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Effect of Crocin on the Spermatogenesis Indices of Mice Testis: A Histopathological and Histomorphological Study

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CROCIN, the chief component of *Crocus Sativus* L. (Saffron), is well recognized for its antioxidant action by scavenging reactive oxygen species (ROS), particularly superoxide anion. In this research, 32 adult NMRI male mice were divided into four groups; control and the three experimental groups as crocin at 4, 20, and 100 mg/kg, and all doses were given to mice by gavage for six weeks. For histological study, tissue sections were stained by hematoxylin and eosin (H&E) and Masson's trichrome stains. The spermatogenesis indices include the repopulation index (RI), spermatogenesis index (SI), tubular differentiation index (TDI), and Sertoli cell index (SEI) were measured. Data was evaluated by one-way ANOVA, using SPSS software. The results of this study showed that crocin (100 mg/kg) significantly reduced the diameter of seminiferous tubules and the number of sperm in seminiferous tubules. The statistical analysis for TDI and SI in experimental groups presented that there was no significant difference in various doses of crocin, compared with the control group while, RI Data analysis showed that all administrated doses of crocin cleared significance to reduce it, compared with the control group. Also, the number of Sertoli cells significantly reduced in all treated groups with crocin.

We concluded that crocin at the high dose of 100 mg/kg has some side effects on the reproductive system of mice while is relatively safer at doses of 4 and 20 mg/kg. Therefore, lower doses are suggested for further studies and possible clinical trials.

Keywords: Crocin, Mice, Histomorphology, Spermatogenesis Indices, Reproduction.

Introduction

In the past years, the rising trend of herbal medicines has been widely spread, due to their protective factors against many diseases [1]. Estimating the toxicity and side effects of herbal medicines on experimental animals help to identify their pharmacologically active components, and also illustrate their possible toxicity and side effects on numerous tissues of the human body [2].

Although modern medicine has an excellent ability to treat many diseases, long-term use of synthetic drugs usually brings reversible and sometimes irreversible side effects. This shortcoming has led to the adoption of certain traditional medical practices as a safe alternative for the treatment of chronic diseases [3]. Saffron is the red stigma of *Crocus sativus L.* that belongs to the family iridaceous, *Crocus sativus L.* and is widely

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planted in many countries of the world such as Iran, India, Pakistan, Italy, China, Japan and Azerbaijan. Saffron is an important, delicate spice with a flavor and golden color and smell like sea air. It is used in cooking, medicine, cosmetics, perfumery, dye and clothes [4-6]. The chemical components of saffron extract showed that it has four main active ingredients namely safranal, crocin, picrocrocin and crocetin. Crocin belongs to the family of monoacid disaccharide esters of polyene dicarboxylic acid namely crocetin. Safranal and picrocrocin are monoterpene aldehydes. Picrocrocin is a compound responsible for the saffron bitter taste; safranal is an essential volatile factor [7,8]. Crocetin and crocin are important components in dye raw materials [9]. Many studies have shown that saffron and its components can be used for many purposes such as sexual stimulants. The exocrine part of testes contains seminiferous tubules that produce spermatozoid. The several stages of reproductive cells division in seminiferous tubules are called spermatogenesis. As long time ago, in traditional medicine, saffron has been used to treat infertility and sexual potential stimulant. Crocin is one of the saffron's powerful metabolites, which plays the main role in protecting the testicular tissues against toxic substances and maintaining its functions in the reproduction system [10].

In addition, the therapeutic indication of crocin can improve perturbed endocrine function caused by cadmium exposure [11]. In diabetic rats, lipid abnormalities, blood glucose, testicular damage and sperm characteristics [12]. Crocin can protect these biological systems through its antioxidant effects, reduce oxidative stress (OS) and reduce the rate of apoptosis [13].

Crocin can alter the levels of hormones such as androgens and estrogen in the blood serum and induce apoptosis in prostate cancer through interception, cell cycle progression, and inhibition of cell proliferation. Through interception, cell cycle progression inhibits cell proliferation and induces apoptosis in prostate cancer [14].

Considering the antioxidant and anti-inflammatory effects of crocin and numerous reports on its ability to improve the spermatogenesis process, we decided to investigate the effect of long-term use of different oral doses of crocin on testicular spermatogenesis indices in mice.

Material and Methods

Chemical and preparation

In this experimental study, 2 g of Crocin powder (Crocin $C_{44}H_{64}O_{24}$) was purchased from Abo-Ali Company, Mashhad, Iran. It was diluted with 200 ml distilled water as a stock solution to prepare treatment doses (4, 20 and 100 mg/kg) for administration. The doses and administration route of chemicals used in the present study were chosen according to previous studies with Crocin [24].

