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Protective Role of Walnut Seeds Extract and Vitamin E against Testicular Toxicity Induced by Cyclophosphamide in Male Rats

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aim: The protective role and antioxidant effect of Walnut seeds extract (WSE) and/or vitamin E (Vit. E), and their combination against testicular damage and oxidative stress induced by anticancer drug cyclophosphamide (CP), were studied on male rats.
Study Design: Forty five rats were randomized into five equal groups, of nine rats each. Group I was assigned as a negative control and orally received saline solution. The other four groups were given CP orally (dissolved in saline solution) at a daily dose of 5 mg/kg of body weight (b. wt) for six weeks. Group II was used as a positive control and groups III, IV and V were received WSE (400 mg/kg of b. wt), Vitamin E (200 mg/kg b. wt) and their combination in daily doses for six weeks, respectively, four hours after CP administration.
Methods: Initial and final weight of rat, food intake were recorded and weight gain and feed efficiency ratio of rats were calculated and recorded. At the end of experimental, rats were anaesthetized by diethyl ether; testes and accessory sexual organs were dissected out and weighed. Semen was separated and analyzed, serum levels of testosterone, FSH and LH were determined. The right testes were used for estimating testicular antioxidant capacity. The left testes

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were preserved in 10% formalin solution untills processing for histopathological examination.
Results: Oral administration of WSE, vitamin E and their combination to intoxicated rats caused increases in the weights of testes, seminal vesicles, sperm motility, count and viability. Serum testosterone and levels of FSH and LH were increased. There were significant increases in the activities of testicular antioxidant enzymes, and alleviation of testicular degeneration and necrosis induced by CP. These effects were amplified by combination of WSE with vitamin E.
Conclusion: Combination of Walnut seeds extract with vitamin E exhibited antioxidant activity and protective effect against reproductive toxicity induced by CP in male rats. Therefore, intake of Walnut seeds in food with vitamin E as food supplement may be beneficial for male patients suffering from infertility problems.

Keywords: Walnut seeds; vitamin E; cyclophosphamide; testes; testosterone; sperms; antioxidant; histopathology.

1. INTRODUCTION

Infertility is one of the major health problems in life and approximately about 30% of this problem is due to male factors, that play an important role in impair male reproductive capacity causing transient or permanent infertility [1]. In addition to, diseases such as coronary heart diseases, diabetes mellitus and liver diseases have deleterious effects on spermatogenesis and production of normal sperm [2,3]. It has been reported that aging, diet, insufficient intake of vitamins, especially vitamin E, smoking and lifestyle changes lead to the reduction in immune defenses and antioxidant activity, lowering semen quality and impairing fertilizing capacity [4]. However, intake of natural antioxidants with vitamins E and C can protect sperm DNA from oxidative stress [5,6].

Cyclophosphamide (CP) drug is a widely used as an antitumor and immune suppressant for the treatment of various cancers as acute and chronic leukemia, multiple myeloma, lymphoma and breast cancer [7]. In spite of its therapeutic importance, it has adverse effects on male reproductive toxicity as reported in CP treatment in the humans and experimental animals [5,8]. Previous studies on male rats have confirmed the potential of CP to cause oligospermia, azoospermia and histological alterations in the testis [5] as well as impaired fertility and decreasing weight of reproductive organs [9,10]. Adult male patients treated with CP have demonstrated diminished sperm count and absence of spermatogenic cycles in the testicular tissue [11].

Natural polyphenolic compounds from vegetables, fruits and nuts are linked with many biological effects, including free radicals scavenging properties and antioxidant activity [12]. Walnut is one among popular consumed nuts and have antioxidant properties related to its higher contents of many polyphenolic compounds especially flavonoids, gamma-tocopherol, and catechins [13] melatonin, ellagic acid, vitamin E, carotenoids, and other polyphenolic compounds [14,15]. Ellagic acid is the most abundant polyphenolic compound in Walnut seeds that has high antioxidant, anti-hyperlipidemic, anti-inflammatory and anti-carcinogenic activities [16].

Vitamin E (alpha-tocopherol) is one of the fat soluble vitamins that regulate oxidation processes in the body [17]. It is required for maintaining the integrity of cell membrane and skin by protecting them from harmful oxygen free radicals [18] and improves male fertility and semen quality in hypercholesterolemic rats [19].

