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Chemoprotective effect of *Crataegus monogyna* aqueous extract against cyclophosphamide-induced reproductive toxicity

Ali Shalizar Jalali*, Shapour Hassanzadeh and Hassan Malekinejad

Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

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***Correspondance:**
Ali Shalizar Jalali, DVM, PhD Candidate
Department of Basic Sciences,
Faculty of Veterinary Medicine,
University of Urmia, Urmia, Iran
E-mail: ali_shalizar@yahoo.com;
a.shalizar@mail.urmia.ac.ir

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Abstract

Cyclophosphamide (CP) is extensively used as an antineoplastic agent for treatment of various cancers, as well as an immunosuppressive agent. However, despite its wide spectrum of clinical uses, CP is known to cause several adverse effects including reproductive toxicity in humans and experimental animals. *Crataegus monogyna* is one of the oldest medicinal plant has been shown to be cytoprotective by scavenging free radicals. The present study was conducted to assess whether *Crataegus monogyna* fruits aqueous extract with anti-oxidant properties could serve as a protective agent against reproductive toxicity during CP treatment in a rat model. Male Wistar rats were categorized into four groups. Two groups of rats were administered CP at a dose of 5 mg in 5 mL saline kg^{-1} per day for 28 days by oral gavages. One of the groups received *Crataegus monogyna* aqueous extract at a dose of 20 mg kg^{-1} per day orally four hours after cyclophosphamide administration. A vehicle-treated control group and a *Crataegus monogyna* control group were also included. The CP-treated group showed significant decreases in the body and organ weights and spermatogenic activities as well as many histological alterations. CP treatment also caused a significant decrease in sperm count and motility with an increase in dead and abnormal sperms. Moreover, significant decrease in serum levels of testosterone and increased serum concentrations of FSH, LH, LDH, CPK and SGOT were observed in CP-treated rats. Notably, *Crataegus* coadministration caused a partial recovery in above-mentioned parameters. These findings indicated that *Crataegus* might be partially protective against CP-induced reproductive toxicity.

Introduction

Cyclophosphamide (CP), is a widely used cytotoxic alkylating agent with antitumor and immunosuppressant properties. It is used for treatment of chronic and acute leukemia, multiple myeloma, lymphomas, rheumatic arthritis and systemic lupus erythematosus and in preparation for bone marrow transplantation.[1] CP undergoes bioactivation by hepatic microsomal cytochrome P450 mixed function oxidase system to active metabolites that enter the circulatory system. Phosphoramide mustard and acrolein are the two active metabolites of cyclophosphamide.[2] The antineoplastic effects of cyclophosphamide are associated phosphoramide mustard, whereas acrolein is linked to toxic side effects like cell death, apoptosis, oncosis and necrosis.[3] In spite of its therapeutic importance, a wide range of adverse effects including reproductive toxicity has been demonstrated following cyclophosphamide treatment in humans and experimental animals.[4] Adult male patients treated with CP have demonstrated diminished sperm counts and an absence of spermatogenic cycles in their testicular tissue.[5] Previous studies on male rats have confirmed the potential of CP to cause oligospermia, azoospermia and histological alterations in the testis and epididymis.[6,7] Decrease in weight of reproductive organ, impaired fertility, growth and development of next generation was also observed in CP treated male rats.[8] Although, the precise mechanism by which CP causes testicular toxicity is poorly understood, numerous studies have shown that CP exposure can disrupt the redox balance of tissues leading to oxidative stress.[9-11] It has been reported that oxidative DNA damage is caused by hydroperoxide derivative of CP through generation of H_2O_2.[12] Further, spermatozoa are more susceptible to peroxidative damage because of high concentration of polyunsaturated fatty acids and low

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antioxidant capacity.[13] Also, acrolein has been found to interfere with the tissue antioxidant defense system and produces highly reactive oxygen free-radicals that are mutagenic to mammalian cells.[14] Consequently, from these aforementioned studies, combination of the drug delivery together with potent and safe antioxidant may be the appropriate approach to reduce CP-induced reproductive toxicity.