Animals and treatment

In this research, 32 adult NMRI male mice weighing 30-40g were purchased from the Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Animals were kept at 22 ± 2°C under the controlled environmental condition of the animal house 12/12 hours light/ dark cycle and free access to food and water. This study was approved by the Research Ethics Committee (Approval ID: IR.UM.REC.1400.162) in accordance with the instruction for the care and use of laboratory animals prepared by Ferdowsi University of Mashhad, Mashhad, Iran.

The animals were divided randomly into four groups of eight each, group I as control received normal saline, groups II, III and IV as treatment groups, received crocin at 4, 20 and 100 mg/kg body weight, respectively. All doses were given to mice by oral gavage for six weeks. Oral gavage (dosing) is used when a specific volume of an agent needs to be administered directly into the stomach. In this study, 18-20gauge gavage needle about 1 to 1.5 inches in length with a rounded tip was selected. The amount of gavage was regulating every day according to the diverse weight of each animal. Mice body weights were measured weekly to adjust the doses of crocin administration. The recorded data were analyzed and presented in the tables and graphs.

Sample collection

At the end of the experiment, the mice were euthanized by carbon dioxide (CO_2) for taking samples. All experimental protocols were approved by the Ethical Committee of Ferdowsi University of Mashhad, Mashhad, Iran (Approval ID: IR.UM.REC.1400.162).

Weight, length and width of testis

The testes were exactly dissected and removed from each mouse and weighed separately using analytical balance. The caliper device was used for measuring its length and width, and then

placed in buffered formalin 10% for 24 – 48 hours for histological studies.

Tissue preparation for histomorphological study

After fixation, tissue samples transferred to the histology laboratory of Ferdowsi University of Mashhad for tissue preparation. All tissues, placed in the tissue processor for dehydration by alcohol, clearing with xylene, and embedding by paraffin wax. After preparing of paraffin tissue blocks by paraffin dispenser, tissue sections (5µm) were prepared by microtome and stained with hematoxylin- eosin and Masson trichrome.

Masson trichrome staining was used to detect connective tissue and distinguish seminiferous tubules for measuring their diameter.

For histological study we used light microscope (Olympus DP25, Tokyo, Japan) and photograph were taken using a high-resolution digital camera (Olympus, Tokyo, Japan). In this study, four parameters, including spermatogenesis index (SI), repopulation index (RI), Sertoli cell index (SEI) and tubular differentiation index (TDI), were investigated. For measuring of these parameters, 200 seminiferous tubules cross sections were recorded. The TDI is the percentage of tubules that contained three or more differentiated spermatogenic cells. The RI is the ratio of active to inactive spermatogenic cells. For the SI, the ratio of seminiferous tubules containing sperm to the tubules without sperm was calculated. The percentage of the Sertoli cells was expressed as the number of Sertoli cells to the number of total spermatogenetic cells.

Morphometric assessment of seminiferous tubules

From each section, seminiferous tubule diameter and area (essentially from circular tubular cross sections) was determined using pre-calibrated measuring eyepiece. About 100 sections of seminiferous tubules were selected randomly and measured for each group. The tubular diameter was measured at ×400 magnification. The diameter of the seminiferous tubule was measured across the minor axes (transverse or width) and the mean diameter was obtained .

Statistical analysis

Statistical analysis was performed by SPSS v.18.0., and differences among group were evaluated by one-way analysis of variance (ANOVA). Tukey›s test was used to compare the two groups according to the equality of variances in different groups and the James-Howell post hoc test was used for inequality of variances in different groups. The results were described as the Mean ± SD, and were considered significant at $p < 0.05$.

Results

Effect of crocin on the body weight

Table 1 presents the mean and standard deviation of body weight on different days of the experiment. Although crocin reduced the weight of the animals, there was no significant difference in mice's body weight between the control group and all treated groups with crocin after six weeks.

Effect of crocin on the weight of testis

The results revealed that after six weeks of treating male mice with crocin at the doses of 4, 20, and 100 mg/kg, a slightly decreased in testis weight was observed in the treated group with crocin at 100 mg/kg. No significant differences were observed in the weight of testis between the groups (Table 2).

Effect of crocin on the testis width and length

The results showed that, no significant differences in mice's testis width and length between the control group and all treated groups with crocin after six weeks (Fig. 1 and 2).

TABLE1. Effect of various dosages of crocin on the mice body weight (g)

Day / Group	1	9	17	25	33	41
Control	38.37±3.701	40.12±2.85	39.12±5.668	39.87±5.817	41.5±5.928	42.37±5.344
Crosin 4mg/kg	34.37±3.42	36.87±4.155	37.5±3.891	38.62±4.207	38.5±3.928	39.37±4.241
Crosin 20mg/kg	37.2±2.712	37.87±2.748	39.625±1.847	39.5±2.507	40.5±2.507	41.5±2.673
Crosin 100mg/kg	36.5±3.162	35.75±3.059	38.375±2.2	38.5±1.773	39.62±2.446	39.25±1.753

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

TABLE 2. Effect of various dosages of crocin on mean testis weight and diameter of seminiferous tubules in mice.

