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Lycopene treatment prevents hematological, reproductive and histopathological damage induced by acute zearalenone administration in male Swiss mice

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ABSTRACT

Zearalenone (ZEA) is a mycotoxin commonly found as a contaminant in cereals. ZEA toxicity targets mainly the reproductive system, and oxidative stress plays an etiological role in its toxic effects. Therefore, the present study aimed to investigate the effect of lycopene, a potent carotenoid antioxidant, on markers of oxidative stress in liver, kidney and testes, and on reproductive, hematological and histopathological parameters after ZEA administration. Adult Swiss albino male mice received lycopene (20 mg/kg, p.o.) for ten days before a single oral administration of ZEA (40 mg/kg, p.o.), and 48 h thereafter tissues (liver, kidney, testes and blood) were collected for biochemical, hematological and histological analyses. Lycopene prevented ZEA-induced changes in hematological parameters (increased number of leukocytes, segmented neutrophils, sticks, eosinophils and monocytes and decreased number of red blood cells (RBC), number of lymphocytes and platelets). Moreover, lycopene prevented the reduction in the number and motility of spermatozoa and the testicular tissue damage induced by ZEA. In addition, lycopene prevented the decrease in glutathione-S-transferase activity in kidney and testes and increased glutathione-S-transferase activity *per se* in the liver, kidneys and testes as well as superoxide dismutase activity in the liver. In summary, lycopene was able to prevent ZEA-induced acute toxic effects in male mice, suggesting that this antioxidant carotenoid may represent a promising prophylactic strategy against ZEA toxicity.

1. Introduction

Zearalenone (ZEA) is a fungal toxin produced by the fungi *Fusarium culmorum* and *Fusarium graminearum* (Placinta et al., 1999; Zinedine et al., 2007). Given ZEA is commonly found in widely-used ingredients for many human and animal food, such as maize, barley, wheat, oats, sorghum and sesame seeds (Schollenberger et al., 2006; Zinedine et al., 2007), the presence of ZEA in food appears as an important health issue, and therefore the study of ZEA toxicity is of fundamental importance for the understanding and management of its toxic effects.

The chemical structure of ZEA resembles that of naturally occurring estrogens (Gromadzka et al., 2008), and in fact this mycotoxin binds to estrogenic receptors (ER) (Boyd and Wittliff, 1978; Greenman et al., 1979). Accordingly, ZEA produces various estrogen-disrupting effects at relatively low concentrations, including infertility, reduced serum testosterone concentrations and sperm counts, enlargement of ovaries and uterus, reduced incidence of pregnancy, and decreased progesterone levels in animals (Zinedine et al., 2007). In this context, the reproductive system has been regarded as a major target of ZEA toxicity (Tiemann and Danicke, 2007; Minervini and Dell'Aquila, 2008). For instance,

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chronic exposure to ZEA caused alterations in the reproductive tract of laboratory (mice, rats and pigs) and farm animals (Minervini and Dell'Aquila, 2008).

In addition to its estrogen-like effects, it has been proposed oxidative stress plays an important role in ZEA toxicity. In fact, it has been demonstrated that ZEA and its metabolites elicit lipid oxidation in several cell lines (Kouadio et al., 2005; Hassen et al., 2007; Othmen et al., 2008), and that acute exposure to ZEA decreased glutathione-S-transferase activity in mice kidney and testes (Boeira et al., 2012). In addition, it has been shown that incubation with antioxidant vitamins A, E or C reduced the formation of DNA adducts induced by ZEA in renal cells (Szkudelska et al., 2002). Altogether, these results reinforce the idea that oxidative stress contributes to ZEA-induced toxicity and suggest that antioxidants could be a potential therapeutic approach to prevent or slow the progression of the toxic effects induced by acute ZEA exposure.

Lycopene, the red pigment of tomato and other red fruits and vegetables, is a carotenoid with high antioxidant potential (Stahl and Sies, 2003). Several studies have suggested lycopene as a potential agent for prevention of some types of cancers, particularly prostate cancer (Giovannucci, 1999; Giovannucci et al., 2002; Shami and Moreira, 2004; Schwarz et al., 2008; Ozten-Kandas and Bosland, 2011). In addition, lycopene has been suggested as an alternative treatment for sperm toxicity after chemotherapy (Atessahin et al., 2005, 2006; Sonmez et al., 2011). Moreover, lycopene also displays protective properties in chronic cardiovascular disorders as well as respiratory and digestive epithelial cancers (Rao, 2002; Voutilainen et al., 2006; Dahan et al., 2008). However, to the best of our knowledge, there is no information available on the potentially beneficial effects of lycopene against ZEA toxicity. Therefore, the present study aimed to evaluate the effects of lycopene on reproductive, hematological, histopathological and oxidative stress parameters in the liver, kidney and testes of male Swiss albino mice following acute exposure to ZEA.