Hawthorn (*Crataegus*), found in northern temperate regions such as East Asia, Europe, and Eastern North America, is a genus of the Rosaceae family. The two most common species used are *Crataegus laevigata* (syn *Crataegus oxyacantha*) and *Crataegus monogyna*. Hawthorn was first mentioned as a drug in the Tang-Ben-Cao (659A.D.), which is the world's earliest officially published pharmacopoeia.[15] Independent studies have shown that extracts of *Crataegus* (from several parts of the plant including fruits) are rich in proanthocyanidins and flavonoids [16,17]and many of these phenolic compounds have been shown to be cytoprotective by scavenging superoxide anion, hydroxyl radical, hydrogen peroxides and reducing lipid peroxidation.[18-20] Based on above findings, the present study was undertaken to assess whether *C. monogyna* fruits aqueous extract with anti-oxidant properties could serve as a protective agent against reproductive toxicity during CP treatment in a rat model.

Materials and Methods

Plant material. The ripe fruits of *C. monogyna* were collected from its natural habitat around the city of Urmia in West Azerbaijan province, northwestern Iran. The identification of collected plants was confirmed scientifically at the research laboratories of the Department of Agriculture of West Azerbaijan province.

Preparation of the aqueous extract. After collection, the fruits were dried for 7–10 days in the shade at room temperature. The dried fruit were then ground and the powder was stored in cloth bags at 5 °C until transfer to the laboratory for extraction. The method for preparing dry water-soluble plant powders has been previously described.[17] Briefly, dried plant material (25 g) was stirred in 250 mL of distilled water for 15 min at 100 ˚C, followed by rapid filtration through a crude cellulose filter and then Whatman #1 filter paper. The resulting filtrate was freeze-dried and the powder was stored at −18 °C in a desiccant until required. The average (w/w) yield was 12.4 %.

Animal model. Adult sexually matured male (4 months of age weighing 177.75 ± 7.68g) albino rats of Wistar strain were obtained from animal Resources Center of Veterinary Faculty of Urmia University. They were housed in a specific pathogen-free environment under standard conditions of temperature (25 ± 2 °C), relative humidity (50 ± 10 %) and light (12 h light/12 h dark). They were fed with a standard pellet diet and had free access to water. Animals were checked daily for occurrence of any toxic signs. All ethical themes of the studies on animals were considered carefully and the experimental protocol was approved by Institute Review Board.

Experimental protocol. After 7 days of acclimation to the environment, the rats were randomly divided into four groups of six animals each (n = 6): control group (Cont), *Crataegus* group (Cr), Cyclophosphamide group (CP) and Cyclophosphamide-*Crataegus* group (CPCr). The two experimental groups (CP and CPCr) were gavaged cyclophosphamide (Endoxan®, Baxter Oncology Gmbh, Germany) at a dose of 5 mg in 5 mL saline kg^{-1} per day, which is in correspondence to the therapeutic dose. The controls were given a similar amount of distilled water. The group (Cr) was gavaged *C. monogyna* aqueous extract at a dose of 20 mg kg^{-1} per day. The (CPCr) group also received the same dose of the extract four hours after cyclophosphamide administration. The treatment period was 28 days. The protocol for this study, including doses and duration of treatment for CP and *Crataegus*, were all designed according to previous studies.[9, 21]

Sampling. Animals were euthanized by CO_2 exposure in a special device following anesthesia with ketamine (75 mg kg^{-1}, IP) 24 hours after the last *Crataegus* treatment. Blood was collected without anticoagulant for serological analyses. Testes, epididymides and accessory sex glands were quickly dissected out, cleared of adhering connective tissue and weighed on a Mattler Basbal scale (Delta Range, Tokyo). Testes were freshly cut with frozen section and periodic acid shiff (PAS) special staining technique was conducted for histological evaluation.

