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Original Article

Effect of *Ginkgo biloba* extract on cyclophosphamide-induced reproductive toxicity and oxidative stress in male albino mice

Saber Sakr[1], Hoda A. Mahran[1], Nahed S. Basily[2] and Mona E. Saif[2]

[1]Zoology Department, Faculty of Science, Menoufia University, Shebin El-kom, Egypt.
[2]Department of Histopathology, National Organization for Drug Control and Research, Egypt

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ABSTRACT

The present study was carried out to investigate the possible protective effect of *Ginkgo biloba extract (EGb)* on reproductive toxicity induced by the anticancer drug cyclophosphamide (CP) in male albino mice. Animals were divided into four groups. G1 was considered as control. G2 was given EGb at a dose level of 100 mg/kg body weight/day, for 4 weeks. G3 was orally given CP at a dose level of 6.5 mg/ kg body weight/ day for 4 weeks. G4 was orally administered EGb and CP daily for 4 weeks. Testes were removed and stained with H&E for histological examinations. Testosterone and LH were measured in the sera. MDA, CAT and SOD were measured in the testicular tissue. The results showed that treating animals with CP caused degeneration of seminiferous tubules and loss of the spermatogenic cells. The interstitial tissue appeared with different vacuoles, blood hemorrhage and Leydig cells were degenerated. The sperm counts decreased and the sperm abnormalities increased. Biochemical results showed a decrease in levels of testosterone, LH, SOD and CAT activities and an increase in MDA. Treating mice with CP and EGb caused an improvement in the pathological alterations and increased the number of sperms. Moreover, testosterone, LH, SOD and CAT increased, while MDA decreased. The results of this study indicated that EGb protected mice against CP induced reproductive toxicity.

Key words: Cyclophosphamide, *Ginkgo biloba*, testis, Histology, Antioxidant

INTRODUCTION

Cancer treatment often includes more than one approach, and the strategy adopted depends largely on the nature of the cancer and how far it has progressed. Chemotherapy involves the use of low molecular weight drugs to selectively destroy a tumor (cytotoxic effect) or at least limit its growth (cytostatic effect) [1]. Cyclophosphamide (CP) is a nitrogen mustard alkylating agent from the oxazophorines group [2]. It is used to treat Hodgkin's disease, lymphomas, leukemia, Wegener's granulomatosis, severe rheumatoid arthritis, and lupus erythematosus. It is also used in combination with other drugs to treat breast cancer, leukemia, and ovarian cancer. The drug also has immunosuppressant action when it has used in smaller doses [2]. In spite of CP therapeutic importance, a wide range of adverse effects were recorded. Sweetman [3] reported many side effects; including hemorrhagic cystitis, alopecia and hyperpigmentation of skin may develop after high or prolonged dosages and can be life-threatening. Reproductive toxicity has been demonstrated following CP treatment in patients and in experimental animals [4,5]. Rezvanfar *et al.* [6] found that after CP (6 mg/kg/day, by gavage for 28 days) treatment in male rats, testicular and plasma lipid peroxidation increased, while total antioxidant power and plasma testosterone content decreased. In addition, impaired spermatogenesis, fertility, and decreased sperm quality associated with increased DNA damage and decreased chromatin quality was detected. Turk *et al.* [7] reported that treating rats with CP caused significant decreases in epididymal sperm concentration and motility and significant increases in MDA levels.

Recently, plants considered as an important source for the discovery of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants [8]. *Ginkgo biloba* Linné is a tree belongs to family Ginkgoaceae. It is thought to have been preserved by priests in China and Japan who cultivated it on temple grounds [9]. The tree has a long medicinal history being recorded as early as 2800 BC in the Chinese literature [10]. Besides, *Ginkgo biloba*

***Corresponding Author Address:** Dr. Saber A. Sakr, Faculty of Science, University of Menoufia, Shebin El-kom, Egypt; E-mail: sabsak@yahoo.com*