The present was designed to clarify whether or not Walnut seeds extract only and together with vitamin E can alleviate cyclophosphamide-induced testicular toxicity and oxidative stress in male rats.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant seeds

Dried Walnuts seeds (*Juglans regia* L., Family *Juglandaceae*) without external husks were purchased from a local store of Spices, Grains and Oils, Cairo, Egypt. The dried seeds were grinded using a mill into a fine powder and kept until alcohol extraction.

2.1.2 Cyclophosphamide

Cyclophosphamide (*Endoxan*®) was purchased from a local pharmacy in the form of 500 mg

vials. It is manufactured by Baxter BioPharma Company for Pharmaceuticals, USA. It is dispensed as a crystalline white powder and freshly prepared in 0.9% sodium chloride saline solution. Cyclophosphamide was given orally in a dose five mg/kg/day for six weeks as recorded by Kim et al. [8].

2.1.3 Rats

Forty five mature male albino rats of Sprague Dawley strain weighing 170 - 180 g and 8–10 weeks old were purchased from Laboratory Animal Colony Helwan Egypt. Rats were maintained under controlled hygienic conditions at controlled room temperature of 23ºC and 55% humidity with 12-hr light / 12-hr dark schedule. Rats were fed on basal diet and water was provided *ad libitum*. Rats were allowed to acclimatize to the laboratory environment for seven days before start of the experiment.

2.2 Methods

2.2.1 Preparation of basal diet

Basal diet was prepared according to the method of Reeves et al. [20]. It was consisted of 20% protein (casein), 10% carbohydrate, 4.7% fat (corn oil), 2% choline chloride, 1% vitamin mixture, 3.5% salt mixture and 5% fibers. The remainder was corn starch up to 100%.

2.2.2 Preparation of walnut seeds extract

Walnut seeds extract was prepared by soaking 200 g of dry powdered seeds in one liter of 90% ethyl alcohol and kept in a refrigerator with daily shaking for 3 days. The ethanol was then evaporated under reduced pressure using rotatory evaporator connected with vacuum pump and the water bath was adjusted at 45ºC. Twenty grams of the obtained semisolid extract were suspended in Tween 80 (suspending agent) and 98 ml of distilled water were gradually added to obtain 20% liquid extract as described by Shalaby and Hamowieh [21].

2.2.3 Experimental design

Forty five mature male rats were randomized into five groups, of nine animals each. Group I received saline solution (vehicle) and kept as negative control group. Group Π was given orally cyclophosphamide (dissolved in saline solution) in a daily dose five mg/kg of b. wt for six weeks and served as a positive control group. Groups III, IV and V were orally given Walnut seeds extract (WSE) in a dose of 400 mg/kg of b. wt, Vitamin E in a dose of 200 mg/kg of b. wt, and WSE concomitantly with Vit E in daily doses for 6 weeks, respectively, four hours after CP administration.

Feed intake was calculated daily and body weight was recorded weekly. Feed efficiency ratio was calculated as FER = weight gain (g) /feed intake (g).

At end of the experiment, the rats were euthanized by prolonged exposure to diethyl ether anesthetic and blood samples were withdrawn by cardiac puncture into clean centrifuge tubes. Blood was left standing for 10 minutes to clot and then centrifuged at 3000 rpm for 15 minutes for separating the serum that used for estimation of testosterone, FSH and LH levels. Semen samples were collected from cuda epididymis by cutting the tail of epididymis (cuda epididymis) and squeezing it gently on a clean glass slide. The semen was used for evaluating epididymal sperm parameters. The testes, seminal vesicles and prostate glands were dissected out and weighed. The left testes were quickly taken on ice bags and kept frozen till preparation of homogenate that used for determination of the activity of tissue antioxidant enzymes. The right testes were preserved in 10% formalin solution till processed for histopathological examination.

2.2.4 Semen analysis

The sperm progressive motility and count were determined according to the method described by Bearden and Fluquary [22]. Microscopic examinations of the seminal smears stained with Eosin Nigrosin stain were also carried out to determine the percentages of sperm viability and abnormality as described by Amann et al. [23].

2.2.5 Assay of sex hormones

Serum testosterone concentration was determined using radioimmunoassay (RIA) method which was intended for the quantitative determination of total testosterone in the serum based on the competitive binding principal. Briefly, the unknown or standards serum samples were incubated with radioactive iodine (I^{125}) labeled testosterone in antibody-coated tubes. After incubation, the liquid contents in the tubes were withdrawn and the bound radioactivity was determined using gamma

counter and serum total testosterone levels were then calculated as described by Wilke and Utley [24]. Serum levels of FSH and LH hormones were determined using enzyme-linked immunosorbent assay (ELISA) with specific commercial kits.

