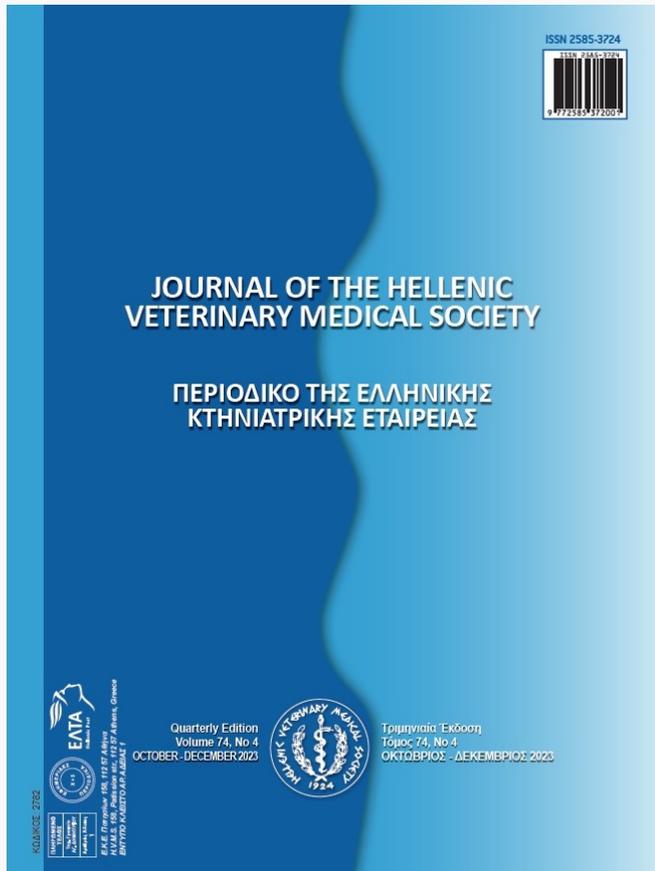


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Ultrastructure of mice testes affected by orally administered crocin of crocus sativus

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ABSTRACT: In this study, thirty-two adult male mice were divided into four groups control and experimental treatment groups received crocin at 4, 20, and 100 mg/kg daily for six weeks. As result, the testosterone level was increased significantly from 18.5 ng/dl in the control to 49.3 ng/dl in the group that received crocin at 20 mg/kg. The regular features of seminiferous tubules with increased Sertoli cells have been observed in the 20mg/kg crocin group.

In the group that received crocin at 100 mg/kg, many of the seminiferous tubules were atrophic, spermatogenic cells were discontinuous in the same areas while the space among them has been increased, and fewer interstitial tissue between seminiferous tubules was observed. These changes were markedly advanced by increasing the number of atrophied seminiferous tubules.

Electron microscopic results showed the large mitochondria with normal crista and dilated rough endoplasmic reticulum in spermatid cells of the 20 mg/kg crocin group.

In the 100 mg/kg crocin group, most of the cell cytoplasm showed vacuolated mitochondria with patchy matrix and distorted vision cristae, with many lysosomes, lipid droplets, and vacuoles. Some sperms had abnormal swollen nuclear and irregular wavy acrosomal systems.

The results of the present study showed that crocin at 20 mg/kg has a protective effect on the germinal epithelium of seminiferous tubules while at 100 mg/kg had destructive effects on the histological structure of the treated mice testicles.

In conclusion, treating healthy male mice with crocin 20 and 100 mg/kg for six weeks increased the testosterone concentration while 100 mg/kg damaged the testicle's structure.

Keywords: Crocin, Mice, Testis, Ultrastructure, Histology

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INTRODUCTION

Medicinal plants and natural supplements were used from old in many regions such as Mesopotamia, Egypt, India, Iran China, etc., and because of their lesser side effects and safety have been widely used over the past decades and are still used.

Numerous studies have revealed that the diversity of active compounds including phenols, glycosides, alkaloids, and terpenes which originated from primary metabolites of plants possess pharmaceutical effects (Hadizadeh et al., 2010; Khayatnouri et al., 2011; Lari et al., 2015).

One of the important wide spread traditional herbs in Iran is Saffron (*Crocus sativus*), which belongs to the iridaceous family (Hadizadeh et al., 2010; Khayatnouri et al., 2011; Lari et al., 2015; Licón et al., 2010).

Saffron (*Crocus sativus*) has been widely used as herbal medicine, spice, flavoring, and food coloring since ancient times. Crocin is the main component of saffron, a carotenoid with antioxidant properties.. Saffron is used in food color and taste, cosmetics, and also used in some other industries (Khayatnouri et al., 2011; Modaresi, Messripour, Asadi et al., 2008). Many studies described its ability to recover normal reproductive function in males having health-related problems (Khorasani et al., 2021; Roshankhah et al., 2019; Khayatnouri et al., 2016). We designed the current experiment to investigate the effects of daily doses of crocin administration on some reproductive parameters of male mice.

Analysis studies reported the active ingredient to contain four main compounds, including crocin, crocetin, picrocrocetin, and safranal along with other materials (Chen et al., 2015; Dianat et al., 2018; Moghaddasi, 2010; Roshankhah et al., 2017). Saffron properties rely on these compounds while crocetin and crocin are the main integral agents responsible for its coloring (Hosseinzadeh et al., 2009; Tong et al., 2018).

Studies have reported the various effects of saffron and its constituent as a potential therapeutic plant, anti-inflammatory, antioxidant, and anti-tumor (Bakshi et al., 2009; Ghafarzadeh et al., 2019; Rashedinia et al., 2015). Crocin is a chemical extraction carotenoid, with high solubility in water. It was used as a strong inhibitor in many kinds of cancers (Sun et al., 2011). It might have anti-cancer activities against ovarian

carcinoma cells, fibrosarcoma, osteosarcoma, and leukemia (Abdullaev & Espinosa-Aguirre, 2004), and may have cancer preventive and cancer therapeutic profit in human breast cancer (Lu et al., 2015).

In one interesting study its application as a sexual potential stimulant and its significant value in prostate cancer treatment by reducing androgen effects in testicular tissue has been reported (Salahshoor et al., 2016; Sefidgar et al., 2019). It can also prevent experimental benign prostatic hyperplasia by inhibiting prostate cell proliferation, inflammation, and angiogenesis (Qar et al., 2022). Crocin has been reported as a protecting agent which ameliorates testicular toxicity and improves perturbation endocrine function induced by exposure to cadmium (Abo Edraet al., 2018). The outcomes of the other investigation appeared that crocin could be a valuable agent to reduce testicular tissue damage following Ischemia-Reperfusion (I/R) injury (Ganjiani et al., 2021) and diabetes (Ataei et al., 2019).

Also crocin treatment improved diabetic testopathy and impairment of seminiferous tubules induced by high-fat diet and streptozotocin. In conclusion, diabetes mellitus in male rats caused pathological changes in testis, and that crocin treatment improved these deficits by increasing the number of sperms and sperm production. (Mirzaee et al., 2019).

It also improves the height of the epithelium in testicular tissue after mice were exposed to atrazine for 23 and 75 days (Fani et al., 2018). Phytochemical constituents of saffron “crocin” and “safranal” were assessed for their aphrodisiac potential and erectile dysfunction of mice treated especially at doses 160 and 320 mg/kg, in addition, the antiproliferative effects on prostate cancer cell line via a dose-dependent style (Pratap & Rajender, 2012).

Also, crocin is one of the most important constituents of saffron to reduce sterility or fertility resulting from the side effect of cyclophosphamide in treated men. The antioxidant effects of crocin on the testis significantly increase sperm quality by increasing the antioxidant capacity of testicular tissues and blood serum (Bakhtiary et al., 2014; Jalili et al., 2015). Crocin and other saffron ingredients are very important for the reproductive system to activate sexual desire and treat impotency. They can be used as an activating generator of sperms, also considered useful for uterus ulcers, regulating the menstrual cycle and dysmenorrhea, to cure uterus pain if used in low concentrations.

It has been shown that the use of high concentrations during pregnancy may cause constrictions of the uterus (Moallem et al., 2016; Cao et al., 2014).

The common effective dose of crocin 20 mg/day, is considered safe for animal administration and non-toxic for human consumption (Bathai & Mousavi, 2010).