leaves extract (EGb) has been used in the treatment of cerebrovascular and peripheral vascular disorders[3]. In addition, ginkgolides A, B, C and M have been shown to check the platelet activating factor thereby preventing the bronchoconstriction, hypotension, cutaneous vasodilatation and finally the release of inflammatory compounds. Extract of *Ginkgo biloba* has antioxidant and hepatoprotective effects and can inhibit liver fibrosis in rat of non-alcoholic steatohepatitis and carbon tetra chloride[11,12].*Ginkgo biloba* leaves extract has also beneficial effects on the nervous system. When EGb was administrated to albino mice, it was found to be effective in preventing some functional and morphological deterioration in cisplatin-induced peripheral neuropathy [13]. Yoo *et al*.[14] reported that repeated intake of EGb enhanced cell proliferation and neuroblasts differentiation in the mouse hippocampal dentate gyrus and consumption of EGb may be helpful to increase endogenous neurogenesis in adults. EGb administration to cisplatin-treated rats effectively alleviated all of the cisplatin induced reproductive toxicity that accompanied with increased germ-cell degeneration and increased germ-cell apoptosis[15]. The present work was aimed to study the effect of EGb on CP-induced reproductive toxicity in male albino mice.

MATERIALS AND METHODS

Healthy adult male Swiss mice of an average body weight 28±3g were used in the present study. They were purchased from the animal house of The National Organization for Drug Control and Research. Animals were kept in the laboratory under almost constant condition of temperature (25 ± 1°C) for 2 weeks before and throughout the experimental work. They were fed on standard rodent diet manufactured specially for laboratory purposes as well as some vegetables and supplied with tap water during the experimental period.

Animals were divided into 4 groups:

First group: This group served as a control (non-treated) group.

Second group: Animals in this group were orally given EGb at a dose level of 100 mg/kg body weight/day, for 4 weeks. *Ginkgo biloba* leaves extract (EGb) was obtained in the form of Tanakan (EGb761) produced by Amriya for Pharmaceutical Industries, Egypt under license of Beaufour-Ipsen International, Paris, France.

Third group: Animals in this group were orally given CP. CP was obtained in the form of powder (Endoxan injection vial) manufactured by Baxter Oncology, Germany. It was dissolved in distilled water and given to experimental mice orally at a dose level of 6.5 mg/ kg body weight/ day, equivalent to the therapeutic dose for humans [16], for 4 weeks.

Fourth group: In this group, animals were orally given CP (6.5 mg/kg body weight) then after 2 hours they were orally given EGb (100 mg/kg body weight) daily for 4 weeks.

Histological study: Animals were dissected after 3 & 4 weeks of the experiment. For histological examination, testes were immediately removed, and were fixed in alcoholic Bouin's fluid. Specimens were dehydrated through ascending series of alcohol, cleared in xylene and embedded in molten paraplast. Sections of 5 micrometer thickness were cut by using rotary microtome, and mounted on clean glass slides without using any adhesive media. Sections were stained with Ehrlich's hematoxylin and counter stained with eosin.

Morphometric study: The diameter and germinal epithelial height of seminiferous tubules were measured. The epithelial height was measured from the spermatogenic cells on the inner surface of the basement membrane through the most advanced cell types lining the lumen of the tubules. Measurement takes place at histopathology lab in NODCAR using a computerized image analysis system "*Image Proplus version 5*". For analysis, eight fields per each testis section were taken at X 100 (10 objective X 10 ocular).

Sperm study (count and abnormalities): Sperm suspensions were prepared from cauda epididymides of mice by mincing the cauda in 2 ml of phosphate buffered physiological saline. The resulting suspension was pipetted and filtered through an eighty micrometer stainless steel mesh to remove the tissue fragments. An hour after the sperm diffusion in saline solution, sperm count was performed in hemocytometer chamber (Nubauer Bright Line Improved, 0.10mm). A fraction of each suspension was then mixed (10:1) with 1% eosin Y in distilled water and 30 minute later smears were made, allowed top dry in air, and were mounted under a cover-slip with DPX. For each suspension 1000 sperm were examined at 400-fold magnifications by light microscopy; a total of 3000 sperm were thus examined for each group [17].

Biochemical studies: For biochemical study, blood samples were collected in clean dry centrifuge tubes according to the retro-orbital plexus method[18]. Blood samples were left to clot at room temperature, and then centrifuged at 3000 round per minute (rpm) for 20 minutes. Luteinizing hormone (LH) concentration in serum was measured by enzyme-linked immunosorbent assay (ELISA), according to Kosasa [19]. Testosterone

concentration in serum was measured by ELISA assay according to Sizonenko [20] using immunoassay kit produced by Monobind Inc, USA. In addition fresh tissue samples of testis were homogenized in cold distilled water until a uniform suspension was obtained. The homogenate was centrifuged and the clear supernatant was separated. Catalase (CAT) activity was measured according to the method of Aebi [21]. Superoxide dismutase (SOD) activity was determined according to the method of Minami and Yoshikawa [22]. Malondialdehyde (MDA) was determined according to Ohkawa *et al*.[23].

