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Aluminum Induced Reproductive Dysfunction in Male Rats: The Ameliorative Effect of Saffron Extract

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ABSTRACT

The current study aimed at evaluating the reproductive toxic effect of $AlCl_3$ and the possible ameliorative role of saffron extract in albino rats by using histological, immunohistochemical and biochemical studies. Male albino rats were divided into four groups. The first group served as control. The second group was orally administered with saffron extract at a dose level of 27 ml/kg b.w, 5 days/week for 6 weeks. Animals of the third group were orally given $AlCl_3$ at a dose level of 20 mg/kg bw daily for 6 weeks and those of the fourth group were given $AlCl_3$ (20 mg/kg b.w.) followed by saffron extract (27 ml/kg b.w.) daily for 6 weeks. $AlCl_3$ treatment induced histological changes in testicular tissue including degeneration of germ cells, hemorrhage and congestion of blood vessels, appearance of giant cells and decrease in the diameter and germinal epithelial height of the seminiferous tubules. Immunohistochemical results revealed decrease in expression of PCNA and increase in caspase-3. Moreover, a reduction in level of testosterone and LH was recorded. The combined administration of $AlCl_3$ with saffron extract caused an improvement in the histological, immunohistochemical as well hormonal level. In conclusions, the administration of saffron extract might suppress the toxicity of $AlCl_3$ by the antioxidant activity of its constituents.

INTRODUCTION

Metals are widely distributed in the environment and may have serious effect on human. Aluminum is the most widely distributed trivalent cation found in its ionic form in most kinds of animal and plant tissues and in natural water everywhere. It is the third most prevalent element and the most abundant metal in the earth's crust, representing approximately 8% of total mineral components (Shafer and Mundy, 1995). Aluminum enters the human body via food, air, water and drugs, aluminum ware and containers and is present in many manufactured foods such as processed cheese, baking powders, cake mixes, frozen dough, pancake mixes and pharmaceutical products, especially antacids (Nayak, 2002). Exposure to aluminum was found accompanied by several symptoms include colic, dementia, esophagitis, gastroenteritis, kidney and liver damage (Pennington, 1987, Kandiah and Kies, 1994). Aluminum has been associated with several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Kawahara, 2005, Bondy, 2010). It also affects other organs like the skeletal system, bone, blood cells, liver and kidney (Mestaghanmi *et al.*2002, Stella *et al.*2005). Various studies demonstrated that aluminum induced toxic effects in male reproduction (Guo *et al.* 2005, Thirunavukkarasu *et al.*2010).

Recently, interest has increased considerably in using plants in medicinal materials to replace synthetic ones, which are being restricted due to their side effects. Saffron, the dried stigma of the *Crocus sativus*, has been extensively used as a spice and food colorant because of its color and taste (Winterhalter and Straubinger, 2000). Saffron extracts were found have many medicinal activities including hypolipemic, anti-inflammatory, antioxidant, antidiabetic and anticarcinogenic effects (Abdullaev, 2002, Hosseinzadeh and Younesi, 2002, Mohajeri *et al.*2008, Hosseinpour *et al.*, 2010). It also showed hepatoprotective effects against liver damages induced by CCl_4 in mice (Iranshahi *et al.* 2011) and has protective effect on cisplatin induced nephrotoxicity in rat (Naghizadeh *et al.*2008).It has been observed that water extract of saffron eliminate oxidative stress in renal ischemia-reperfusion damage in rats and ensure protection against oxidative damages (Hosseinzadeh *et al.*, 2005). It also protects the liver cells of rats against oxidative damages (Giaccio, 2004). Sakr *et al.* (2014) reported that saffron extract ameliorates sodium valproate-induced cytogenetic and testicular damage in rats. The present study was designed to investigate the role of saffron extract against $AlCl_3$- induced reproductive toxicity in male albino rats.

MATERIALS AND METHODS

Preparation of saffron extract

Saffron, the dried stigmas of *Crocus sativus* flower were obtained from Al-alawy market, Jeddah, Saudi Arabia. One gram of saffron was soaked in 100 ml distilled water. After 2 hours it was homogenized in the same distilled water, stirred for 1 hour and filtered. The residue was re-extracted with fresh distilled water. This aqueous extract was lyophilized and stored at 4°C until further use (Premkumar *et al.*2003).

Aluminum chloride

Aluminium chloride ($AlCl_3$) was obtained from Sigma Chemical Co. (St Louis, Mo, USA). It was dissolved in distilled water to obtain the desired concentration.

Animals and treatments:

Adult male Wistar rats (*Rattus norvegicus*) weighing 160±10g were obtained from the animal house of the National Organization for Drug Control and Research, Egypt. They were maintained on a standard rodent diet composed of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminized starch. Maintenance of animals and experimental procedures was approved by the animal ethical committee of Faculty of Science, Menoufia University, Egypt in accordance with the guide for care and use of laboratory animals. Animals were divided into four groups:

***Group1*:** Animals were served as controls.

Group 2: Rats were orally administered with saffron extract at a dose level of 27 ml/kg b.w, 5 days/week for 6 weeks (Sakr et al., 2014).