Sperm characteristics. In order to assess the sperm motility, one caudal epididymis was placed in 1 mL of Ham's F10 medium. Cauda was cut into 2–3 pieces and incubated at 37 °C for 10 min in CO_2 incubator to allow sperm to swim out of the epididymal tubules. One drop of sperm suspension was placed on a microscope slide, and a cover slip was placed over the droplet. At least 10 microscopic fields were observed at 400× magnification using a phase contrast microscope, and the percentage of motile sperm was evaluated microscopically within 2–4 min of their isolation from the epididymides and was expressed as a percentage of motile sperm of the total sperm counted.[22]

The epididymal sperm count was determined by hemocytometer. After dilution of epididymal sperm to 1:20 in Ham's medium, approximately 10 μL of this diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 min in a humid chamber to prevent drying. The cells sediment during this time and were counted with a light microscope at 400×. The sperm count was expressed as number of sperm per milliliter.[23]

A 20 μL of sperm suspension was mixed with an equal volume of 0.05 % eosin-Y. After 2 min incubation at room temperature, slides were viewed by bright-field microscope with 400× magnification. Dead sperms appeared pink and live sperms were not stained. Two hundred sperms were counted for each sample and viability percentages were calculated. For the analysis of morphological abnormalities, sperm smears were drawn on clean and grease-free slides, and allowed to dry in air overnight. The slides were stained with 1 % eosin-Y/5 % nigrosin and examined at 400× for morphological abnormalities such as amorphous, hook less, bicephalic, coiled or abnormal tails.[24]

Biochemical parameters. Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by enzyme-linked immunosorbent assay (ELISA) as described in the instructions provided by manufacturer's kits (Monobind Inc., USA) as well as testosterone (Demeditec Diagnostics GmbH, Germany). The activities of serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and glutamic oxaloacetate transaminase (SGOT) were measured using an automatic blood chemistry analyzer (BT3000 Plus; Biotecnica Instruments, Italy).

Histological parameters. For each testis, five vertical sections from the polar and the equatorial regions were sampled[25] and an unbiased numerical estimation of the following histological parameters was determined using a systematic random scheme.

Tubule differentiation index (TDI) and spermiation index (SPI): 200 cross-sections of seminiferous tubules were randomly analyzed in each rat (one hundred per testis) for the calculation of tubule differentiation index (TDI) and spermiation index (SPI). TDI is the percentage of seminiferous tubules containing at least three differentiated germ cells.[26] SPI is the percentage of seminiferous tubules with normal spermiation.[27]

Statistical analysis. Results are expressed as mean ± SD. Differences between groups were assessed by the analysis of variance (ANOVA) using the SPSS software package for Windows. Statistical significance between groups was determined by Tukey multiple comparison post hoc test and the *p*-values less than 0.05 were considered to be statistically significant.

Results

Clinical signs and, body and organ weight changes. All animals survived the experimental period. CP treated animals showed general signs of deterioration such as piloerection, hair loss, lethargy, hunched posture, shivers and low activity. The absolute and relative weights of testes and epididymides as well as seminal vesicles and ventral prostate weights were significantly lower than those of controls after CP treatment, whereas daily administration of *Crataegus* caused significant increase in the absolute and relative weights of testes, absolute weight of epididymides and seminal vesicles and ventral prostate weights of cyclophosphamide-crataegus group in comparison with CP group but relative weight of epididymides was not protected by *Crataegus* co-administration. Absolute and relative weights of testes and absolute weight of epididymides as well as seminal vesicles weight increased significantly in *Crataegus* group compared to control (Table 1).

Sperm characteristics. Treatment of male rats with CP caused a significant decrease in the sperm concentration and motility, while dead and abnormal sperms increased compared to those of control (Table 2). Co-administration of *C. monogyna* fruits aqueous extract caused a significant increase in semen quality and minimized toxic effects of CP.