Statistical analysis: Data were expressed as mean ± Standard error (SE). The significance of differences among group's means was evaluated by using one-way ANOVA. SPSS program (version 18) for windows software was used.

RESULTS

Testis of control animals or those treated with EGb for 3 or 4 weeks showed normal seminiferous tubules with sequence of spermatogenic cells (spermatogonia, spermatocytes and sperms). The tubules are separated with delicate intertubular connective tissue contains small blood vessels and groups of Leydig cells (Fig. 1). Examination of sections of testes of mice treated with CP for 3 weeks showed histopathological alterations. In these specimens, multiple scattered seminiferous tubules were degenerated and appeared with irregular basement membrane. The spermatogenic cells were less compact and loosely arranged with vacuoles separating the cells from each other. Cytoplasmic vacuolization of spermatogonia was a common feature in the examined specimens (Fig. 2) and the intertubular veins were congested (Fig.3). All the above mentioned changes became more obvious in testes of animals treated for 4 weeks. Specimens which examined from these animals revealed marked degeneration in seminiferous tubules, some of them contained only the spermatogonia, while others were completely degenerated (Figs. 4&5). Animals treated with EGb in combination with CP for 3 weeks revealed a less pronounced pathological alterations as compared to CP treated group. Animals coadministered with CP and EGb for 4 weeks exhibited advanced degree of improvement in their testicular tissues. In these specimens, testicular tubules configuration, cellularity, and spermatic density and orientation were markedly improved. In addition, most of interstitial cells were within normal shape and size (Fig. 6).

Morphometric results: Data obtained from image analysis denoted no statistical difference in seminiferous tubule diameter ($P<0.05$) between CP treated group and the other groups. On the other hand, a significant reduction in height of spermatogenic layer was detected in CP-treated animals compared to control group. Animals coadministered with CP and EGb showed a significant increase in the height of the germinal epithelia compared to CP-treated animals (table 1).

Sperm results

i.Sperm count: Data in figure (7) showed that the means of sperm count in either control or EGb treated group were nearly similar; there was no significant difference among those groups. When mice were administered with CP for 4 weeks, marked reduction in sperm count was detected. Treating animals with CP and EGb significantly elevated sperm count over that in CP treated group ($P< 0.05$).

ii. **Sperm abnormalities:** Various abnormalities in sperm morphology were observed in the treated animals. The determinant abnormal characteristics of the spermatozoa head abnormalities included amorphous and head lack the usual hook. The tail abnormalities included folded and short tail. Data in Fig. (8) showed that there was no statistical difference in sperm abnormalities between the control and EGb treated group either for 3 or 4 weeks. However, CP treated group showed significant increase in sperm head and tail abnormalities ($P<0.05$) over the control group. Animals treated with CP and EGb showed significant reduction in sperm abnormalities when compared to CP treated group.

Biochemical results

i. Change in testosterone and LH: The means of serum testosterone levels in different groups were presented in figure (9). There was no statistical difference in testosterone level between control and EGb treated groups. Testosterone level significantly ($P< 0.05$) decreased in sera of mice treated with CP for 3 and 4 weeks, when compared to the control group. When animals treated with both CP and EGb, significant elevation in testosterone level was observed as compared to CP treated mice. Data in figure (10) showed that control group and EGb treated group were nearly similar in their LH level at the same duration. Mice treated with CP for 3 or 4 weeks showed significant reduction in serum LH level ($P< 0.05$) when compared to control group. Treating animals with both CP and EGb revealed elevation in serum LH level when compared to CP treated group. When mice administered with CP and EGb, a significant elevation in LH level ($P< 0.05$) was detected as compared to CP treated group.

ii. Testicular MDA, SOD and CAT: Data in table (2) showed no statistical difference in the testicular

MDA level between control and EGb treated groups. Treating animals with CP either for 3 or 4 weeks significantly elevated MDA level when compared to control group. Co-exposure of mice to CP and EGb for 3 or 4 weeks was capable of reducing the elevated MDA level significantly in testes, when compared to CP treated group. No significant difference in testicular CAT activity was recorded between control and EGb treated mice. CP- treatment either for 3 or 4 weeks led to significant reduction in CAT activity as compared to control group ($P<0.05$). A time dependent significant elevation in CAT activity was observed after treatment with CP and EGb ($P<0.05$) as compared to CP treated group. SOD activity was gradually reduced after treating mice with CP either for 3 or 4 weeks as compared to control group. Combined treatment with both CP and EGb for 3 or 4 weeks elevated SOD activity in testes as compared to CP treated group (table 2).