Group 3: Animals of this group were orally given $AlCl_3$ at a dose level of 20 mg/kg b.w. daily for 6 weeks **(**Khatab et al.2010).

Group 4: Animals of this group were given $AlCl_3$ (20 mg/kg b.w.) followed by saffron extract (27 ml/kg b.w.) daily for 6 weeks.

Histological study

For histological study, animals were sacrificed after 6 weeks; testes were immediately removed and fixed in 10% neutral formalin for 24 hours**.** After fixation, specimens were dehydrated in ascending series of ethyl alcohol, cleared into two changes of xylene, infiltrated in three changes of molten paraffin wax with melting point of 58- 60 ^{o}C and then embedded in molten paraffin blocks. Sections of 5 microns thickness were cut by using rotary microtome and mounted on clean slides. Sections were stained with Ehrlich's haematoxylin and counterstained with Eosin and examined under light microscope.

Immunohistochemical study

For Immunostaining methods of PCNA and Caspase-3, slides were deparaffinized and rehydrated in a series of graded alcohol concentrations. Then rinsed in phosphate-buffered saline (PBS) containing 0.1% Tween-20. Antigen retrieval was performed by placing slides in sodium citrate solution (PH 6.0) at 90°C. Avidin (0.001% in PBS) and biotin (0.001% in PBS) was blocked in each section by using Avidin/biotin blocking solutions, where sections were incubated and rinsed with PBS between steps. Sections were incubated with monoclonal primary rat antibodies (Neo Markers, Cat.#Ms-113-P, Fremont, CA, USA), at appropriate dilution (1:200) in antibody diluent, directed against rat PCNA or Caspase (each antibody was used separately to react on different slides) at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in peroxidase blocking solution (3% H_2O_2 in PBS) at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated with biotinylated secondary antibody in PBS at room temperature. For detection, sections were incubated with horseradish peroxidase (HRP)-streptavidin solution at room temperature. Slides were washed in PBS-Tween 20 and were incubated in peroxidase substrate solution "3,3-diaminobenzidine tetrahydrochloride (DAP) until adequate color was developed. Sections were counterstained with hematoxylin, dehydrated through graded alcohol series, clear in xylene and mounted with DPX **(**Ramos-Vara, 2011**).**

Image analysis:

Digital images were analyzed by a semi-quantitative scoring system (Image J software, Java based application for analyzing images). The brown-stained immunohistochemical expressions of PCNA and caspase-3 were analyzed by measuring the percentage colored stained area per field area in five randomly high power fields at magnification of 400X.

Biochemical analysis:

For biochemical analysis, blood samples were collected in clean centrifuge tubes. Blood samples left to clot at room temperature and then serum separated by centrifugation at 3000 rpm for 20 minutes. The collected serum stored at -18 -20ºC until analysis. Testosterone and Luteinizing hormone LH were estimating by enzyme-linked immunosorbent assay (ELISA) according to the methods of Sizonenko (1987) and Kosasa (1981), respectively.

Statistical Analysis:

Results were expressed as mean ± standard deviation (SD). The significance of differences means was evaluated by using independent sample t-test. All statistical analysis was performed using SPSS statistical version 16 software package.

RESULTS

Histological observations

Examination of testes of control rats revealed that it formed of the seminiferous tubules which contain normal germ cells (spermatogonia, spermatocytes, spermatids and spermatozoa) and Sertoli cells (Fig.1A). Animals given saffron extract for 6 weeks showed normal structure of the testes (Fig.1B). Rats treated with $AlCl_3$ showed different histopathological alterations. The blood vessels were enlarged and congested (Fig.1C) and most of the tubules showed that the germ layers were detached from the basal lamina (Fig.1D). The intertubular spaces showed blood hemorrhage (Fig.2A). Vacuoles of different sizes were observed among the germ layers (Fig.2B). A severe reduction of spermatogenic cells with appearance of giant cells were abundant (Fig.2C). These histopathological alterations were improved in animals given $AlCl_3$ and saffron extract. In these specimens, the germ cells increased and the seminiferous tubules appeared somewhat normal.

Morphometric results

Treating rats with $AlCl_3$ caused a significant decrease in the diameter of the seminiferous tubules and in their epithelial heights. On the other hand, animals exposed to $AlCl_3$and saffron extract showed a highly significant increase ($P < 0.001$) in the diameters of the tubules and the epithelial heights in compare with $AlCl_3$-treated group. However, No

significant change was observed in the diameter of the seminiferous tubules and their epithelial heights between control and saffron treated rats (Figs.3&4).

