Health Services Vocational Collage

Protection against the cisplatin-induced testis injury by administering Ceratonia siliqua L. extract in Sprague dawley rats

Gulsah Yildiz Deniz[1]

Esra Laloglu[2]

Selim Comaklı[3]

Arzu Gezer[1]

Nimet Yigit[1]

1) Vocational school of health services, Ataturk university, 25240, Erzurum, Turkey

2) Faculty of medicine, Biochemistry department, Ataturk university, 25240, Erzurum, Turkey

3) Faculty of veterinary medicine, Pathology department, Ataturk university, 25240, Erzurum, Turkey

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Corresponding author: Gulsah Yildiz Deniz

Vocational School of Health Services, Ataturk University, 25240 Erzurum, Turkey

e-mail:gulsah.ydeniz@gmail.com

Abstract

The purpose of this study was to show the possible ameliorative effect of Ceratonia siliqua L. (CS) extract on the cisplatin (CP) induced testis damage in rats. The physiological saline was applied to the control group and CS was injected in two different doses for two groups, that is, the low-dose CS group and the high dose group. The CP injection was divided into three groups (CP group, CP + low dose CS group, CP + high dose CS group). At the end of the experiment, the animals were sacrificed and their testis tissues were removed for the biochemical and histopathological investigation. Total antioxidant status (TAS) and total oxidant status (TOS) levels were determined and finally the histopathological changes int he testis tissues were examined using hematoxylin-eosin staining method. It was found that applying CS extract could revert the level of fertility hormones and suppress the histomorphological alterations and DNA damages. CS provided the evidence that it might have a therapeutic role in free radical mediated diseases.

Key words: Chemotherapy, Ceratonia siliqua, Cisplatin, Cu / Zn SOD, Sprague dawley, Testis.

Introduction

CP is a drug used in the treatment of many tumors such as the neck, endometrium, kidney, bladder, head, lung, and ovary (Pabla et al., 2008). However, CP can damage the testicles and cause infertility (Sabanegh et al., 2009). Today, the infertility is a problem that is increasing in prevalence. About 40% of the infertility problems are associated with men (De Keersmaeker et al., 2004). Furthermore, after the chemotherapy and radiotherapy treatment, the rate of infertility increases even more in the cancer patients (Colpi et al., 2004; Dohle, 2010). In recent years, the natural products and dietary antioxidants have been widely used to protect the organism against the CP induced toxicity (Behling et al., 2006; Darwish et al., 2018). In both preclinical and clinical studies, CS has been shown to be effective in preventing the side effects such as liver toxicity, gastrointestinal toxicity, and pancreatic toxicity (Souli et al., 2015; Ydjedd et al., 2017; Rtibi et al., 2017). Ceratonia siliqua L. (CS) is an evergreen tree grown in the Mediterranean climate. It grows in small or large groups around Antalya, Mersin, and Muğla in Turkey (Pazır F, & Alper, 2018) and contains carbohydrates, tannins, polyphenols, and dietary fiber (Papagiannopoulos et al., 2004). CS is used as a raw material in the pharmaceutical, cosmetics, and food industry. Since carop (Ceratonia siliqua L.) is sweet and has a chocolate-like taste, the carop pods have long been used as a cocoa substitute in the food production (Durazzo et al., 2014). In the experimental and clinical studies conducted on C. silique, it has been observed that most of the pharmacological effects are mainly due to the antioxidant activity occurring by means of its ability to cleanse the free radicals and/or prevent the lipid peroxidation (Kumazawa et al., 2002). It has been revealed that carop selectively protects the vital tissues such as liver, gastric mucosa, and kidney (Hsouna et al., 2011; Rtibi et al., 2015).

In folk medicine, people generally claim that carob is an incredible source of energy and it has the power to increase the sexual power and desire. Besides, there are some subject matter experts who say that since it is effective against the impotency, people regularly consume it. It is said to be very effective against the sperm loss. However, no study has been conducted yet to examine whether carob has these effects.

Therefore, this study sought to evaluate the protective effects of the methanolic extract of carob in the rat model having CP induced testis injury. In the light of the information in the literature, we hypothesized that CS might have the protective activity against the CP induced testicular toxicity.

Material and Method

Chemicals

Cisplatin [high performance liquid chromatography (HPLC) grade≥ 98%] were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All drug and chemicals were freshly prepared before each administration.