2. Materials and methods

2.1. Animals and reagents

Male Swiss albino mice (25–30 g in weight and 90 days old) were used. Animals were housed in groups of 5 in Plexiglas cages (41 cm × 34 cm × 16 cm) with the floor covered with sawdust. They were kept in a room with light–dark cycle of 12 h with the lights on between 7:00 and 19:00 h and temperature controlled (20–25 °C) and received water and food *ad libitum*.

The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources (process #068/2012) of the Federal University of Santa Maria, Brazil.

Zearalenone (ZEA), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), epinephrine bitartrate salt, glycine, trizma base, were purchased from Sigma (St. Louis, MO, USA). Lycopene was obtained from commercially available capsules (Equaliv Licopeno®; the purity of lycopene in each capsule is 100% and represents 7% of total content and the remaining 93% consist of excipient). All other chemicals were analytical grade from local suppliers. The stain used for the blood smear (Quick Romanowsky) was purchased from Laborclin (Pinhais, PR, Brazil). ZEA and lycopene were dissolved in olive oil, immediately before administration.

2.2. Experimental design and sampling

2.2.1. Treatment

Treatment schedule is depicted in Fig. 1. Mice were weighed and randomly divided in two groups receiving lycopene (20 mg/kg) or olive oil (10 ml/kg) by gavage for ten days. In the eleventh day each group was again randomly divided in two groups receiving ZEA (40 mg/kg – 8% of LD_{50}) or olive oil (10 ml/kg) by gavage. Forty eight hours after ZEA or vehicle administration the animals received a dose of pentobarbital (180 mg/kg, i.p.), and blood was collected by cardiac puncture into tubes containing heparin (1 UI/μl). The liver, kidneys and testes were removed, weighed, and homogenized in Tris–HCl 50 mM pH 7.4 for the determination of enzymatic indicators of oxidative stress. The epididymides were weighed and used for determining the number and motility of spermatozoa. One of the testes was fixed in 10% formalin for histopathological examination, as described below.

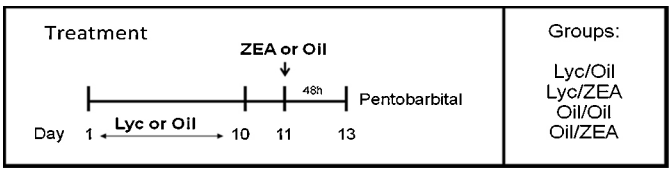

Fig. 1. Schematic presentation of the experimental protocol. Mice were weighed and randomly divided in four groups which received lycopene (Lyc) (20 mg/kg) or olive oil (10 ml/kg) by gavage for ten days. In the eleventh day mice received ZEA (40 mg/kg) or olive oil (10 ml/kg) by gavage. Forty eight hours after ZEA or vehicle administration the animals received a dose of pentobarbital (180 mg/kg, i.p.), and blood and major organs were collected for biochemical and histopathological analysis.

2.3. Blood cells

Total leukocyte count was performed using 25 μl of blood and 500 μl of Turkey solution in a Neubauer chamber with the aid of optical microscope (Nikon Eclipse 50i) at 40× magnification. The same technique was applied for differential counts of neutrophils (segmented and sticks), eosinophils, basophils, lymphocytes and monocytes in blood smears (5 μl of blood). After counts, slides were stained (Quick Romanowsky) and viewed under a microscope according to the method described by Failace et al. (2009). Red blood cells (RBC) were measured using a blood counter (Mindray, BC-2800 Vet model) with adapted dilutions.

2.4. Number and motility of spermatozoa

Assessment of spermatozoa count and motility was performed according to Freund and Carol (1964). Briefly, the cauda epididymides were homogenized in 2 ml of warmed (37 °C) saline solution (0.9% NaCl). An aliquot (10 μl) of the diluted spermatozoa suspension was transferred to a standard hemocytometer counting chamber and was allowed to stand for 5 min. Cells settled in the 5-min period were counted with the help of light microscope (Nikon Eclipse 50i) at 200× magnification.

2.5. Antioxidant enzymes

2.5.1. Glutathione S-transferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig et al. (1974). The reaction mixture contained a 50 μl aliquot from supernatant (1000 × *g*/10 min/4 °C) of liver, kidney or testes, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM CDNB (1-chloro-2,4-dinitrobenzene). GST activity was expressed as nmol CDNB/min/mg of protein.