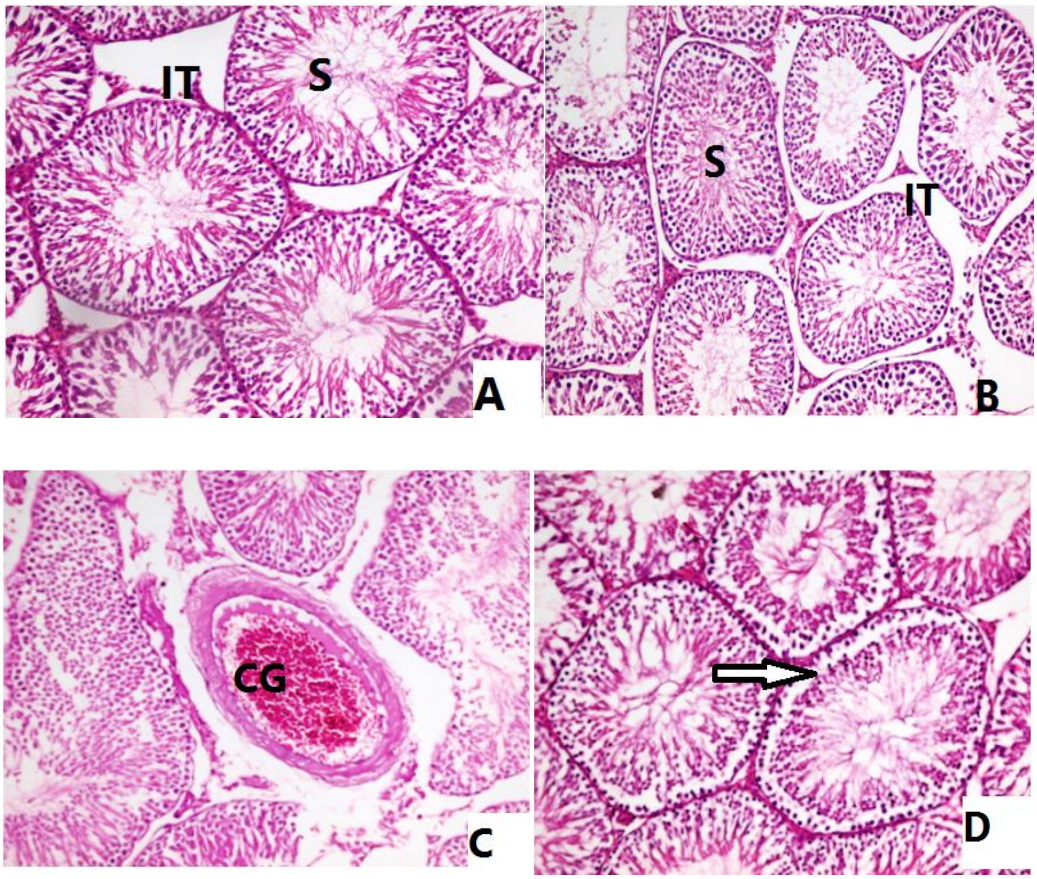

Fig. 1 A) A photomicrograph obtained from testis of a control rat showing normal seminiferous tubules, different stages of spermatogenic cells, spermatozoa (S) and interstitial tissue (IT), B) A photomicrograph obtained from testis of a rat treated with saffron extract showing normal structure of seminiferous tubules, C) A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ showing congested and enlarged blood vessel (CG), D). Section in testis of a rat treated with $AlCl_3$ showing separation of germ layers from underline basement membrane (arrow), (H&E, X400).

