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Ginger and Atorvastatin Protect Against Diazinon-Induced Testicular Damage in Rats Through Regulation of Oxidative Stress, Inflammation, and Apoptosis

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Abstract

DIAZINON (DZ), an organophosphorus pesticide that is commonly used in agriculture, has been associated with testicular toxicity. This study explored the protective effects of ginger extract (GE) and atorvastatin (ATR) on DZ-induced testicular damage in rats. 49 male rats were divided into 4 main groups initially. In the first 3 groups (n=7/each), rats received either saline (control group) or GE or ATR daily while the rats in the fourth group (n=28) were orally gavaged with diazinon (DZ) (Intoxicated groups). The rats in the fourth group were further subdivided into 4 equal groups (n=7/each) where saline or GE or ATR or both GE and ATR were administered for 30 days. The rats were sacrificed after 30 days of treatment, and serum samples and tissues were collected for analysis. In DZ intoxicated rats, there was a significant decrease in testosterone, FSH, and LH levels. In addition, testicular malondialdehyde (MDA) levels was increased, while glutathione (GSH) and catalase (CAT) levels were decreased. DZ also induced degeneration and necrosis in seminiferous tubules with marked increase in caspase-3 expression in cytoplasm. However, the administration of both GE and ATR in intoxicated rats alleviated DZ induced oxidative changes and reduced testicular degeneration more effectively than using either one alone. GE and ATR provided a synergistic protective effect against testicular damage induced by DZ in rats, suggesting their potential as a therapeutic strategy for combating organophosphorus poisoning. This protective effect could be attributed to the reduction of lipid peroxidation and the mitigation of damage caused by oxidative stress.

Keywords: Oxidation; Inflammation; Testicle; Organophosphorus; Atorvastatin, Ginger, Diazinon.

Introduction

Pesticides are widely used in agriculture and veterinary services, and they pose a threat on public health. Their misuse could lead to severe environmental pollution and major health hazards for humans. Organophosphorus pesticides (OPs) are among the largest and most toxic chemical pesticide groups affecting both animals and humans [1, 2]. While pesticides could enhance human and animal nutrition by increasing the availability and shelf life of food and reducing costs, their usage often results in toxicity to both humans and animals, particularly through accidental occupational exposure [3].

Diazinon (DZ), a type of organophosphorus pesticide, is widely used as an antiparasitic agent in veterinary medicine to control external parasites such as mites and ticks. It is also commonly used in agriculture to manage insect infestations [4]. DZ can enter the body through direct contact, ingestion, or inhalation and is primarily processed and eliminated by the liver and kidneys [5]. The toxicity of DZ is due to its ability to generate free radicals and reactive oxygen species, leading to oxidative stress and lipid peroxidation in various mammalian tissues. It has been shown that DZ triggers inflammation in nervous and hepatic tissues by stimulating the production of tumor necrosis factor-alpha (TNF-α) [6, 7]. DZ also

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induces DNA fragmentation in cells, leading to genotoxicity and apoptosis [8]. The lipophilic nature of DZ enables it to interact with the phospholipid bilayer of cell membranes in many visceral organs [9]. The liver and kidneys are typically the most affected organs by DZ toxicity, which can also damage reproductive systems and cause both hematological and biochemical alterations [10]. Recent studies have highlighted the effectiveness of natural products in reducing the side effects of xenobiotics in complementary and alternative medicine [11-15]. This has led to increased interest in using these natural products and medicinal plants to discover new pharmacologically active compounds [16, 17]. Spices contain phytochemicals that are beneficial for health and provide protection against various chronic diseases [18]. Ginger, or Zingiber officinale, is a widely used spice globally and is recognized in traditional medicine for its therapeutic benefits in treating diseases like cardiovascular disease, diabetes, ulcers, Alzheimer's, depression, and cancer [9, 19-22]. The ginger rhizome is packed with numerous bioactive substances, including volatile oils, oleoresin, gingerols, and their derivatives. It also contains a variety of other compounds such as acid resins, vitamins (vit B3, B6 and C, folic acid, inositol, choline, and pantothenic acid), gingerol, sesquiterpenes, volatile oils, and trace elements like calcium, magnesium, phosphorus, and potassium [23, 24]. Ginger root enhances testosterone production through its antioxidant properties and boosts the activity of antioxidant enzymes, thus protecting reproductive organs from oxidative stress and lipid peroxidation [25].