Groups	Testis weight(g)	Seminiferous tubule diameter(µm)
Control	0.121±0.016	47.328±12.562
Crocin 4mg/kg	0.12±0.024	41.879±8.429
Crocin 20mg/kg	0.113±0.025	33.98±3.503
Crocin 100mg/kg	0.104±0.019	28.809±7.216*
Degree of freedom	3	3
Chi-square value	3.726	73.012
P-value	0.239	0.000

* Significant difference with $p<0.05$

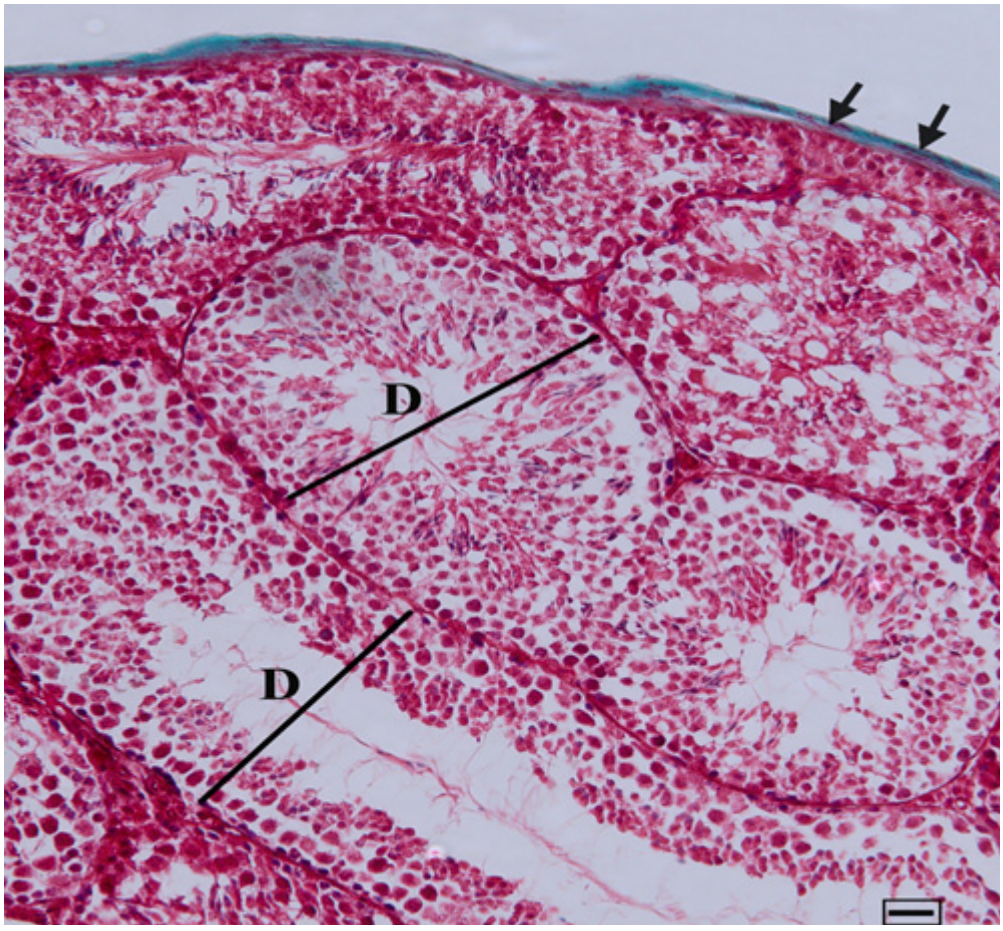

Fig. 1. Photomicrograph of a Masson's trichrome stained section of testis of a control group. Measurement of transverse diameter (D) of seminiferous tubules. Arrow shows the capsule of testis. Bar = 5µm.