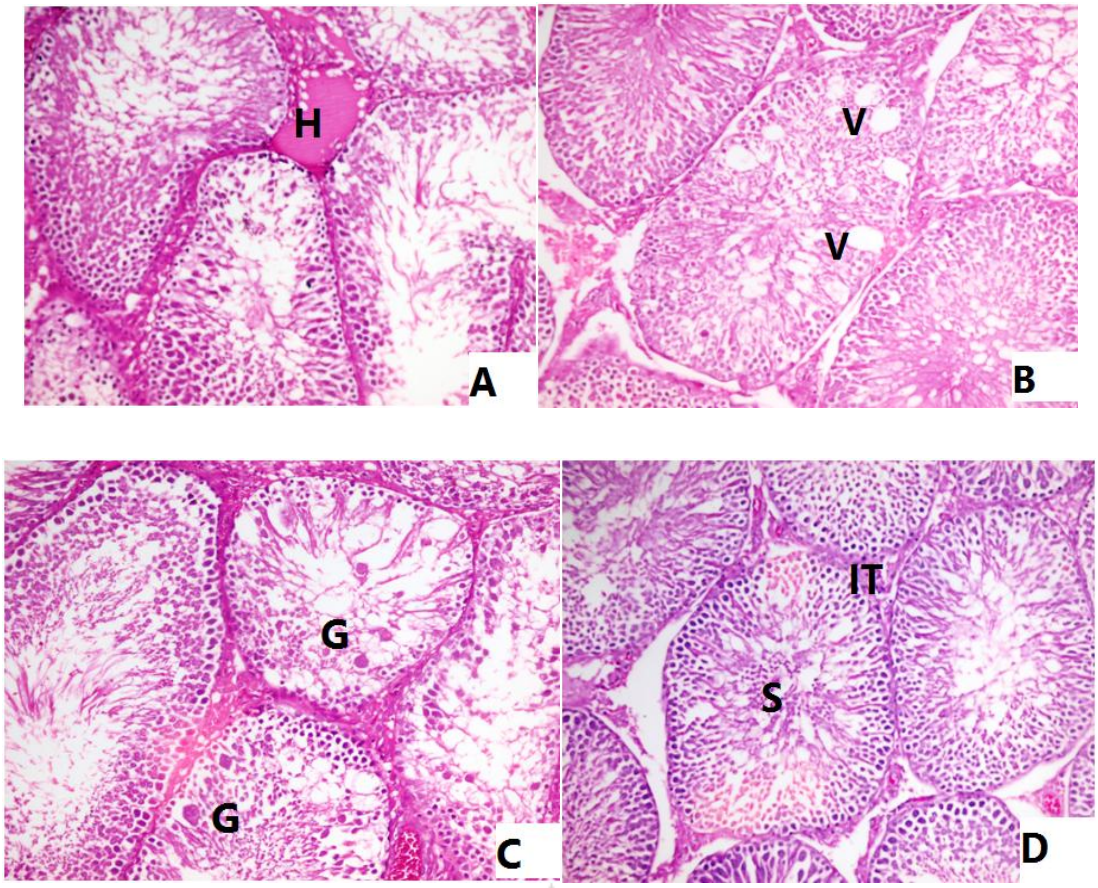

Fig.2. A). A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ showing interstitial haemorrhage (H), B). Seminiferous tubules of a rat treated with $AlCl_3$ showing vacuoles (V), C). Giant cells (G) in seminiferous tubules of a rat treated with $AlCl_3$,

D). A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ and saffron showing an improvement in the structure of seminiferous tubules with an increase in spermatozoa (S), (H&E, X400).

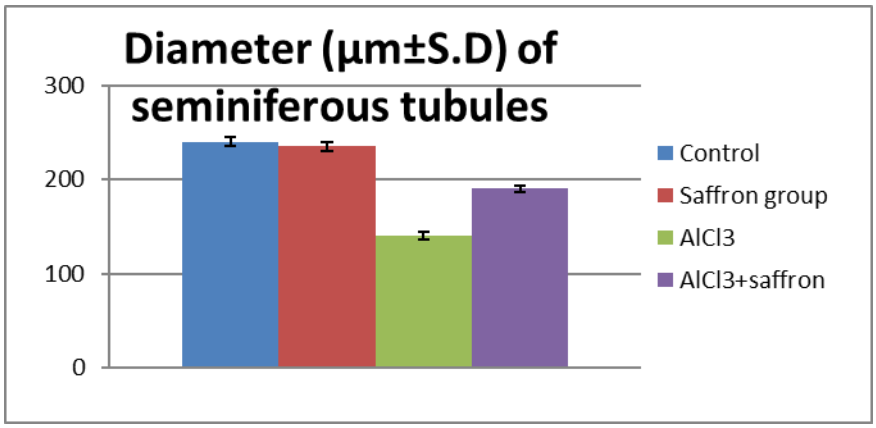

Fig.3. Effect of different treatments on the diameters of seminiferous tubules.

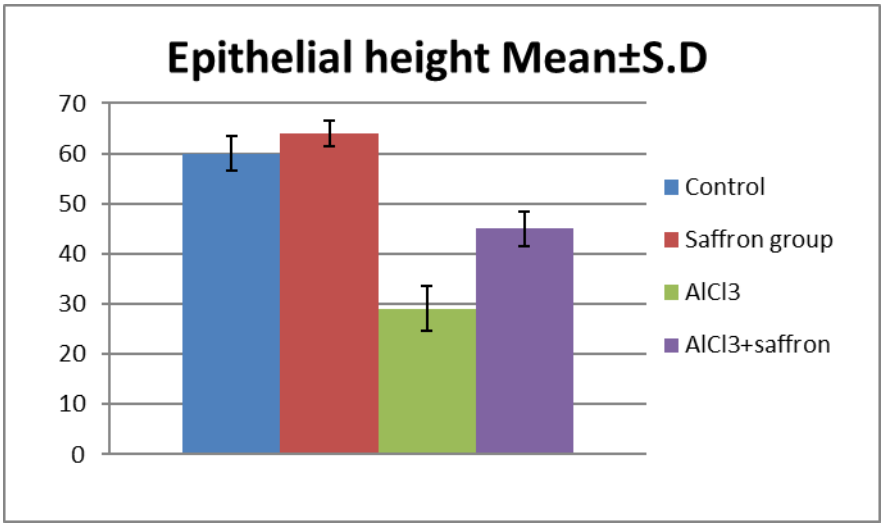

Fig. 4. Effect of different treatments on epithelial height of seminiferous tubules.