Atorvastatin (ATR) is recognized for its efficacy in treating hypercholesterolemia, along with its antioxidant and anti-inflammatory properties [26]. The effectiveness of this potent synthetic statin in reducing cholesterol synthesis is mainly attributed to its ability to inhibit the enzyme 3-Hydroxy 3-Methyl Glutaryl Coenzyme A (HMG-CoA) reductase and to increase the uptake of hepatic Low-Density Lipoprotein (LDL) through its receptors [27, 28]. At low doses, ATR has beneficial pleiotropic effects, functioning as an antioxidant and reducing inflammation [29]. However, higher doses of atorvastatin have been associated with several adverse effects, including nephrotoxicity and testicular damage [30]. The current study aimed to investigate whether ginger (GE) and/or atorvastatin (ATR) could mitigate the testicular damage caused by diazinon (DZ) toxicity in rats.

Material and Methods

Chemicals

Diazinon-60® was purchased from Drug Pharmaceuticals (EPC, Cairo, Egypt). GE was obtained in 400 mg tablet form from MEPACO Company (Ismailia, Egypt) and ATR was purchased in 40 mg tablets from Delta Pharma Company (Cairo, Egypt). These tablets were grounded using a blender and dissolved in water before use. The dose was calculated based on the BW of rats.

Experimental design

Forty-nine apparently healthy Wister Albino male rats (4-6 weeks old), weighing between 185 and 200 g, were purchased from the Egyptian Organization for Biological Products and Vaccines. They had 7 days acclimatization period in a controlled environment at 25°C with a 12:12 h light/dark cycle, with unrestricted access to water and commercial feed. After acclimation period, the rats were subdivided into four main groups. Group 1 rats (n=7) were given saline (5 ml/kg orally); Group 2 rats (n=7) were administered GE (100 mg/kg/day)[15]; Group 3 rats (n=7) received ATR (20 mg/kg/day)[15]; remaining rats (n=28) were orally gavaged with diazinon (DZ) (20 mg/kg/day)[15] to induce toxicity (DZ intoxicated rats). These rats were further subdivided into 4 Groups (n=7/group) where rats received either saline (Group 4) or GE (group 5) or ATR (Group 6) or both GE and ATR (Group 7). All treatments were administered orally (via gavage) once daily for 30 days. The experimental protocol was approved by the Institutional Ethical Committee of the Faculty of Veterinary Medicine, Benha University (Approval No BUFVTM 06-08-22).

Blood sampling and tissue harvesting

Following anesthesia of rats with isoflurane (2.5%), blood was drawn from the retro-orbital plexus a day after the last dose of treatment with GE, ATR or both in the experiment using a capillary tube. The serum was separated by centrifugation of the blood at 1200 g for 15 minutes and stored at -20°C for further biochemical analyses. Rats were sacrificed by cervical dislocation and testicular tissues were rapidly excised, rinsed in saline, homogenized in phosphate buffer (pH 7.4), and centrifuged at 1200 x g for 20 minutes at 4°C. The supernatants were stored at -20°C for oxidative stress assessment. For histological and immunohistochemical analysis, sections of the tissue were preserved in formalin.

Hormone level analysis

Testosterone, FSH, and LH levels were measured using specific ELISA kits following the manufacturers' instructions [31].

Oxidative stress markers

The serum levels of malondialdehyde (MDA), and activities of catalase (CAT) and glutathione reductase (GSH) were measured based on previous studies [32, 33].