2.5.2. Superoxide dismutase (SOD) activity

SOD activity was determined in liver, kidney and testes, according to the method described by Misra and Fridovich (1972). Briefly,

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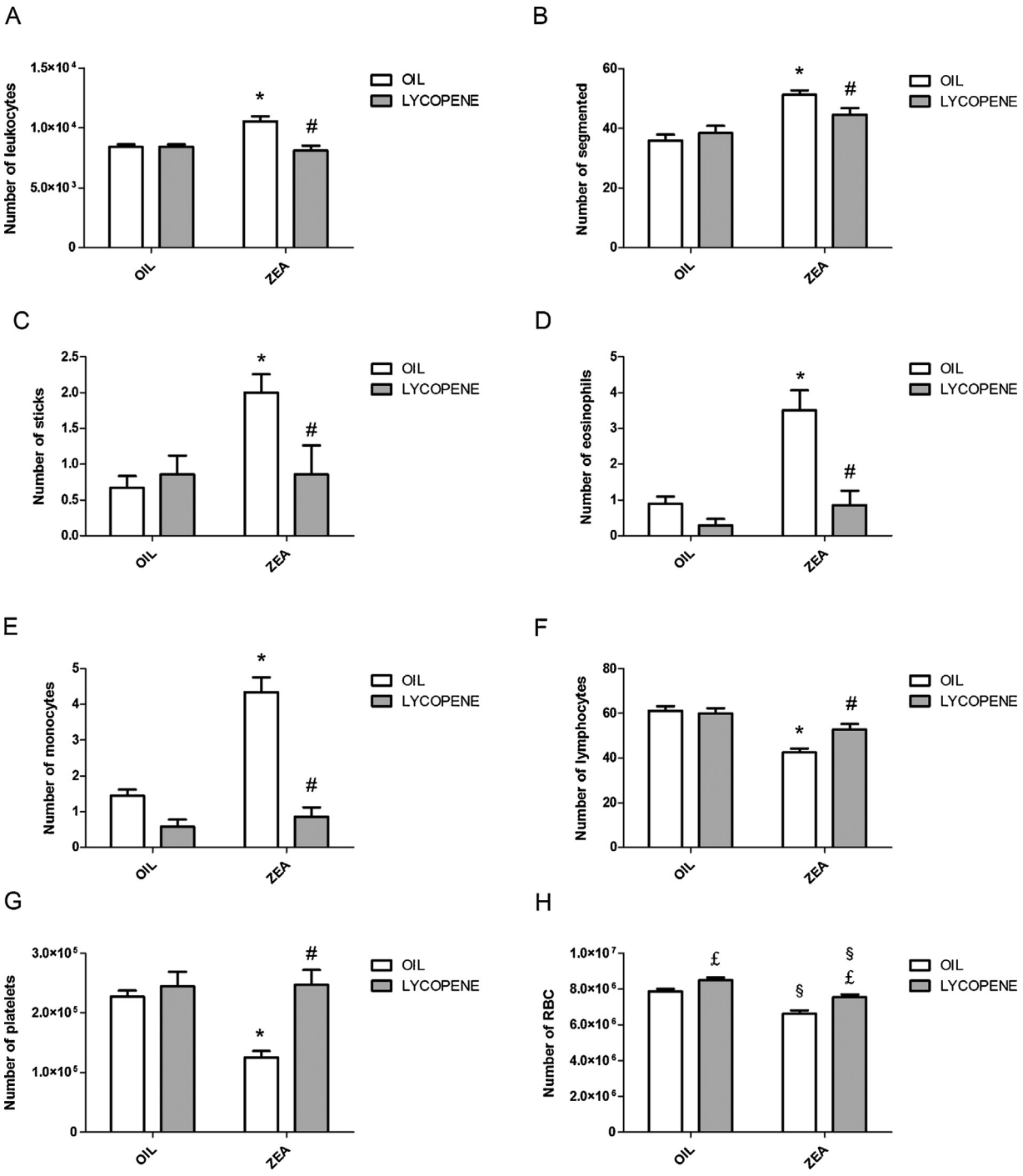