Animals

Fifty six adult male Sprague dawley rats (weighing 230 - 240 g) obtained from the Medical Experimental Application and Research Center, Atatürk University, Turkey, were used. The animals were housed inside polycarbonate cages in an air-conditioned room (22 °C ± 2 °C) under a 12 hour light dark cycle. Standard rat feed and water were provided ad libitum.

Experimental groups

A total of 42 male Sprague dawley rats (230 - 240 g) were used in the study. The rats were randomly divided into six groups (7 rats in each group).

I. Control group (n = 7); On the 10th day of the study, the physiological saline was injected.

II. CP Group (n = 7); On day 10, 7 mg/kg of CP was injected (i.p.).

III. Low dose CS Group (n = 7); 14 days (100 mg/kg) (i.p.).

IV. High dose CS group (n = 7); 14 days (200 mg/kg) (i.p.).

V. CP + low dose CS group (n = 7); (100 mg/kg) was given i.p. for 14 days and 7 mg/kg CP was injected on day 10.

VI. CP + high dose CS group (n = 7); (200 mg/kg) was given i.p. for 14 days and 7 mg/kg CP was injected on day 10.

The dose and duration of CP were determined according to the study carried out by Ademiluyi et al., Although several researchers chose different doses of CS in their studies, we preferred the doses used by Rtibi et al. (2017) The approval of Committee for Institutional Animal Care and Use was taken from Atatürk University Local Board of Ethics (with protocol number: 2, 22.02.2018).

Collection of samples

One day after the last treatment, the rats were killed after sevoflurane inhalation (SevoFlo; Abbot Laboratories, Chicago, IL, USA). Both testes were removed. The right testis was fixed in 10% buffered formalin for the histopathological examination.

Preparation of carob extract

The carob pods were collected in August 2017 in Antalya and Mersin (Southern part of Turkey). The plant material was later dried in an incubator at 50 °C for 72 h and then powdered in an electric blender (Moulinex Ovatio 2, FR). The powder mixture containing the carob pods (90%) and seeds (10%) was dissolved in distilled water. It was filtered through a colander (0.5 mm

mesh size). The carob pods aqueous extract was immediately used for in vivo experiments.

Biochemical analysis

The blood samples taken from the animals were collected in the gel-activated tubes for evaluation of FSH, LH and testosterone, then centrifuged at 4000 xg for 10 minutes at 4 °C (Roche Diagnostic, commercial kits in COBAS device).

Preparation of tissue homogenates

The testicular tissue samples obtained from each group were first perfused with PBS / heparin. The frozen tissues were homogenized in a TissueLyser II grinding Jar set (Qiagen, Hilden, Germany). 100 mg of ground tissue was transferred to an eppendorf tube with TissueLyser II and homogenized by adding 1 ml of PBS homogenate buffer, and then the samples were centrifuged.

Measurement of tissue total antioxidant status and total oxidant status levels

TOS and TAS of each sample, taken from the supernatant, were measured by colorimetric methods using the commercially available kits (Rel Assay Diagnostics, Bursa, Turkey). The results of TAS (Erel, 2004) and TOS (Erel, 2005) in the tissues were expressed as mmol/mg protein and mol/mg protein, respectively (Erel, 2004; Erel, 2005).

Histopathological evaluation

The tissue samples were fixed in 10% buffered neutral formalin solution and placed in the fixation solution for 48 h and in water overnight. Then, the tissue specimens were dehydrated by passing through increasing concentrations of alcohol, cleared by passing through xylol, and finally embedded in paraffin blocks. Paraffin sections of 3 - 5 μm slice thickness were cut from each specimen and placed on slides. They were deparaffinized, rehydrated through a graded series of ethanol, and rinsed in distilled water. The specimens were stained with haematoxylin eosin (H & E) stain to evaluate the tissue morphology and integrity. The high-resolution pictures of the samples (× 100 and × 200) were taken under bright field using an Olympus B X 60 microscope.

Immunofluorescence analysis

Paraffin sections of 5 μm slice thickness were cut from each specimen, placed on slides, and passed through xylol and alcohol series. The slides were washed with phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated by 3% H2O2 for 10 min. Then, the samples were placed in the microwave oven and processed at 500 watts for 5 min with an antigen in the tissues. Then the tissues were incubated with antibody (cat no. LS B9346, dilution 1/100; Lifespan, USA) at 37 °C for 30 min for apoptosis. The cross sections washed with PBS after the incubation were used for Goat Anti Rabbit IgG H & L TR (catalog number: ab6719, dilution 1/50, Abcam, UK) secondary antibodies for 45 min. At the end of the process, the surfaces of the cross-sections were coated with 4 6, 6 diamidino 2 phenylindole (DAPI) florescence medium. The fluorescence staining was expressed in three categories, that is; negative, intermediate, and severe.