Histopathology and Immunohistochemistry

Testicles were fixed in 10% buffered neutral formalin, embedded in paraffin, and sectioned at 4 µm. The sections were stained with Hematoxylin and Eosin for histological examination after deparaffinization. The stained sections were mounted and histologically examined. Regarding immunohistochemistry steps, tissue sections were heated at 60°C for 1 hr, followed by deparaffinization, rehydration, and antigen retrieval in a 10 mM citrate buffer (pH 6.0) using a steamer for 50 minutes, with a subsequent gradual cooling period. The slides were then treated with 3% hydrogen peroxide to remove endogenous peroxidase activity, rinsed, and blocked with goat serum. The tissue sections were incubated overnight at 4°C with either rabbit anti-cleaved caspase-3 (Asp175; 1:100; Cell Signaling Technology, Danvers, MA, USA) or anti-Ki67 (Ab16667; 1:200; Abcam, Cambridge, UK) or anti-TNFa (Ab6671; 1:100; Abcam, Cambridge, UK). On the following day, biotinylated secondary antibodies (Goat Anti-Rabbit IgG H&L horseradish peroxidase [HRP], Abcam, USA) were applied for 30 minutes, incubated with diaminobenzidine (DAB) for 3 minutes and counterstained with hematoxylin. Sections were subjected to dehydration steps using a series of ethanol and xylene before being mounted. Positive staining was identified by brown cytoplasmic coloration (DAB). Quantitative evaluation of DAB positive cells was performed using Image Pro Plus 7.0 software (Media Cybernetics, Silver Spring, MD, USA)

Statistical analysis

Data were presented as mean ± SE. Statistical analysis was performed using Graph Pad Prism. One-way ANOVA and Duncan's post hoc test were employed for comparisons among multiple groups, with significance set at $P<0.05$.

Results

Effect on Serum Sex Hormone Levels

There was no change in the level of serum sex hormones (testosterone, FSH and LH) in control, GE and ATR groups. In DZ intoxicated rats, there was a marked reduction in the levels of testosterone, FSH, and LH compared to control rats. Treatment of DZ intoxicated rats with both GE and ATR upregulated these altered hormone levels, although they remained lower than those of the control group. This finding indicated that the combination of GE and ATR provided better protection against Diazinon-induced reproductive damage than either agent alone (Figure 1).

Effect on Oxidative Damage Parameters

No significant changes in the level of oxidative damage parameters (MDA, GSH and CAT) were noticed in control, GE and ATR groups. There was a marked increase in malondialdehyde (MDA) level and a significant reduction in glutathione (GSH) and catalase (CAT) levels in the testicular tissues of DZ intoxicated rats. Treatment of DZ intoxicated rats with either GE or ATR alone showed significant mitigation of the adverse effects of DZ on testicular MDA, CAT, and GSH levels, although differences from control levels persisted. Reduction in the level of MDA with upregulation in the levels of GSH and CAT was reported in GE and ATR co-treated DZ intoxicated groups. DZ intoxicated rats co-treated with both GE, and ATR showed improved outcomes in their testicular oxidative damage compared to DZ intoxicated groups treated with GE or ATR alone (Fig. 2).

Histopathology

No significant microscopic alterations were observed in the seminiferous tubules or Leydig cells of the control, GE, and ATR groups (Fig. 3A-C). In contrast, DZ intoxicated rats showed marked testicular tissue damage, characterized by a reduced number of spermatogenic cell layers and the presence of degenerated or necrotic cells within the seminiferous tubules (Fig. 3D). Additionally, DZ-intoxicated rats exhibited moderate to severe vascular congestion, intertubular edema, and necrosis in some Leydig cells (Fig. 3E). Co-treatment with GE, ATR, or a combination of both resulted in minimal degenerative changes in the seminiferous tubules of DZ-exposed rats, compared to those treated only with DZ (Fig. 3F-I). DZ-intoxicated rats treated with GE showed only mild degenerative changes, in form of cytoplasmic vacuolation in a few seminiferous tubules, while the majority of seminiferous tubules appeared nearly normal without significant microscopic abnormalities (Fig. 3F). Additionally, some degenerated tubules in this group displayed spermatogenic cells with a higher mitotic index (Fig. 3G). In rats co-treated with ATR, mild vacuolation was noted in the cytoplasm of spermatogenic cells in a few rats, with no evidence of degeneration in the rest (Fig. 3H). Most testes in this group also showed mild interstitial oedema. Finally, in DZ-intoxicated rats co-treated with both ATR and GE, there were no significant microscopic changes detected in the seminiferous tubules or Leydig cells in most specimens (Fig. 3I).