Administration of crocin have been increased testosterone levels in serum and the activity of the Leydig cells which is the main part of the steroidogenesis process of Leydig, and it may be due to reducing the hypophyseal-hypothalamic sensitivity, it seems that crocin antioxidants effects can be ameliorated sexual hormone concentration (Asadi et al., 2014; Modaresi et al., 2008).

Our result in the anothe work revealed that serum testosterone levels significantly increased in both crocin groups (20mg/kg) and (100mg/kg) compared with the control group, which clears that crocin administration ameliorates the endocrine testicular function. However, the mechanism by which the serum testosterone level increased by crocin administration provided miscellaneous information (Rahim Hussein Al-Fartwsy et al., 2022).

The effectiveness of crocin is explained by the increase of testosterone, FSH, and LH, This active substance affects the hypothalamus-pituitary axis, increasing the concentration of the LH hormone, which

in turn increases the activity of the leydig cells and releases testosterone hormone (Khazdair et al., 2015).

The exact mechanism by which crocin alters hormones synthetics, especially testosterone and spermatogenesis which in turn affected the male reproductive system is not yet fully understood. Therefore, this study aimed to elucidate long-term oral administration of crocin with different dosages on histomorphology and ultrastructure changes of testicular tissue in mice.

MATERIAL AND METHODS

Chemicals

Crocin powder ($C_{44}H_{64}O_{24}$) was purchased from Abo-Ali Company, Mashhad, Iran (Figure 1). In this experimental study, 2 g crocin powder was first dissolved in 200 ml distal water to prepare different doses of crocin solution, 4 mg/kg, 20 mg/kg, and 100 mg/kg for administration. Figure 1 shows the chemical structure of crocin (Mard et al., 2017). Buffer formalin(Merck,Germany), Ethanol (MojallaliCompany, Iran), Hematoxylinandeosin (Merck,Germany), Local anesthetic cream (lidocaine, Abureyhan, Iran) were used. ELISA testosterone kit was purchased from Cayman Chemical, USA

Animals

Thirty-two adult NMRI male mice (3 months) weighing 30-40 grams were obtained from the Fac-

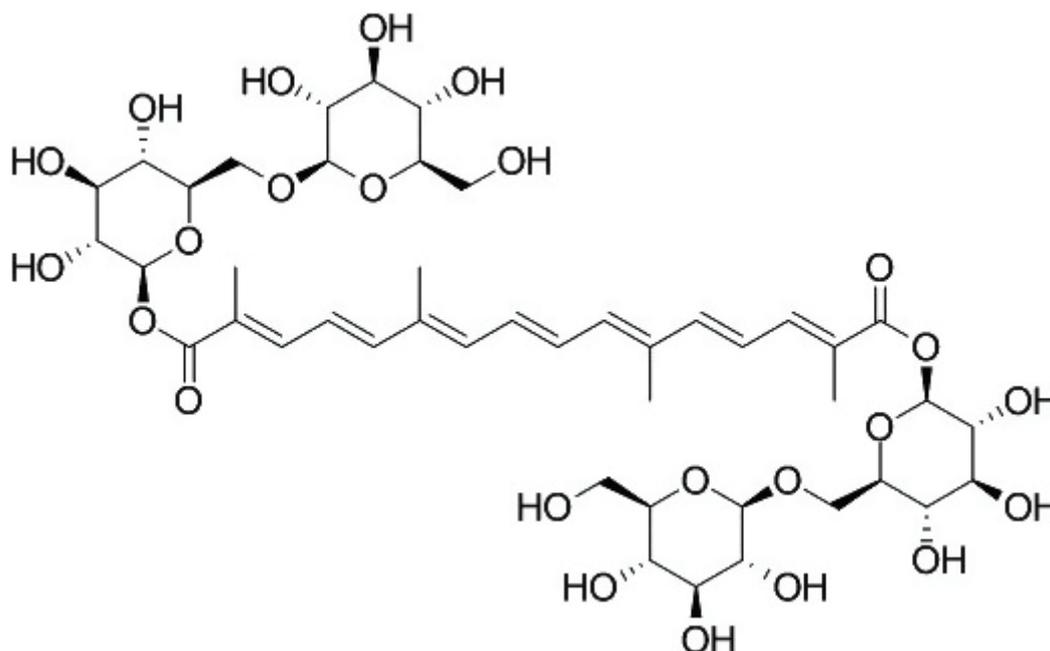


Figure 1. Chemical structure of crocin ($C_{44}H_{64}O_{24}$)

ulty of Pharmacy, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran and housed in the Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. The animals were kept in the animal house for two weeks at a temperature of 22 ± 2 °C under the controlled environmental condition of 12/12 hours light /dark cycle and were allowed tap water and standard rodents diet (Behparvar company, Karaj, Iran). According to the National Institute of Health, all experimental protocols were performed, Guide for the Care and Use of laboratory animals (Council, 2010).

Ethical Approval

This study was approved by the Research Ethics Committee (IR.UM.REC.1400.162) following the instruction for the care and use of laboratory animals prepared by the Ferdowsi University of Mashhad, Mashhad, Iran.

Experimental design

The mice were randomly divided into four groups of 8 each, and their weights were measured individually and recorded before the start of dosing. The first group received daily normal saline as the control group. The second, third, and fourth groups received daily crocin at doses of 4, 20, and 100 mg/kg respectively (Bukhari & Manzoor, 2018; Bathaie & Mousavi, 2010). All dosages were given to mice by gastric intubation (gavage) with a force-feeding needle and continued once daily for six weeks. The amount of gavage (in 1 ml solution) was regulated every four days according to the diverse weight of each mouse. The animals were weighed for their body weight analyses at weekly intervals.

Blood samples for testosterone assay

Blood sample: Blood samples were collected from the tail vein (1 ml) of animals. At first, local anesthetic cream (lidocaine, Abureyhan, Iran) was applied on the surface of the tail 30 min before the experiment, disinfected by alcohol, and then a 23G needle was inserted into the blood vessel and blood was collected. The serum was isolated by centrifugation at 3000 rpm for 10 minutes immediately and kept at -20 °C. Using the ELISA method Testosterone kit of Cayman Chemical, USA was utilized for the experiment.

ELISA Method: The normal range was calibrated and then 25 µl serum samples were taken in the well plates. 100 µl of enzyme conjugate was added to each well. After that, it was left for incubation at 37°C in

the incubator for 1 hour. Then, the wells were washed with 300 µl distilled water at least 3 times and blotted. Then, 100 µl TMB solution was added as substrate in each well plate and was again left for incubation for 15 minutes for the color. Finally, 100 µl stop solution was added to each well to stop the reaction. Reading was taken at 630 nm through Merck ELISA reader in ng/ml value.

Finally, animals were sacrificed under deep CO₂ anesthesia at the end of the period for taking their tissue samples.

Testis weight

At the end of the treatment period (42 days), the peritoneal cavity of animals was opened through a lower transverse abdominal incision and their testes were carefully removed, weighed individually, and recorded.

Tissue preparation

Light microscope

The left testis was carefully removed for each mouse and fixed in 10% buffered formalin for 24-48h. After fixation, tissue samples were transferred to the histology laboratory of the Ferdowsi University of Mashhad for tissue preparation. All of the tissues were placed in autotechnicon tissue processor for dehydration by ascending degrees of alcohol, clearing using xylene, and embedding by paraffin wax. After preparing paraffin, tissue blocks were prepared by paraffin dispenser, and tissue sections (5 µm) were prepared by microtome and then stained with Hematoxylin-eosin and Masson's trichrome. The light microscope (Olympus DP25, Tokyo, Japan) was used for histological observations, and photos were taken using a high-resolution digital camera (Olympus, Tokyo, Japan).

Electron microscope

The right testis was carefully removed from each mouse and a small piece of it (1 mm³) was fixed in 2.5% glutaraldehyde (pH 7.4) in 0.1 M phosphate buffer and then post-fixed for 1h with 2% aqueous osmium tetroxide at 4 °C. Then dehydrated by different grade of ethyl alcohol, cleared in toluene for 8 min and embedded in Epon resin. Semithin sections of 1 µm thickness were cut using an ultramicrotome and stained with 0.5% toluidine blue to be examined under the light microscope. Ultrathin section (50-60 nm thickness) was obtained by ultramicrotome from the

selected blocks, mounted on copper grids for double staining uranyl acetate and lead citrate for 20 min and examined by transmission electron microscope (Leo 912 AB, Zeiss, Germany).