Fig. 2. Effect of ZEA (40 mg/kg, p.o.) and lycopene (20 mg/kg, p.o.) on blood cell count. (A) Number of leukocytes ($F(1,25) = 16.28$, $P = 0.0005$), (B) segmented neutrophils ($F(1,25) = 4.77$, $P = 0.038$), (C) sticks ($F(1,25) = 5.75$, $P = 0.024$), (D) eosinophils ($F(1,25) = 8.92$, $P = 0.0062$), (E) monocytes ($F(1,25) = 24.79$, $P = 0.0001$), (F) lymphocytes ($F(1,25) = 6.21$, $P = 0.019$), (G) platelets ($F(1,25) = 7.70$, $P = 0.010$) and (H) red blood cells (RBC) (lycopene $F(1,25) = 22.31$, $P = 0.001$ and ZEA factors $F(1,25) = 43.04$, $P = 0.001$). Data are mean + S.E.M. for $n = 6$–9 animals in each group. *Indicates a significant difference ($P < 0.05$) compared with olive oil group. #Indicates a significance difference ($P < 0.05$) compared with ZEA olive oil group. £Indicates a significance difference ($P < 0.05$) between lycopene and the respective vehicle-treated controls. §Indicates a significance difference ($P < 0.05$) between ZEA and the respective vehicle-treated controls.

the supernatant fraction (20–60 μl) was added to a medium containing glycine buffer (50 mM; pH 10.5) and epinephrine (1 mM). Reaction was started by adding epinephrine to the medium, and the reaction was spectrophotometrically followed at 480 nm for 300 s at 30 °C. One unit (U) of SOD was defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50%, and SOD activity was expressed as U/mg protein.

2.5.3. Catalase (CAT) activity

CAT activity was determined in liver, kidney and testes, according to the method proposed by Aebi (1984). Briefly, the reaction medium consisted of 30 mM hydrogen peroxide in 50 mM potassium phosphate buffer (pH 7.0). The reaction was spectrophotometrically followed at 240 nm for 120 s at 37 °C. CAT specific activity was expressed as first-order rate constant k corrected per

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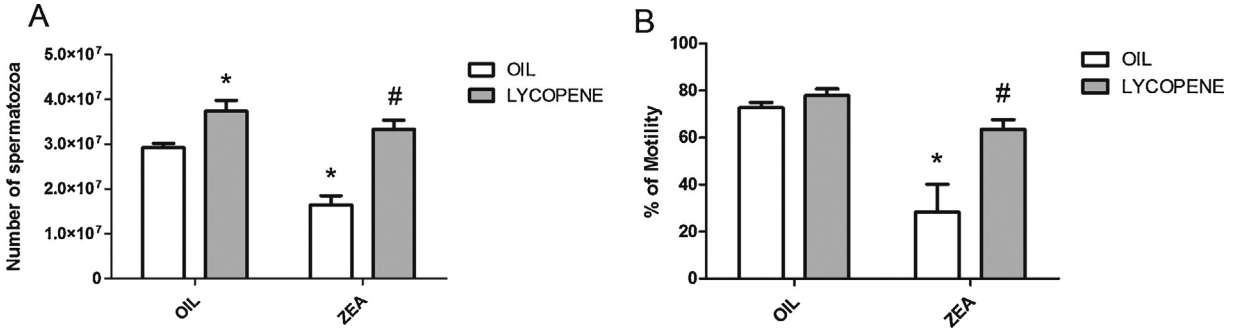

Fig. 3. Effect of ZEA (40 mg/kg, p.o.) and lycopene (20 mg/kg, p.o.) on (A) number of spermatozoa in epididymis A: ($F(1,25) = 5.13$, $P = 0.03$) and (B) percentage of motile spermatozoa ($F(1,25) = 7.18$, $P = 0.012$). Data are mean + S.E.M. for $n = 6–9$ animals in each group. *Indicates a significant difference ($P < 0.05$) compared with olive oil group. #Indicates a significant difference ($P < 0.05$) compared with ZEA olive oil group.

mg of protein. Appropriate controls for non-enzymatic decomposition of hydrogen peroxide were included in the assays.

2.6. Histopathologic examination

Testes were fixed in 10% formalin, dehydrated in ethanol, cleared in xylol and embedded in paraffin. After microtome sectioning at 4 μm, slices were stained with hematoxylin and eosin. Light microscopy was used for the evaluations, according to the method described previously by Brandão et al. (2009). The structural integrity of the testis, seminiferous tubules and interstitial tissues were evaluated, as well as morphology of spermatogonia, spermatocytes, spermatids and mature spermatozoa.

2.7. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976), using bovine serum albumin (1 mg/ml) as standard.

2.8. Statistical analysis

Data were analyzed by two-way ANOVA and *post hoc* analyses were carried out by the Newman–Keuls test. When appropriate, data were transformed ($Y = \text{Log}(Y)$) to meet ANOVA requirements. A probability of $P < 0.05$ was considered significant, and all data are reported as mean and S.E.M.