Immunohistochemistry

In control rats and those treated with GE or ATR, there was negligible to null cleaved caspase-3 staining in the spermatogonia and spermatocytes of the seminiferous tubules and interstitial Leydig cells (Fig. 4A-C). Diazinon-treated rats exhibited moderate to strong caspase-3 staining in the spermatogonia and interstitial cells across several testicular sections (Fig. 4D-E). Rats treated with Diazinon and co-treated with either GE, ATR, or both showed reduced caspase-3 staining in their

spermatogenic cells within the seminiferous tubules (Fig. 4 F-H).

Caspase-3 positivity was significantly low in the control, GE, and ATR-treated rats compared to those exposed to Diazinon, with a notable decrease in caspase-3 expression observed in DZ-intoxicated rats that received co-treatment with GE, ATR, or both (Fig. 5A). Additionally, DZ-intoxicated rats exhibited higher levels of TNF-α compared to the control, GE, and ATR groups, but co-treatment with GE, ATR, or both resulted in a reduction in TNF-α levels (Fig. 5B). The control, GE, and ATR-treated rats displayed high levels of Ki67 expression, while DZ-intoxicated rats showed significantly lower Ki67 expression. However, co-treatment with GE, ATR, or both restored Ki67 expression levels in DZ-intoxicated rats (Fig. 5C).

Discussion

In mammals, reactive oxygen species (ROS) are generated due to exposure to xenobiotics and metabolic byproducts. ROS induces severe damage in cell structures when their production exceeds the body's ability to neutralize them [34]. Diazinon (DZ), a commonly used organophosphorus insecticide (OPI) in both veterinary and agricultural industries, poses significant health risks, primarily due to ROS-induced damage. Studies have shown that this oxidative stress can potentially be moderated by reducing the overall level of oxidative stress in the body [35, 36]. In this study, we explored how natural compounds such as ginger (GE) and synthetic compounds as ATR could mitigate the harmful effects of DZ, especially on testicular tissues.

DZ exposure is known to impair male reproductive health by reducing spermatogenesis and androgen levels [37]. Our study recorded similar findings reflected by a significant decrease in testosterone in the DZ-treated rats and this could be attributed to the damage to testicular cells and the reduction in spermatogonia vital for sperm production. Our findings are consistent with a prior study showing similar oxidative stress responses in DZ-exposed rats, including increased levels of malondialdehyde (MDA) and decreased activities of antioxidants such as superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) [5]. Previous studies have shown that DZ intoxication induces damage in seminiferous tubules and decrease in spermatogenesis, potentially due to elevated levels of luteinizing hormone (LH), which can degrade Sertoli cells and germinal cells [4, 38]. In our study, DZ intoxicated rats showed elevated LH and follicle-stimulating hormone (FSH) levels, indicative of reproductive toxicity. Histopathologically, DZ intoxication was accompanied with significant necrosis and degeneration within the testes, affecting both the structure and function of germinal epithelium and interstitial tissues.

Interestingly, co-treatment of DZ intoxicated rats with GE or ATR improved testicular function, suggesting their potential in protecting cell membranes, and aiding in the regeneration of damaged cells. In our study, GE decreased the oxidative damage markers in testicular tissues and increased the levels of reproductive hormones and improved the histological picture of testicles in the DZ intoxicated rats. The protective potential of ginger against such toxic effects is attributed to its rich composition of bioactive compounds such as gingerols, shogaols, and zingerone. These molecules exhibit significant free radical scavenging activity, which may mitigate the oxidative damage prompted by diazinon exposure [39]. Specifically, gingerols have been shown to enhance the antioxidant defences by upregulating the activity of key enzymatic antioxidants like superoxide dismutase and glutathione peroxidase, thus providing a biochemical barrier against ROS [40]. In addition to its antioxidant activity, ginger's anti-inflammatory effects are mediated through the inhibition of NF-kB signaling pathways, which are typically activated in response to oxidative stress and can lead to inflammation and cellular damage in testicular tissue [41]. By blocking NF-kB signaling pathway, ginger could potentially reduce the synthesis of inflammatory cytokines and attenuate the inflammatory response induced by diazinon. Furthermore, the anti-apoptotic properties of ginger involve the modulation of key regulators of apoptosis, such as Bcl-2 and caspase pathways. This modulation can prevent the apoptosis of spermatogenic cells, maintaining their function and viability even under the stress of pesticide exposure [20].