Immunohistochemical observations

Examination of sections of stain immunohistochemically for caspase-3 showed that caspase-3 was expressed in cytoplasm of few Leydig cells in tests of control and saffron treated animals (Fig. 5a)**.** Testicular tissue obtained from $AlCl_3$-treated rats for 6 weeks showed strong expression of caspase-3 in Leydig cells in comparison with the control group (Fig. 5b). Treatment of animals with $AlCl_3$ and saffron extract decreased the expression of caspase-3 (Fig. 5c). Image analysis revealed significant increase in expression of caspase-3 and this expression was decreased after treatment with $AlCl_3$ and saffron extract (Fig. 6). Concerning the expression of PCNA, results in figure 7a revealed strong expression in spermatogonia of control rats whereas a decreased expression of PCNA was recorded in rats treated with $AlCl_3$ (Fig.7b). Animals treated with $AlCl_3$ and saffron extract showed an increase in the expression of PCNA compared to $AlCl_3$-treated rats (Fig.7c). Figure 8 showed the area percentage of PCNA expression in testes of animals of different groups after 6 weeks of treatment.

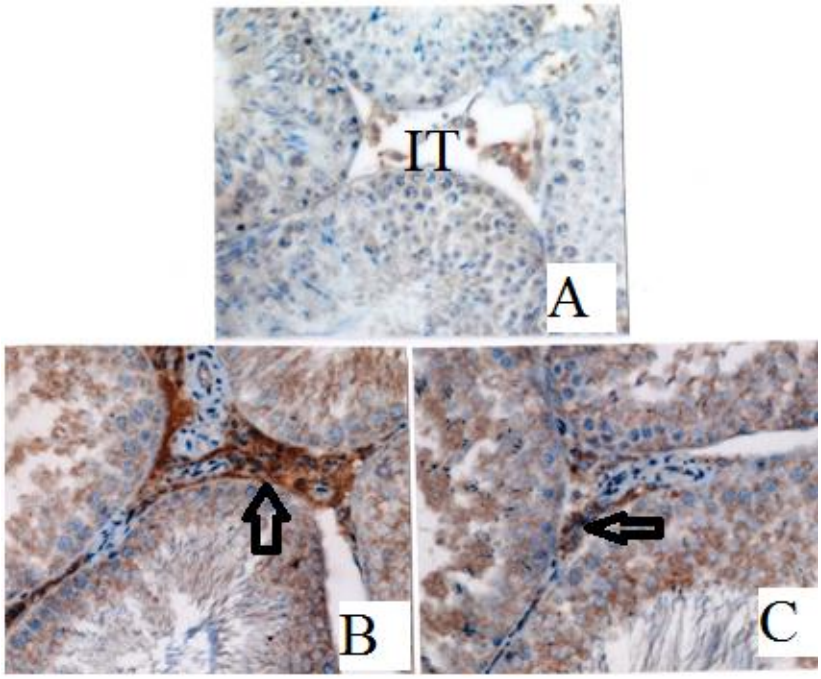

Fig. 5. A) A Photomicrograph obtained from testis of a control rat showing expression of casepase-3 in cytoplasm of Leydig cells (IT), B) A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ showed an increase in expression of caspase-3 in most cytoplasm of Leydig cells (arrow), C) A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ and saffron showed slight expression of caspase-3 in the cytoplasm of Leydig cells (arrow), (caspase-3 immunostaining, counterstained with hematoxylin, X400)

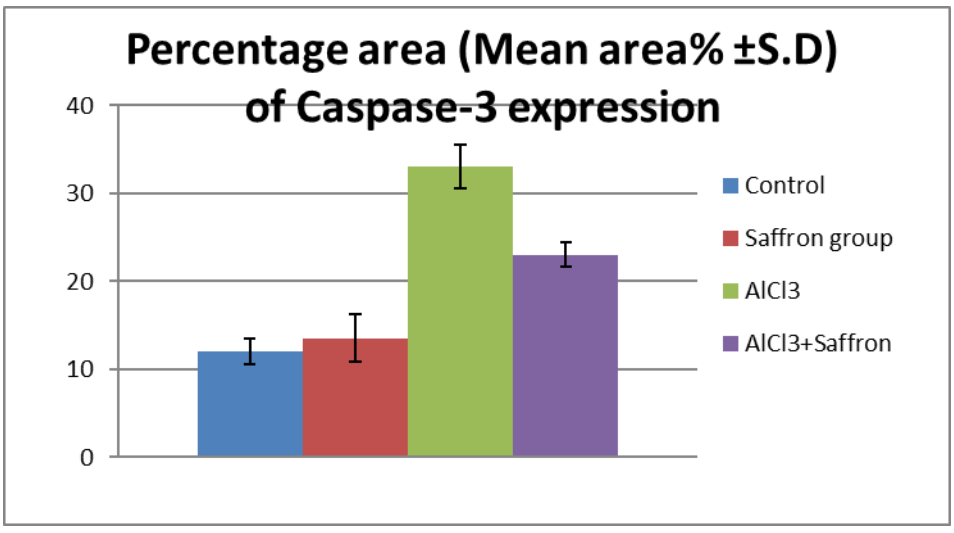

Fig. 6. Percentage area of caspase-3 in testes of different groups.