2.2.6 Assay of tissue antioxidant enzymes

Testis tissues (one gram of each rat) were collected at the end of experiment. Testes specimens were washed in ice-cooled 0.9% NaCl and homogenized in ice-cooled 10 ml of 1.15% solution of potassium chloride in 50 mM potassium phosphate buffer solution (pH 7.4) to yield 10% (W/V) homogenate. The homogenates were centrifuged at 4000 rpm for 5 min at 4℃. The supernatants were collected and used for determination of tissue antioxidant enzymes. The activity of enzymes superoxide dismutase (SOD), glutathione peroxidase superoxide (GPx), and catalase (CAT) were determined according to Nishikimi et al. [25], Paglia and Valentine [26], and Aebi [27], respectively.

2.2.7 Histological examination

The fixed specimens of the testes were then trimmed, washed and dehydrated in ascending grades of alcohol. These specimens were then embedded in paraffin, sectioned at 4-6 microns thickness, stained with Hematoxylen and Eosin (H&E) then examined microscopically.

2.3 Statistical Analysis

Data were presented as means ± standard errors (SE). The statistical analysis was performed using computerized statistical package of social sciences (SPSS) program (SPSS. 20 software version) with one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests.

3. RESULTS

Oral administration of cyclophosphamide in a daily single dose (5mg/kg of b.wt) to rats for 6 weeks induced significant ($P< 0.05$) decreases in body weight gain, food intake and feed efficiency ratio (FER), compared with the negative control rats (normal rats). Oral administration of Walnut seeds extract (WSE) and/or, vitamin E; and their combination to rats inflicted with testicular damage caused significant ($P<0.05$) increases in body weight gain, food intake and FER compared with the positive control group. There was a marked increase in body weight gain, feed intake and feed efficiency ratio in treated rats with a mixture of the WSE and vitamin E as recorded in Table 1.

Weights of testes and seminal vesicles were decreased significantly in untreated rats (positive group) compared with the normal rats (negative group). Injured rats in the testicular and treated with WSE and/or Vit. E and their combination had significant ($P <0.05$) increased in weights of testes and seminal vesicles compared with the positive (CP-intoxicated) control group. The non significant higher in testes weights and seminal vesicles were founded in treated rats with a mixture of the WSE and Vit. E. In addition there was no significant changes in the weight of prostate glands were observed as recorded in Table 2.

As recorded in Table 3, orally administration of Cyclophosphamide to male rats for 6 weeks induced significant decrease in sperm count, motility and viability; and an increase in sperm cell morphology compared to the negative control group. Examination of seminal smears of CP-intoxicated rats showed azoospermia (absence of sperms) in some smears and oligospermia (decreased number of sperms) in the others. The most frequently seen sperm abnormalities were

Table 1. Effect of walnut seeds extract and/or vitamin E and their combination on body weight gain, (BWG), feed intake (FI) and feed efficiency ratio (FER) in cyclophosphamide (CP)-intoxicated rats

Groups	Parameters as Mean ± SE				
	Initial body weight (g)	Final body weight (g)	BWG (g)	FI (g)	FER
Group I: Negative control	175.0±3.24^{a}	255.0±6.7^{a}	42.8±3.6^{a}	19.0±2.2^{a}	0.343^{a}
Group II: CP (5 mg/kg)	180.0±4.24^{a}	210.5±5.7^{c}	17.0±1.6^{d}	16.9±1.2^{c}	0.142^{c}
Group III: WSE (400 mg/kg)	175.0±3.34^{a}	230.5±3.8^{b}	31.7±3.2^{c}	16.0±1.1^{b}	0.283^{b}
Group IV: Vit. E (200 mg/kg)	170.0±2.44^{a}	235.0±5.8^{b}	37.7±2.2^{b}	17.5±1.4^{b}	0.312^{b}
Group V: WSE + Vit. E	170.0±2.85^{a}	240.5±4.6^{b}	42.7±3.2^{b}	18.0±1.2^{b}	0.329^{b}

Means ± SE with different letters superscripts in each column are significant at $P < 0.05$

Table 2. Effect of walnut seeds extract and/or Vit. E; and their combination on relative weights of male rats' sexual organs