DISCUSSIONS

In the present study, several histopathological alterations were seen in testes of mice after CP treatment. These alterations included reduction in height of epithelial lining of the seminiferous tubules, degenerative change in the spermatogenic cells as well as Leydig cells, and congestion of the blood vessels. These changes may be due to the oxidative stress induced by CP metabolites on testicular tissue. The same results were also detected by many investigators in different animal models [24-27]. In this respect, Rezvanfar *et al.* [6] reported that CP induced a marked reproductive toxicity in rats including disintegration of seminiferous tubules, impaired spermatogenesis, increased testicular and plasma LPO and decreased total antioxidant power through induction of free-radical toxic stress. Similarly, Ilbey *et al*.[28] observed that CP caused excessive production of oxygen-derived free radicals in testes. Agarwal *et al.* [29] denoted that reproductive cells and tissues remain stable when the balance between free radical production and scavenging anti-oxidants is maintained. Ceribasi *et al*.[30] suggested that the damage observed after CP treatment in the histological structure of testes of rats may be elucidated with the direct or indirect effect of CP, which later induces lipid peroxidation that is a chemical mechanism capable of disrupting the structure and function of testis. Recently, Khan and Jena [31] observed that CP induced degeneration of spermatogenic cells, significant increase in sperm head abnormality and decline in sperm count in mice. The authors attributed these changes to direct cytotoxic effects of the drug on the highly proliferating germ cells.

In the present study, significant reduction in sperm count and marked increase in the sperm abnormalities of both head and tail were observed after CP treatment. Similarly, Elangovan *et al.* [5] recorded a reduction in sperm count and motility after CP treatment in mice and considered it as indication of drug toxicity. Many investigators agreed that damage of sperms might be attributed to the presence of large quantities of polyunsaturated fatty acids in the sperm plasma membrane and low concentrations of scavenging enzymes in sperm cytoplasm, which make spermatozoa particularly susceptible to the damage induced by excessive reactive oxygen species[7,29,30]. Another explanation was conducted by Ilbey *et al.* [28] who reported that increased morphological defects and production of abnormal sperms may be as a result of direct toxicity of CP, because cellular DNA is a primary target of CP in its anti-neoplastic and toxic activity.
Serum hormonal analysis of animals treated with CP denoted significant reduction in levels of LH and testosterone. The toxic effect of CP on Leydig cells may lead to disruption of its role in testosterone production. In this respect, Debnath and Mandal [32] showed that increased generation of free radicals is one of the possible mechanisms involved in CP-induced Leydig cell dysfunction. In addition, Cao *et al*.[33] reported that reduced expression of key enzymatic and nonenzymatic antioxidants in Leydig cells resulted in excessive oxidative stress and enhanced oxidative damage in these cells that resulted in a decline in testosterone secretion. On the other hand, Rezvanfar *et al*.[6] attributed the reduction in plasma testosterone to the direct toxic effect of CP on the structure of Leydig cells. Elangovan *et al*.[5] recorded reduction in testosterone level and attributed this to degeneration of spermatogonia.

In the current study, CP induced marked reduction in antioxidant enzymes activities (CAT and SOD) and elevation in oxidative stress marker (MDA) in both liver and testes of mice. In the same way, many investigators denoted significant reduction in antioxidant enzymes activities, and testicular toxicity after CP administration to different animals [34-36]. Oxidative damage was observed by Motawi *et al*.[34] in testes of CP-administered rats. This was indicated by decreased serum total antioxidant capacity level, abnormal alterations in GSH-Px and glutathione reductase activities, levels of GSH, MDA, and nitric oxide in testes. In addition, many testicular markers (sorbitol dehydrogenase, gamma-glutamyl transferase, acid and alkaline phosphatases, serum testosterone level and hemoglobin absorbance) were also abnormal.
Examination of testes of mice exposed to combined treatment with CP and EGb revealed marked

improvement in the histopathological alterations compared with those treated with CP. Similarly. EGb treatment effectively alleviated all histological abnormalities induced by doxorubicin in testicular tissue of rats [37], and protected from cadmium chloride induced histological alterations [38]. Furthermore, EGb administration to cisplatin-treated rats effectively alleviated all of the cisplatin induced reproductive toxicity that accompanied with increased germ-cell degeneration and increased germ-cell apoptosis. This confirms the essential antioxidant potential of EGb [15]. Elevation in sperm count and reduction in sperm abnormalities was observed in the current study after EGb treatment. Due to the essential antioxidant potential of EGb, it was able to elevate epididymal sperm count and motility induced in rats by different anticancer drugs [37,15].