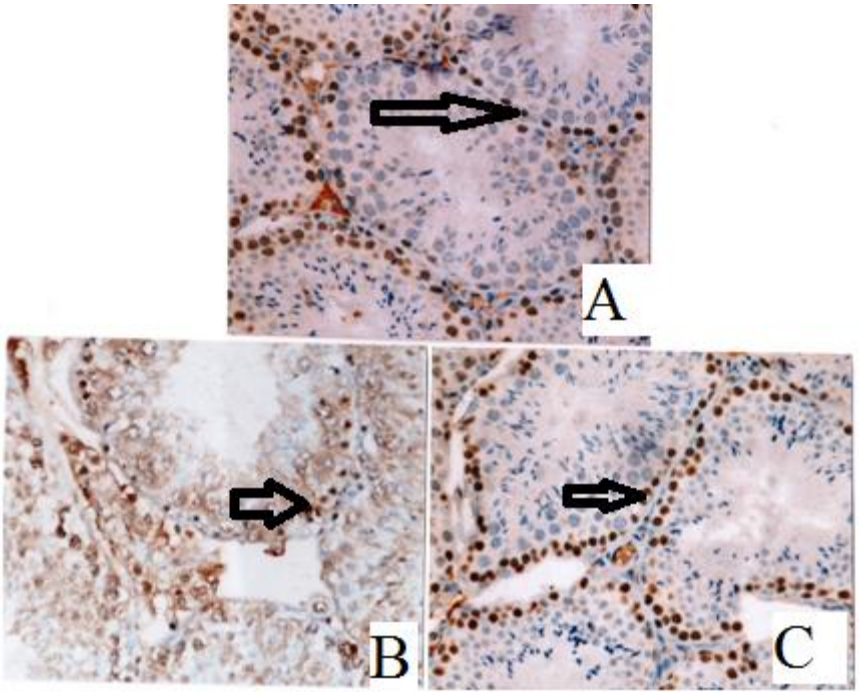

Fig.7. A) A Photomicrograph obtained from testis of a control rat showing expression of PCNA in nuclei of spermatogonia (arrow), **B)** A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ showed a weak expression of PCNA in most nuclei of spermatogonia, **C)** A Photomicrograph obtained from testis of a rat treated with $AlCl_3$ and saffron showed an increase in expression of PCNA in the nuclei of spermatogonia, (PCNA immunostaining, counterstained with hematoxylin, X400).

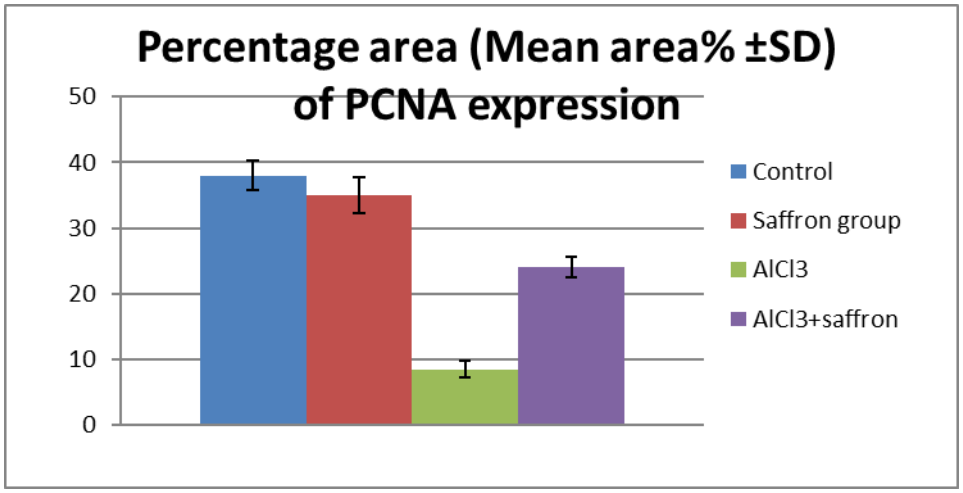

Fig. 8. Percentage area of PCNA in testes of different groups.

Biochemical results

Figure 9 & 10showed the change in serum testosterone and LH. Rats treated with $AlCl_3$ for six weeks revealed a significant decrease in serum level of testosterone and LH when compared with control group. On the other hand, animals treated with $AlCl_3$ and saffron extract showed a marked increase in levels of these hormones, in comparison with $AlCl_3$ group. There were no significant differences in serum level of testosterone and LH in control and saffron group during the experimental period.