Statistical Analysis

Data recording and analysis was carried out using SPSS 20.0 for Windows (SPSS

Inc., IL, USA). The descriptive data were expressed as mean ± standard deviation. Kolmogorov Smirnov test was used for assessing the compatibility with normal distribution of TAS and TOS kidney homogenate levels and the serum FSH, LH and testosterone results. Since all results were normally distributed, comparisons of them among the groups were made using the parametric one way ANOVA, while degree of significance of differences between the groups was determined using the post hoc LSD test.

Results

Effect of CS treatment on male reproductive hormones of rat

In order to investigate the protective effects of CS on the basic toxicity induced by CP, we created an animal model of CP exposure and administrated different doses of CS to the rats. The reproductive hormones such as testosterone (T), luteinizing hormone (LH) and follicle stimulating hormone (FSH) are considered as the imperative serological biomarkers to estimate testicular toxicity. CP inoculation significantly ($P < 0.05$) decreased the concentration of serum T, LH and FSH (Fig.1A, 1B, 1C). These hormones were ameliorated significantly ($P < 0.05$) by i.p. administration of CS compared to CP group. However, the CS alone group showed the same serum hormone concentration as the control group. The CP + high dose CS treatment was more effective ($P < 0.05$) than the CP + low dose CS treatment in preventing CP intoxication, which signified the protective role of CS.

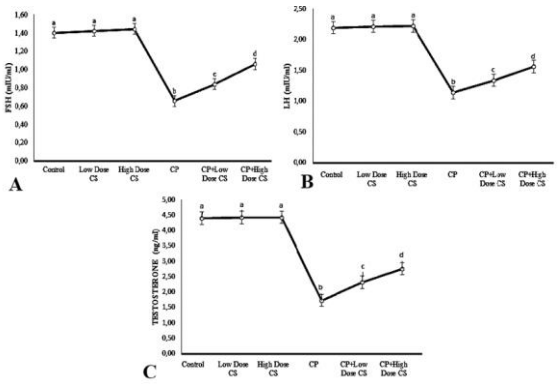

Figure 1. The effects of CS on serum FSH, LH and Testosterone levels after the CP treatment. Data are presented as mean ± SD (n =7). Different letters indicate significant differences between the studied groups at $P < 0.05$.

TOS and TAS levels

The differences between the control, CP, and CP + high dose CS groups were found to be statistically significant ($P < 0.05$) in terms of TAS and TOS levels. The TAS level in the CP group was significantly lower compared to the control group (Figure 2A). When we compared the CP group with the CP + high dose CS group, we found that the mean TAS level significantly increased, but it did not approach the control group exactly. Serum TOS levels were almost identical in the control, low dose CS, and high dose CS groups, but were significantly different in the CP group ($P < 0.05$). Furthermore, serum TOS levels of the CP group were significantly higher than the control and the CS groups ($P < 0.05$) (Figure 2B). Plant extracts significantly reduced this high TOS level but could not reduce it to the control level.

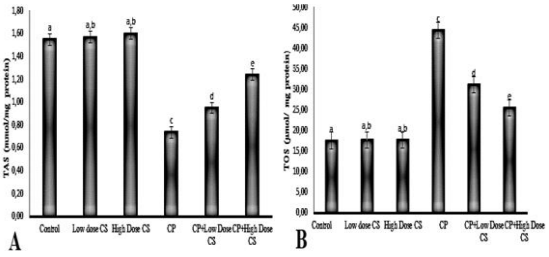

Figure 2. The effects of CS on testis TAS and TOS levels after the CP treatment. Data are presented as mean ± SD (n =7). Different letters indicate significant differences between the studied groups at $P < 0.05$

Histopathological analysis

The histo architecture of the testicular tissue of the control groups exhibited a normal structure and normal cells (Figure 3A). In the high-dose CS group, a near control image was obtained (Figure 3B). There was no difference between the low dose CS and the high dose CS group (Data not shown). In the CP group, the testes showed a significant reduction in the seminiferous tubular diameter and also showed an abnormal spermatogenesis and maturation compared to the control group (Figure 3C). The rats treated with CP + high dose CS showed an improved histological appearance compared to the CP group (Figure 3D).

