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Protective Effect of Ghrelin on Oxidative Stress and Tissue Damages of Mice Testes Followed by Chemotherapy With Cyclophosphamide

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Abstract

Objectives: Cyclophosphamide (CP) is one of the common medications used as chemotherapy and immune-suppressive agent in organ transplantation. Despite numerous clinical applications of this drug in cancer treatment, it causes adverse effects on body tissues, especially the male reproductive organs by increasing oxidative stress. The present study aimed to analyze the effects of ghrelin, as an antioxidant substance, on testicular damages induced by CP.

Materials and Methods: Forty male mice were randomly divided into 4 groups: 1) control; 2) CP; 3) CP + ghrelin; and 4) ghrelin. CP (100 mg/kg body weight) was injected intraperitoneally once a week and ghrelin (80 µg/kg body weight) was administered daily for 5 weeks. After 5 weeks, the testicles were removed and we investigated histological changes and testicular oxidative stress markers including malondialdehyde, superoxide dismutase, glutathione peroxidase, and total antioxidant capacity.

Results: Our results showed that CP increased malondialdehyde level and decreased glutathione peroxidase, superoxide dismutase, and the total antioxidant capacity ($P<0.05$). Furthermore, degenerative changes in the testicular tissue were observed in CP group. The aforementioned factors were improved in the group that was treated with ghrelin ($P<0.05$).

Conclusions: The results of this study revealed that ghrelin decreases the damages caused by CP in testicular tissue of mice by reducing lipid peroxidation and increasing total antioxidant capacity.

Keywords: Chemotherapy, Cyclophosphamide, Oxidative stress, Ghrelin, Testes

Introduction

Cyclophosphamide (CP) is an alkylating and cytotoxic agent that is used in cancer therapy; it acts as an immune-suppressive agent in organ transplantation as well (1). CP is well absorbed from the digestive system and is widely distributed in tissues and body fluids and leads to the alkylation of DNA molecules (2,3). CP is bound between two strands of DNA and applies its cytotoxic effect by breaking the strands of DNA and inhibiting protein synthesis (2,3). Despite the wide clinical applications, CP causes many side effects in humans and laboratory animals. The most important side effects include decreased blood cells, increased levels of uric acid, reduced gonadal function and causing amenorrhea (2,4). A damaged testicular germ cell is one of the main adverse effects of chemotherapy drugs that have been proved in some experimental and clinical studies. Some of the reported adverse effects of aforementioned drugs on the male reproductive system are decreased testicular weight, transient oligospermia, changed histological and biochemical parameters of the testes (1,5).

However the exact mechanism of CP-induced reproductive toxicity is unknown, based on the results of the previous studies, administration of CP can disrupt redox balance in tissues and thus results in physiological and biochemical dysfunction caused by oxidative stress (1,3). According to some available evidence, administrated antioxidant during chemotherapy plays a crucial role in detoxification of oxidant agents created by aforementioned treatment (1,3). In this regard, Mohammadnejad et al showed that the use of GnRH can reduce the adverse effects of cisplatin in the testicular tissue (6). Lu et al also suggested that administration of Zn (II)–curcumin with antioxidant properties improved sperm parameters in mice treated with CP (1). Therefore, it is reasonable that reduction of CP-induced oxidative stress by an antioxidant can lower the resultant reproductive toxicity (1,7).

Ghrelin is a hormone with antioxidant effects that is primarily produced in the stomach (8). Initially, it was thought that ghrelin is an appetite-related hormone, but now we know that in addition to food intake and glucose in metabolic disorders, it plays a key role in brain

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practices including memory, learning, neuroprotection, reproduction and cardiovascular actions (9-12). Recent studies have shown that ghrelin is expressed in rats and humans' Sertoli and Leydig cells, ghrelin receptor (GHS-R) type 1a has also been observed in these cells (13). Therefore, it appears that ghrelin regulates the seminiferous tubules function directly. The accumulation of data demonstrates that ghrelin removes free radicals and thus reduces testicular damage caused by various factors such as cadmium and cisplatin (14,15). According to the antioxidant effects of ghrelin and since it has the receptor in the testes, the aim of this study is to investigate the effects of ghrelin on oxidative stress and histological damages in mice testes followed by chemotherapy with CP.