Groups	Mean ± SE of relative weight of sexual organs (g/100 g b.wt)		
	Testes	Seminal vesicles	Prostate glands
Group I: Negative control	2.55±0.03[a]	0.85±0.02[a]	0.35±0.01[a]
Group II: CP (5 mg/kg)	1.10±0.01[c]	0.42±0.03[c]	0.36±0.02[a]
Group III: WSE (400 mg/kg)	2.15±0.04[b]	0.75±0.03[b]	0.35±0.03[a]
Group IV: Vit. E (200 mg/kg)	2.35±0.01[b]	0.78±0.02[b]	0.37±0.02[a]
Group V: WSE + Vit. E	2.40±0.04[b]	0.81±0.04[b]	0.36±0.04[a]

Means ± SE with different letters superscripts in the same column are significant at P < 0.05

Table 3. Effect of walnut seeds extract and/or vitamin E and their combination on semen picture of cyclophosphamide (CP) - intoxicated rats

Groups	Count (10^6/ml)	Sperm parameters as Mean ± SE		
		Motility (%)	Viability (%)	Abnormality (%)
Group I: Negative control	74.50±5.22[a]	90.00±4.15[a]	80.0±4.6[a]	10.50±1.15[b]
Group II: CP (5 mg/kg)	25.50±5.45[d]	55.00±5.67[d]	40.6±4.6[d]	18.50±2.18[a]
Group III: WSE (400 mg/kg)	55.60±5.34[c]	65.00±4.17[c]	50.6±6.9[c]	9.50±2.15[b]
Group IV: Vit. E (200 mg/kg)	66.50±4.55[b]	75.00±5.55[b]	65.6±4.6[b]	8.30±1.42[b]
Group V: WSE + Vit. E	68.80±4.75[b]	80.00±4.45[b]	77.6±5.6[b]	7.20±1.35[c]

Means ± SE with different letters superscripts in the same column are significant at P <0.05

Table 4. Effect of walnut seeds extract or vitamin E (Vit. E) and their combination on serum levels of testosterone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH) in cyclophosphamide (CP) - intoxicated rats

Groups	Serum levels of hormones		
	T (ng/mL)	FSH (ng/mL)	LH (ng/mL)
Group I: Negative control	23.5±1.4[a]	155.0±9.3[a]	3.7±0.2[a]
Group II: CP (5 mg/kg)	15.5±2.3[d]	96.66±7.2[d]	1.5±0.1[d]
Group III: WSE (400 mg/kg)	19.2±1.6[c]	122.50±3.5[c]	2.2±0.4[c]
Group IV: Vit. E (200 mg/kg)	21.1±1.4[b]	140.75±3.4[b]	3.3±0.3[b]
Group V: WSE + Vit. E	22.2±1.5[b]	145.82±4.2[b]	3.5±0.3[b]

Means ± SE with different superscripts in the same column are significant at P < 0.05

bent and coiled tails, detached head and double head. Concomitant administration of Walnut seeds extract and vitamin E improved sperm parameters as evident by significant increases in sperm count, motility and viability percentages and a decrease in percent of sperm cell abnormalities when compared to CP-intoxicated rats.

Data in above Table 4 showed that treated rats with cyclophosphamide in a dose 5 mg/kg b. wt for six weeks have significant (P < 0.05) decreases in serum testosterone, FSH and LH levels compared with the negative control group. Oral administration of Walnut seeds extract and/or vitamin E alone; and in combination, significantly normalized serum testosterone FSH and LH levels when compared to the positive control group (CP- intoxicated rats).

The recorded results in Table 5 revealed that cyclophosphamide in a dose 5 mg/kg b.wt produced significant decreases in activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzymes in testicular tissue as compared to the negative control group. Administration of Walnut seeds extract and/or vitamin E; and their combination to CP- intoxicated significantly increased activities of SOD, GPx and CAT enzyme in the testicular tissue when compared to CP-intoxicated positive control group.

Histopathological examination of testes for normal control rats showed normal histological structure of mature functioning seminiferous tubules associated with complete spermatogenic series and the lumen was filled with mature spermatozoa (Fig. 1). Large vacoulations, degeneration and necrosis of germ cell lining of somniferous tubules were showed in testes of cyclophosphamide (CP)-intoxicated rats (positive rats) (Fig. 2) in addition to azoospermia (absence of sperms) was showed in other rats (Fig. 3). Microscopic examination of testis sections of rats given orally Walnut extract showed mild degeneration and necrosis associated with interstitial edema as illustrated in Fig. 4. However, testes of rats given vitamin E revealed almost normal histological architecture with normal spermatogenic series as shown in Fig. 5. In rats given Walnut extract with vitamin E concomitantly, normal testes histological architecture with normal spermatogenic series and the lumen of seminiferous tubules was filled with spermatozoa as shown in Fig. 6.

