Protective Effect of Fennel Oil on Cyclophosphamide Inhibited Spermatogenesis and Induced Oxidative Stress in Albino Rats

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Abstract

Introduction: Cyclophosphamide (CPA) is an anticancer drug. Fennel (Foeniculum vulgare Mill) essential oil is a traditional medicine used against many diseases.

Aim. The present work studied the effect of fennel oil against testicular damage and oxidative stress induced by the anticancer drug, cyclophosphamide (CPA) in albino rats.

Methods. Animals were divided into 4 groups: group1, control, group2, orally given fennel oil, group3 treated with CPA and group4 treated with CPA and fennel oil. The testes were removed for histological and immune histochemical preparation. Blood was collected and sera were prepared for hormonal and biochemical analysis.

Results. The results revealed that CPA caused histological alterations in the testis including decrease in diameter and germinal epithelial height of the seminiferous tubules, degeneration of germ cells, cytoplasmic vacuolation and congestion of blood vessels. Cell proliferation marker was decreased and apoptotic marker caspase-3 was decreased. Biochemical results revealed decrease in the hormones LH and testosterone. Moreover, the serum activity of the antioxidant enzymes, SOD, CAT was decreased and the lipid peroxidation marker, DMA was increased. Treating rats with CPA and fennel oil caused an improvement in the histological structure of the testis. There was an increase in LH ,testosterone,SOD and CAT, while MDA level decreased.

Conclusion. It is concluded that administration of fennel oil exhibited protective effects against CPA-induced reproductive toxicity in male rats. The protective effect of fennel oil might be due to induction of antioxidant defense systems by one or more of its constituents.
Introduction

Cyclophosphamide (CPA) is a nitrogen mustard alkylating agent, and anti-neoplastic drug. It is used to treat Hodgkin’s disease, lymphomas, leukemia, granulomatosis, severe rheumatoid arthritis, and lupus erythematosus. It is also used in combination with other drugs to treat breast cancer, leukemia, and ovarian cancer. The drug also has immunosuppressive action when it has used in smaller doses (1). CPA acts by modifying and cross linking purine bases in DNA, thus inhibiting DNA, RNA and protein synthesis and death of rapidly dividing cells. CPA has been found to cause many toxic effects including haemorrhagic cystitis, pulmonary fibrosis, irreversible azospermia in man, gastrointestinal bleeding and hepatotoxicity(2). Cardiotoxicity is a major problem with people treated with higher dose regimens (3). In addition, CPA was found to affect male reproduction. Wetzel (4) Indicated that men exposed to CPA may develop oligospermia or azoospermia associated with increased gonadotropin release. Turk et al., (5) reported that CPA impaired spermatogenesis and androgenesis and induced germ cells apoptosis.

Medicinal plants contain phytochemicals and numerous chemical compounds, which are used in treatment of different diseases. Fennel plants (Foeniculum vulgare) is a medicinal plant belongs to the family Apiaceae (Umbelliferae) (6). This herb is traditionally used as treatment for colic, wind, irritable bowel, kidneys, spleen, liver, lungs, suppressing appetite, breast enlargement, promoting menstruation, improving digestive system, milk flow and increasing urine flow (7). The chief component of fennel, anethole, had anticarcinogenic and anti-inflammatory effects through modulation tumor necrosis factor–induced cellular processes (8) and antimicrobial properities (9). Furthermore, fennel has a bronchodilatory effect (10) as well as immunomodulatory activities by enhancing natural killer cell functions, the effectors of the innate immune response (11). Fennel extracts was found to increase male fertility and is consider as a novel medicine for treatment of infertility (12,13). In view of these considerations, the present work investigated the effect of fennel oil on cyclophosphamide-induced testicular toxicity in albino rats.