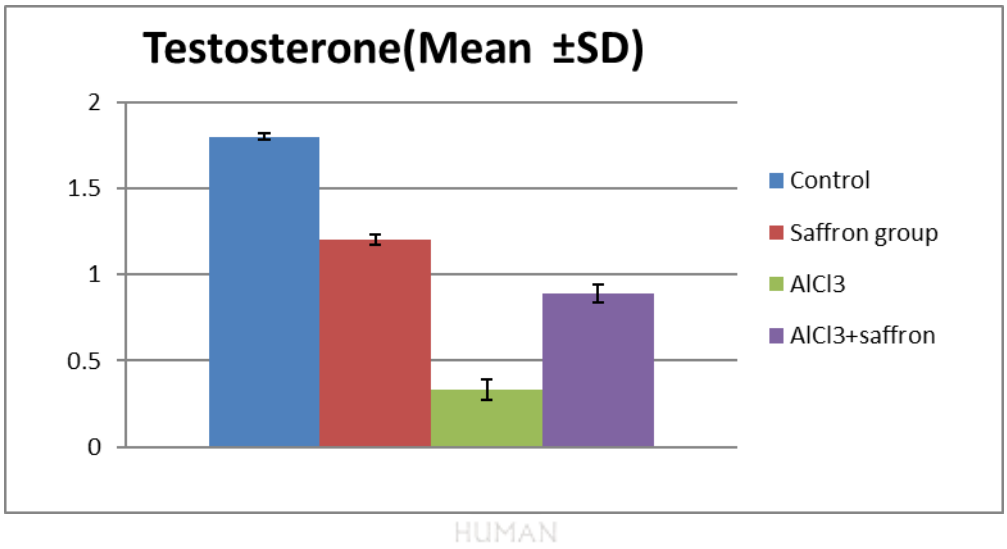

Fig. 9. Effect of different treatments on serum level of testosterone

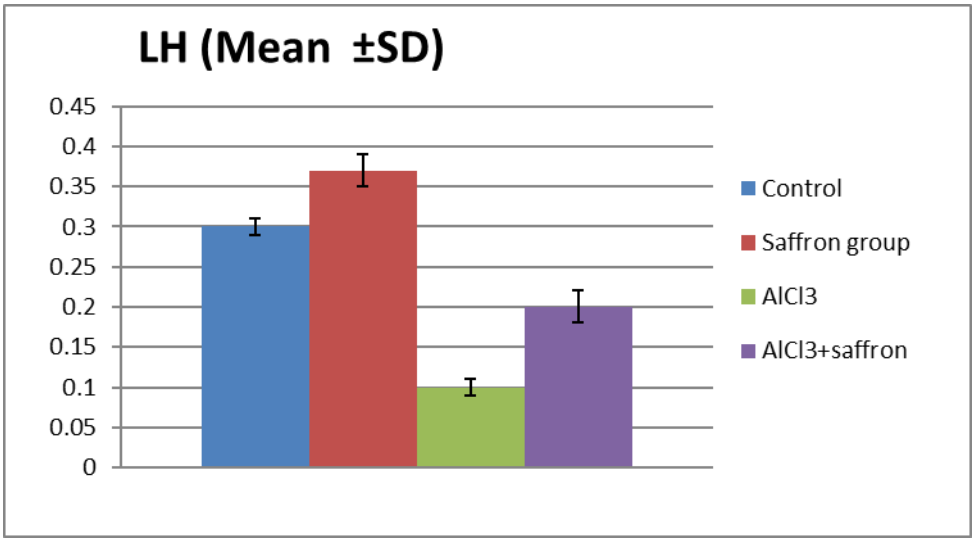

Fig. 10. Effect of different treatments on serum level of LH.

DISCUSSION

Aluminium (Al) has for a long time been considered as a toxic element. Results obtained in the present study revealed that $AlCl_3$ induced many histological alterations in the testis. These changes include degeneration of germ cells, hemorrhage and congestion of blood vessels, appearance of giant cells and decrease in the diameter and germinal epithelial height of the seminiferous tubules. Similarly, Khatab (2007) reported that treating rats with $AlCl_3$ induced histological and ultrastructural changes in the testis. The histological changes were severe damage of germ cells and the electron microscopy observations include atrophy of the tubular membrane, mitochondria, endoplasmic reticulum, golgi apparatus and nucleus. The multinucleated giant cells appeared in the cytoplasm of the treated rat testis. Mayyas, *et al.*, (2005) reported male mice ingested $AlCl_3$ showed different histological changes in the testes. Chinoy, *et al.* (2005), showed that administration of sodium fluoride together with $AlCl_3$ to mice for 30 days, caused inhibition of spermatogenesis and formation of giant cells. Khatab *et al.* (2010) showed that $AlCl_3$ treatment caused histological alterations, sex organs relative weight, sperm concentration, motility and viability, and serum testosterone concentration in rats.

