotonics ImageJ 1.41a software by densitometry method. The positive staining severity was assessed as the ratio of the stained area to the entire field assessment.

5.10. Statistical analysis

Statistical analysis for the obtained data was performed using version 19 SPSS (Chicago, USA). The normality of data was evaluated using the K-S (Kolmogorov-Smirnov) test, and then results with normally distributed was checked by One-Way ANOVA and followed by Tukey's procedure and are presented as the mean ± standard deviation (M±SD). Statistically significant differences were accepted as $p < 0.05$.

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Conflict of interest statement: There is no conflict of interest in this study and publication.

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