Materials and Methods

Chemical and Plant used:

1-Endoxan (Cyclophosphamide):

Endoxan was obtained as tablets from Baxter Oncology Halle, Germany. Each tablet contain 50mg cyclophosphamide. Endoxan was dissolved in distilled water and orally given by gastric tube at a dose level of 15 mg/kg body weight once a week for six weeks (14).

2- Fennel Oil:

Fennel essential oil (FEO) was purchased from a localmarket at shebin El-Kom, Menufyia Governement (ElMasry Everline company). The main oil components of oil were found to be trans-anethol (84.1-86.1%),fenechone (7.13 – 8.86 %), limonene (3.0–3.3%),and methyl chavicol (2.5–2.7 %). Fennel essential oil was given at a dose level of 1mg/kg body weight once a week for six weeks (15).

Experimental Animals:

Forty eight healthy adult Wister male albino rats, three months age weighting 150±10 g were purchased from experimental rat house localized in Helwan. Animals were kept in plastic cages (each contained six animals) in the animal house for two weeks before the experimental work. Animals were kept at 25 ± 2 °C with relative humidity of 50-60% and on 12h light/ 12h dark cycle. They received a standard diet composed of 50% barley, 20% yellow corn, 20% dry milk, 10% different vegetables and tap water. The study and all procedures were approved by the Animal Care and Bioethics Committee, Menoufia University, Egypt (Approval No. MNSH173). Animals were divided into four groups

Group 1 (Control group): Animals of this group (12 rats) were served as control group and were kept without any treatment and were given standard diet and tap water.

Group 2 (Fennel oil group): Animals of this group (12 rats) were orally given fennel oil at a dose level of 1 mg/kg body weight once a week for six weeks.

Group 3 (CPA group): Animals of this group (12 rats) were orally treated with endoxan at a dose of 15mg/kg body weight once a week for six weeks.

Group 4 (CPA+Fennel oil group): Animals were
given endoxan and then after two hours they were given fennel oil, with the same doses of group 2 and 3.

**Histological Studies:**

For histological study, testes were immediately removed after 3 and 6 weeks, and fixed in 10% formalin for 24 hours. Specimens were dehydrated in ascending series of ethyl alcohol, cleared in two changes of xylene, infiltrated in three changes of molten paraffin (melting point 58-60 °C) and then embedded in molten paraffin blocks. Paraffin sections (5 micron thickness) were sectioned using a rotary microtome and mounted on clean glass slides. Sections were stained with Ehrlich’s hematoxylin and counter stained with eosin for histological examination (16).

**Immuno Histochemical Studies:**

For Immunostaining methods of kip67 and Caspase-3, slides were deparaffinized in xylene and rehydrated in a series of alcohol concentrations, then rinsed in phosphate-buffered saline (PBS) containing 0.1% tween-20. Antigen retrieval was performed by placing slides in sodium citrate solution (PH 6.0) at 90°C. Avidin (0.001% in PBS) and biotin (0.001% in PBS) were blocked in each section by using Avidin/biotin blocking solutions, where sections were incubated and rinsed with PBS between steps. Sections were incubated with monoclonal primary rat antibodies (Neo Markers, Cat.#Ms-113-P, Fremont, CA,USA), at appropriate dilution (1:200) in antibody diluent, directed against rat Ki-67 or Caspase (each antibody was used separately to react on different slides) at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in peroxidase blocking solution (3%H2O2 in PBS) at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in biotinylated secondary antibody in PBS at room temperature. Slides were washed in PBS-Tween 20. For detection, sections were incubated in horse radish peroxidase (HRP)-streptavidin solution at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in peroxidase substrate solution “3,3-diaminobenzidine tetra hydro chloride (DAP)” until adequate color was developed. Slides were washed in PBS-Tween 20. Sections were counterstained with hematoxylin, dehydrated through garded alcohol series, clear in xylene and mounted with DPX (17).

