Evaluation of antioxidant effects of crocin on sperm quality in cyclophosphamide treated adult mice

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Abstract

Cyclophosphamide (CP) is one of the anti-neoplastic drugs. Despite its numerous clinical applications, it has devastating effects on the testicles and declines the sperm quality in treated patients. This study was aimed to investigate the protective effect of crocin in improving the toxicity induced by CP in reproductive system. In this study, 24 male adult mice (6 to 8 weeks) were randomly divided into three groups, control group received normal saline (0.1 mL, IP, daily), the CP group received CP (15 mg kg\(^{-1}\), IP, weekly) and the CP + crocin group received CP along with crocin (200 mg kg\(^{-1}\), IP, daily). After 35 days of treatment, animals were sacrificed. The samples of epididymis in human tubal fluid medium incubated for 30 min in 5% CO\(_2\) for flotation of sperm. Sperm were obtained from caudal epididymis using dissecting method. Then, the parameters of sperm quality including sperm count, motility, viability, DNA damage, nuclear maturation, and sperm morphology were evaluated. In CP group, the sperm count, motility, viability, nuclear maturation and sperm morphology were significantly decreased compared to control group (\(p<0.05\)) and in the CP + crocin group all of these parameters significantly increased compared to CP group (\(p<0.05\)). The percentage of sperm with DNA damage in the CP group significantly increased compared to other groups (\(p<0.05\)). The results of this study indicated that the crocin was able to suppress free radicals and enhance the quality of sperm in CP treated animals.

Key words: Crocin, Cyclophosphamide, Mice, Sperm quality

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Introduction

One of the current drugs used in chemotherapy is cyclophosphamide (CP), in addition to its beneficial therapeutic effects, by disrupting of antioxidant system in tissues and increasing the oxidative stress, decreases fertility in treated patients. According to previous studies, CP may negatively affect testis tissue and epididymis. It can also affect the sperms generation in testes and sperm maturation in the epididymis. Patients treated with this drug for four months or more have experienced varying states of oligosperma and azoosperma. Drugs with alkylating properties have the most deleterious effects on the testes. One of the major side effect of CP as an alkylating agent and a cytotoxic substance is impairment of sperm quality and fertility. Acrolein, a toxic metabolite of CP, interferes with the body's antioxidant system and a high level of reactive oxygen species (ROS) is produced. Compounds with antioxidant properties enable the body to fight against conditions caused by ROS or free radicals. Thus, prescription of antioxidant compounds during chemotherapy in order to reduce oxidative stress due to CP and increase fertility parameters seems necessary. Enzymatic and non-enzymatic antioxidants serve as an important biological defense against environmental pollutants. Various enzymatic and non-enzymatic antioxidants as a stress biomarker in liver and kidney of rat have investigated. The antioxidant enzymes included superoxide dismutase, catalase, glutathione reductase, glutathione-S-transferase, and glutathione peroxidase have been studied. Recent pharmacological studies have demonstrated that saffron extracts as non-enzymatic antioxidants have antitumor effects, radical scavenger properties and hypolipemic effects. Among the constituents of saffron extract, crocin is mainly responsible for these pharmacological activities.

Saffron cultivated in Iran and other countries such as India and Greece, has been used in traditional medicine as an anti-cough and anti-sputum. Compounds in saffron have numerous health benefits, such as anti-nociceptive, anti-inflammatory, anti-convulsants, antidepressants in animals and human. One of the main ingredients of saffron is crocin, and it is a water-soluble carotenoid unit used in the treatment of patients with neurological degeneration and loss of memory. Pharmacological studies have shown this substance is a free radicals scavenger. Whereas, the effect of crocin on the reproductive potential of patients treated with CP has not been studied, this research was aimed to evaluate the protective effect of crocin on semen parameters such as average number, motility status, viability, DNA damage and nuclear maturation of sperm to be done.

Materials and Methods

Animals and treatment groups. In this study, 24 adult male mice of NMRI rat aged 8 to 12 weeks were kept up in standard conditions of temperature 22 ± 2 °C, 30 to 60 % humidity and the light period of 14 hr light and 10 hr of darkness. Animals were randomly divided into three groups: Control, CP and CP + crocin groups, as below:

1. Control group received normal saline (0.1 mL, daily, IP).
2. Cyclophosphamide group received only CP (Baxter, Frankfurt, Germany; 15 mg kg⁻¹, weekly, IP).
3. Cyclophosphamide + crocin group received crocin (Sigma-Aldrich, St. Louis, USA; 200 mg kg⁻¹, daily, IP) in addition to CP (the same dose).