Data Analysis

The final results are reported as mean \pm SD for eight animals in each group. The statistical analysis was carried out on a computer using the SPSS version 24 software (SPSS/IBM, Chicago, IL) (ANOVA) and Tukey's test ($P < 0.05$) to determine the significance of individual differences between the control and other treatments groups.

RESULTS

Effects of crocin on the body and testis weight

The administration of various doses of crocin for six weeks caused no statistically significant difference in the body and testis weight. The mean body weight was 38-41 g on the first day and it was increased to 40-43 g at the end of the experiment (Table 1). By comparing the weight of the right and left testis

we concluded that there is no significant difference but on the left side, it was decreased in the control (0.12 ± 0.024 g) to crocin 100mg/kg (0.104 ± 0.019 g) group (Figure 2).

Effects of crocin on the level of serum testosterone

Crocin groups (20 mg/kg) showed a significant increase in testosterone levels in blood serum compared to the control group. No significant difference in testosterone level was observed in the crocin group with a dose of 4 and 100 mg/kg compared to the control group. Although the level of testosterone increased in the crocin group with a dose of 100 mg/kg, but it was not significantly different from the control group. (Figure 3).

Testis histology by light microscopy

Histopathology study showed that mouse testes in the control group had normal testicular architecture with regular seminiferous tubular morphology and the presence of spermatogonia A & B, spermatocytes, spermatid, and spermatozoa arranged from the basal compartment to the lumen compartment. Most semi-

Table1: Effect of various dosages of crocin on the mice body weight (g)

Day Group	1	9	17	25	33	41
Control	38.37 \pm 3.701	40.12 \pm 2.85	39.12 \pm 5.668	39.87 \pm 5.817	41.5 \pm 5.928	42.37 \pm 5.344
Crocin4mg/kg	34.37 \pm 3.42	36.87 \pm 4.155	37.5 \pm 3.891	38.62 \pm 4.207	38.5 \pm 3.928	39.37 \pm 4.241
Crocin20mg/kg	37.2 \pm 2.712	37.87 \pm 2.748	39.625 \pm 1.847	39.5 \pm 2.507	40.5 \pm 2.507	41.5 \pm 2.673
Crocin100mg/kg	36.5 \pm 3.162	35.75 \pm 3.059	38.375 \pm 2.2	38.5 \pm 1.773	39.62 \pm 2.446	39.25 \pm 1.753

Data were represented as Mean \pm SD for eight mice each group. No Significant difference with $p < 0.05$

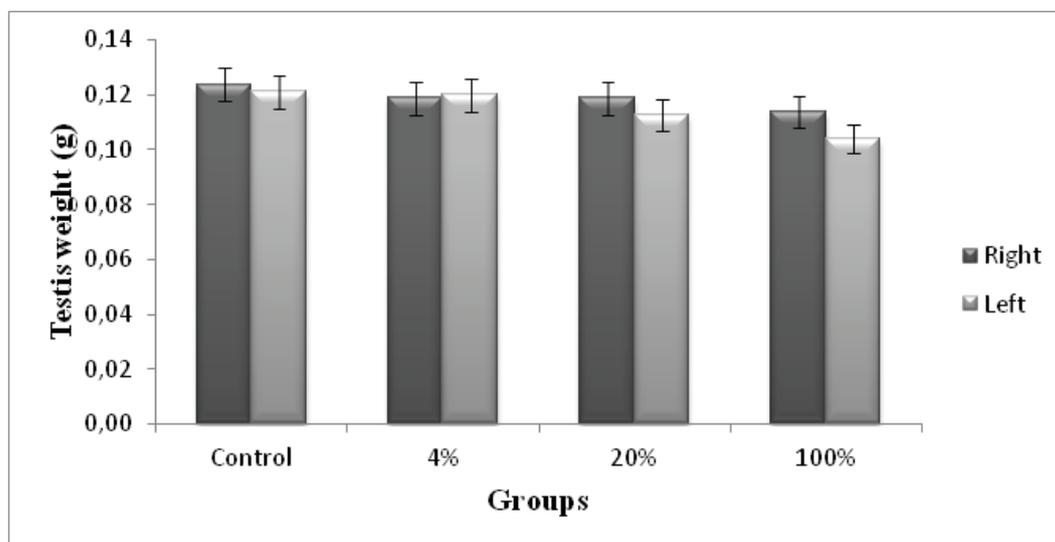


Figure 2. Effect of crocin on testis weight. No Significant difference with $p < 0.05$

No Significant difference with $p < 0.05$

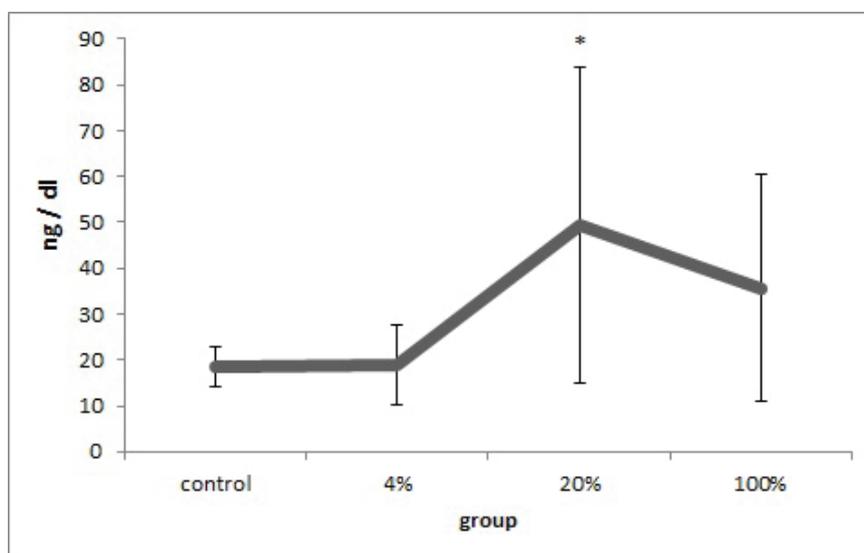


Figure 3. Effect of crocin on the level of testosterone. *significant with $P < 0.05$.

niferous tubules appear obnoxious appearance of mature sperms in the lumen. The seminiferous tubules also contain Sertoli cells which are resting on the basement membrane between spermatogonia, which ovoid nuclei having elucidated chromatin and prominent nucleoli. The primary spermatocytes, which are spherical cells with euchromatic nuclei were characterized by their large size, located next to the spermatogonia. The rounded spermatids were characterized by their small size and small rounded centrally located nuclei. All stages of spermiogenesis occur with the cells intimately associated with the surfaces of adjacent Sertoli cells. Outside the tubules are found flattened myoid cells (Figure 4A, 5A). Intercellular bridges connected the spermatogenic cells each to other. The blood vessels can be seen between the seminiferous tubules in the interstitial tissue were surrounded by single Leydig cells or in clusters shapes (Figure 6A).

Section of seminiferous tubules with interstitial tissue in the group at 4mg/kg crocin, appeared to disorganization of cellular elements within the seminiferous tubules and the spermatozoan population reduced compared with the control group. The disorganization of the germ cells was with increased intercellular spaces between the primary spermatocyte and spermatid. The tubules appear in the section differently, as they are spaced from some of them, and voids can be seen in the interstitial tissue that surrounds the tubule. Also, some tubules contained small numbers of Sertoli cells and mature sperm. with interstitial tissue organized which have blood vessels (Figures 4B, 5B, 6B).

The 20mg/kg crocin group showed regular features of seminiferous tubules, interstitial tubule tissues were well organized when compared to Crocin 4mg/kg group. Some primary spermatocyte cells may appear in the stage of mitosis division (Figure 4C). there is limited spermatogenesis and some contain spermatogenesis cells of different sizes and the hyperchromatic nuclei of primary spermatocyte, with a distinct appearance of active spermatogonium and resting on a basement membrane. the average number of Sertoli cells was significantly increased in this group whose peaks are associated with clusters of elongated spermatids which crowded with nuclei more elongated and irregularly shaped present next to the Sertoli cells resting on the basement membrane can be seen (Figure 5C). One of the most important features diagnosed in this group is the increased interstitial tissue between the seminiferous tubules. Numerous spindle-shaped flat myoid cell-surrounded tubules can be seen (Figure 6C).