**Biochemical Analysis:**

For biochemical analysis, blood samples were collected in clean centrifuge tubes. Blood samples left to clot in room temperature and then serum separated by centrifugation at 3000 rpm for 20 minutes. The collected serum stored at -18 -20 °C until analysis. The activity of superoxide dismutase (SOD) was estimated according to (18), the activity of Catalase (CAT) was estimated according to (19). Glutathione (GSH) was determined according to (20). Malondialdehyde (MDA) was assayed according to (21). Testosterone and Luteinizing hormone LH were estimating according to the method of (22).

**Statistical Analysis**

Data were expressed as mean ± standard deviation (SD). The significance of differences means was evaluated by using independent sample t test. All statistical analysis was performed using SPSS statistical version 16 software package.

**Results**

**Histological Observations**

The testis of control animal is formed of a number of seminiferous tubules each contain the different stages of spermatogenesis (spermatogonia, 1ry, 2nd spermatocytes and spermatozoa). Between the seminiferous tubules, the interstitial region, there are blood vessels and the interstitial cells (Leydige cells) (Fig.1-A). Testes of animals treated once a week with fennel oil (1ml/kg) for three and six weeks showed nearly normal histological structure (Fig 1-B).

Testes of animals examined after three weeks of treatment with cyclophosphamide exhibited a distinct histological change when compared with control group. The spermatogenic layers were degenerated and appeared with less compact spermatogenic cells. The basement membrane appeared in some tubules irregular and others were separated from the underline. Within the intertubular connective tissue, haemorrhage (Fig 1-C) and congested blood vessels were seen (Fig 1-D). Testis of animals treated with cyclophosphamide for six weeks showed exfoliated germ cells to the center of the tubules (Fig 2-A), Marked degeneration of the spermatogenic cells and appearance of vacuoles were observed (Fig 2-B). The interstitial connective tissue was degenerated and the number of germ cells was...
Fig. 1  A) A photomicrograph obtained from testis of a control rat showing normal seminiferous tubules, different stages of spermatogenic cells, spermatozoa (Sp) and interstitial tissue (IT), (H&E). B) A photomicrograph obtained from testis of a rat treated with fennel oil for six weeks showing normal structure of seminiferous tubules, (H&E). C) A Photomicrograph obtained from testis of a rat treated with CPA for three weeks showing interstitial haemorrhage (h), and separation of germ layers from underline basement membrane (arrow), (H&E). D) A Photomicrograph obtained from testis of a rat treated with CPA for three weeks showing congested and enlarged blood vessel, (H&E).
reduced (Fig 2-C). Testes of rats treated with cyclophosphamide and fennel oil for 3 weeks showed an improvement in the histological appearance of the testicular tissue, but intertubular haemorrhage was rarely seen. After 6 weeks of treatment with cyclophosphamide and fennel oil, the histological picture of the testes appeared better than testes of animals treated only with cyclophosphamide. There was an increase in number of spermatogenic cells (Fig. 2-D).

Morpho Metrical Results:

Figure (3 &4) showed that the diameter and epithelial height of seminiferous tubules of CPA-treated animals for 3 weeks were nearly similar to control animals. A significant decrease was seen in diameter and epithelial height of seminiferous tubules of the CPA treated groups for 6 weeks. Animals treated with CPA followed by fennel oil for 6 weeks showed a significant increase in diameter and epithelial height when compared with CPA groups.

Immuno Histochemical Results:

1- The Expression of Ki-67

In control group, Ki-67 was expressed in the nuclei of the spermatogonia as brown color. On the other hand, the negative nuclei of the spermatocytes are stained blue with hematoxylin (Fig 5-A). Animals given fennel oil for six weeks showed expression of Ki-67 in the nuclei of the spermatogonia which is nearly similar to control group (Fig 5-B). A decrease expression of Ki-67 immunore activity was observed in most of the nuclei of the spermatogonia after six weeks of treatment with CPA (Fig 5-C). Testis sections obtained from rats treated with CPA followed by fennel oil for six weeks, showed expression of Ki-67 in cytoplasm of leydige cells (Fig7-C). Testis sections obtained from rats treated with CPA followed by fennel oil for six weeks, showed a decrease in expression of casepase-3 immune reactivity in the cytoplasm of Leydige cells (Fig 7-D). The data figure (8) showed the percentage area of casepase-3 expression in cytoplasm of Leydige cells showed a significant increase in comparison with control group. There was no significant difference in the expression of casepase-3 in cytoplasm of Leydige cells when compared with CPA groups.