In this study, atorvastatin has shown effectiveness in reducing oxidative stress and improvement in the level of sex hormones and testicular histological pictures. It has been shown that atorvastatin can significantly enhance the enzymatic and non-enzymatic antioxidant defence systems in the testis [42]. This includes upregulation of catalase and superoxide dismutase activities, which directly neutralize the harmful effects of ROS, thereby mitigating oxidative stress [43]. Furthermore, atorvastatin's ability to inhibit the biochemical pathway leading to cholesterol synthesis may also contribute to reduced lipid peroxidation levels, a key factor in protecting cell membrane integrity against oxidative attacks. This lipid modulation can help stabilize cell membranes and prevent the apoptosis of spermatogenic cells [42]. Additionally, ATR has been shown to down-regulate the expression of inflammatory cytokines through the inhibition of the NF-kB pathway, further reducing the inflammatory response within the testis that is often triggered by pesticide exposure [44].

Furthermore, the elevated TNF-α levels seen in DZ-intoxicated rats compared to controls reflect an inflammatory response triggered by Diazinon exposure. TNF-α is a key pro-inflammatory cytokine, and its increased expression is consistent with findings of pesticide-induced inflammation [45]. The ability of GE and ATR to reduce TNF-α levels suggests their role in counteracting inflammatory responses. Previous studies have reported that ginger possesses anti-inflammatory properties by inhibiting pro-inflammatory cytokines like TNF-α and IL-6 [46], while atorvastatin has been shown to attenuate inflammation by modulating cytokine production and reducing oxidative stress [47]. Ki67 is a marker for cell proliferation, and the significant reduction in its expression in DZ-treated rats indicates impaired spermatogenesis. This reduction is consistent with findings that Diazinon negatively affects testicular tissue, leading to a decline in spermatogenic cell proliferation [48]. However, the restoration of Ki67 expression in DZ-intoxicated rats co-treated with GE, ATR, or both highlights their potential in supporting recovery of spermatogenic activity. This recovery may be attributed to their ability to reduce oxidative stress and inflammation, creating a more favorable environment for normal cell division and proliferation.

Our results suggest that GE and ATR could help alleviating the oxidative stress and reproductive harm caused by exposure to toxic chemicals like DZ. The study demonstrated that the combination of GE and ATR provides a synergistic protective effect on testicular tissues in rats, primarily by reducing lipid peroxidation, inflammation and damage induced by oxidative stress and promoting the proliferation of testicular cells.

Conclusion

In conclusion, these findings suggest that ginger and atorvastatin could be promising adjuvant therapeutic agents against Diazinon-induced testicular damage. Their combined effects in reducing apoptosis, minimizing inflammation, and enhancing cell proliferation point to their potential utility in treating pesticide-related reproductive toxicity. GE and ATR combination counteract DZ-induced apoptosis and oxidative stress more effectively than single agent alone. These findings support the potential of integrating natural and synthetic therapies to safeguard reproductive health against chemical insults, illustrating a promising avenue for future therapeutic strategies against environmental toxicants. alone. These findings support the potential of integrating natural and synthetic therapies to safeguard reproductive health against chemical insults, illustrating a promising avenue for future therapeutic strategies against environmental toxicants.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Faculty of Veterinary Medicine, Benha University, Egypt (Approval No BUFVTM 06-08-22).