In the present work, significant elevation in serum LH and testosterone levels in mice treated with both CP and EGb was recorded. The elevation in testosterone level could be explained on the bases of the ability of EGb to preserve Leydig cells and the increased production of LH. This was in agreement with Yeh *et al*. [39], who reported that EGb administration induced significant production of testosterone in rat Leydig cells *in vitro*. de Souza *et al*. [38] denoted that EGb was also able to preserve Leydig cells volume and morphology, and elevate serum LH concentration.

Biochemical data obtained in the present work recorded marked elevation in the activities of antioxidant enzymes (CAT and SOD) and reduction in the level of the oxidative stress marker (MDA) in testes of mice treated with both CP and EGb. Similarly, EGb protected against the oxidative actions of doxorubicin by increasing levels of SOD, GSH-Px, and GSH, while depressed lipid peroxidation products (MDA) in testes of rat [37]. He *et al.* [40] and Liu *et al*. [41] recorded an elevation in the activities of SOD and GSH-Px, and reduction in MDA content in rats treated with both CCl_4 and EGb. They assumed that EGb is able to ameliorate liver injury induced by CCl_4 in rats by suppressing oxidative stress. Furthermore, Boveris *et al*. [42] showed that EGb was able to limit lipid peroxidation and scavenge lipid radicals both *in vitro* and *in vivo* and actively protect microsomal membranes from oxidative damage. Tozan *et al.* [43] demonstrated that EGb, by balancing the oxidant-antioxidant status, protects against naphthalene-induced oxidative organ injury. In this respect, Yao *et al.* [44] added that pretreatment of ethanol-treated rats with EGb was able to reduce GSH depletion, lipid peroxidation, and inhibited the inactivation of SOD, CAT, and GSH-Px. In addition, EGb was able to treat diet-induced steatohepatitis in rats by elevating activities of SOD, and decrease levels of MDA due to its antioxidant and hepatoprotective effects [11]. Standard *Ginkgo biloba* extract, EGb 761, contains 22-27% flavonoids (ginkgo flavone glycosides) and 5-7% terpenoids (ginkgolides and bilobalides) [45]. The antioxidant effect of EGb has been linked to its main constituents, flavonoids and terpenoids, which can scavenge free radicals and reduce levels of reactive oxygen species [46]. Liu *et al*. [41] reported that due to the antioxidant potential of EGb, it was able to prevent oxidative stress- related diseases including cancers, cardiovascular diseases, degenerative diseases and central neural system disorders. In conclusion, the present study recorded that EGb administration succeeded to alleviate testicular tissue damage induced in mice after CP administration and this is may be due to its antioxidant activity.

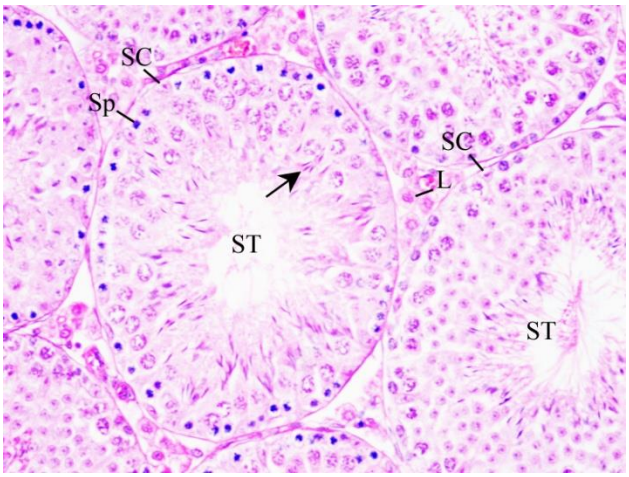

Fig. (1). Section in testis of a control mouse showing seminiferous tubules (ST), spermatogonia (Sp), Sertoli cells (SC), spermatozoa (arrow), and interstitial Leydig cells (L), (H&E, X 400).