Biochemical Results

1- Change in serum testosterone and LH hormones

The change in serum testosterone and LH are seen in Figures 9&10. Animals treated with CPA for three and six weeks showed a significant decrease in serum level of testosterone and LH when compared with control group. On the other hand, animals treated with CPA for three and six weeks followed by fennel oil showed a marked increase in levels of these hormones, in comparison with CPA group. There was no significant differences in serum level of testosterone and LH in control and fennel group during the experimental period.

2- Lipid peroxidation marker and antioxidant enzymes:

Treating Animals with CPA for three and six weeks showed a significant increase in serum MDA activity. On the other hand animals treated with CPA and fennel oil showed a significant decrease in serum MDA level. The present results showed that testosterone and LH were reduced in rats treated with CPA. These results are in agreement with results of some investigators.
Fig. 2 A) A Photomicrograph obtained from testis of a rat treated with CPA for six weeks showing exfoliated cells (arrows), (H&E). B) A Photomicrograph obtained from testis of a rat treated with CPA for six weeks showing appearance of vacuoles (arrows) and degenerated spermatogenic cells, (H&E). C) A Photomicrograph obtained from testis of a rat treated with CPA for six weeks showing degenerative interstitial tissue and reduced and n degenerated spermatogenic cells, (H&E). D) A Photomicrograph obtained from testis of a rat treated with CPA followed by fennel oil for six weeks showing advanced degree of improvement of seminiferous tubules and increase of spermatogenic layers, (H&E).
Fig. 3. Change in diameter (μm±S.D) of seminiferous tubules in different experimental groups. (*) significant at P < 0.05 compared with control group. (**) significant compared with CPA group.

Fig. 4. Change in germinal epithelial height (μm±S.D) of seminiferous tubules in different experimental groups. (*) significant at P < 0.05 compared with control group. (**) significant compared with CPA group.
Fig. 5A) A Photomicrograph obtained from testis of a control rat showing expression of Ki-67 in nuclei of spermatogonia as brown color. B) A Photomicrograph obtained from testis of a rat treated with fennel oil for six weeks showing normal expression of Ki-67. C) A Photomicrograph obtained from testis of a rat treated with CPA for six weeks showed a weak expression of Ki-67 in most nuclei of spermatogonia. D) A Photomicrograph obtained from testis of a rat treated with CPA followed by fennel oil showed an increase in expression of Ki-67 in the nuclei of spermatogonia, (Ki-67 immunostaining, counter stained with hematoxylin).
Fig. 6: The percentage area (Mean area% ± S.D) of Ki-67 expression in rat testis of different experimental groups. (*) significant at P < 0.05 compared with control group. (**) significant compared with CP group.
Fig. 7. A) A Photomicrograph obtained from testis of a control rat showing expression of casepase-3 in cytoplasm of leydige cells as brown color (casepase-3 immunostaining, counter stained with hematoxylin). B) A Photomicrograph obtained from testis of a rat treated with fennel oil for six weeks showing expression of casepase-3 in cytoplasm of the leydige cells, (casepase-3 immunostaining, counter stained with hematoxylin). C) A Photomicrograph obtained from testis of a rat treated with CPA for six weeks showed an increase in expression of casepase-3 in most cytoplasm of leydige cells (arrow), (casepase-3 immunostaining, counter stained with hematoxylin). D) A Photomicrograph obtained from testis of a rat treated with CPA followed by fennel oil for six weeks showed low expression of casepase-3 in the cytoplasm of leydige cells (arrow), (casepase-3 immunostaining, counter stained with hematoxylin).
Fig. 8: The percentage area (Mean area% ± S.D) (of Caspase-3 expression in rat testis of different experimental groups. (*): significant at P < 0.05 compared with control group. (**): significant compared with CPA group.