3. Results

3.1. Hematological parameters

Treatment with lycopene for ten days did not change significantly blood parameters (leukocytes, segmented neutrophils, sticks, eosinophils, monocytes, lymphocytes and platelets number) (Fig. 2). On the other hand, a hematotoxic effect of ZEA and the protective effect of lycopene were evident after 48 h of exposure to a single dose of the mycotoxin. Statistical analysis revealed that ZEA significantly increased the number of leukocytes (Fig. 2A), segmented neutrophils (Fig. 2B), sticks (Fig. 2C), eosinophils (Fig. 2D) and monocytes (Fig. 2E). In addition, ZEA decreased the number of lymphocytes (Fig. 2F) and platelets (Fig. 2G). Interestingly, lycopene was able to prevent the changes in the number of all blood cells analyzed. ZEA and/or lycopene treatments did not change the count of basophils (data not shown).

Treatment with ZEA alone caused a significant decrease in RBCs (Fig. 2H). Mice treated with lycopene for ten days prior to ZEA exposure exhibited prevention in this parameter.

3.2. Reproductive parameters

Fig. 3 depicts the effects of ZEA and lycopene on reproductive parameters in male mice. Acute administration of ZEA significantly reduced the number (Fig. 3A) and motility of living spermatozoa

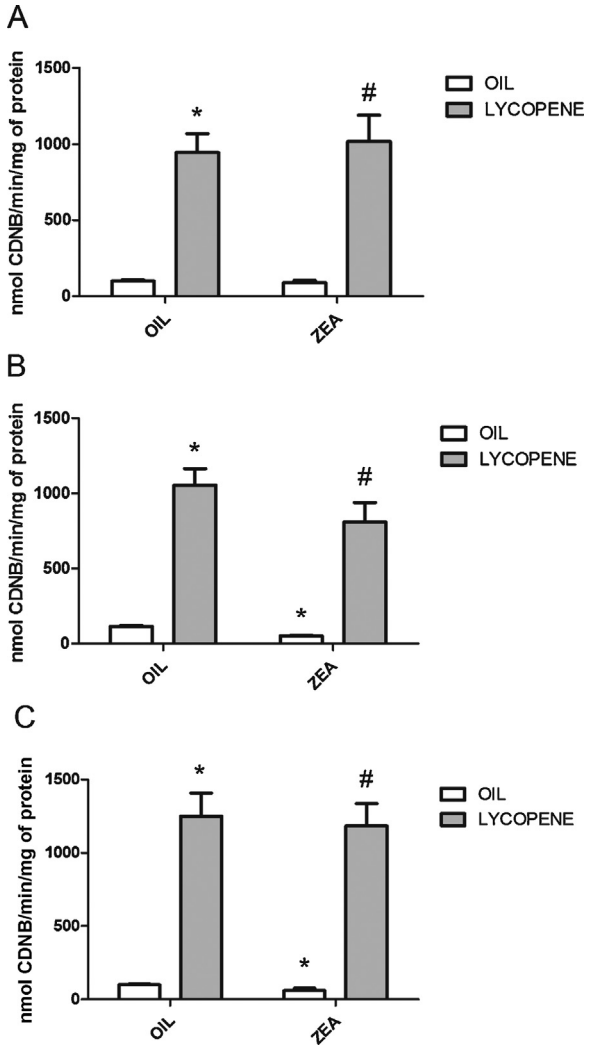

Fig. 4. Effect of ZEA (40 mg/kg, p.o.) and Lycopene (20 mg/kg, p.o.) on GST activity in (A) liver ($F(1,25) = 0.40$, $P = 0.530$), (B) kidney ($F(1,25) = 5.17$, $P = 0.031$) and (C) testes ($F(1,24) = 3.97$, $P = 0.05$). Data are mean + S.E.M. for $n = 5–9$ animals in each group. CDNB (1-chloro-2,4-dinitrobenzene). *Indicates a significant difference ($P < 0.05$) compared with olive oil group. #Indicates a significant difference ($P < 0.05$) compared with ZEA olive oil group.