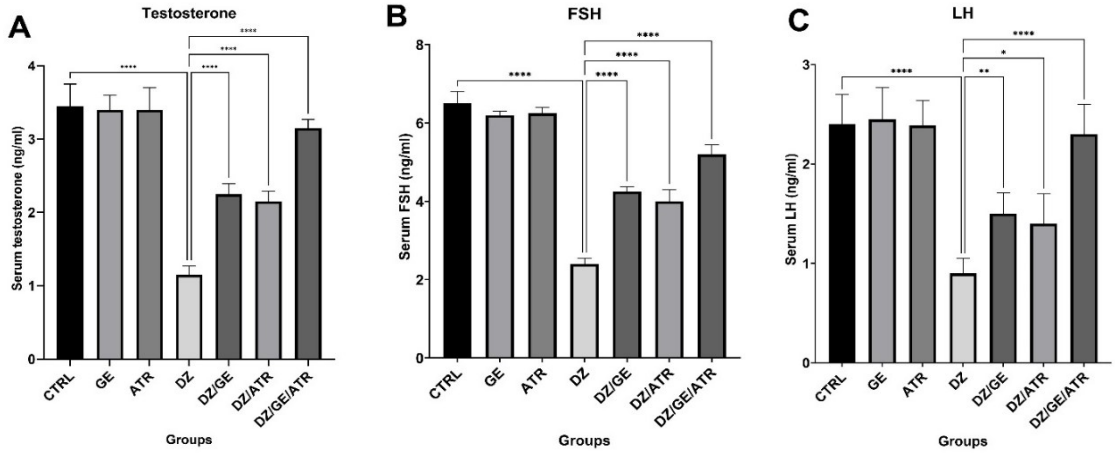

Fig. 1. The effects of GE, ATR and DZ alone or in combination, on serum sex hormone levels in rats (n=7/group). The statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ****p < 0.0001.

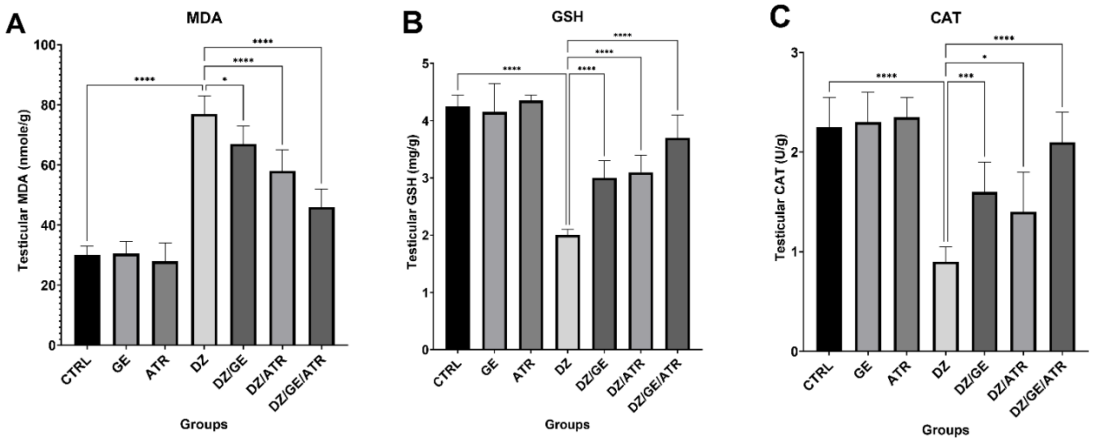

Fig. 2. The effects of GE, ATR and DZ alone or in combination, on testicular oxidative stress markers in rats treated with DZ. Statistical significance is indicated as follows: *p < 0.05, ***p < 0.001, ****p < 0.0001.