Fig. 9: Effect of different treatments on serum testosterone level (ng/ml) after three and six weeks of treatment. (*): significant at P < 0.05 compared with control group. (**: significant compared with CPA group.
Fig. 10: Effect of different treatments on serum LH level (mIU/ml) after three and six weeks of treatment. (*): significant at P < 0.05 compared with control group. (**:): significant compared with CPA group.

Fig. 11: Effect of different treatments on serum MDA level (nmol/ml) after three and six weeks of treatment. (*): significant at P < 0.05 compared with control group. (**:): significant compared with CPA group.
Fig. 12: Effect of different treatments on serum CAT level (µmol/sec/ml) after three and six weeks of treatment. (*): significant at $P < 0.05$ compared with control group. (**): significant compared with CPA group.

Fig. 13: Effect of different treatments on serum SOD level (nmol/ml) after three and six weeks of treatment. (*): significant at $P < 0.05$ compared with control group. (**): significant compared with CPA group.
Fig. 14: Effect of different treatments on serum GSH level (U/ml) after three and six weeks of treatment. (*): significant at P < 0.05 compared with control group. (**): significant compared with CPA group (Fig 9). A significant decrease in CAT, SOD and GSH activities were recorded in sera of rats treated with CPA for 3 and 6 weeks. Animals treated with CPA and fennel oil showed a significant increase in these enzymes (Fig. 11-14).

Discussion

In the present study, results revealed that treating rats with CPA induced many histological alteration in the testis and these alterations were more prominent in animals treated for six weeks and include degeneration of sperm cells, hemorrhage and congestion of blood vessels. Moreover, the diameter and germinal epithelial height of the seminiferous tubules were decreased. These results are in agreement with many studies which reported that a period of 3 to 6 weeks of CPA treatment leads to testicular toxicity. It was reported that the administration of CPA once a week for 5 weeks caused oligospermia, azoospermia, testicular damage (23) and germ cell toxicity in mice (24). Sakr et al. (25) indicated that CPA-treated mice showed many histological changes including the appearance of irregular seminiferous tubules, reduction in the number of spermatogenic cells, degeneration of Leydig cells and appearance of intertubular hemorrhage. El-Seedy et al. (26) reported that a marked increase in sperm abnormality induced by CPA in mice proved the ability of this drug to interfere with different stages of spermatogenic cells. They concluded that these abnormalities may be resulted directly from DNA damage or at specific levels of differentiation of spermatocytes. Reduction in diameter of seminiferous tubules and germinal epithelial height was recorded in testes of rats given CPA. The recorded decrease in the height of germinal epithelium is attributed to the inhibition of spermatogenesis by CPA. Morphometrical parameters such as diameters of the seminiferous tubules and tubular lumen can give information about the testicular damage degree as a consequence of germ cell death (27).

Immunohistochemical observations revealed decrease in expression of Ki-67 and increase of caspase-3. Antigen Ki-67 is a nuclear protein that is associated with cellular proliferation. Furthermore, it is associated with ribosomal RNA transcription (28). Inactivation of antigen Ki-67 leads to inhibition of ribosomal RNA synthesis (29). Caspase-3 is a marker of the early phase of apoptosis (30), and is essential for certain processes associated with the formation of apoptotic bodies (31). Thus, CPA inhibited spermatogenesis via decrease of cell proliferation and increase of apoptosis.
(23,24). The reduction in these hormones is due to the effect of active metabolites such as acrolein which break DNA, affect RNA and protein synthesis (32). Degeneration of interstitial tissue was observed in the present work. Interstitial tissue plays an important role in the testis as it contained the Leydig cells which are the main testosterone producer. When the testosterone levels are diminished, the process of spermatogenesis is affected.