4. DISCUSSION

The present study was done to investigate the protective role and antioxidant effect of Walnut seeds extract and/or vitamin E and their combination against cyclophosphamide (CP)-induced testicular injury and oxidative stress in male rats.

Administration of cyclophosphamide (CP) to male rats in dose 5 mg/kg for 6 weeks induced testicular toxicity and oxidative stress. The toxicity of cyclophosphamide on male reproduction was characterized by the decreases in weight of the testes and seminal vesicles; lowered semen quantity and quality; and decreased in serum testosterone, FSH and LH levels. In addition, there were lowered testicular antioxidant capacity and incidence of degeneration and necrosis in testicular tissue. These effects were similar to those reported by Akram et al. [10] and Kim et al. [8]. The previous authors reported that cyclophosphamide (CP) decreased weight of reproductive organs, lowered semen quality and impaired fertility in male rats. In addition to the toxic effects of CP it caused oligospermia, azoospermia and histological alterations in the testis and epididymis and reduced male fertility [5]. Adult male patients treated with CP have demonstrated diminished sperm count and absence of spermatogenic cycles in the testicular tissue [11,28]. The oxidative stress induced by CP as reported in testicular tissue of treated rats in this study was agreed with that previously demonstrated by Abraham and Rabi, [29] and Nitharwal et al. [30]. These authors concluded that exposure of rats to CP can disrupt the reduction–oxidation (redox) balance in the tissues leading to oxidative stress. Moreover, Libey et al. [31] and Motawi et al. [32] reported that CP caused testicular atrophy, decreased epididymal sperm count and motility and decreased plasma testosterone in male rats. Meistrich et al. [33] indicated that the testicular toxicity and oxidative stress induce by CP could be attributed to its direct cytotoxic effect on the testes because of oxidative stress and/or indirectly via decreasing serum testosterone, FSH and LH levels in rats.

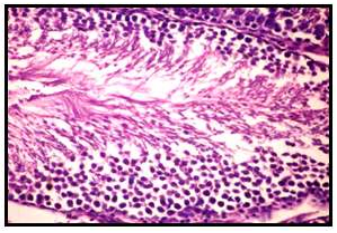

Fig. 1. Testis of a negative control rat showing normal histological structure of mature functioning seminiferous tubules associated with complete spermatogenic series. (H&E, X 400)

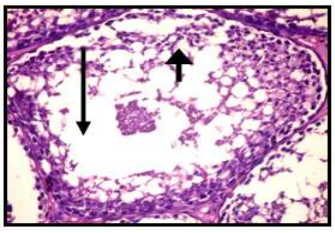

Fig. 2. Testis of a positive control rat given cyclophosphamide showing large vacoulations (Long arrow), degeneration and necrosis (Short arrow) of germ cell lining of seminiferous tubules and oligospermia. (H&E, X 400)

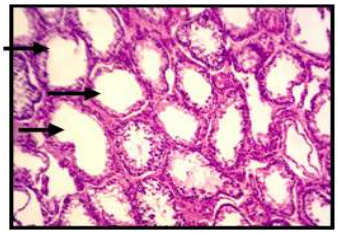

Fig. 3. Testis of a positive control rat given cyclophosphamide showing Vacoulations, degeneration and necrosis of germ cell lining of somniferous tubules and azoospermia (Arrows). (H&E, X 200)

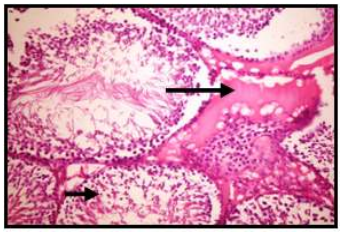

Fig. 4. Testis of a rat given Walnut seeds extract in a dose of 400 mg/kg showing mild degeneration of germ cell lining of somniferous tubules (short Arrow) associated with interstitial edema (long Arrow). (H&E, X 400)

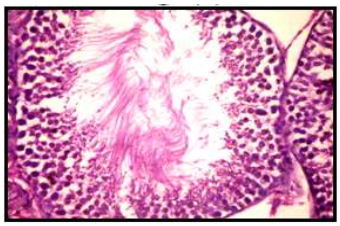

Fig. 5. Testis of a rat given vitamin E in a dose of 200 mg/kg showing almost normal histological structure of seminiferous tubules with the lumen filled with spermatozoa. (H&E, X 400)