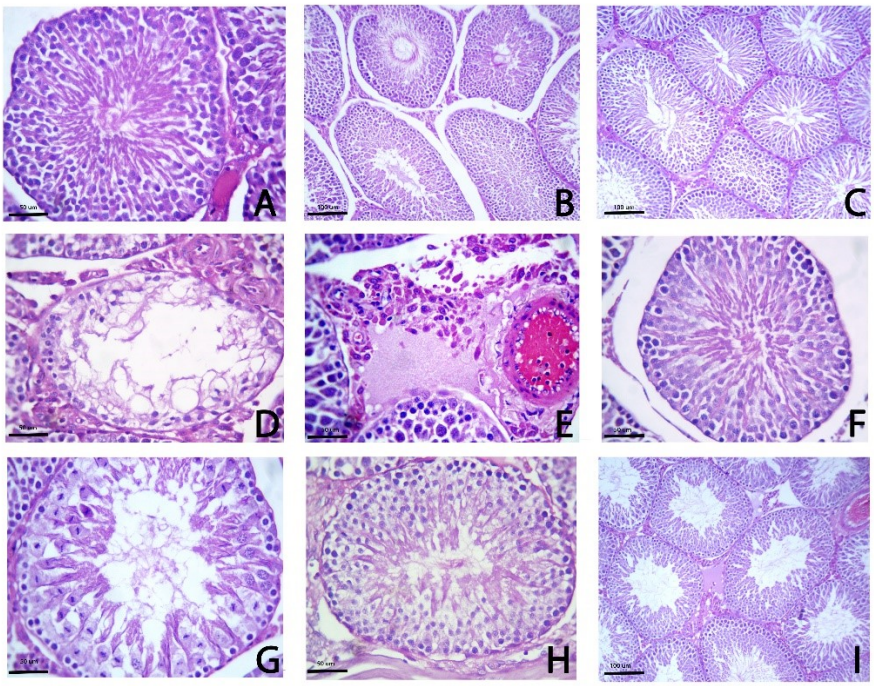

Fig. 3. Histopathological examination of rat testicular tissue stained with hematoxylin and eosin (H&E). Normal architecture with well-structured seminiferous tubules and typical spermatogenesis is observed in control (A), GE (B), and ATR (C) groups. (D) Severe microscopic alterations are evident in DZ-treated rats, including vacuolation, necrotic spermatogenic cells, and (E) interstitial edema with necrotic Leydig cells. (F-G) DZ administered rats co-treated with GE show minimal degeneration and a higher mitotic index. (H) Mild vacuolation is present in DZ rats co-treated with ATR. (I) Rats receiving both GE and ATR display organized spermatogenic layers with mild interstitial edema. (A, D-H X20; B, C, I X10)

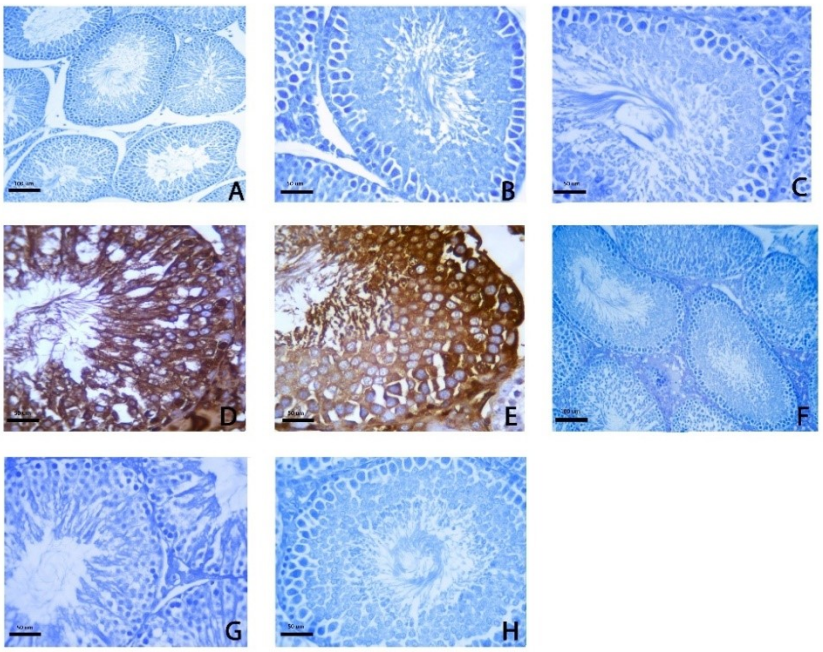

Fig. 4. Cleaved caspase-3 immunostaining in testicular sections of different treatment groups. (A-C) Minimal to no cleaved caspase-3 staining observed in control (CTRL), ginger extract (GE), and atorvastatin (ATR) treated rats. (D-E) Strong positive staining in Diazinon (DZ) treated rats, showing increased apoptosis across the seminiferous tubules. (F-H) Reduced caspase-3 staining in DZ-exposed rats co-treated with GE (F), ATR (G), and the combination of both (H). (A, F X10; B-E, G, H X20)