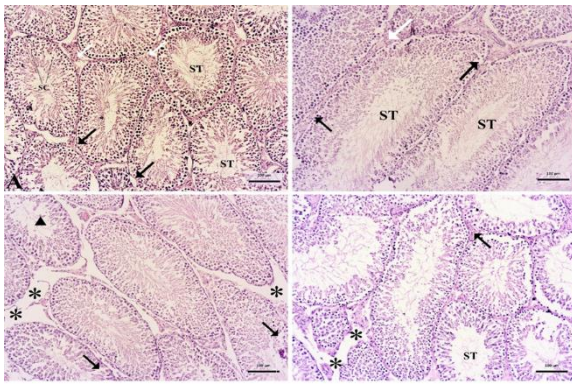

Figure 3. Hematoxylin eosin staining of rat testes. Testicular structure was observed as normal in the control group and the CS groups (at 100 mg/kg, 200 mg/kg, data not shown). (A) Control group: seminiferous tubules (ST), leyding cell (white arrows), spermatogonia (black arrows), sertoli cells (SC); (B) high dose CS group: leyding cell (white arrows), spermatids (black arrows); (C) CP group: degenerative alteration (arrows) was seen in seminiferous tubules, empty tubuli or tubuli containing scarce number of spermatozoa (arrow head), interstitial dilation (*); (D) CP + high dose CS group: degenerative alteration (ST), interstitial dilation (*) and vascular bleeding (black arrow) (H&E, Scale bars: 100 μm).

Cu / Zn superoxide dismutase expression in the rat testis

In each study group, Cu / Zn SOD level was measured to determine the effect of CS treatment on the antioxidative and oxidative parameters. No difference was found between the control group and the CS treated groups in terms of the distribution pattern and cellular identity of Cu / Zn expressing cells and, therefore it was described in general terms (Figure 4A). The CP treated group showed a low immunoreactivity for Cu / Zn SOD (Figure 4B). In the CP + high dose CS group showed a higher immunoreactivity for Cu / Zn SOD compared to only CP treated group (Figure 4C). Furthermore, there was no significant difference in the Cu / Zn SOD expression in the low dose CS-treated group after the CP administration compared to the high dose CS treated group (data not shown) (original magnification × 10).

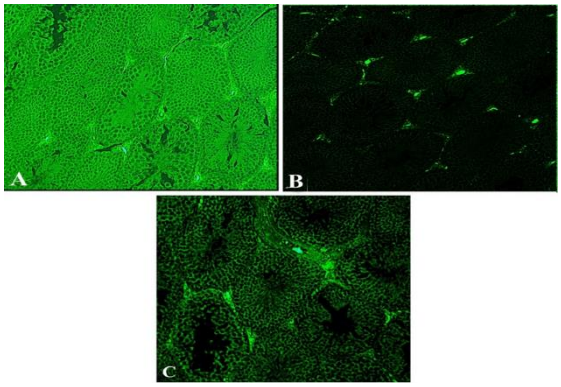

Figure 4. (A) Control group: Cu / Zn superoxide dismutase expression positive, (B) CP alone group: low Cu / Zn superoxide dismutase expression, (C) CP + high dose CS group: intermediate cleaved Cu / Zn superoxide dismutase expression. No difference was observed between the low-dose and the high dose CS groups after CP administration in terms of Cu / Zn SOD expression (FITC).

Discussion

Carob plant is of great importance because of its various pharmacological activities (Gulay et al., 2012). In this study, the protective effect of the carob extract on the testis cells was evaluated in vitro, then the acute toxicity of the carob on male rats was evaluated and the effective doses were determined (100-200 mg/kg). Our findings showed that the carob extract was not toxic against the testis cells. This result was consistent with the data obtained from the liteature review and the information obtained from the local people. Fruits have a potential antioxidant effect on the organisms and are capable of reducing or preventing the effects of free radicals, even at low concentrations (Maxwell et al., 1995). Thus, the immunoexpression of the Cu / Zn SOD enzymes in the antioxidant balance x free radicals in testis injury induced by CP was evaluated in the animals treated with CS. Consistent with the literature, in this study, the CS treatment was found to reduce the oxidative stress in the testis, as seen in significant increases in Cu / Zn SOD expression (Soyman et al., 2018). Therefore, the aforementioned pathways may be responsible for the attenuation of CP induced oxidative stress. These findings suggest that CS may act as a testis protectant against the CP therapy through its high Zn content and ability to scavenge ROS.