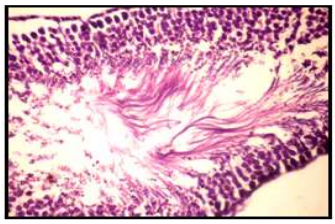

Fig. 6. Testis of a rat given both Walnut seeds extract and vitamin E showing normal histological structure of seminiferous tubules with the lumen filled with spermatozoa. (H&E, X 400)

Table 5. Effect of walnut seeds extract and/or vitamin E and their combination on testicular superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in cyclophosphamide (CP) - intoxicated rats

Groups	Parameters as Mean ± SE		
	SOD (U/mg protein)	GPx (nmol/min/mg protein)	CAT (nmol/min/mg protein)
Group I: Negative control	18.2±1.3[a]	242.0±7.2[a]	360.5±6.1[a]
Group II: CP (5 mg/kg)	9.0±1.1[d]	192.1±5.3[d]	277.5±5.3[d]
Group III: WSE (400 mg/kg)	11.0±1.1[c]	185.3±3.9[c]	283.3±8.3[c]
Group IV: Vit. E (200 mg/kg)	15.0±1.5[b]	233.2±4.6[b]	310.6±7.0[b]
Group V: WSE + Vit. E	16.2±1.3[b]	235.2±4.6[b]	315.5±4.2[b]

Means ± SE with different superscripts in the same column are significant at P < 0.05.
GPx unit = nmol of GSH utilized/min/mg protein
CAT unit = nmol of H_2O_2 utilized/min/mg protein

As founded in the current study, oral administration of Walnut seeds extract (WSE) to cyclophosphamide (CP) - intoxicated rats induced protective effects against male reproductive toxicity and oxidative stress induced by CP. The protective effect WSE on male fertilizing capacity manifested by significant increases in weight of male sexual organs; percentages of sperm count, motility and viability and serum testosterone, FSH and LD levels. There were also marked increases in the activity of antioxidant enzymes in testicular tissue and partial alleviation the testicular degenerative changes induced by CP administration to rats. This protective effect of WSE against CP-induced testicular injury was partially similar to the obtained results by Owumi et al. [34] who found that Walnut (Juglans nigra) ameliorated the toxic effect induced by arsenite on sperm morphology and quality in male rats. Robbins et al. [35] reported that consumption of Walnuts added to Western-style diet improved sperm vitality, motility, and morphology in men.

The antioxidant activity of Walnut in testicular tissue that reported in this study was in agreement with that demonstrated in previous studies by Bhatia et al. [36]; Mishra et al. [37]; and Negi et al. [38]. Mishra et al. [37] founded that Walnut revealed the best antioxidant properties followed by almonds cashew nut, chironji and the least phenolic content was found in raisins. Negi et al. [38] reported that walnuts contain high phenolic content and exhibits antioxidant activity, and potent antiproliferative activity, so Walnuts seeds may act as a cancer chemopreventive agent.

The mechanism underlying the protective effect of Walnut seeds extract on testicular damage induced by cyclophosphamide could be attributed to its antioxidant protection and free

radicals scavenging property [15]. The antioxidant effect of Walnut seeds extract could be due to its high content of antioxidant polyphenolic compounds [37,38].

Results of this study demonstrated that the protective effect of Walnut seeds extracts on male fertility in rats was intensified by its coadministration with vitamin E. This could be explained by addition antioxidant effect of vitamin E. It was reported that vitamin E acts as a powerful antioxidant in rats [17], normalize the damaging effect of oxidative stress induced by reactive oxygen species [18] in rats and improve male fertility and semen quality in hypercholesterolemic rats [39].

5. CONCLUSION

In conclusion, cyclophosphamide (CP) induces reproductive toxicity in male rats manifested by decreased weights of sexual organs; lowered semen quantity and quality; decreased serum testosterone, FSH and LH levels; decreased testicular tissue antioxidant capacity and incidence of testicular degeneration necrosis. However, oral concomitant administration of Walnut seeds extract and vitamin E produces protective effect and antioxidant activity against CP - induced reproductive toxicity in male rats. The protective effect could be attributed to the antioxidant property of both Walnut seeds and vitamin E.

6. RECOMMENDATIONS

The present study recommends that intake of Walnut seeds toghter with vitamin E as a food supplement may be beneficial for male patients suffering from infertility problems. Moreover, isolation of bioactive constituents from Walnut seeds is necessary to search for safe natural antioxidants that can be developed for use as food preservatives instead of the least safety synthetic antioxidants.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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