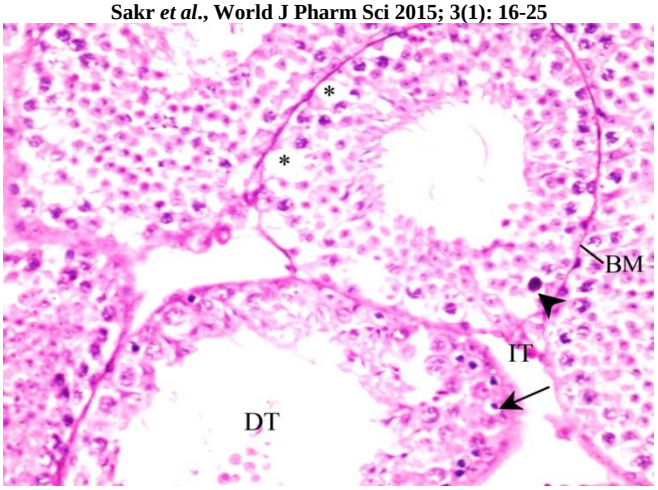

Fig. (2). Seminiferous tubules of a mouse treated with CP for 3 weeks showing irregular basement membrane (BM), less compact spermatogenic cells separated by vacuoles (*), pyknotic nucleus (arrow head), cytoplasmic vacuolization in spermatogonia (arrow), degenerated tubule (DT), and degenerated intertubular connective tissue (IT), (H&E, X 400).

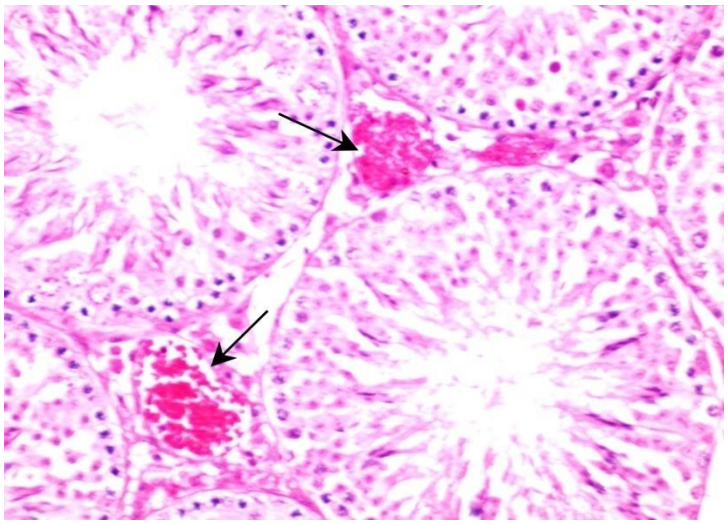

Fig. (3). Testicular tissue of a mouse treated with CP for 3 weeks showing congested intertubular veins (arrows), (H&E, X 400).

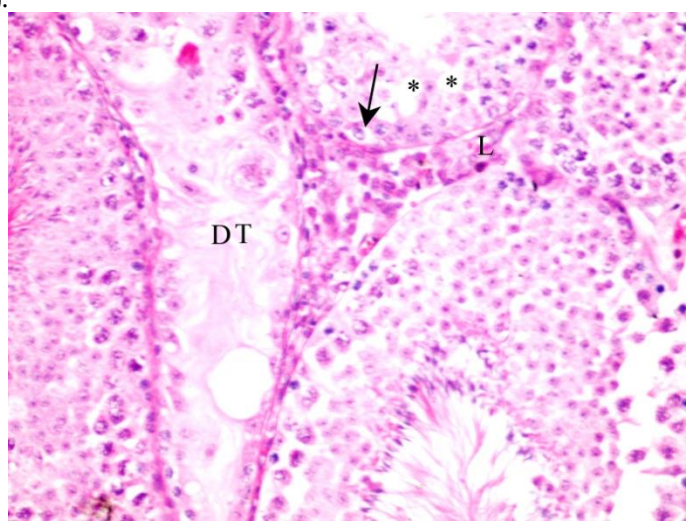

Fig. (4). Testis of a mouse treated with CP for 4 weeks showing degenerated seminiferous tubules(DT), cytoplasmic vacuolization (arrow), less compact spermatogenic cells separated with vacuoles (*), and degenerated Leydig cells(L), (H&E, X 400).