Examination of sera of animals treated with CPA in the present study revealed a significant increase in the lipid peroxidation marker, MDA.

It has been reported that CPA treatment resulted in elevated MDA levels because of the excessive generation of free radicals (33-35) and generally, it is accepted that the increased lipid peroxidation is one of the toxic manifestations of CPA administration in testis. (36).

A reduction in the activities of the antioxidant enzymes (superoxide dismutase, catalase). Similarly, Manda and Bhatia, (37) reported that fifteen days oral administration of CPA induced depletion in the levels of glutathione peroxidase, catalase and super oxide dismutase. Reduction of these enzymes was also recorded in various tissues as a result of CPA treatment (38-40). CPA toxic effects on testis were mainly attributed to oxidative stress on seminiferous tubules and Sertoli cells, impairing spermatogenesis and androgenesis, and inducing germinal cells apoptosis (5).

The current study, for the first time, showed that treating rats with CPA followed by fennel oil revealed nearly normal appearance of testicular tissue and increased the number of germ cells. In addition, animals treated with CPA and fennel oil caused an increase in expression of Ki-67 and decrease in expression of caspase-3. Sakr et al. (41) obtained the same results in liver of rats treated with CPA and fennel oil. They added that this result is due to antiproliferative and antioxidant effect of fennel oil. Fennel oil, in the present work, caused a decrease in caspase 3, the marker of apoptosis. Similarly, Ibrahim (12) reported that fennel oil resulted in amelioration in testicular tissue lesions and decrease of apoptosis in rats exposed to tobacco smoke. Fennel treatment was found to improve sperm quality, and spermatogenic cells apoptosis in obese rats (13). The level of testosterone and LH increased. Ibrahim (42) showed that there were significant increases over the control in testosterone level in the serum of rats treated with fennel oil. The author added that the improvement effect of fennel oil on testicular function as indicated by the testosterone may be attributed to the powerful active components of the fennel oil.

Decrease in lipid peroxidation marker, MDA and increase in antioxidant enzymes, SOD, CAT and GSH was recorded in sera of animals treated with CPA and fennel oil. Several reports indicated the antioxidant effect of fennel oil. Pretreatments with fennel oil significantly inhibited the frequencies of aberrant metaphases, chromosomal abractions, micronuclei formation, and cytotoxicity in mouse bone marrow cells induced by CPA and antagonized the reduction of CPA-induced SOD, CAT, and GSH activities and inhibited increased MDA content in the liver of mice (43).

Sheweita et al. (44) reported that fennel oil restored changes in activities of antioxidant enzymes SOD, CAT, GR, GST, and GPx caused by CPA to their normal levels compared to control mice. Mohamad et al., (45) have demonstrated that fennel oil acts like antioxidants due to its ability to inhibit lipid peroxidation. Moreover, it has been revealed that oil extracted from this fennel herb has a protective tetrachloride in rat liver (46). Fennel essential oil has physiologic antioxidant activities including the radical scavenging effect, inhibition of hydrogen peroxides H2O2 and Fe chelating activities where it can minimize free radical which initiate the chain reactions of lipid peroxidation (47).

(48). Studies have shown that antioxidants have a widespread effect on reproduction. These protect spermatozoa from ROS producing abnormal spermatozoa, prevent DNA fragmentation, improve semen quality in smokers, reduce cryodamage to spermatozoa, block premature sperm maturation, and stimulate spermatogenesis (49). It is concluded that administration of fennel oil exhibited protective effects against CPA-induced reproductive toxicity in male rats. The protective effect of fennel oil might be due to induction of antioxidant defense systems by one or more of its constituents.

References