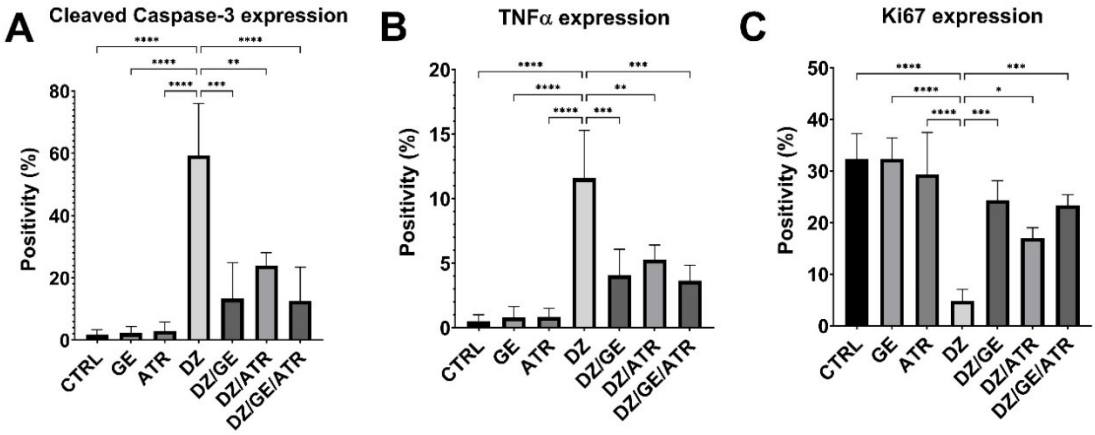

Fig. 5. Expression levels of cleaved caspase-3 (A), TNF-α (B), and Ki67 (C) in testicular tissues across different treatment groups. Statistical significance is denoted by asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

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الزنجبيل وأتورفاستاتين يحميان من تلف الخصية الناجم عن الديازينون في الفئران

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الملخص

لقد ارتبط الديازينون (DZ) وهو مبيد آفات فوسفوري عضوي شائع الاستخدام في الزراعة بسمية الخصية. لقد استكشفت هذه الدراسة الآثار الوقائية لمستخلص الزنجبيل (GE) وأتورفاستاتين (ATR) على تلف الخصية الناجم عن تسمم ال DZ في الفئران. لقد تم تقسيم 49 فأرا من الذكور إلى 4 مجموعات رئيسية في البداية. تلقت الفئران إما محلول ملحي (المجموعة الضابطة) أو GE أو ATR يوميا في أول 3 مجموعات (العدد = 7 فئران/ بكل مجموعه) بينما تم حقن الفئران في المجموعة الرابعة (العدد = 28) عن طريق الفم بالديازينون (DZ). لقد تم تقسيم الفئران في المجموعة الرابعة إلى 4 مجموعات متساوية (العدد = 7 / لكل منهما) حيث تم إعطاء محلول ملحي أو GE أو ATR أو كلاهما لمدة 30 يوما. تم ذبح الفئران بعد 30 يوما من بدايه العلاج وتم جمع عينات الدم والأنسجة لتحليلها. كان هناك انخفاض كبير في مستويات هرمون التستوستيرون ، FSH ، و LH في الفئران المسممه ب DZ ، بالإضافة إلى ذلك ، تمت ملاحظه زيادة في مستويات malondialdehyde (MDA) بالخصية ، بينما انخفضت مستويات الجلوتاثيون (GSH) والكاتليز (CAT). لقد تسبب ال DZ أيضا في التنكس والنخر في الأنابيب المنوية مع زيادة ملحوظة في تعبير caspase-3 في السيتوبلازم. ومع ذلك ، فإن إعطاء كل من GE و ATR في الفئران المسممه ب DZ قد خفف من التغيرات المؤكسدة التي يسببها ال DZ وقلل من تنكس الخصية بشكل أكثر فعالية من استخدام أي منهما بمفرده. لقد قدمت GE و ATR تأثيرا وقائيا تآزريا ضد تلف الخصية الناجم عن ال DZ في الفئران ، مما يشير إلى إمكاناتها كاستراتيجية علاجية لمكافحة التسمم بالفوسفور العضوي. من المرجح أن يعزى هذا التأثير الوقائي من GE , ATR من الحد من بيروكسيد الدهون وتخفيف الأضرار الناجمة عن الإجهاد التأكسدي.

الكلمات الدالة: أكسدة ، التهاب ، الخصية ، الفوسفور العضوي ، أتورفاستاتين ، زنجبيل ، ديازينون