Biochemical findings. Administration of CP alone significantly increased serum level of CPK, LDH and SGOT compared to control rats (Table 3). Also, serum concentrations of FSH and LH were significantly elevated, while serum level of testosterone decreased by CP treatment (Table 4). The administration of *C. monogyna* fruits aqueous extract along with CP significantly restored serum marker levels towards the control value.

Histopathologic findings. CP induced drastic morphologic changes in the testis (Fig. 1B). Shrunken seminiferous tubules showed severe germ cell aplasia and basement membrane thickening as well as rupture,

Table 1. Effect of cyclophosphamide and *Crataegus monogyna* fruits aqueous extract on body weight and weights of testis, epididymis, seminal vesicles and ventral prostate.

	Control	CP	*Crataegus*	CP + *Crataegus*
Final Body Weight (BW, g)	226.33 ± 5.35	182.00 ± 6.06[a]	229.66 ± 4.92[b]	211.33 ± 5.00[a,b]
Absolute weight (g)				
Testes	2.01 ± 0.065	1.49 ± 0.040[a]	2.14 ± 0.017[a,b]	1.81 ± 0.043[a,b]
Epididymides	1.15 ± 0.044	0.85 ± 0.010[a]	1.21 ± 0.023[a,b]	1.01 ± 0.037[a,b]
Relative weight (per BW, %)				
Testes	0.88 ± 0.011	0.82 ± 0.004[a]	0.93 ± 0.014[a,b]	0.85 ± 0.008[a,b]
Epididymides	0.50 ± 0.019	0.46 ± 0.023[a]	0.52 ± 0.010[b]	0.47 ± 0.017[a]
Seminal vesicles (mg)	644.33 ± 37.84	447.00 ± 25.98[a]	739.83 ± 31.88[a,b]	557.83 ± 16.75[a,b]
Ventral Prostate (mg)	191.83 ± 6.17	164.00 ± 6.09[a]	190.33 ± 12.70[b]	177.00 ± 4.19[a,b]

The values are expressed as mean ± S.D. (n = 6).
[a] Significant differences as compared with the control group at $P < 0.05$.
[b] Significant differences as compared with the cyclophosphamide group at $P < 0.05$.

vacuolization, edematous fluid accumulation and fibrosis in interstitial and peritubular tissue. In these specimens, Leydig cells were degenerated and appeared with pyknotic nuclei. Moreover, Sertoli cells lost their junction with germ cells and looked amorphous with irregular and smaller nuclei. Administration of *Crataegus* along with CP restored these changes towards normality (Fig. 1D).

Histological parameters. As seen in Table 5, CP treatment induced deletion of germ cells during spermatogenesis, which resulted in a dramatic decrease in TDI. Due to the germ cells deletion, the SPI was greatly decreased in the CP-treated animals. *Crataegus* coadministration significantly attenuated the CP-induced germ cell loss from seminiferous tubules.

Table 2. Effect of cyclophosphamide and *Crataegus monogyna* fruits aqueous extract on epididymal sperm characteristics.

	Control	CP	*Crataegus*	CP + *Crataegus*
Sperm count (10^6/mL)	77.83 ± 10.02	13.33 ± 3.26^{a}	71.50 ± 5.16^{b}	50.00 ± 5.79^{a,b}
Motility (%)	82.73 ± 2.33	44.06 ± 2.08^{a}	80.92 ± 1.62^{b}	57.53 ± 1.62^{a,b}
Dead sperms (%)	9.20 ± 0.76	41.58 ± 1.83^{a}	8.33 ± 0.75^{b}	27.50 ± 2.04^{a,b}
Abnormal sperms (%)	7.45 ± 0.87	34.95 ± 1.25^{a}	7.20 ± 0.91^{b}	24.79 ± 1.46^{a,b}

The values are expressed as mean ± S.D. (n = 6).
[a] Significant differences as compared with the control group at $P < 0.05$.
[b] Significant differences as compared with the cyclophosphamide group at $P < 0.05$.

Table 3. Effect of cyclophosphamide and *Crataegus monogyna* fruits aqueous extract on serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and glutamic oxaloacetate transaminase (SGOT) activities.