Leydig cells show fewer changes within the interstitial tissue the blood vessels are observed in wide ranges. Interstitial cells of Leydig are normal shapes but in some areas seem to be grouped too much appear around dilated blood vessels these cell has large cytoplasm full of lipid droplets. The seminiferous tubules appeared with cells of an organized shape, and spermatogenic cells spread from the basement membrane to the lumen which is full of mature sperms (Figure 6C). Crocin 20mg/kg can affect the spermatogenesis and steroidogenesis processes of Leydig cells.

In the group treated with crocin at 100mg/kg many

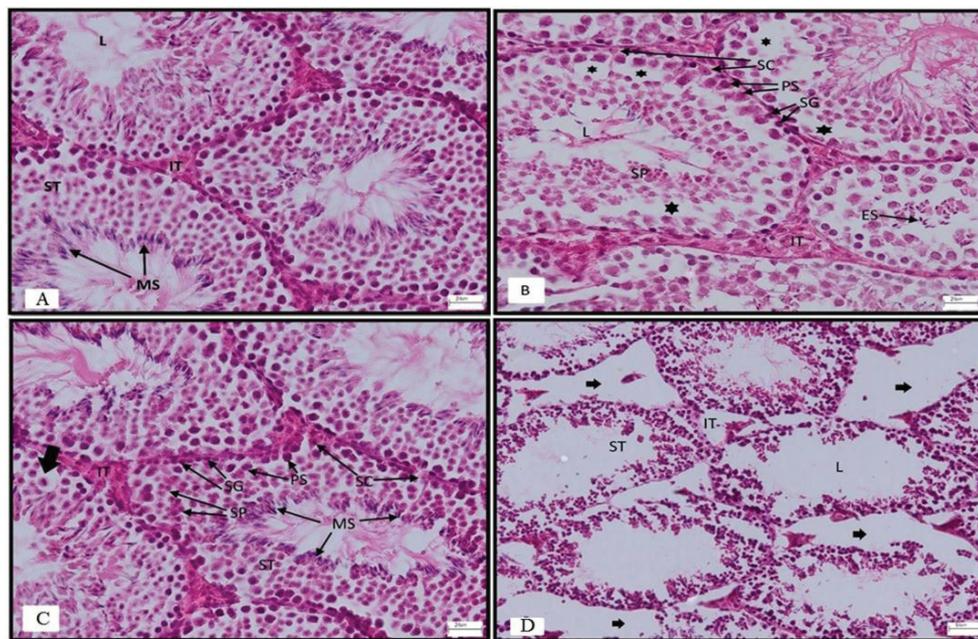


Figure 4. Photomicrograph of section of the mice testis in control group (A, $\times 400$) and experimental treatment groups received crocin at dose of 4(B, $\times 400$), 20(C, $\times 400$), and 100(D, $\times 200$) mg/kg. H&E staining. Normal arrangement of spermatogenic and Sertoli cells on basement membrane in control group (A). Some seminiferous tubules show the presence of focal empty areas of spermatogenic cells between the primary spermatocyte and spermatid (Black star) in treatment group 4mg/kg(B). Some primary spermatocytes appeared in the stage of mitosis division (arrowhead) in treatment group 20mg/kg(C). Many of the seminiferous tubules were atrophic and increased spaced between tubules (arrowhead) in treatment group 100mg/kg(D).

Lumen(L), Seminiferous tubules (ST), Mature sperm (MS), Interstitial tissue (IT), Sertoli cell (SC), Primary Spermatocyte (PS), Spermatogonia (SG), Spermatid (ES)

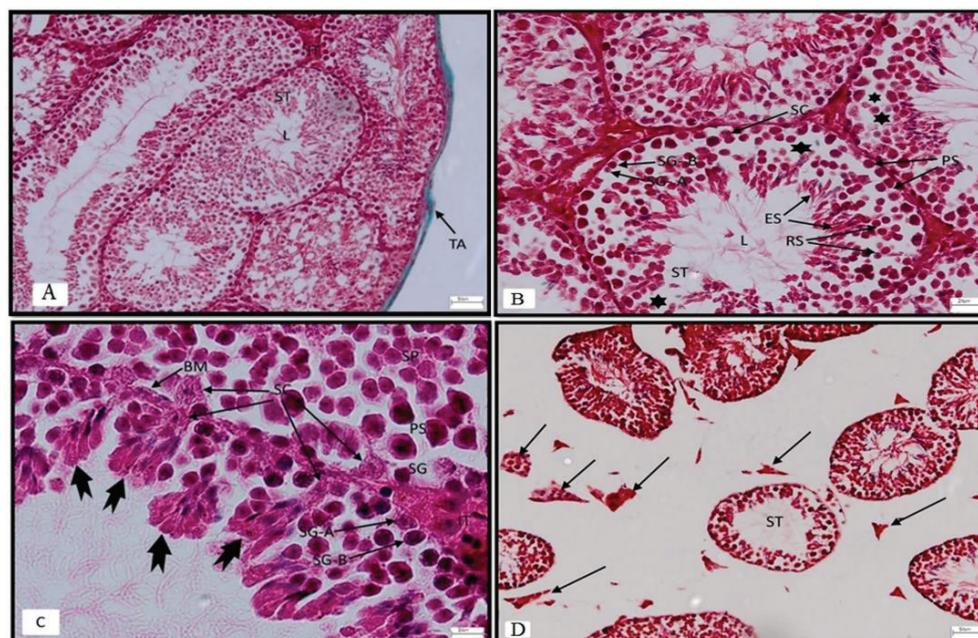


Figure 5. Photomicrograph of section of the mice testis in control group (A, $\times 200$) and experimental treatment groups received crocin at dose of 4(B, $\times 400$), 20(C, $\times 1000$), and 100(D, $\times 200$) mg/kg. Masson trichrome staining. Testis surrounded by tunica albuginea and normal arrangement of spermatogenic and Sertoli cells on basement membrane in control group(A). Many Seminiferous tubules appeared wide space between them (Black star) in treatment group 4mg/kg(B). Sertoli cells were increased on the basement membrane, between spermatogonium and spermatid cells looks like clusters at the apical of lumen (arrowhead) in treatment group 20mg/kg (C). Many tubules had completely disorganized and the interstitial tissue was separated (arrow) in treatment group 100mg/kg(D).

Tunica albuginea (TA), Lumen(L), Seminiferous tubules (ST), Sertoli cell (SC), Primary spermatocyte (PS), Spermatogonia (SG), Spermatid(ES), Round spermatid(RS), Basement Membrane (BM).

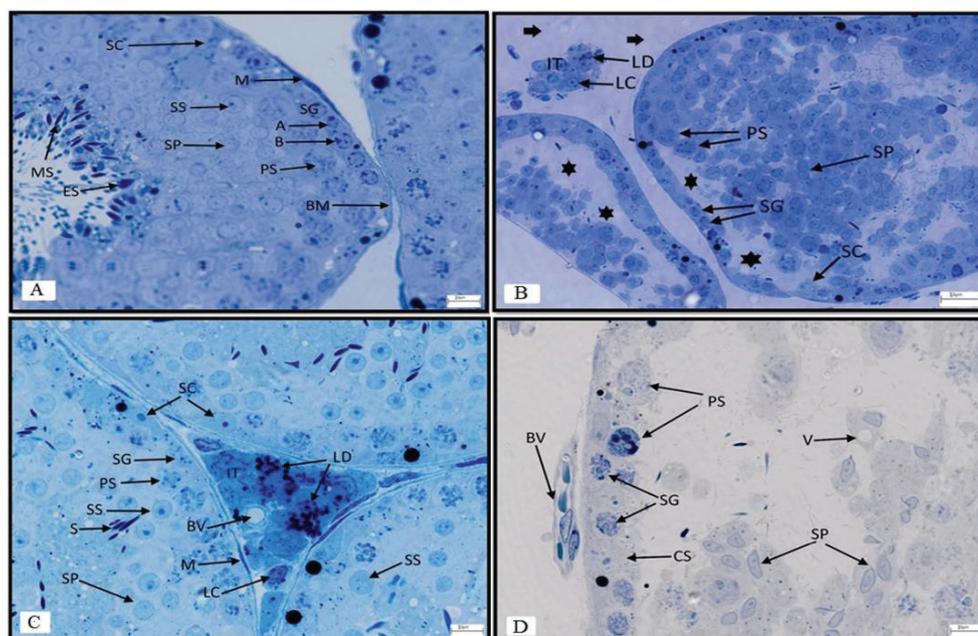


Figure 6. Photomicrograph of section of the mice testis in control group (A) and experimental treatment groups received crocin at dose of 4(B), 20(C), and 100(D) mg/kg. Toluidine blue staining, $\times 1000$

Normal arrangement of spermatogenic and Sertoli cells on basement membrane in control group (A) and focal empty areas between spermatogenic cells (black star) in treatment group 4mg/kg (B). Seminiferous tubules contained spermatogenic and Sertoli cells with interstitial tissue in between them, the blood vessels surrounded by Leydig cells and lipid droplets in treatment group 20mg/kg (C).