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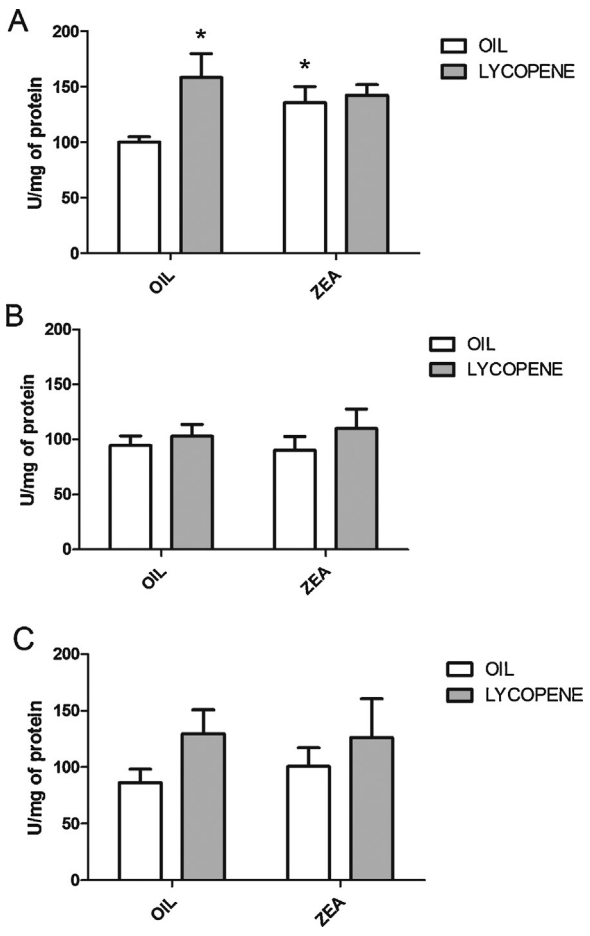

Fig. 5. Effect of ZEA (40 mg/kg, p.o.) and Lycopene (20 mg/kg, p.o.) on SOD activity in (A) liver ($F(1,25) = 4.29$, $P = 0.048$), (B) kidney ($F(1,25) = 0.028$, $P = 0.8683$) and (C) testes ($F(1,23) = 0.609$, $P = 0.4431$). Data are mean + S.E.M. for $n = 5$–8 animals in each group. *Indicates a significant difference ($P < 0.05$) compared with olive oil group.

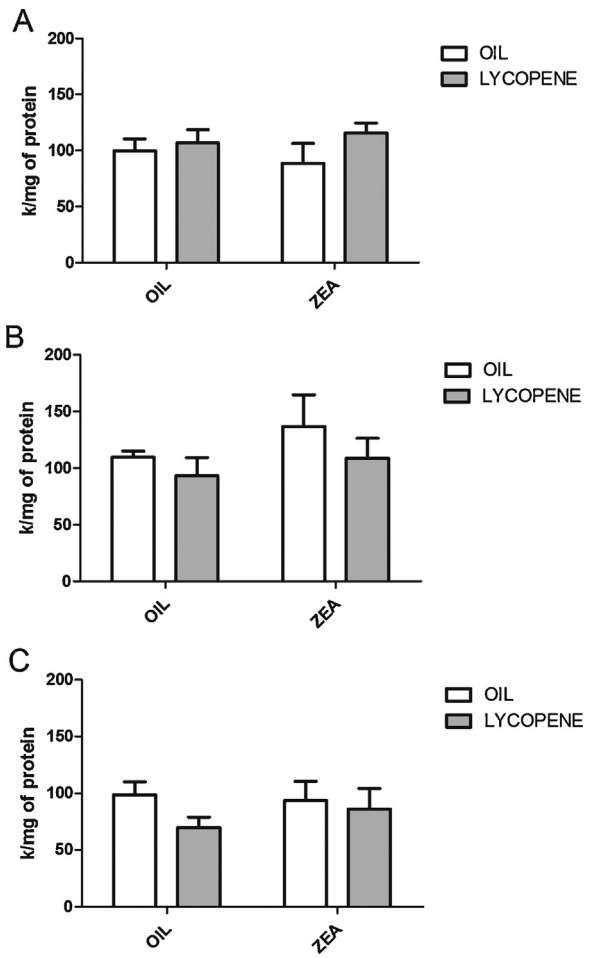

Fig. 6. Effect of ZEA (40 mg/kg, p.o.) and Lycopene (20 mg/kg, p.o.) on CAT activity in (A) liver ($F(1,25) = 1.17$, $P = 0.2898$), (B) kidney ($F(1,25) = 0.0044$, $P = 0.9476$) and (C) testes ($F(1,24) = 0.4503$, $P = 0.5086$). Data are mean + S.E.M. for $n = 5$–9 animals in each group.

(Fig. 3B). Interestingly, lycopene increased sperm count *per se*, but did not affect motility of living spermatozoa. In addition, lycopene was able to prevent the deleterious effects of ZEA on sperm count and motility.