	Control	CP	*Crataegus*	CP + *Crataegus*
LDH (IU/l)	283.16 ±17.13	461.33 ± 49.09^{a}	254.50 ± 52.89^{b}	367.66 ± 45.37^{a,b}
CPK (IU/l)	244.16 ± 12.60	403.00 ± 9.85^{a}	252.50 ± 9.56^{b}	332.50 ± 12.12^{a,b}
SGOT (IU/l)	92.00 ± 11.98	189.66 ± 11.84^{a}	98.50 ± 15.85^{b}	139.83 ± 9.55^{a,b}

The values are expressed as mean ± S.D. (n = 6).
[a] Significant differences as compared with the control group at $P < 0.05$.
[b] Significant differences as compared with the cyclophosphamide group at $P < 0.05$.

Table 4. Effect of cyclophosphamide and *Crataegus monogyna* fruits aqueous extract on serum concentrations of sex hormones

	Control	CP	*Crataegus*	CP + *Crataegus*
FSH (mIU mL^{-1})	0.25 ± 0.04	0.44 ± 0.05^{a}	0.29 ± 0.03^{b}	0.36 ± 0.04^{a,b}
LH (mIU mL^{-1})	0.28 ± 0.03	0.57 ± 0.06^{a}	0.32 ± 0.04^{b}	0.47 ± 0.04^{a,b}
Testosterone (ng mL^{-1})	6.66 ± 0.28	3.79 ± 0.29^{a}	6.46 ± 0.51^{b}	4.98 ± 0.13^{a,b}

The values are expressed as mean ± S.D. (n = 6).
[a] Significant differences as compared with the control group at $P < 0.05$.
[b] Significant differences as compared with the cyclophosphamide group at $P < 0.05$.

Table 5. Effect of cyclophosphamide and *Crataegus monogyna* fruits aqueous extract on tubule differentiation index (TDI) and spermiation index (SPI)

	Control	CP	*Crataegus*	CP + *Crataegus*
TDI (%)	91.08 ± 3.24	15.91 ± 3.15^{a}	92.16 ± 4.23^{b}	67.83 ± 5.01^{a,b}
SPI (%)	92.16 ± 2.31	13.66 ± 1.57^{a}	89.91 ± 4.22^{b}	58.83 ± 6.51^{a,b}

The values are expressed as mean ± S.D. (n = 6).
[a] Significant differences as compared with the control group at $P < 0.05$.
[b] Significant differences as compared with the cyclophosphamide group at $P < 0.05$.

Discussion

Many drugs used for cancer chemotherapy are known to produce toxic side effects in multiple organ systems including the testes. In a clinical context, testicular stem cell damage in patients exposed to chemotherapeutic drugs for a limited duration could result in long-term infertility or genetic alterations.[28] A strategy to diminish the side-effects of anticancer drugs with preservation of their chemotherapeutic efficacy is necessary. Effective anticancer and immunosuppressive therapy with CP is severely limited by reproductive toxicity as documented in a variety of species.[4] An oxidant mechanism may be involved in the reproductive toxicity, wherein CP and its metabolite acrolein cause inactivation of microsomal enzymes and result in increased reactive oxygen species