Sperm sampling. After 35 days of treatment, mice were anesthetized and euthanized with 25 mg kg⁻¹ ketamine (Alfasan, Woerden, The Netherlands) and the abdominal skin was sterilized with 70% ethanol. After euthanasia by 100 mg kg⁻¹ ketamine (Alfasan, Woerden, The Netherlands), the epididymis was removed and transferred to Petri dish 6 cm containing medium 1 mL of human tubal fluid medium (HTF; Sigma-Aldrich, St. Louis, USA) and 4 mL of bovine serum albumin (BSA; Sigma, St. Louis, USA) that previously its temperature was balanced by incubator (5% CO₂, 37 °C) and then, by making a few incision in the epididymis and 30 min incubation at 37 °C in 5% CO₂, spermatozoa released from epididymis.

Sperm count. For sperm counting at 1:20 dilution from sperm samples were prepared. For this purpose 10 µL of the sperms were added to 190 µL of distilled water, and then 10 µL of the diluted sperm was dropped on a Neubauer slide and the average number of sperms were counted.

Sperm motility. The medium (10 mL) of containing sperm was placed on the Neubauer slide and under a light microscope with a magnification of 20× the percentage of sperm motility was evaluated.

Sperms viability. Semen sample (20 µL) of was placed onto a clean slide and then 20 µL of eosin solution was added to it, after 30 sec, 20 µL of nigrosin solution was added. Then, from the desired solution, smear was prepared and after drying slides and using a light microscope with 40× magnification percentage of alive sperm (colorless) and dead sperm (red color in head) were determined.

DNA strand damage. The semen samples were washed three times with phosphate buffered saline (PBS) and after removal of the supernatant, the sediment was achieved by using PBS to a final concentration. Then, smears were prepared from the medium containing sperm and after drying in a laboratory environment, for 30 min, was placed into in acetone - ethanol (1:1) container.
Results

Sperm count. The results showed a significant difference between average number of sperms in CP group compared to control and CP + crocin groups (p < 0.05), (Table 1).

Sperm motility. The results for mean percentage of motile sperms in the studied groups indicated a significant decrease of this parameter in the CP group compared to the control and CP + crocin groups (p < 0.05), (Table 1).

Sperm viability. Results of alive sperms using eosin-nigrosin staining indicated a significant decrease of sperm viability in CP group compared with the other groups and a significant increase in CP + crocin group compared to the other groups was found (p < 0.05), (Table 1).

DNA integrity. Sperms observed with green nuclei were normal, and sperms with yellow, orange, or red nucleus depending on the extensive of damage, recognized as sperms with DNA damage (Fig. 2). A significant increase in the mean percentage of sperm with damaged DNA in CP group compared with the control group and CP + crocin group were observed (p < 0.05), (Table 2).

Sperm chromatin condensation. After aniline-blue staining the mean numbers of immature sperms in all groups were calculated (Fig. 1). The mean percentage of immature sperms in CP group compared to control and CP + crocin groups showed a significant increase (p < 0.05), (Table 2).

Sperm morphology. In this study, the percentage of

Table 2. Percentage of sperm chromatin condensation and DNA disintegration in different groups. Data are presented as mean ± SE.

| Groups                        | Chromatin condensation | DNA integrity of sperms** |
|-------------------------------|------------------------|---------------------------|
| Control                       | 2.00 ± 0.71            | 2.00 ± 0.41               |
| Cyclophosphamide              | 41.75 ± 3.75**         | 36.00 ± 2.79**            |
| Cyclophosphamide + crocin     | 18.50 ± 1.55**         | 10.50 ± 1.19**            |

ab indicate significant difference with control and cyclophosphamide groups respectively (p < 0.05). *Aniline blue positive and **Acridine orange positive.

Table 1. Different parameters of sperm quality. Data are presented as mean ± SE.

| Groups                        | Sperm count (×10⁶ per mL) | Motility (%) | Viability (%) | Normal sperms (%) |
|-------------------------------|---------------------------|--------------|---------------|-------------------|
| Control                       | 32.00 ± 0.65              | 61.00 ± 2.12 | 68.50 ± 0.64  | 92.25 ± 0.85      |
| Cyclophosphamide              | 15.87 ± 1.28**            | 35.77 ± 2.75**| 40.00 ± 3.03**| 61.75 ± 0.85**    |
| Cyclophosphamide + crocin     | 28.75 ± 2.18**            | 51.50 ± 3.12**| 53.25 ± 1.89**| 77.00 ± 3.29**    |

a,b indicate significant difference with control and cyclophosphamide groups, respectively (p < 0.05).
sperm with normal morphology was calculated. Results showed a significant decrease of this parameter in CP group compared with the other groups, and crocin in the CP + crocin group significantly increased this parameter ($p < 0.05$), (Table 1).