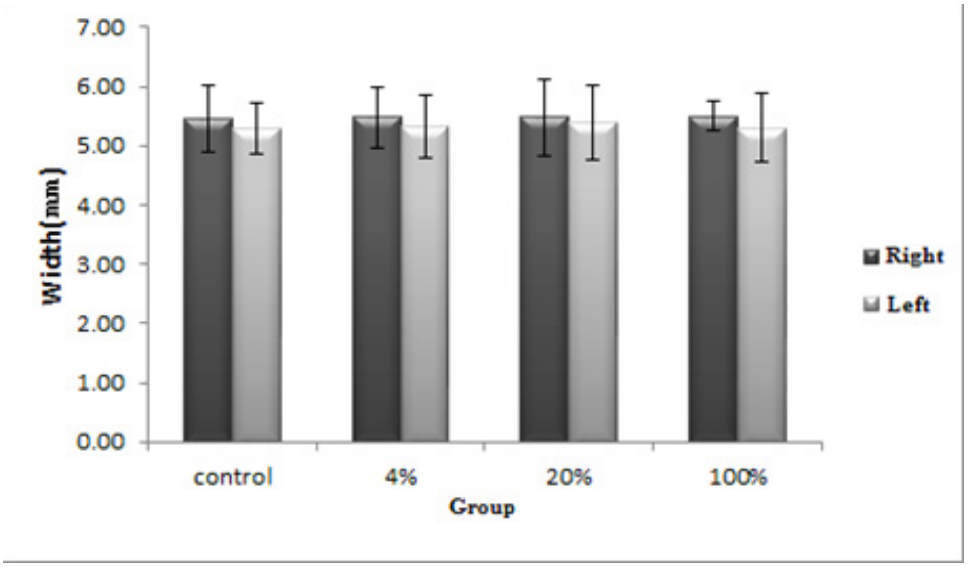

Fig. 2. Effect of crocin on the width of mice's testis: Control (C), crocin at 4 mg/kg (4%), 20 mg/kg (20%), and 100 mg/kg (100%) administration.

Effect of crocin on the epithelium thickness and diameters of seminiferous tubule

Germinal epithelium of seminiferous tubules composed of 4 to 8 cell layers. The thickness of the germinal layer's epithelium did not show any significant differences among all groups after measuring 100 seminiferous tubules' thickness (Fig. 3), but the diameter of seminiferous tubules showed a significant decrease between the control and the treated group with crocin at 100mg/kg (Fig. 4 and Table 2).

Effect of crocin on the spermatogenesis indices

As shown in Fig.s 6A and 6B in the control group, cellular layers of the walls of the seminiferous tubules are clear and normal and their cells are arranged spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, spermatozoa, and sertoli cells. Most of the seminiferous tubules contain mature sperm in the lumen.

Histological results in the group treated with 4 mg/kg crocin showed that the seminiferous tubules are somewhat arranged and contain a smaller number of Sertoli cells and are organized with interstitial tissue which has blood vessels. The seminiferous tubules lined by spermatogenic cells and Sertoli cells irregularly and some seminiferous tubules have focal empty areas between primary spermatocyte and spermatid (Fig. 6C).

The treated group with 20 mg/kg crocin showed regular features of seminiferous tubules and interstitial tubules tissues were well organized in comparison to the treated group with 4 mg/kg crocin. The seminiferous tubules appeared with cells in an organized shape, spermatogenic cells spread from the basement membrane to the lumen which was full of mature sperms (Fig. 6D).

Histological observation of testicular tissue after six weeks crocin administration at 100 mg/kg revealed that the numbers of spermatozoa in the lumen of the seminiferous tubules were significantly reduced as compared to the control group. Also, many of the seminiferous tubules were atrophic, and spermatogenic cells were discontinuous in some areas also the space increased between them, with lesser interstitial tissue (Fig. 6E).

The statistical analysis for TDI and SI in different groups, showed no significant difference in various doses of crocin administration compared with the control group. Also, RI analysis showed that a significant decrease between the control and treated groups; these results are presented in Table 3.

The number of Sertoli cells significantly decreased in all treated groups with crocin in comparison to the control group (Fig. 7).

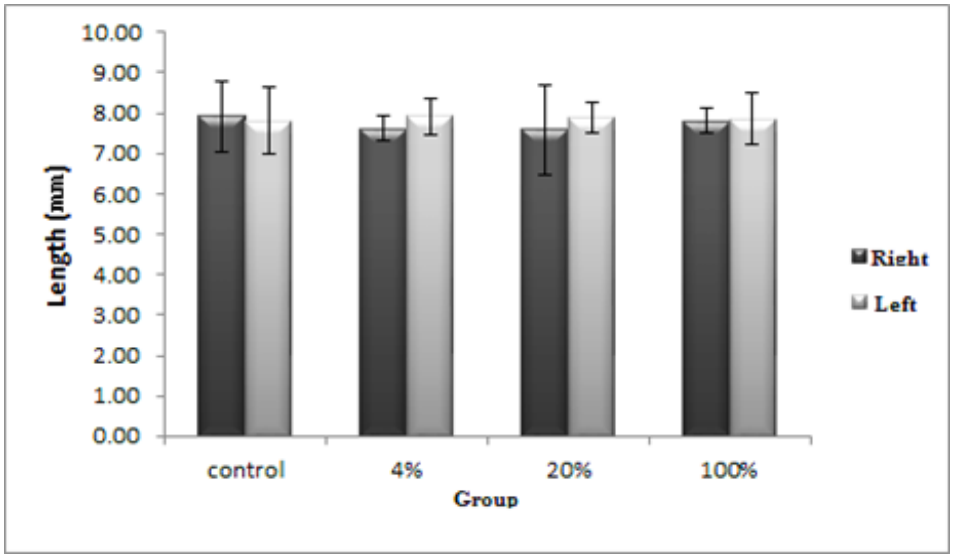

Fig. 3. Effect of crocin on the length of mice's testis: Control (C), crocin at 4 mg/kg (4%), 20 mg/kg (20%), and 100 mg/kg (100%) administration.