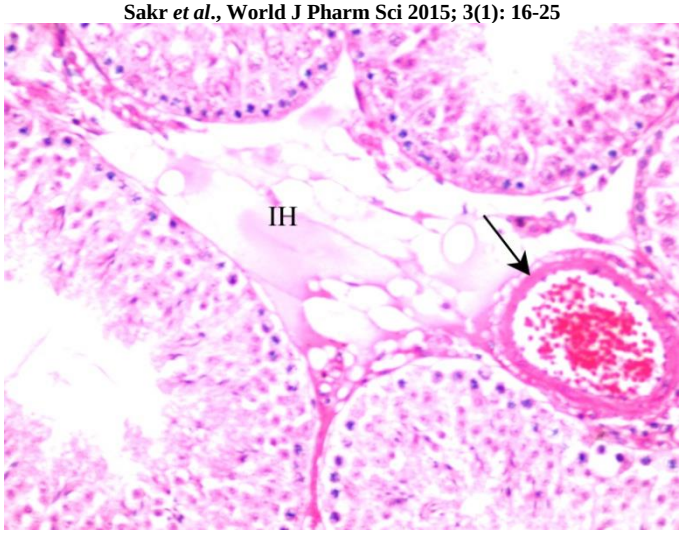

Fig. (5). Testis of a mouse treated with CP for 4 weeks showing congested intertubular arteriole (arrow) and intertubular hemorrhage (IH), (H&E, X 400).

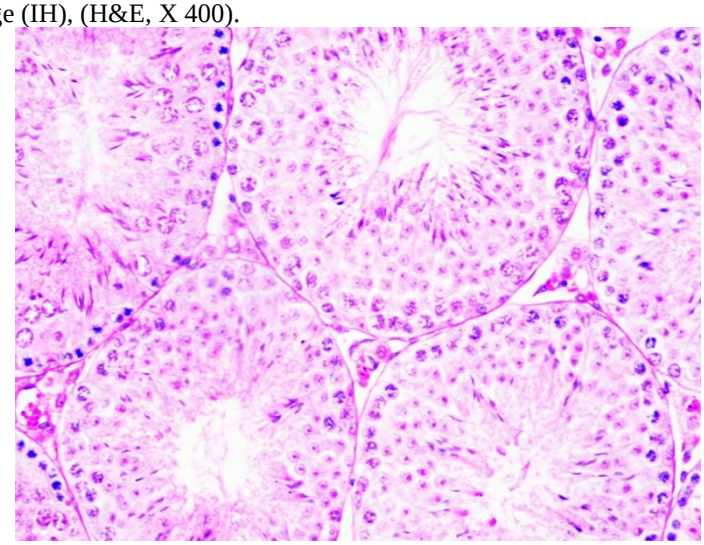

Fig. (6). Testis of a mouse treated with CP + EGb for 4 weeks showing seminiferous tubules with compact spermatogenic cells, and nearly normal Leydig cells (H&E, X 400).

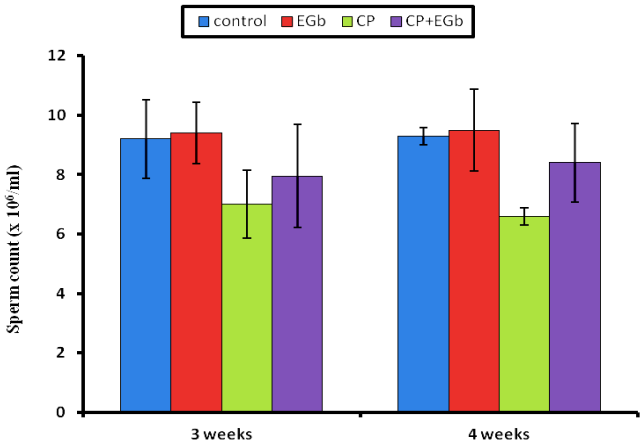

Fig. (7). Means of sperm count (x10^6/ml) in the different experimental groups.

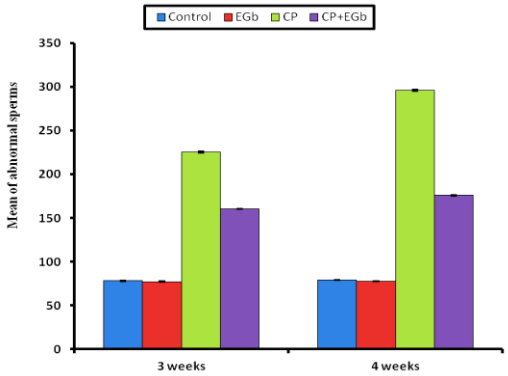

Fig. (8). Means of abnormal sperms/ 1000 examined sperm in the different experimental groups.