Abnormal architecture of seminiferous tubules with decreased germinal epithelium in treatment group 100mg/kg (D).

Primary Spermatocyte (PS), Spermatid (SP), Sperm (S) or Mature Sperm (MS), Spermatogonia (SG), Spermatogonium A (A), Spermatogonium B (B), Lipid droplets (LD), Myoid cells (M), Blood vessels (BV), Basement membrane (BM), Interstitial tissue (IT), Sertoli cell (SC), Leydig cells (LC), Vacuolated cytoplasm (V).

of the seminiferous tubules were atrophic, spermatogenic cells were discontinuous in the same areas and increased spaced between them, with fewer interstitial tissue between them (Figure 4D). Spermatogonia and primary spermatocytes have a small size with small heterochromatic nuclei, The changes were markedly advanced by an increase in the number of atrophied seminiferous tubules, and total loss of spermatogenic cells inside the tubules. Interstitial tissue was widely separated and there is also an irregular arrangement (Figures 4D, 5D). Leydig cells appeared abnormal architecture and others were crowded with fat droplets around dilated blood vessels. Some tubules contained little numerous spermatogenic and mature sperm in the lumina of tubules. Also, some tubules appeared devoid of spermatogenic cells and mature sperm. There are many abnormally shaped spermatids in addition to have vacuolated cytoplasm. an irregular arrangement with few sertoli cells and spermatogenic cells (Figure 6D).

Testis histology by electron microscopy

Histopathology study by electron microscopy showed that in the control group seminiferous tu-

bules contained spermatogenic cells, formed of type A and B spermatogonia (type A small size possesses a large oval nucleus containing finely granular nucleoplasm with scanty cytoplasm, type B bigger size is characterized by a spherical nucleus dark containing numerous chromatin clumps and sprinkled nucleolar fragments), and Sertoli resting on the basement membrane.

The cytoplasmic type B spermatogonia contained several normal mitochondria with crista. The primary spermatocytes were large and possess large rounded central nuclei that have condensed chromatin and are located next to the spermatogonia.

Sertoli cells contained large nuclei and connected with germ cells by numerous intercellular junctions (Figure 7A, B). Round spermatids were identified by their acrosomal vesicle over the anterior part of the round nucleus which seemed smaller than that spermatocyte nucleus. In a round spermatid, the cytoplasm contained smooth endoplasmic reticulum, and mitochondria near the nucleus, and numerous lysosomes, lipid droplets, centrioles situated peripherally. The elongated spermatids were found next to the

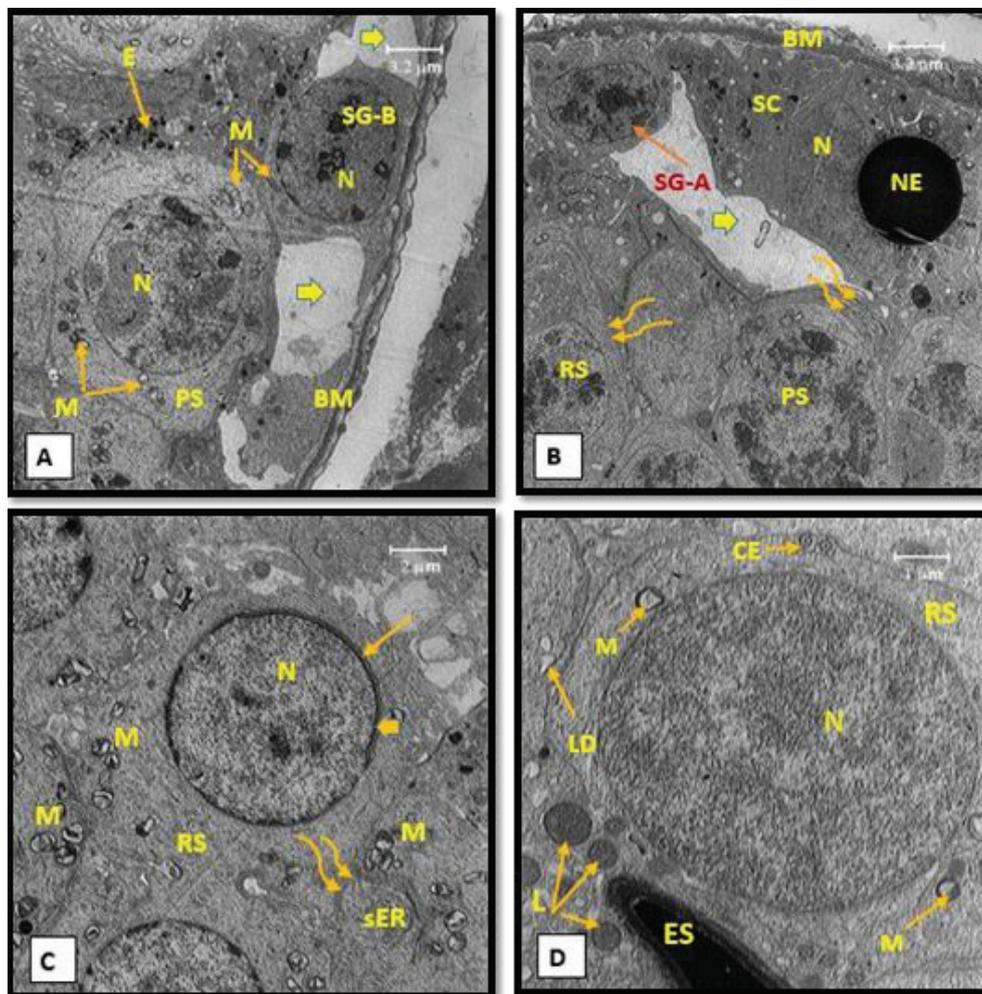


Figure 7. Ultrastructure of the section of a seminiferous tubule of control mice. A. Spermatogonia type B (SG-B) (N) basement membrane (BM), and Primary Spermatocyte (PS). Mitochondria (M), Electron dense (E), Intercellular spaces (arrowhead). B. Spermatogonia type A (SG-A), and Sertoli Cell (SC) Nuclei (N) and nucleolus (NE), intercellular junctions (curved arrow), Primary Spermatocyte (PS), Round Spermatid (RS) have Acrosomal Vesical (arrowhead), the nuclear membrane is thickened (arrow), Mitochondria (M) and smooth Endoplasmic Reticulum (sER). D. RS with Nucleus, Mitochondria (M) centriole (CE) located peripherally, and Lysosomes (L), Lipid Droplets (LD), The Elongated Spermatid (ES) near round spermatids. Magnifications: A 3.2 μm ; B 3.2 μm ; C 2 μm ; D 1 μm .

lumen of seminiferous tubules and identified by their elongated distinctly stained nuclei (Figure 7C, D).

In the group treated with crocin at 100 mg/kg seminiferous tubules contained types A and B spermatogonia and Sertoli cells resting on a thick irregular basement membrane.

Wide intercellular space was observed between types A and B spermatogonia and Sertoli cells. The types A spermatogonia were observed with oval nuclei containing marginated coarse clumps of heterochromatin with little cytoplasm and few mitochondria. Spermatogonia appear larger than spermatogonia and have a round nucleus with chromatin granules in variable size (Figure 8A). The hyperchromatic nuclei of primary spermatocyte cells were randomly distribut-

ed. Intercellular bridges were observed normal among spermatogenic cells.

Mitochondria aggregated in the cytoplasm, while many of them appeared normal with tubular crista, and with the smooth endoplasmic reticulum, and lysosomes (Figure 8B).