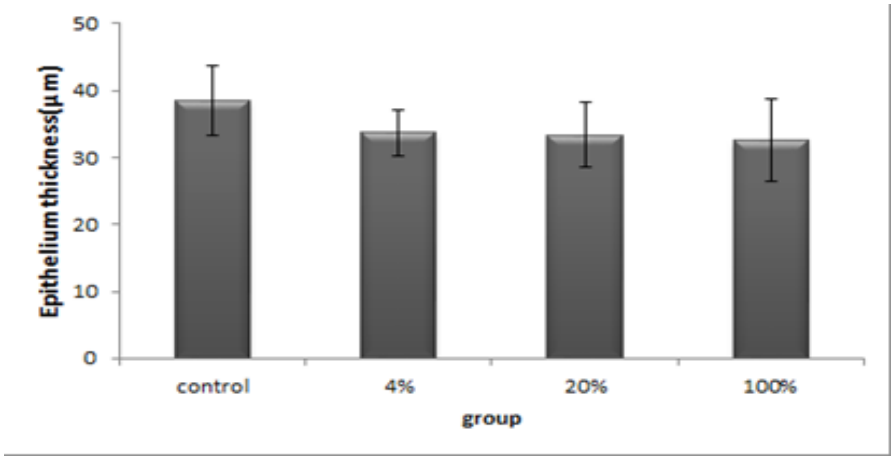

Fig. 4. Effect of crocin on the seminiferous epithelium thickness of the mice's testis: Control (C), crocin at 4 mg/kg (4%), 20 mg/kg (20%), and 100 mg/kg (100%) administration.

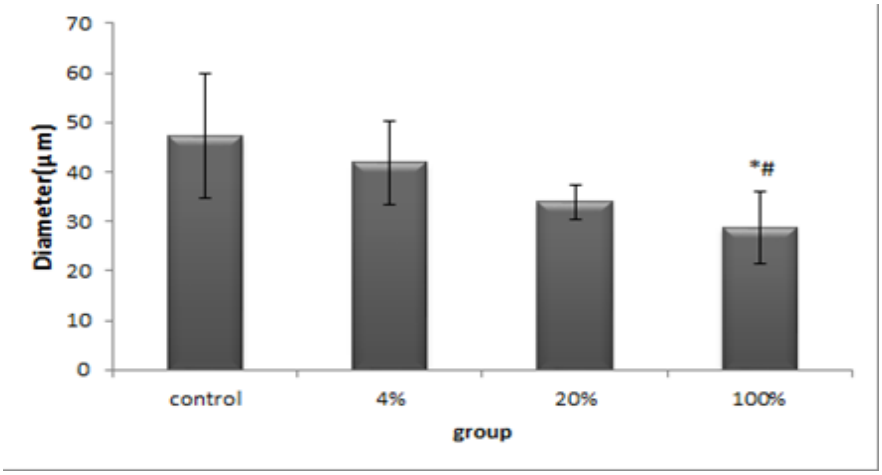

Fig. 5. Effect of crocin on the diameter of seminiferous tubules in mice: Control (C), crocin at 4 mg/kg (4%), 20 mg/kg (20%), and 100 mg/kg (100%) administration. *# Significant difference with $p<0.05$.

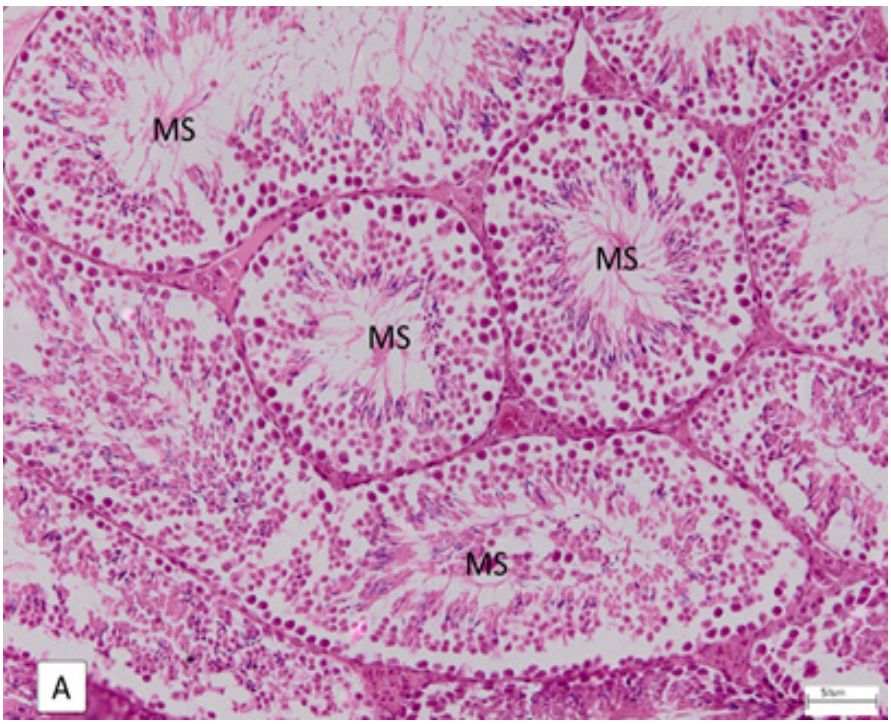

Fig. 6A. Histological structure of mice testis in control group. Mature sperm (MS). Hematoxylin and Eosin staining. Bar = 50µm.