Materials and Methods

Animals and Groups Studied

In this experimental study, 40 Balb/c male mice aged 2 months were examined. Animals were purchased from Animal House of Tabriz Medical University and were kept under standard conditions (with a light/dark cycle of 12 hours and a temperature of 22-24˚C). The mice were divided randomly into 4 groups, each of which consisting of 10 mice, and were treated as follows: 1) control (Con); 2) CP; 3) CP + ghrelin (CP + Gh) and 4) ghrelin (Gh).

Cyclophosphamide (Endoxan, Germany) (100 mg/kg body weight) was injected intraperitoneally (ip) once a week for 5 weeks. Ghrelin (Innovagen, Sweden) (80 µg/kg body weight) was also administered ip daily for 5 weeks. The dosage of CP and ghrelin was chosen based on previous studies (1,16).

Sampling

After 5 weeks, the mice were weighed and killed by cervical dislocation and the testicles were immediately removed. After weighing, the right testicles were immediately frozen in liquid nitrogen for the analysis of oxidative stress markers including glutathione peroxidase (GPx), malondialdehyde (MDA), total antioxidant capacity (TAC) and superoxide dismutase (SOD) and were kept at a temperature of -80˚C until testing was completed. In addition, to evaluate histological changes, the left testicles were fixed in formalin (10%).

Assessment of Tissue Lipid Peroxidation

For this purpose, the testicular tissue was homogenized in 1 ml of 1.15% KCL. The level of tissue lipid peroxidation was measured according to MDA level. Measuring the level of malondialdehyde was based on the reaction of thiobarbituric acid (TBARS). The level of tissue MDA was measured based on the method described by Kaya (17).

Assessment of Tissue Superoxide Dismutase and Glutathione Peroxidase Activity

To measure cytosolic enzymes the testicular tissue was homogenized in 1.15% KCL. The tissue SOD activity was measured via the method described by Paoletti and using a spectrophotometer (17). Moreover, the enzymatic activity of GPx was measured using Randox kit (UK) according to the manufacturer's protocol (17).

Assessment of Tissue Total Antioxidant Capacity

The total antioxidant capacity of testicular tissue was measured using Randox kit (UK). $ABTS^{+}$ cation formation followed by its inhibition by the sample antioxidant compounds is the base of total antioxidant capacity assessment (17).

Histological Analysis and Maturation of Seminiferous Tubules

To evaluate the histological changes of the seminiferous tubules of testes after fixation, the testicles were dehydrated with an ascending ethanol sequence, cleared with xylene, and embedded in paraffin. Then, 5 µm sections were prepared and stained with hematoxylin-eosin (H&E). Spermatogenesis in the seminiferous tubules was evaluated using the Johnsen score (Table 1). For this, 50 seminiferous tubules were randomly selected from each slide and Johnsen score (scale of 1–10 based on the level of spermatogenesis) was calculated for each tubule. Then the mean Johnsen score of each case was calculated (18,19).

Statistical Analysis

All statistical analysis was carried out using the SPSS, version 11.5 (SPSS Inc, Chicago, Illinois, USA). All of

Table 1. Johnsen Score

Score	Level of Spermatogenesis
10	Full spermatogenesis
9	Slightly impaired spermatogenesis
8	Less than five spermatozoa per tubule
7	No late spermatids; many early spermatids
6	Few early spermatids; arrest of spermatogenesis at the spermatid stage
5	Many spermatocytes
4	Few spermatocytes; arrest of spermatogenesis at the primary spermatocyte stage
3	Spermatogonia only
2	No germ cells; Sertoli cells only
1	No seminiferous epithelial cells; tubular sclerosis

Table 2. Effects of Ghrelin on Body and Testes Weights of Mice Treated With Cyclophosphamide

Weight	Groups			
	Con (Group 1)	CP (Group 2)	CP + Gh (Group 3)	Gh (Group 4)
Body weight at the beginning of experiment (g)	23.40 ± 1.50	23.10 ± 1.19	23.80 ± 1.13	23.30 ± 1.63
Body weight at the end of experiment (g)	30.50 ± 1.31	20.03 ± 1.12 +	25.54 ± 1.64 *	31.06 ± 1.47
Testes weight at the end of experiment (mg)	97 ± 0.94	50 ± 1.40 +	83 ± 0.96 *	100 ± 0.36

Data are mean ± SE. + $P<0.05$ compared with control (Con) group; * $P<0.05$ compared with cyclophosphamide (CP) group.

the values were expressed as mean ± standard error (SE). The calculated data tested for parametricity using Kolmogorov-Smirnov (K-S) test. Finally, the data were analyzed using one-way ANOVA followed by Tukey tests. The results with $P<0.05$ were considered statistically significant

Results

The Effect of Ghrelin and Cyclophosphamide on Body and Testis Weight

Body and testis weight changes in different groups were displayed in Table 2. The results of this study showed that CP reduced body and testis weight significantly when compared to the control group ($P<0.05$). In the treatment group, ghrelin suppressed body and testicles weight loss ($P<0.05$). However, administration of ghrelin in Gh group did not make any significant change in body and testicles weight compared to the control group.