The round spermatid showed an acrosomal system and was seen spreading acrosomal vesicles over its round nucleus where the nuclear membrane was thickened. With rigorous intercellular adhesion and intercellular junction, dilated smooth endoplasmic reticulum could be seen (Figure 8C). The late stage of spermatogenesis elongated spermatid with an elongated electron-dense nucleus surrounded by an acrosomal system, which was diffuse next to the luminal

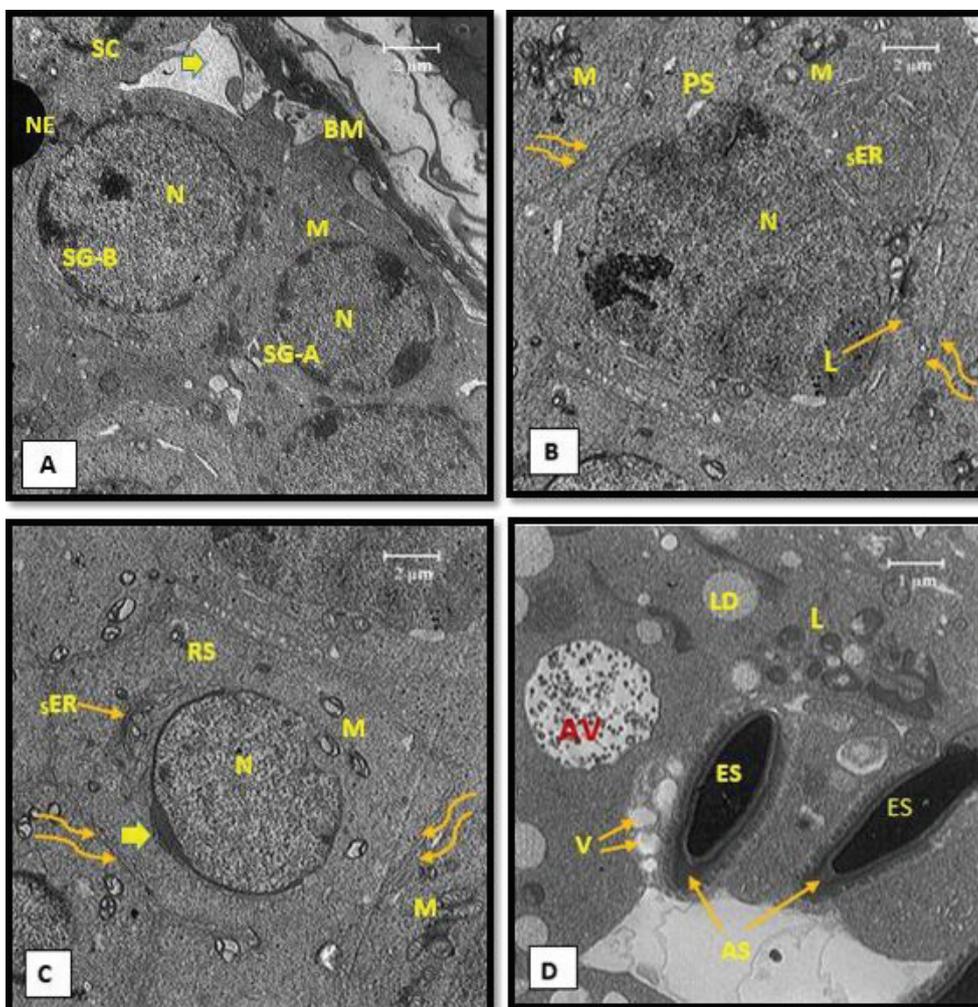


Figure 8. Ultrastructure of the section of a Seminiferous Tubule of Crocin 4mg/kg group Mice. A. Spermatogonia type A (SG-A), B (SG-B) and Sertoli Cell (SC) Basement Membrane (BM) Wide Intercellular Space (arrowhead), Sertoli Cell (SC) Contained Nucleolus (NE), Mitochondria (M), Nucleus (N). B. Primary Spermatocyte Cell (PS), Nuclei (N), Intercellular Bridges (curved arrow), Mitochondria (M), Smooth Endoplasmic Reticulum (sER), Lysosomes (L). C. Round Spermatid Cells (RS) have a round Nucleus (N) with Acrosomal Vesical (arrowhead), rigorous Intercellular Adhesion and Intercellular Junction (curved arrow), Smooth Endoplasmic Reticulum (sER), Mitochondria (M). D. Elongated Spermatid (ES) with Elongated Electron-dense Nucleus surrounded by Acrosomal System (AS), the luminal part of seminiferous tubules contained cytoplasmic Autophagic Vacuoles (AV), lipid droplets (LD), numerous of the lysosome (L) and cytoplasmic Vacuoles (V) could be seen. Magnifications: A 2 μm ; B 2 μm ; C 2 μm ; D 1 μm .

part of seminiferous tubules, and contained cytoplasmic autophagic vacuoles, lipid droplets, numerous of lysosome and cytoplasmic vacuoles could be seen (Figure 8D).

An electron micrograph of a group treated with crocin at 20 mg/kg showed irregular contours of seminiferous tubules, with an apparent decrease in germ cell numbers, spermatogonia and Sertoli cells separated on the thickened basement membrane, Sertoli cells possess a large disorganized shape nucleus with the dark indented nucleolus, the cytoplasm process numerous vacuoles, some degenerated mitochondria, many electron-dense particles, lysosomes and their tight junction which were distorted between

Sertoli and spermatogenic cells. Many primary spermatocytes showed nuclei with the dilated nuclear membrane and swollen mitochondria (Figure 9A, B). Round spermatids with a round nucleus and four elongated spermatids (at the late of spermatogenesis) could be seen (Figure 9C). Also, in their cytoplasm abnormally enlarged mitochondria, cytoplasmic autophagic vacuoles, and elongated spermatid with an elongated electron-dense nucleus surrounded by an acrosomal system at the one end of the nucleus have been observed.

Sperms are formed of arrangement mitochondria in the middle piece surrounded by the fibrous sheath, then covered by the plasma membrane, the end piece