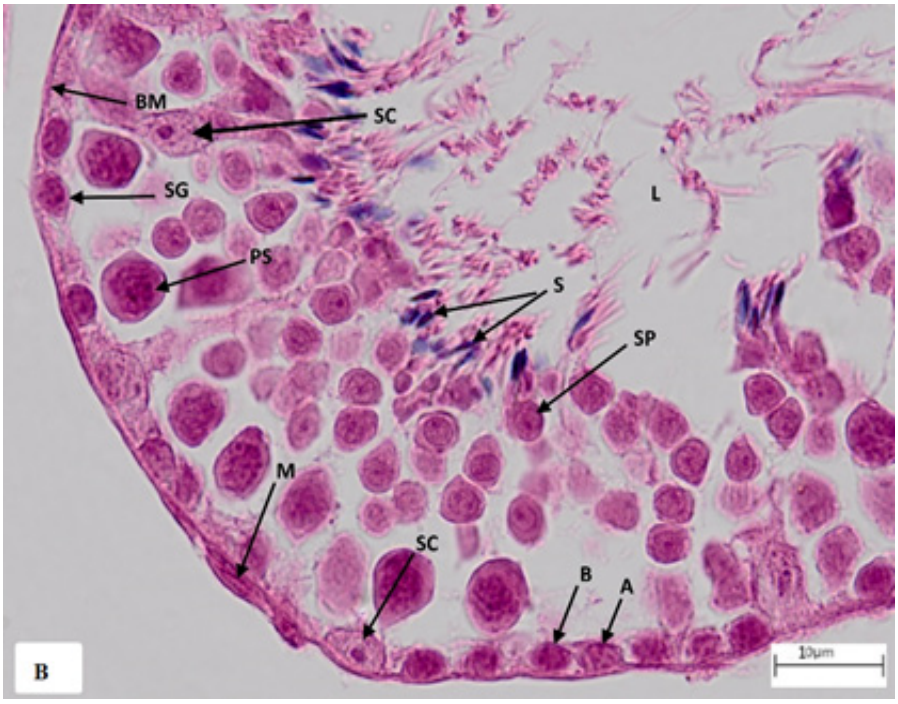

Fig. 6B. Histological structure of mice testis in control group. Normal arrangement of spermatogonia A & B (SG) and Sertoli Cells (SC) resting on the basement membrane (BM). Typical structure of primary spermatocyte (PS), spermatid (SP) (round cells early-stage), sperm (S) or mature sperm (MS) at the apical of SC. The sperm tail in the lumen (L). Myoid cells (M). Hematoxylin and eosin staining. Bar = 10µm.

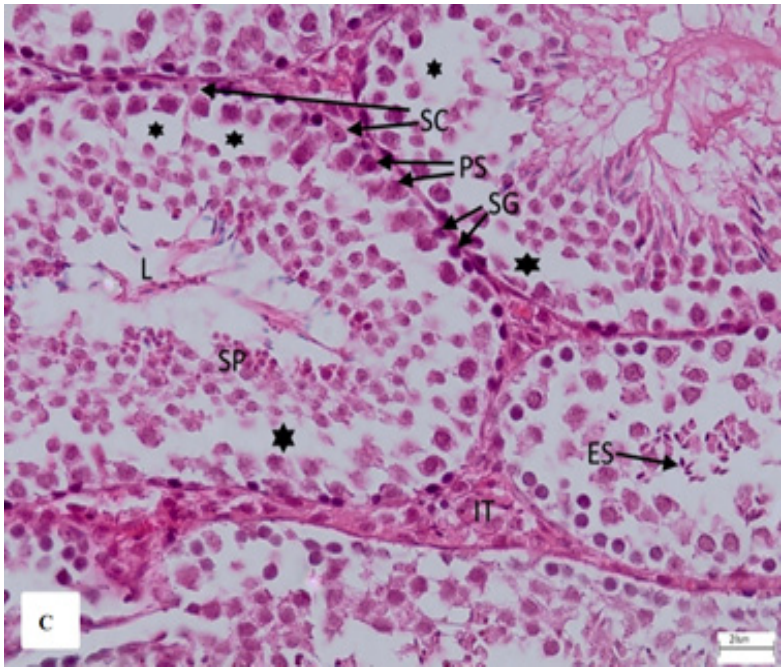

Fig. 6C. Histological structure of mice testis in the group treated with crocin at 4 mg kg, show that many seminiferous tubules were contained spermatogenic and Sertoli cells (SC) with interstitial tissue (IT) between them, and some seminiferous tubules show the presence of focal empty areas (black stare) of spermatogenic cells between primary spermatocyte (PS) and spermatid (ES). Hematoxylin and Eosin staining. Bar = 20µm.