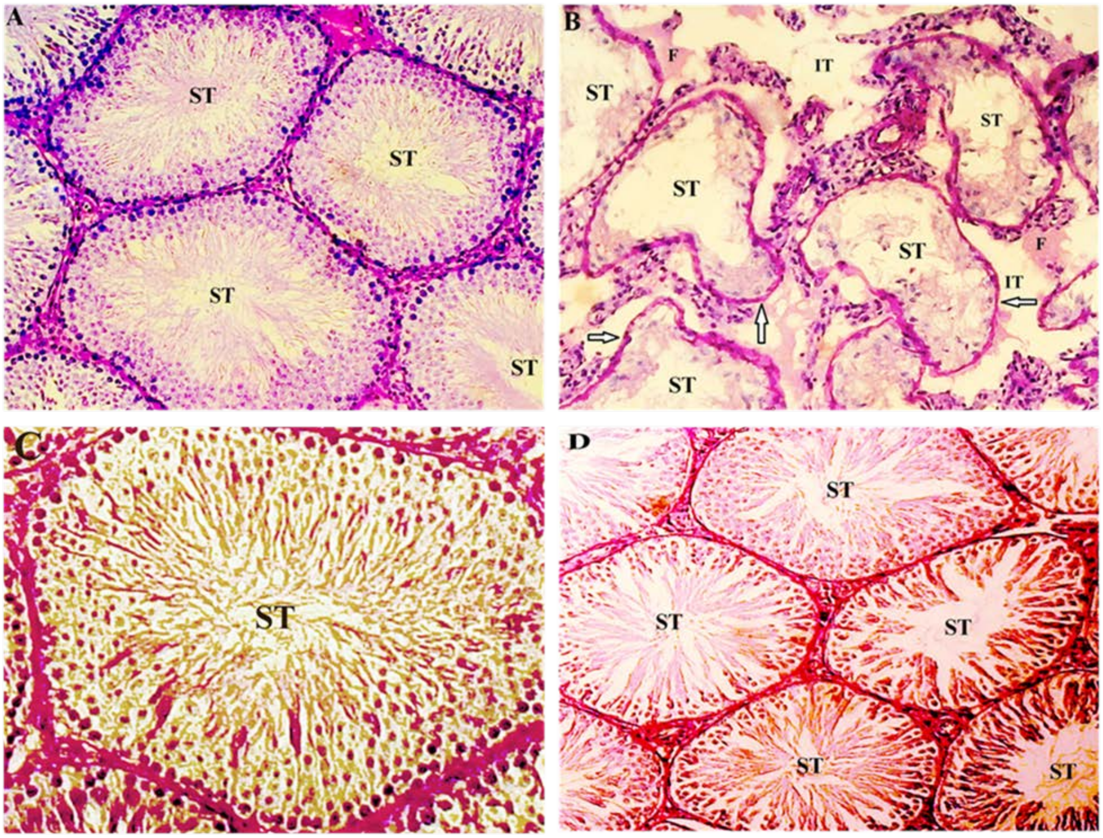

Fig 1. Photomicrographs of testicular sections of control **(A)**, Cyclophosphamide **(B)**, *Crataegus* **(C)** and Cyclophosphamide + *Crataegus* **(D)** treated rats. Testes from control group exhibit a normal feature of seminiferous epithelium (ST) and interstitial tissue (IT) with active spermatogenesis **(A)** ×400, as well as Crataegus-treated rats **(C)** ×800. However, a testis from a Cyclophosphamide treated rats reveals markedly shrunken seminiferous tubules with severe germ cell aplasia and basement membrane thickening (arrows). Note Rupture, vacuolization, edematous fluid accumulation **(F)** and interstitial space widening in intertubular connective tissue **(B)** 400×. *Crataegus* cotreated animals display nearly normal histoarchitecture **(D)** 400×. PAS staining technique.