**Discussion**

Although CP is known for its anti-neoplastic properties, there are several reports of its cytotoxic impacts via enhancing the oxidative stress. In this regard, Lear *et al.*, showed that chronic administration of CP resulted in an increased lipid peroxidation in rats.$^{31,32}$ On the other hand, Crocin is used for treatment of dyslipidemia and atherosclerosis.$^{33}$ The antioxidant agents of crocin result in considerable inhibition of free radical generation in different tissues.$^{34}$ Considering the CP's impact on antioxidant status, we hypothesized that crocin with antioxidant properties could inhibit the CP-dependent damages. In present study we showed that CP significantly reduced the sperm count, motility and viability. Accordingly, a remarkable elevation in sperm abnormality was observed in CP-treated animals. Meanwhile, administration of crocin as a potent antioxidant compound down-regulated the CP-induced damage and enhanced the sperm motility and viability.

Our preliminary data showed that CP significantly decreased sperm count and motility. Reduction of sperm count, mainly attributed to beginning of testicular tissue damage. While decreased sperm motility largely depended on oxidative stress-induced derangements. Accordingly, the first sign of increased ROS content is remarkable reduction in sperm motility.$^{34-39}$ The increased levels of ROS considerably influences sperm enzymatic content, and increases phospholipids peroxidation, which ultimately reduces fluidity of cell membrane and sperm motility.$^{40,41}$ However, administration of crocin resulted in significant enhancement in sperm count as well as motility. Therefore, we could suggest that crocin might up-regulated the antioxidant status, which in turn elevated CP-induced damage in testicular tissue and up-regulated the CP-reduced sperm motility.

Observations demonstrated that CP-treated animals exhibited a remarkable increase in sperm abnormality. In order to understand how CP induced this impairment, one should note that in a physiologic conditions during the maturation process, a part of the sperm cytoplasm remove as residual bodies by Sertoli cells, otherwise the cytoplasmic droplets will be remain at the middle piece of sperm. Thus, the sperms with cytoplasmic droplets did not complete their maturation process.$^{42-44}$ Therefore, it is logical to hypothesis that CP exerts its degenerative impact partly via influencing the Sertoli cells physiologic function, which could be partly attributed to CP-induced oxidative stress. On the other hand, the crocin-received animals showed a significantly reduction in percentage of abnormal sperms. Thus, we can assume that crocin provokes the Sertoli cells physiologic activity partly by enhancing the antioxidant status.

Reportedly, there is a positive correlation between increased ROS generation and degenerative changes in all nucleic bases, including removal and unpairing of complementary bases, deformation and changes in cross-linking of DNA and chromosomal rearrangement.$^{45,46}$ As a result, these impairments lead to a severe DNA damage at sperm level. In close relation with these findings, our data showed that CP increased sperm DNA damage and crocin significantly decreased the percentage of sperms with DNA disintegrity. However, the protective role of chromatin condensation against free radicals should not be forgotten. Accordingly, most of the DNA damage happens during intermediate stages of spermiogenesis when replacement of protamine-histone occurs.$^{47}$ Moreover, CP affects the protamine-DNA binding processes and therefore results in alkilation of protamine. In correlation with these reports, CP-treated animals showed a remarkable reduction in percentage of sperms with condensed chromatin. Contrarily, crocin up-regulated protamination process. Thus, we can hypothesis that crocin protected the DNA content of the sperms via provoking the protamination.

The sperm plasma membrane integrity and controlled lipid peroxidation play important role in sperm viability. Accordingly, increased lipid peroxidation associated with continuing oxidative stress lead to severe damage at plasma membrane level.$^{48,49}$ As it is mentioned previously CP results in severe lipid peroxidation by elevating the oxidative stress.$^{32,33}$ Light microscopic evaluation of sperm viability showed that CP-treated animals exhibited significantly higher percentage of death sperms comparing to crocin-received group. Thus, we can suggest that crocin reduced CP-induced mortality partly by promoting antioxidant status and reducing lipid peroxidation.

In conclusion, our data suggested that crocin as an antioxidant compound was able to inhibit the CP-induced damage at sperm level. Thus, crocin can be co-administrated with CP in order to protect sperms against CP-dependent derangements.

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