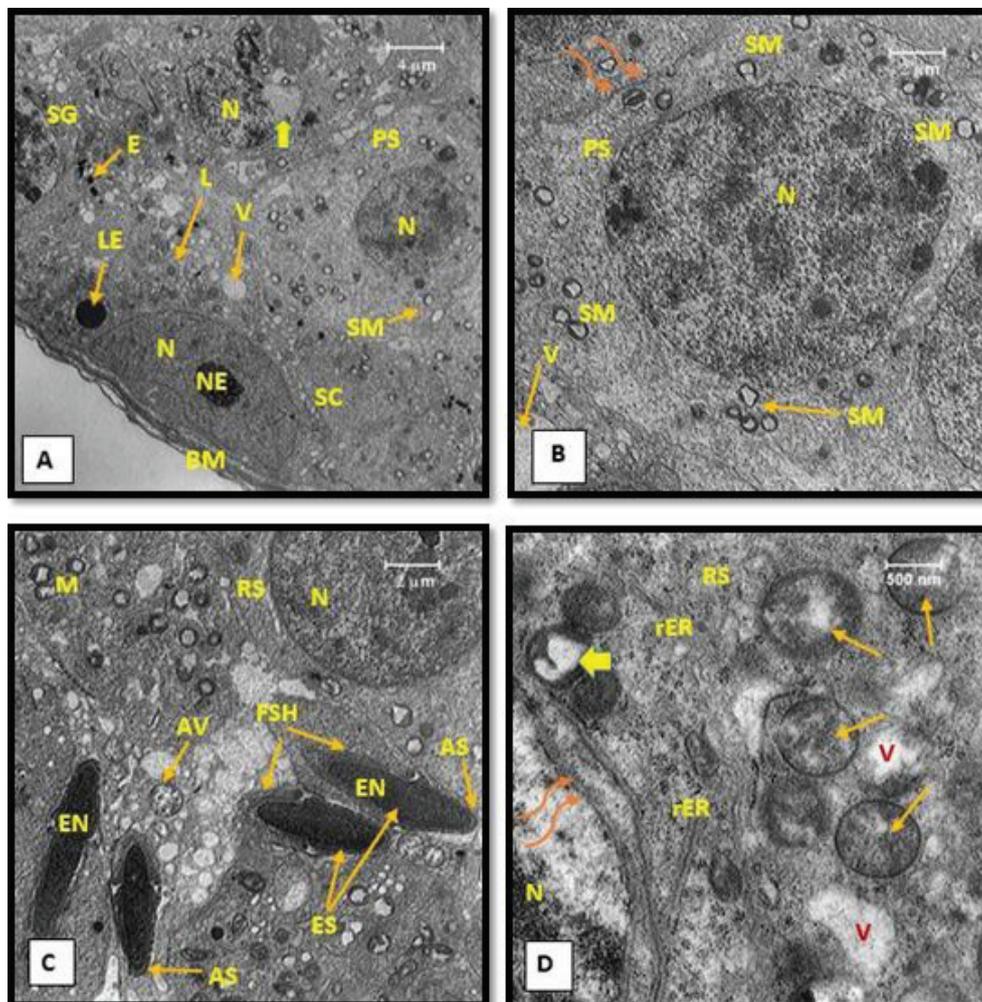


Figure 9. Ultrastructure of section of a Seminiferous Tubule of Crocin 20mg/kg group Mice. A. Spermatogonia cell (SG) and Sertoli Cells (SC) separated on thickened Basement Membrane (BM), Sertoli cell possesses a large nucleus (N) with dark indented Nucleolus (NE), the cytoplasm was contained Vacuoles (V), Large Electron-dense (LE) and small particles (E), Lysosomes (L). Primary Spermatocyte (PS) showed Nuclei (N) with dilated nuclear membrane (arrowhead) and Swollen Mitochondria (SM). B. Primary Spermatocyte (PS) their tight junction was distorted between spermatogenic cells (curved arrow), and Swollen Mitochondria (SM). C. Round Spermatids (RS) with four Elongated Spermatid (ES) could be seen, cytoplasmic Autophagic Vacuoles (AV), Mitochondria (M), elongated spermatid has Elongated Electron-dense Nucleus (EN) surrounded by Acrosomal System (AS), and the Fibrous Sheath (FSH). D. Round Spermatids (RS) cytoplasm contained swollen mitochondria (arrow) some mitochondria have vacuole (arrowhead), dilated rough Endoplasmic Reticulum (rER), Vacuoles (V) contained multiple vesicles and dilated nuclear membrane (curved arrow). Magnifications: A 4 μ m; B 2 μ m; C 2 μ m; D 500 μ m.

of the tail has consisted of the nucleus on the side opposite of the acrosome system (Figure 9C).

Many round spermatids cytoplasm contained swollen mitochondria and showed irregular disruption (of what) with distorted crista. Some of their mitochondria have vacuoles, dilated rough endoplasmic reticulum, and cytoplasmic vacuoles which contained multiple vesicles and dilated nuclear membrane (Figure 9D).

Electron microscopic examination of the group treated with crocin at 100 mg/kg showed that most of the spermatogenic cells were disorganized, with a de-

crease in germ cell number. Appeared spermatogonia and a small number of Sertoli cells resting on a thick hyalinized basement membrane. The spermatogonium type A revealed a small heterochromatic nucleus. The cytoplasmic processes of Sertoli cells contained swollen mitochondria, several vacuoles, and numerous electron-dense bodies. The heterochromatic nuclei of primary spermatocyte cells were randomly distributed, cytoplasmic vacuoles could be seen (Figure 10A) and their tight junctions were distorted (Figure 10A). Round spermatid cells appeared with irregular outlines with round nuclei some round spermatid cells have aggregated chromatin in the nucleus. Most of the

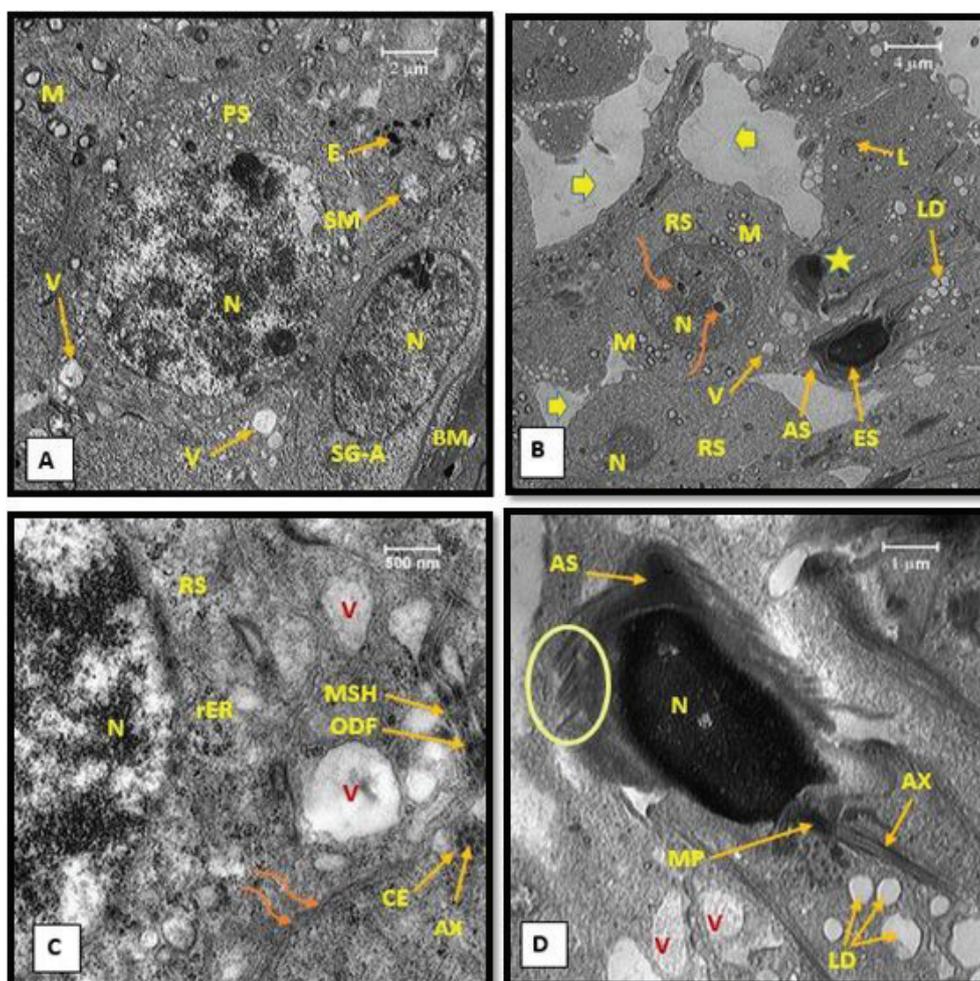


Figure 10. Ultrastructure of the section of a Seminiferous Tubule of Crocin 100mg/kg group Mice. A. Spermatogonia type A (SG-A), Basement Membrane (BM). The cytoplasmic processes of Sertoli Cells contained Swollen Mitochondria (SM), some Vacuoles (V), and numerous Electron-dense bodies (E). Primary Spermatocyte (PS) contain randomly distributed heterochromatic Nuclei (N). B. Round Spermatid cells (RS) appeared with irregular outlines, wide intercellular space between cells (arrowheads), Nucleus (N) have aggregated chromatin (curved arrow), the cytoplasm contains vacuolated Mitochondria (M) with patchy matrix and distorted, many of Lysosome (L), Lipid Droplets (LD), Vacuole (V), Elongated Spermatid cells (ES), have Acrosomal System (AS), some of their appeared abnormal crescent-shaped nucleus (star). C. The cytoplasm showed dilated rough Endoplasmic Reticular (rER) and Centrioles situated peripherally (CE), Outer Dense Fiber (ODF), surrounded by a Mitochondria Sheath (MSH), and abnormal cell junction was observed (curved arrow). D. Sperm has an Axoneme (AX), Middle Piece (MP), and the head of sperm consists of the Nucleus (N). Which is covered by serrated rims acrosome system (circle). Lipid Droplets (LD), and Vacuole (V) are also seen. Magnifications: A 2 μm ; B 4 μm ; C 500 nm; D 1 μm .

cell cytoplasm showed vacuolated mitochondria with patchy matrix and distorted vision cristae, with many lysosomes, lipid droplets, and vacuoles (Figure 10B). The cytoplasm showed dilated rough endoplasmic reticular and centrioles situated peripherally, inter the centriole axoneme situated centrally surrounded by the outer dense fiber which wrapped by a mitochondrial sheath (middle pieces which were formed of the mitochondrial sheath, dense fiber, and microtubules). Abnormal cell junctions between spermatogenic cells were observed (Figure 10C). Elongated spermatid cells were diffused next to the round spermatid, some of their abnormal crescent-shaped nucleus (Figure

10B). Some sperms had abnormal nuclear shapes, swollen nuclei, irregular wavy acrosomal systems, and showed large round heads capped with serrated rims acrosomal systems. The head of the sperm consists of the nucleus which is covered by the acrosomal system, and connected to the middle piece by a short neck. The axoneme begins in the middle piece and is surrounded by outer dense fibers. The axoneme and dense fibers are surrounded by a sheath of mitochondria (Figure 10D).