generation and lipid peroxidation.[29] In the present study, reduction in body weight, weight of the testis, epididymis and accessory sex glands and histological changes in testis were indicative of drug toxicity. Because weight of the testis largely depends on the mass of the differentiated spermatogenic cells [30], the marked reduction in organ weight by CP can be explained by diminished number of germ cells, atrophy of Leydig cells and a significant lower rate of spermatogenesis as confirmed by our findings. Reduction in the weight of testes and accessory reproductive organs in CP-treated animals reflect the reduced availability of androgens.[31] Increased generation of free radicals is one of the possible mechanisms involved in CP-induced Leydig cell degeneration resulted in marked reduction of serum testosterone.[32] Moreover, significant increase in serum LH levels certainly indicates disturbance in Leydig cell function.[33] Chemotherapy can result in long-term or permanent azoospermia, the mechanism of which is most likely the death of germ cells[34] and histological parameters such as tubule differentiation and spermiation indices can also give information about degree of testicular damage as a consequence of germ cell death. In general, massive germ cell loss caused by anticancer drugs is followed by a sharp decline in testicular histological parameters.[35] As shown in the present study, depletion of seminiferous epithelium and the consequent decrease of histological measurements caused by cytotoxic agents were confirmed in our report Structural development and maturation of germ cells and spermiation are important functions of Sertoli cells.[36] Therefore, a potential explanation for failure of spermiogenesis in the CP-treated males is disruption of testosterone dependent junction of Sertoli cells with germ cells leading to their disorganization and separation. Additionally, FSH elevation can be an indication of spermiogenesis failure related to various causes including: testicular failure; genetic abnormalities and toxic exposure such as radiation, chemotherapy and heat.[37] Moreover, it indicates the abnormal Sertoli cell function resulted in reduced inhibin secretion.[38] In the present study, epididymal sperm count and motility decreased by CP treatment while the number of dead and abnormal sperms increased, confirming a previous report that CP induced an epididymis specific effect on sperm count and motility.[39] The decreased sperm count clearly shows the elimination of sperm cells at different stages of development and points to free radical attack through CP metabolism. In fact, oxidative damage to polyunsaturated fatty acids of cell membranes has long been considered to result in the impairment of membrane fluidity and permeability. This, results in damage of germ cells, spermatozoa and mature sperm.[40] It has also been reported that CP causes an increase in apoptosis at specific stages of germinal cycle.[41] Hence; the decrease in epididymal sperm count observed in CP-treated rats might reflect the spermatogenic cell death. The significant reduction in sperm motility may be due to the toxic effect of CP on the sperm flagellum through rapid loss of intracellular ATP.[27] CP-treated rats showed decrease in testicular tricarboxylic acid cycle enzyme activities[42] and thus impaired energy metabolism. It has been suggested that ATP may serve as an energy source for sperm motility and decrease in energy metabolism may be one of the limiting factors responsible for loss of sperm motility in CP-administered rats. A direct toxic effect of CP on the spermatogenesis in the seminiferous tubules may be considered as one of the mechanisms of action of CP in producing abnormal and dead sperms.[4] Spermatozoa are more susceptible to oxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity.[13] Therefore, oxidative stress could play a critical role in the induction of sperm abnormalities through DNA denaturation and fragmentation.[43]

In our study, LDH, CPK and SGOT activities in serum were significantly elevated. These findings suggested that CP may have induced generalized toxicity in rats. There are several reports on the benefit of antioxidants in protecting male reproductive system from deleterious effects of reactive oxygen species and other free radicals generated during CP exposure. It was found that ascorbic acid reduced cyclophosphamide-induced reproductive toxicity[9] as well as alpha-tocopherol-succinate.[44] There is also evidence that Yukmijihwang-tang as a multi-herbal medicinal formula can improve reproductive toxicity of CP through reduction of oxidative stress.[45] Two studies from the same researchers indicated that supplementation with lipoic acid as an antioxidant reduces CP-induced reproductive toxicity by the same mechanism.[46,47] In the present study, it has been shown that *C. monogyna* fruits aqueous extract co-administration was effective in protection or attenuation of testicular damage following CP exposure. Increasing evidences support the fact that *Crataegus* is beneficial where free radicals are known to play a predominant role in toxicity. Previous studies have shown that hawthorn extract reduced the stress conditions and genotoxicity induced by CP in mouse bone marrow cells due to its strong antioxidant activity.[48] Furthermore, it has been revealed that hawthorn extract reduces infarct volume and improves neurological score by reducing oxidative stress in rat brain.[49] In conclusion, the findings of our study indicate that CP can adversely damage the testicular tissue through imposing oxidative stress, while *C. monogyna* fruits aqueous extract co-administration could effectively prevent these adverse effects by effective inhibiting of oxidative processes and efficient scavenging of free radicals.

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