The Effect of Ghrelin and Cyclophosphamide on MDA Level of Testicular Tissue

The results of the comparison of MDA level showed a significant increase in the CP group compared to the control group ($P<0.05$). Administration of ghrelin plus CP significantly reduced MDA level compared to CP group ($P<0.05$). Furthermore, the comparison between the control and Gh group revealed that MDA level in the testicular tissue of the Gh group significantly decreased compared to the control group ($P<0.05$) (Figure 1).

The Effect of Ghrelin and Cyclophosphamide on SOD and GPx Activity of Testicular Tissue

Mean GPx and SOD activity of testicular tissue in the CP group significantly decreased compared to the control group ($P<0.05$). Administration of ghrelin in the CP + Gh group significantly prevented this decreased activity ($P<0.05$). The comparison of GPx and SOD activity between the control and the ghrelin groups did not show any significant change (Figure 2 and 3).

The Effect of Ghrelin and Cyclophosphamide on TAC of Testicular Tissue

We find that CP reduced the total antioxidant capacity of the testicular tissue significantly when compared to the control group ($P<0.05$). Ghrelin administration in the CP + Gh group significantly prevented decreased total antioxidant capacity compared to the CP group ($P<0.05$). Statistical analysis also showed no significant difference between the control and Gh group (Figure 4).

The Effect of Ghrelin and CP on Histological Changes in the Testicular Tissue

In the present study, the testis and seminiferous tubules of the control group had normal histology (Figure 5-A). Histological analysis revealed degenerative changes in seminiferous tubules epithelium such as cell detachment and reduction of the germinal epithelium thickness in the CP group (Figure 5-B). Treatment of mice with ghrelin in the CP + Gh group prevented the damaging effects of

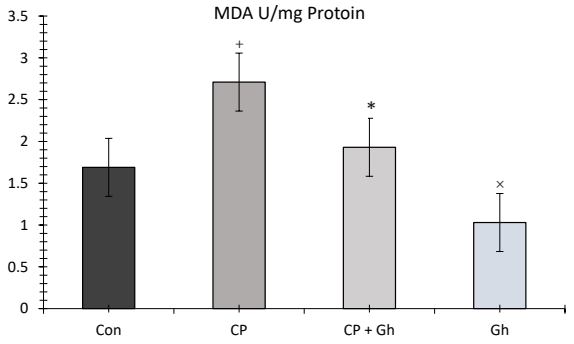

Figure 1. Comparison of the MDA Level in Testes Tissue in Different Groups. Data are mean ± SE. + $P<0.05$ compared with control (Con) group; * $P<0.05$ compared with cyclophosphamide (CP) group and × $P<0.05$ compared with control (Con) group.

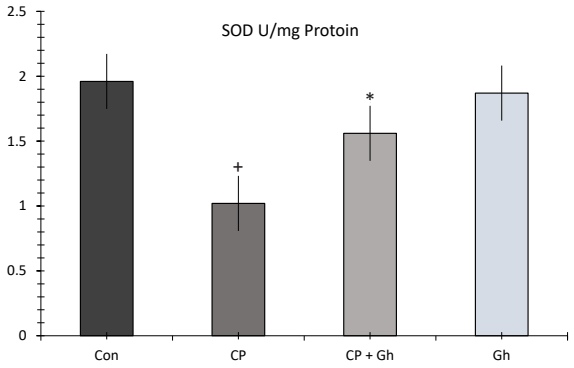

Figure 2. Comparison of SOD Activity in Testes Tissue in Different Groups. Data are mean ± SE. + $P<0.05$ compared with control (Con) group; * $P<0.05$ compared with cyclophosphamide (CP) group.