3.3. Antioxidant enzymes

Statistical analyses revealed that ZEA reduced GST activity in kidneys and testes (Fig. 4), and increased SOD activity in liver (Fig. 5). On the other hand, lycopene increased GST activity in all tissues, and prevented the ZEA-induced decrease in GST activity. Furthermore, lycopene increased hepatic SOD activity *per se*. CAT activity was not altered in any of the tissues evaluated (Fig. 6).

3.4. Histopathological analysis

Histopathological analysis of control and lycopene-treated animals showed germ cells in the seminiferous tubules and interstitial space of normal aspect (Fig. 7A–D). In ZEA-treated animals we detected hypercellularity in seminiferous tubules and also germ cells in different stages presenting pyknotic nuclei and eosinophilic cytoplasm. In addition, reduced intercellular connections typical of tissue injury were found (Fig. 7E and F). Importantly, lycopene prevented the testicular histopathological changes elicited by ZEA in spermatogonia, spermatocytes and spermatids (Fig. 7G and H).

4. Discussion

ZEA is a fusariotoxin occurs worldwide in cereals, animal food and forages (Placinta et al., 1999; Zinedine et al., 2007), and adversely affects reproduction (Creppy, 2002). In addition, ZEA is also associated with several human diseases of unknown etiology (Placinta et al., 1999). ZEA is a common contaminant of food (Abid-Essefi et al., 2011), and therefore studying the protective potential of natural compounds toward ZEA toxicity becomes important. Several experimental studies have evidenced that plant-based diets, in particular those rich in vegetables and fruits, provide a great amount of antioxidant phytochemicals, such as vitamins C and E, glutathione, flavonoids and vegetable pigments such as lycopene. Accordingly, they offer protection against oxidative cellular damage and consequently have been recognized as valuable sources of nutraceuticals (Dimitrios, 2006). In this context, the present study aimed to evaluate the protective effect of lycopene on hematological, reproductive, oxidative and histopathological deleterious effects induced by the mycotoxin ZEA in mice.

We showed that acute ZEA administration caused deleterious hematologic effects (Fig. 2) and strongly reduced the number and motility of spermatozoa (Fig. 3) in male Swiss albino mice. The role of oxidative stress in the toxic effects of ZEA was also investigated. Interestingly, this mycotoxin decreased GST activity in kidneys and testes (Fig. 4) and increased SOD activity in liver (Fig. 5). Histopathological analysis revealed toxic testicular effects induced

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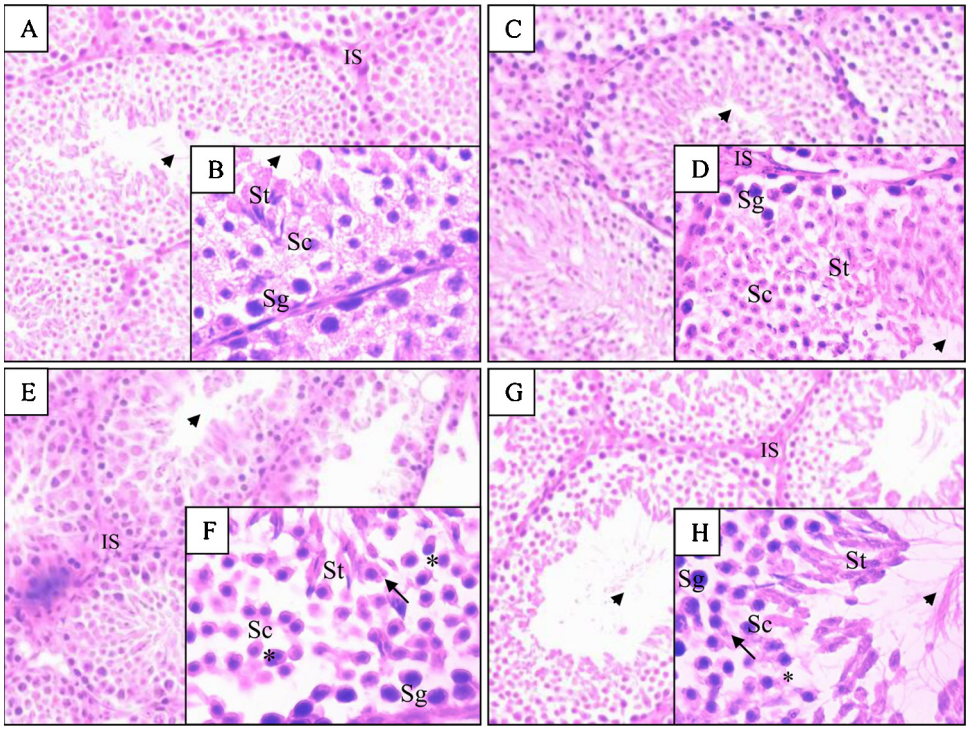