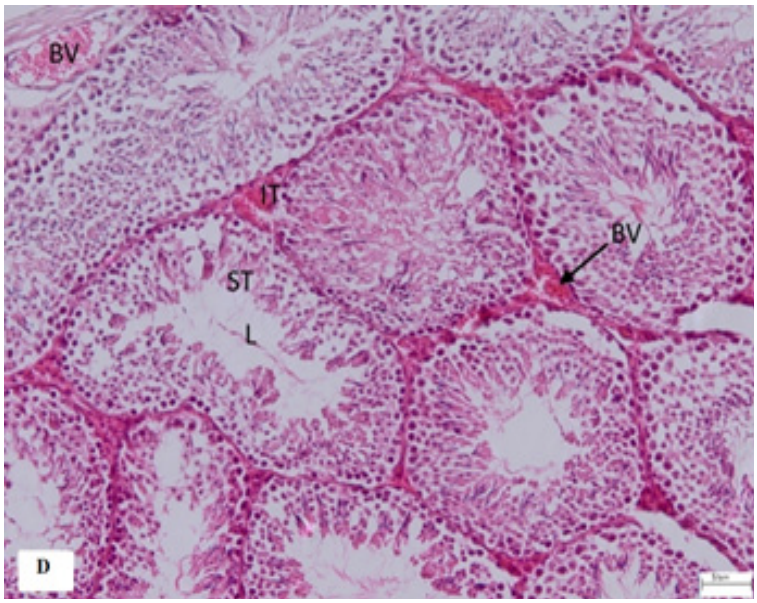

Fig. 6D. Histological structure of mice testis in the group treated with crocin at 20 mg/kg show regular features of seminiferous tubules (ST) containing spermatogenic and sertoli cells, interstitial tissues (IT) were well organized and have extensive blood vessels (BV). Hematoxylin and eosin staining. Bar = 50µm.

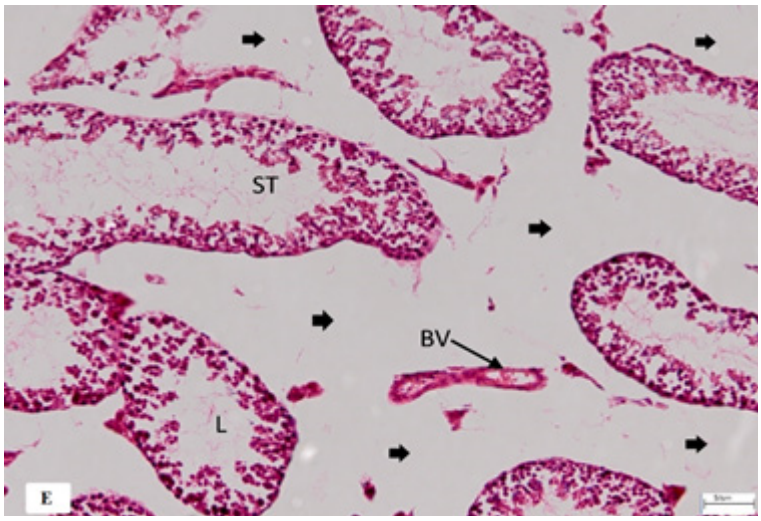

Fig. 6E. Histological structure of mice testis in the group treated with crocin at 100 mg/kg show many of the seminiferous tubules (ST) were atrophic with increased space among them, (arrow head), interstitial issues were widely separated and have little blood vessels (BV). Hematoxylin and eosin staining. Bar = 50µm.

TABLE 3. Comparison of the averages of TDI, SI, and RI after administration of crocin with various dosages.

Groups	TDI	SI	RI
Control group	11.75±1.254	12.125±0.876	90.4±17.01
Crocin 4mg/kg	11.5±1.669	10.563±4.395	42.6±8.56*
Crocin 20 mg/kg	11.415±1.699	8.813±5.542	45.6±16.77*
Crocin 100 mg/kg	12.5±0.053	12.5±0.053	26.6±16.52*
Degree of freedom	3	3	3
Chi-square value	5.755	4.729	12.765
P-value	0.124	0.193	0.005