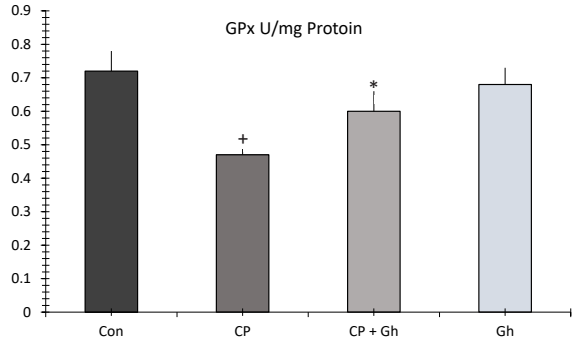

Figure 3. Comparison of GPx Activity in Testes Tissue in Different Groups. Data are mean ± SE. $^{+}$ P<0.05 compared with control (Con) group; * P<0.05 compared with cyclophosphamide (CP) group.

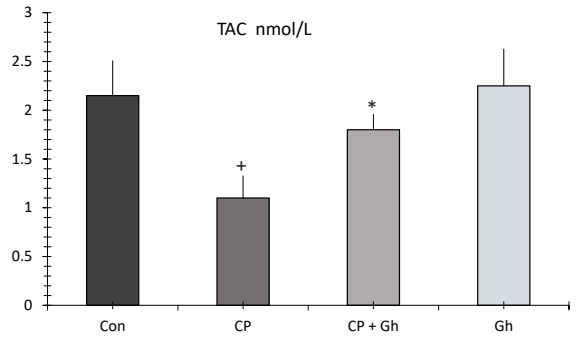

Figure 4. Comparison of the TAC Level in Testes Tissue in Different Groups. Data are mean ± SE. $^{+}$ P<0.05 compared with control (Con) group; * P<0.05 compared with cyclophosphamide (CP) group.

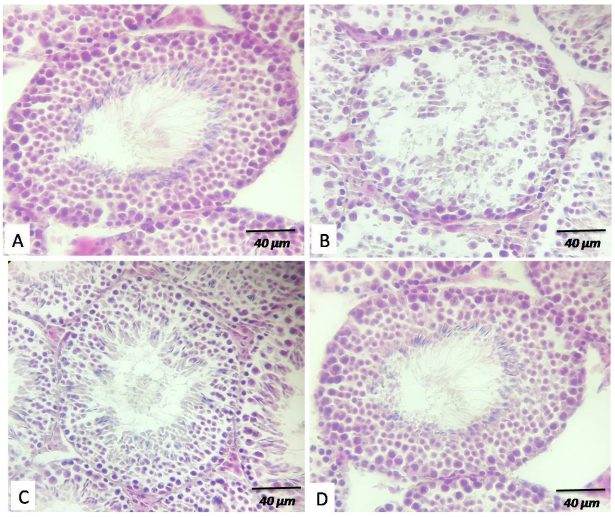

Figure 5. Light Microscopy of Testicular Tissue in Different Groups. Representative photographs of H&E staining in control group (A), Cyclophosphamide group (B), Cyclophosphamide + Ghrelin group (C) and Ghrelin group in mice testis (D) (400X).

Table 3. A Comparison of the Testicular Mean Johnsen Score in Difference Group

Groups	Con	CP	CP + Gh	Gh
MJS	9.12 ± 0.11	3.38 ± 0.25^{+}	6.78 ± 0.53*	9.24 ± 0.31

Abbreviation: MJS, mean Johnsen score.
Data are mean ± SE. $^{+}$ P<0.05 compared with control (con) group; * P<0.05 compared with cyclophosphamide (CP) group.

CP in seminiferous tubules (Figure 5-C). The comparison between the control and Gh group proved no histological changes (Figure 5-D). Statistical analysis showed that the mean Johnsen score (MJS) significantly decreased in CP group as compared with the control group (P < 0.05). The MJS of the CP + Gh group significantly increased when compared with the CP group (P < 0.05). No significant difference was recorded for Gh and control groups (Table 3).

Discussion

The biochemical and histological results of testicular tissue in this study showed that CP results in reproductive toxicity. Accordingly, to access ways to reduce the adverse effects of anti-cancer drugs while maintaining therapeutic efficacy of these drugs is necessary.

The evaluation of the body and testis weight in mice showed that CP caused a significant reduction in body and testis weight. This finding is consistent with the results of previous studies on the effect of CP on the body and sexual organs weight (1,3). One of the reasons for testis weight loss is spermatogenesis anomalies induced by CP. Testicle weight reduction can be caused by oligospermia or azoospermia, and histopathological alterations (20).