Fig. 7. Representative photomicrographs of hematoxylin-eosin stained sections from testis of mice ($n = 4$ in each group) treated with (A–B) olive oil only, (C–D) lycopene plus olive oil, (E–F) olive oil plus ZEA and (G–H) lycopene plus ZEA. Animals that received olive oil or lycopene but not ZEA the germ cells in the seminiferous tubules as well as the interstitial space appear with normal aspect. Note the presence of mature spermatogonia, spermatocyte and spermatids. In contrast, animals treated with ZEA and olive oil display hypercellularity in the seminiferous tubules and germ cells containing pyknotic nuclei (*) and eosinophilic cytoplasm (arrow). Also, intercellular connections were reduced. G and H represent lycopene + ZEA-treated animal showed slight organization in germ cells with spermatogonia, spermatocytes and spermatids. Note the immature spermatids and spermatocytes with eosinophilic (arrow). I, interstitial space; tubular lumen (arrowhead); Sg, spermatogonia; Sc, spermatocyte; St, spermatids. Magnifications are 10× (in A, C, E and G) or 40× (in B, D, F and H), respectively.

by this mycotoxin (Fig. 7). Importantly, treatment with lycopene for ten days prior to ZEA exposure prevented the toxic effects of ZEA on blood cells number, number and motility of spermatozoa and histopathological damage. In addition, lycopene treatment prevented the ZEA-induced decrease in renal and testicular GST activity, but did not alter hepatic SOD activity.

Lycopene, a naturally occurring carotenoid, has received particular attention in recent years because of its high antioxidant activity and free radical scavenging capacity (Cohen, 2002; Atessahin et al., 2006). For instance, lycopene is well known as highly efficient scavenger of singlet oxygen (O_2) and other reactive species. During O_2 quenching, energy is transferred from O_2 to the lycopene molecule, converting it to the energy-rich triplet state. Thus, lycopene may protect *in vivo* against oxidation of lipids, proteins, and DNA (Gupta and Kumar, 2002; Stahl and Sies, 2003). Lycopene has been shown to have the highest antioxidant activity among the carotenoids regarding cell protection against hydrogen peroxide and nitrogen dioxide radical components (Bose and Agrawal, 2007). In addition, lycopene has been reported to attenuate oxidative stress and exert anticancer effects both *in vitro* and *in vivo* (Cohen, 2002; Atessahin et al., 2006). Bose and Agrawal (2007) reported that lycopene elevated the concentrations of GSH, which plays a major role in maintaining high glutathione peroxidase and GST activities. Additionally, Kumar and Kumar (2009) showed that lycopene treatment (2.5, 5 and 10 mg/kg) significantly improved memory and restored GST activity in the striatum, hippocampus and cortex of the brain after 3-NP model of Huntington's disease.

Several antioxidant preparations have been examined for screening of compounds capable of crossing the blood testes barrier and protecting the germinal epithelium and Leydig cells from oxidative stress (Aitken and Roman, 2008). For instance, Turk et al. (2008) showed that ellagic acid, a phenolic antioxidant compound, ameliorated the cisplatin-induced reductions in weights of testes, epididymis, seminal vesicles, and prostate along with epididymal sperm concentration and motility. In addition, it has been reported that the therapeutic antioxidant effect of lycopene (Hekimoglu et al., 2009) and ellagic acid (Turk et al., 2011) on germ cells could serve as promising intervention to oxidative stress-induced infertility problems. In the present study, lycopene prevented the testicular histopathological damage caused by ZEA, as well as the harmful effects of this mycotoxin on sperm count and motility. To the best of our knowledge, this is the first study to evaluate the antioxidant activity of lycopene against testicular ZEA toxicity. To some extend, our present results agree with previous studies on the protective effect of lycopene. For instance, Tang et al. (2007) demonstrated that lycopene prevented the oxidative damage to DNA as well as the metabolic activation of aflatoxin B1 in F344 rats. Moreover, Leal et al. (1999) showed that lycopene prevented the occurrence of oxidative stress in broiler chicks exposed to T-2 toxin.

In conclusion, in the present study we showed that ZEA treatment markedly impaired testicular function as well as caused oxidative stress in Swiss albino mice. Importantly, treatment with lycopene prevented such toxic effects. Our results also indicate that lycopene could represent a preventive approach to minimize the deleterious effects associated with ZEA exposure.

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