CP has been proposed for the clinical use due to its curative effect. The CP treatment is a widely accepted form of chemotherapy for the treatment of testicular malignancies (Krege et al., 2008) however, it is limited due to the development of myelosuppression and genotoxicity which may lead to a secondary cancer (Basu et al., 2015). Generally, infertility is a secondary syndrome that occurs after the chemotherapy and radiotherapy. In recent years, the cryopreservation of semen accompanies the treatment of cancer patients to partially compensate for the possibility of infertility. However, the sperm cryopreservation and thawing reduces the fertility and alters the content and function of various sperm proteins (Ojaghi et al., 2018). Therefore, there is need for the more efficient and harmless alternative approaches. Testosterone (T) has been accepted to play a crucial role in the reproductive function and is mainly regulated by the hypothalamic-pituitary-testicular axis (Tremblay et al., 2015). The generation of male gametes is directly linked with the collective actions

of both the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), the two separate heterodimeric glycoprotein gonadotropin hormones on the testis (Simoni et al., 1999). In order to investigate the effects of CP and CS exposure on testicular testosterone and its underlying mechanism, we observed the levels of crucial hormones, such as T, LH, and FSH. In this study, CS proved to be very successful and effective in increasing the sexual capacity by remarkably enhancing the T, LH and FSH levels (Figure 1). Our results are in concordance with the others (Atesahin et al., 2006; Ilbey et al., 2009).

In this study, we also investigated the effects of TAS and TOS on testis. According to our results, it was found that the measurements of TAS in the biological tissues would enable the researchers to understand wheter the protective effect displayed by the antioxidants reflected an improvement in the endogenous antioxidant defenses and a reduction in the chemotherapy risk. We established that CS administration dose-dependently increased the antioxidant capacity (100 and 200 mg/kg). Especially, 200 mg / kg of CS exhibited a powerful antioxidant activity in testis against the CP exposure. According to our findings, CS could protect the tissue from the harmful effects of CP by increasing the level of reduced TAS. CS increased the cells' antioxidant capacity by stimulating the synthesis of antioxidant enzymes and helped maintain their activity during the oxidative stress (Figure 2).

The CP-induced reproductive toxicity and the recovery of spermatogenic damage depend on the type of drugs, doses of drugs, and the length of therapy (Ishikawa et al., 2004). Recent studies have reported that the toxic effect of CP on the testis results in a variety of anomalies, such as atrophic necrotic seminiferous tubules, germinal epithelium damage, inflammation and damage in the peritubular area (Hussein et al., 2015; Aksu et al., 2016). Our histopathological findings appeared to support the cytotoxic effects of CP. Our results, however, were consistent with those of our previous study, in which cisplatin was found to cause an injury in rat kidneys (Deniz et al., 2020).

However, we determined that CP caused an increase in the nuclear caspase 3 expression in the spermatogonial cells. On the contrary, the expression of caspase 3 decreased in all CP groups treated with the CS extract. In the cisplatin group alone, we observed the degenerated basal lamina, apoptotic spermatogonia, spermatocytes, fragmented spermatogonia, and spermatid. The spermatocytes were swollen and lost their stellar appearance, demonstrating a condensed nucleolus, cytoplasm, and perinuclear area.

As a result, we concluded that carob extract could improve the disorders of male reproductive system caused by CP. This is the first study to evaluate the healing effects of CS on testicular injury caused by CP.

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Authors' Contributions

Conception and design of study: Gulsah YILDIZ DENIZ

Acquisition of data: Gulsah YILDIZ DENIZ

Analysis and/or interpretation of data: Gulsah YILDIZ DENIZ, Esra LALOGLU, Selim ÇOMAKLI

Drafting the manuscript: Gulsah YILDIZ DENIZ

Revising the manuscript critically for important intellectual content: Gulsah YILDIZ DENIZ

Approval of the version of the manuscript to be published: Gulsah YILDIZ DENIZ, Nimet YIGIT, Arzu GEZER

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Conflict of Interest

The authors report no financial or nonfinancial conflict of interest.

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