DISCUSSION

Natural products are of particular interest as pre-

ventive agents due to their low toxicity and potent efficacy. Crocin belongs to carotenoids, which are synthesized in subcellular organelles of plants and are used to prevent and treat various diseases. Crocin is a primary constituent that gives the red color to the culinary spice *Crocus sativus L.* (saffron) (Mohamadpour et al., 2013). Crocin is a well-documented pharmaceutical agent with a wide range of bio-activities such as antioxidant (Hosseinzadeh et al., 2009), antitumoral (Aung et al., 2007), and DNA protective activities (Hosseinzadeh et al., 2008).

Many reports documented crocin ability to restore the induced damage to males' reproductive function by different harmful conditions like diabetes (Khorasani, Ahangarpour, & Khorsandi, 2021), nicotine exposure (Sefidgar et al., 2019), Busulfan induced adverse effects on male fertility parameters (Roshankhah et al 2019), electromagnetic field (Vafaei, Motejaded, & Ebrahimzadeh-Bideskan, 2020), toxic drugs (Bakhtiary et al., 2014), genotoxic and cytotoxic herbicide of male germ cells (Kamali et al., 2020), testicular toxicity induced with chemotherapeutic agents (Mesbahzadeh et al., 2021; Davoodi et al., 2022) and oxidative stress (Domínguez-Rebolledo et al., 2010;).

Diabetes can increase the generation of free radicals and can be harmfully effective in spermatogenesis. Crocin is a carotenoid and is accountable for the red color of saffron. Crocin has shown numerous pharmacological actions such as antioxidant roles and radical scavenging. It seems that, as a strong antioxidant, crocin could compensate for the toxicity induced through STZ and raise the quality of some sperm parameters (Roshankhah et al 2019).

Also, crocin at a concentration of 100 mg/ml can be to decrease the tissue expression index of Bax and significantly increased the tissue expression of Bcl2 as well as antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase compared to diabetic control group; it also improved the damages induced by diabetes in the testicular tissues of the rats (Ataei et al., 2019).

In another research, the results showed that crocin has protective and therapeutic effect testicular damage-induced by cadmium via improving the endocrine testicular function and via its antiapoptotic and antioxidant effect. Also, results showed that the therapeutic effect of crocin is more effective than its protective effect on testicular toxicity induced by cadmium (Abo Edra et al., 2018)

In contrast, some investigations revealed that crocin is not possessed healing power in infertile men with idiopathic oligoasthenoteratozoospermia (OAT) or even has no antioxidant capacity (Safarinejad et al., 2011).

The data of this study showed that the treatment with crocin at 4, 20, and 100 mg/kg in mice did not show any significant differences between the control group and all treated groups after six weeks.

However, Asdaq and Inamdar reported significantly elevated the bodyweight in rats treated with crocin at 19.38 mg/kg and a high dose of saffron at 100 mg/kg administered orally for 5 consecutive days with a reduction in daily diet intake compared to normal fat diet control (Asdaq & Inamdar, 2010).

Moreover, our results showed that the weight of the testes was decreased with high doses (100mg/kg) of crocin, compared to the control group; however, this reduction was not statistically significant.

Crocin increased testis weight and compensated for the testicular loss caused by the nicotine effect. (Sefidgar et al., 2019).

In the present study, we administrated crocin daily for six weeks to healthy male mice in three different doses at 4, 20, and 100 mg/kg. The testosterone levels significantly increased from 18.5 ng/dl in the control animals to 49.3 ng/dl in the animals that received a daily dose of crocin at 20 mg/kg. Our findings are in agreement with previous studies (Modaresi et al., 2008). Crocin may directly affect testosterone concentrations by increasing the steroid biosynthesis process or by changing the testosterone-Luteinizing hormone (LH) feedback mechanism (Modaresi et al., 2008; Salahshoor et al., 2016). Also, crocin eliminates free radicals and reactivates oxygen species (ROS), which prevents free radical chain reactions. In general, the antioxidant effects of crocin stimulate the synthesis of steroid hormones and lead to an overall improvement in the male reproductive system. The positive effects of crocin on many sperm parameters have been reported, which can be attributed to the increase in testosterone due to crocin injection (Asadi et al., 2014).

However, the mechanism by which the serum testosterone level increased by crocin administration provided miscellaneous information. Other researchers stated that the administration of crocin increased the activity of the Leydig cells, and plays a major role

in the steroid production process. One of the possible mechanisms to explain the effect of crocin on increased testosterone levels in serum is that crocin may reduce the hypophyseal-hypothalamic sensitivity to control testosterone feedback on gonadotropin secretion; also, crocin antioxidant effects may modify sexual hormone concentration in male laboratory mice (Modaresi et al., 2008).

Remarkably, the light and electron microscopic observations revealed that crocin at a high dose of 100 mg/kg had destructive effects on the histological structure of the treated mice testicles which appeared to have less seminiferous tubules, spermatogenic cells, and more abnormal spaces. Marked decreases in these testicle weights accompanied the deterioration in their histological structures. The same observations were documented in the rat after treating them with 200 mg/kg of saffron for 28 days (Khayatnouri et al., 2011).

In our study, in the group that received 20 mg/kg crocin, no pathological changes were seen in the testicular tissue, while it was associated with the development of seminiferous tubules. At a low dose of 4 mg/kg, pathological changes were observed in the testicular tissue compared to the control group.

The detrimental effects of crocin may be due to the toxicity resulting either from the high dosages or the prolonged treatment period. Other studies reported crocin-induced embryonic malformation in pregnant mice (Moallem et al., 2016). It seems that the effects of crocin are dependent on its doses and time duration of treatment.

The majority of changes that occur in testicle tissue and spermatogenic procedure following crocin administration are probably due to changes in testosterone amount (Sefidgar et al., 2019). Saffron is also applied as a traditional anticancer drug to on curing advanced prostate cancer due to its prevention effect on testosterone.

The antitumor mechanisms of crocin are apoptosis, inhibition of cell proliferation, and cell cycle progression, reduction of MRP1 and MRP2 gene expression,

inhibition of telomerase activity, microtubule polymerization, and suppression of cyclin D1 and p21Cip1 overexpression. Moreover, this carotenoid can reduce N-cadherin, and beta-catenin expression, increase expression of E-cadherin, and down-regulate matrix metalloproteinases 2 and 9, and urokinase-type plasminogen activator expression/activity in tumor cells. Furthermore, crocin decreases the mRNA expression of certain proinflammatory cytokines associated with cancer (Veisi et al., 2020).

Although several hypotheses have been put forward, the precise mechanisms underlying crocin effects against cancer are not clear yet but these reports imply that crocin also may have a potential inhibitory hormone-related cancers effect.

CONCLUSION

The results of the present study showed that crocin at the low dose of 20 mg/kg has a protective effect on the germinal epithelium of seminiferous tubules while ultrastructure observations of testicles revealed that crocin at 100 mg/kg had destructive effects on the histological structure of this organ in treated mice in a dose-dependent manner. In conclusion, treating healthy male mice with crocin dose of 20 mg/kg for six weeks increased the testosterone concentration while at a high dose of 100 mg/kg induced structural damage to the testicles.

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CONFLICT OF INTEREST

All authors read and approved the submitted paper and declare no conflicts of interest.

ETHICS STATEMENT

The Committee for Ethics and Animal Care of the Ferdowsi University of Mashhad (ACEC) issued an ethical license (IR.UM.REC.1400.162) for conducting this protocol (No. 49430).

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