Immunohistochemical results revealed decrease of expression of PCNA in testes of rats treated with $AlCl_3$ and many positive nuclear reactions were detected in spermatogonia. Similar result was obtained by Afeefy *et al.* (2016) in testes of rats exposed to aluminum hydroxide. The authors added that the aluminum hydroxide had an inhibiting effect on the proliferative activity in the seminiferous tubules. Proliferating cell nuclear antigen (PCNA) is a kind of cyclin protein found in the nuclei of proliferating cells and is differentially expressed during the cell cycle and reaches its maximum level during late G1/S phases and begins to decrease during late G2/M to G1 phases. Therefore, PCNA is considered a useful molecular marker for the assessment of germ cell kinetics (Xue e*t al.* 2007). Expression of caspase-3 was increased in testes of rats given $AlCl_3$. Caspase-3 is a marker of the early phase of apoptosis and is essential for certain processes associated with the formation of apoptotic bodies (Porter and Janicke, 1999).

Apoptosis has been detected in spermatogonia and primary spermatocytes after $AlCl_3$ administration (Abdel-Moneim, 2013) and primarily results from microtubule targeting and mitotic arrest. Saberzadeh *et al.* (2016) reported that Al-maltolate induced apoptosis in PC12 cells.

Serum level of testosterone and LH was declined in $AlCl_3$-treated rats. These results were in line with the results reported by Yousef (2004) who showed that $AlCl_3$ decreased testosterone and was able to generate reactive oxygen species in rabbit's testes. Guo *et al*. (2005) reported that aluminium administration significantly increased nitric oxide (NO) production and decreased both testicular adenosine 3′, 5′-cyclic monophosphate (cAMP) and testosterone levels. Ige and Akhigbe (2012) reported that Al induced reduction in testosterone, FSH, and LH in male rats. They added that this might be associated with calcium channel blocking effect of Al which led to impaired secretion of gonadotropins in the hypophysis (Platt and Busselberg, 1994). It was reported that oxidative stress can induce male infertility and caused an increase in germ cell apoptosis and subsequent hypospermatogenesis. Aluminum is considered to be a nonredox active metal, it promotes biological oxidation both *in vitro* and *in vivo* because of its pro-oxidant activity (Turner and Lysiak, 2008). Yousef and Salama (2009) reported that $AlCl_3$ caused reproductive toxicity in male rats and induced oxidative stress results from the production of excess oxygen radicals in excess. Thus, the observed effect of $AlCl_3$ on reproduction of male rats may be due to its generated oxidative stress.

The present results have revealed that saffron extract ameliorates the reproductive toxicity of $AlCl_3$ in male rats. Treating rats with $AlCl_3$ and saffron extract improved the histological appearance of the testis, increased expression of PCNA and decreased caspase-3. Moreover, the serum level of testosterone and LH was increased. The protective effect of saffron was recorded in several studies. Sakr *et al.* (2014) reported that treating rats with saffron extract ameliorated the histopathological alterations induced by valproic acid. The germ layers increased with appearance of somewhat normal cells as well as increase in number of sperm bundles. Asadi *et al.*, (2014) reported that saffron extract improved semen parameters (sperm concentration, motility and viability in cauda of epididymis) in rats exposed to cadmium. They added that saffron extract prevents the formation of free radicals and lipid peroxidation hence prevent oxidant-induced apoptosis. Heidary *et al.*, (2008) found that prescribing edible saffron is effective on increasing the average number and motility of sperms in nonsmoker infertile men with oligospermia. Modaresi *et al.*,(2008) reported that in mice, saffron consumption resulted in increased FSH, LH, and testosterone serum levels. Treating animals with crocin, one of the saffron constituents, significantly boosted sperm motility and viability; seminiferous tubule diameters; testis weight; and testosterone levels after treatment with nicotine (Salahshoor *et al.*2016) and cyclophosphamide (Bakhtiary *et al.* 2014).

Stigma of *Crocus Sativus* flower was found to contain three main metabolites; Crocins, Picrocrocins and Safranal. In addition to crocin and picrocrocin, anthocyanins, flavonoids, vitamins (riboflavin and thiamine), amino acids, proteins, starch, mineral matter, gums, and other chemical compounds have been found in saffron (Rios *et al.*, 1996). The antioxidant activity of saffron was attributed to these constituents. Xi *et al.*,*(*2007) have shown that crocin is beneficial for sperm cryo-conservation; therefore, it could be helpful in treatment of neurodegenerative disorders due to its great antioxidant activity. Bakhtiary *et al.* (2014) reported that crocin was able to suppress free radicals and enhance the quality of sperm in cyclophosphamide treated animals. Co-treatment of $AlCl_3$ with saffron and honey improved the disrupted liver biochemical markers and alleviated the increase of lipid peroxidation (Shati and Alamri, 2010). Goli *et al.* (2012) reported that saffron petal as the main by-product of saffron production possessed considerable phenolic compounds which showed high antioxidant power. Ethanolic extract of saffron as found to reduce lipid peroxidation, and improved the antioxidant enzyme activities, SOD, CAT, and GSH-related enzymes in liver of rats treated with cisplatin (Mohajeri and Doustar, 2012). In conclusion, our results suggested that saffron compounds with its antioxidant activity was able to ameliorate reproductive toxicity of $AlCl_3$ in male rats.

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