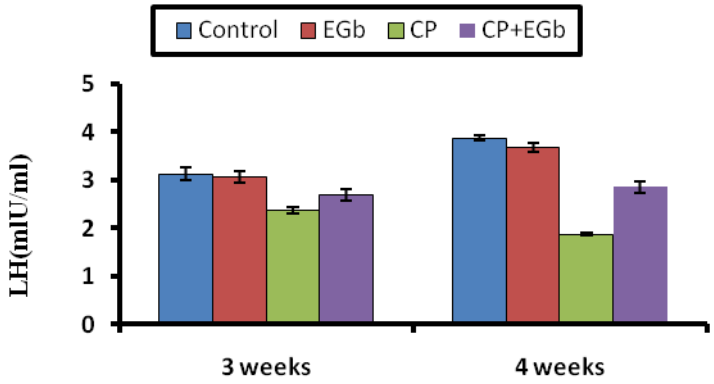

Fig. (9). Serum LH level in the different experimental groups.

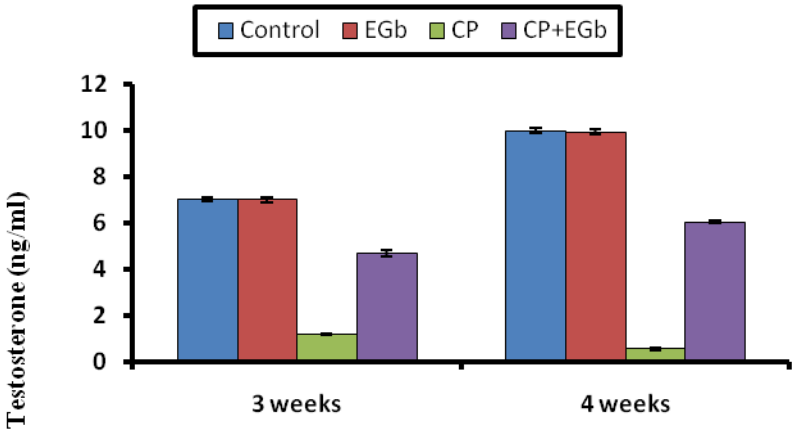

Fig. (10). Serum testosterone level in the different experimental groups.

Table (1). Mean of diameters of seminiferous tubules and epithelial heights (µm) in the different experimental groups.

Parameter / Group	Diameter of seminiferous tubules (µm) Mean ± SE		Epithelial Height (µm) Mean ± SE	
	3 weeks	4 weeks	3 weeks	4 weeks
Control	21.17 ± 0.07	21.16 ± 0.09	4.90 ± 0.07	5.04 ± 0.12
EGb	21.13 ± 0.05	21.19 ± 0.08	4.85 ± 0.05	5.00 ± 0.08
CP	21.24 ± 0.09	21.27 ± 0.06	3.47 ± 0.18*	3.33 ± 0.06*
CP+EGb	21.04 ± 0.04	21.16 ± 0.06	4.53 ±0.11**	4.74 ±0.11**

(*) Significant against control group
(**) Significant against CP group

Table (2). Testicular MDA, CAT and SOD in the different experimental groups.

Parameter / Group	MDA (nmol/g tissue) Mean ± SE		SOD (U /g tissue) Mean ± SE		CAT (µmol/g tissue) Mean ± SE	
	3 weeks	4 weeks	3 weeks	4 weeks	3 weeks	4 weeks
Control	41.78 ± 1.07	42.87±1.19	95.71±1.75	96.46±1.75	1.43 ± 0.06	1.49 ± 0.05
EGb	43.88 ± 1.59	41.33 ± 1.71	96.14 ±1.90	97.36±1.38	1.47 ± 0.05	1.52 ± 0.05
CP	61.78 ±2.11*	65.16 ±2.18*	89.48±1.87*	90.77±1.91*	0.93 ± 0.03*	0.97 ± 0.03*
CP+EGb	45.76±1.42**	46.89±1.48**	93.31 ±2.12	94.21±2.37	1.28 ±0.05**	1.35 ± 0.05**

(*) Significant against control group.
(**) Significant against CP treated group.

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