Histological analysis showed that germinal epithelium thickens in the CP group that experienced degenerative changes. These changes can be deemed as reasons for the reduction in testicular weight. However, administration of ghrelin to mice that were treated with CP prevented their weight loss. Moreover, histological analysis showed a decrease in the harmful effects of CP on the epithelium of seminiferous tubules in the mice treated with ghrelin. In this regard, Garcia et al have suggested that administration of ghrelin in mice treated with cisplatin prevents reduction of body and sexual organs weight (15). The protective effect of ghrelin could be due to its antioxidant property.

Previous studies relate toxicity of CP in the male's reproductive system to oxidative stress that occurs by inactivation of microsomal enzymes exposed to this drug through the increased generation of reactive oxygen species (ROS) and lipid peroxidation (1,4). In line with previous reports, our results also showed a significant increase in the MDA level (as a marker of lipids peroxidation), as well as a decrease in GPx and SOD activity, and TAC in mice treated with CP (1,21). In this regard, Turk et al demonstrated that CP increased the level of MDA in the testicular tissue (21).

GPx as an antioxidant plays a special role in protecting sperm in the testicular tissue and epididymis, and reduction of GPx in the body leads to infertility (22). This enzyme is in the plasma membrane of sperm, sperm nucleus and epididymal fluid which protects sperm against free radicals

and leads to final maturity and the development of sperm (22,23). This enzyme uses glutathione in the process of reducing lipid hydroperoxides and hydrogen peroxide and reduces oxidative damage. Reduced GPx activity suggests the overuse of glutathione reflecting an increased level of MDA and oxidative damage (17). Reduction of GPx activity and an increase of MDA level in the testicular tissue of the mice treated with CP in this study can also be an emphasis on the increase of lipid peroxidation.

Previous studies have suggested that antioxidants neutralize ROS formation during lipid peroxidation and protect the cell and tissue against the oxidative damage (24-26). On this subject, Lu et al have shown that administration of Zn (II)-curcumin that has antioxidant properties leads to the increase of GPx activity and the decrease of MDA level (1). Hence, reducing the level of MDA and increasing the testicular GPx activity in the group treated with ghrelin is likely related to their antioxidant effects.

Our results also showed that CP significantly reduces the activity of SOD compared to the control group. This finding agrees with the results of other studies which suggested that administration of CP decreases the activity of SOD in the testicular tissue (20). SOD is one of the most important enzymes of the body's antioxidant system and neutralizes the toxicity of superoxide through the decomposition of superoxide anion radicals (first radical product of oxygen) to H_2O_2 and prevents the formation of free radicals caused by superoxide (23,27,28). On the other hand, the results of this study showed that treatment of the mice with ghrelin increases SOD activity in the testes of the mice treated with CP. This finding confirms the results of the study conducted by Kheradmand et al in 2009 who reported that administration of ghrelin increases the activity of SOD in the testicular tissue (10).

Antioxidant mechanisms are normally exhibited in the body, including reproductive tissues, and prevent oxidative damage (17). Some treatments such as chemotherapy disrupt the activity of antioxidant system and thereby causing damage to tissues (3,29). Therefore, total antioxidant capacity (TAC) of the testicular tissue was examined in this study. The present results showed a reasonable decrease in the TAC in mice treated with CP, which is likely to happen due to the reduction of antioxidant system activity by CP. Administration of ghrelin as an antioxidant plus CP leads to the increase of the total antioxidant capacity. This finding is consistent with the results of previous studies. It is reported that ghrelin can increase the serum antioxidant capacity in rats under hypoxia (30).

According to the available evidence, testicular freezing and transplantation can be considered as the important step in fertility preservation of children with cancer (13,31). Oxidative stress is one of the most common damages during the freezing-thawing process (31). The previous study indicated that optimization of the freezing-thawing media with the antioxidant can reduce the side effects of freezing-thawing processes, the oxidative damage of this process (31). According to the results, ghrelin could protect testis from oxidative damages resulting from chemotherapy and also it can be used in freezing-thawing processes to improve the fertility preservation.

Conclusion

The results of the present study showed that CP through increased lipid peroxidation and decreased activity of antioxidant enzymes leads to the increased oxidative stress and damage to the testicular tissue. Injection of ghrelin plus CP resulted in the reduction of oxidative stress and damage to the testes through increasing antioxidant activity and decreasing lipid peroxidation. This performance is probably due to the antioxidant property of ghrelin that has been reported in previous studies.

Conflicts of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

All procedures and methods of working with animals were in accordance with the protocol of Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1395.307).

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