* Significant difference with $p<0.05$

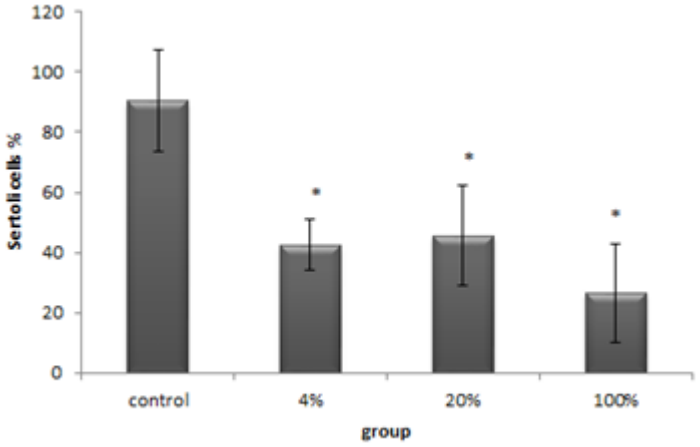

Fig. 7. Effect of crocin on the Sertoli cells of seminiferous tubules in mice. Control (C), crocin at 4 mg/kg (4%), 20 mg/kg (20%), and 100 mg/kg (100%) administration.

*** Significant difference with p<0.05.**

Discussion

The pharmacological and biological properties of crocin (one of the active ingredients of saffron, have a positive effect on the reproductive organs, nervous system, gastrointestinal tract, endocrine, immune system and cardiovascular, and many other organs) have been conducted numerous studies. However, there is no information about its mechanism of action in the body [15, 16]. Administration of crocin can reduce damage caused by exposure to toxic substances [17].

The present research determined the effect of crocin on spermatogenesis indices and testicular histomorphology in healthy and normal mice. The data of this study showed that the treatment with crocin at 4, 20, and 100 mg/kg of the body weight in mice did not show any significant differences from the control group after six weeks. However, Asdaq and Inamdar referred the body weight in diabetic rats treated with a high dose of saffron (100mg/kg) administered orally for 5 consecutive days which was a significant elevation in body weight with a reduction in daily diet intake compared to normal fat diet control [18]. These researchers were used aqueous saffron extract at doses of 50 and 100 mg intraperitoneally in normal and diabetic rats, which at the end of the 5-month period was associated with body weight gain in rats, which is not consistent with our results because saffron has different compounds and one of them is crocin which was used in the present study. In control and treated groups, the body weight of the animals increased from the beginning to the end of the experiment, but in comparison with the weekly body weight alteration in each group, the group that received 100 mg/kg of crocin lost more body weight that others, although, the difference was not significant.

In the present study, crocin decreased testicular weight in different doses which was more evident with increasing dose to 100 mg/kg however this reduction was not statistically significant.

It seems that the impact of crocin on testis weight is dependent on its concentration and duration of exposure. In the present study, the effectiveness of crocin cannot be determined on testicular weight but there is conflicting information about the impact of crocin on the weight of testes. However, other researchers showed that the testicular weight loss was compensated by crocin administration after treatment with nicotine [10].

Accordingly, the efficacy of saffron and its components are used in traditional medicine and could be considered a hopeful fertility agent in the male mice reproductive system [19]. Pratap and Rajender showing that phytochemical constituents of saffron "Crocin " and "safranal" were evaluated for their aphrodisiac potential in male rats and improvement of erectile dysfunction, particularly at doses of 160 and 320mg/kg. Furthermore, the antiproliferative effects of Crocin have been searched on the prostate cancer cell line in a dose-dependent style [20].

Other studies have shown that crocin can improve the side effect of cyclophosphamide in men treated with this medication. The toxic effects of cyclophosphamide can decrease fertility.Crocin significantly improved sperm quality in the mice treated with cyclophosphamide by an increase in the antioxidant capacity of testicular tissues and blood serum [21].

The present study revealed no significant difference in the germinal epithelium height among all groups.

Previous researchers have shown that crocin improved testicular tissue damage. Fani et al. reported that exposure of mice testicular tissue to atrazine for 23 and 75 days reduced the height of the epithelium, while crocin treatment increased that [22].

In the present study, although the seminiferous tubules thickness was reduced, but this reduction was not significant.

Our results revealed that in the group treated with high doses of Crocin (100 mg/kg), Sertoli cells, and spermatozoa decreased in the lumen this could be due to physiologic condition, in which the Sertoli cells' physiologic function is affected during the spermatogenesis through removing the parts of the sperm cytoplasm; therefore, the sperm with cytoplasmic droplets cannot complete its maturation [23].

This study clarified that high-dose administration of Crocin causes a decrease in spermatogenesis indexes in mice, leading to changes in quantities of the TDI, SI, and RI.

In conclusion, the current research presented that higher doses of crocin (100 mg/kg) may harmfully affect the reproductive system by decreasing sperm count, sperm motility, and

testicular injury. But, relatively safe doses of crocin (4 or 20 mg/kg) are essential in clinical trials.

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Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Ethical Approval

All protocols and methodologies were revised and approved by the Ethical Committee at Ferdowsi University of Mashhad, Mashhad, Iran (Approval ID: IR.UM.REC.1400.162)

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