World Applied Sciences Journal 23 (10): 1351-1359, 2013
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2013.23.10.13153

Pumpkin Seed Oil and Vitamin E Improve Reproductive Function of Male Rats Inflicted by Testicular Injury

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Submitted: Jun 9, 2013; **Accepted:** Jul 22, 2013; **Published:** Jul 28, 2013

Abstract: The principal goal of this study was to investigate the protective role of pumpkin seed oil (PSO) and vitamin E (Vit E) against sodium valproate (SVP)-induced testicular damage in male rats. Forty five adult male rats were randomly distributed into 5 groups, of 9 animals each. Group (1) was given orally 1ml distilled water/rat and used as negative control and group (2) was orally given SVP (500 mg/kg/day) during the last week of experiment period to induce testicular damage and used as positive control. The other three protected groups were pretreated by oral administration of PSO at a dose of 40 mg/kg, Vit E at 200 mg/kg and PSO with Vit E respectively for 8 weeks and received SVP during the last week. Blood samples were collected for estimating serum levels of testosterone, Follicle – stimulating hormone (FSH) and Luteinizing hormone (LH). Semen samples were collected for semen analysis. Rats were then sacrificed and male sexual organs were removed and weighed. Tissue lipid peroxidation (LPX) and activity of antioxidant enzymes in testes and histopathology of testes were also performed. The results showed that combination of PSO and Vit E significantly increased serum levels of testosterone, FSH and LH, testes weight and sperm motility, count and vitality and decreased sperm cell abnormalities in rats given SVP. Decreased testicular LPO, increased antioxidant capacity and alleviation of testicular degenerative changes caused by SVP were reported. In conclusion, PSO and Vit E produce protective and antioxidant effects against SVP-induced testicular damage in rats. Therefore, dietary intake of pumpkin seed oil and vitamin E may be useful for male patients who suffer from infertility due to oxidative stress.

Key words: Pumpkin seed oil · Vitamin E · Testosterone · Testes · Sperms · Antioxidant · Histopathogy

INTRODUCTION

Infertility is one of the major health problems in life and approximately about 30 % of this problem is due to male factors [1]. Several factors (as smoking, stress, drinking alcohol and increasing age) and diseases (e.g. coronary heart diseases, diabetes mellitus and chronic liver diseases) can interfere with the process of spermatogenesis, reduce semen quantity and quality and decrease male fertility. Insufficient intake of vitamins has been reported to produce deleterious effects on the process of spermatogenesis and production of normal sperms [2-6]. Moreover, dietary intake of antioxidants from natural products with vitamins E and C can protect sperm DNA from oxidative stress in the rat testis [7]. Pumpkin seed oil (PSO) is a natural product commonly used in folk medicine for the treatment of hypertension and atherosclerosis [8]. It is rich in many antioxidants and beneficial nutritional components such as essential fatty acids, amino acids (especially tyrosine and L-phenylalanine), phytosterols (e.g. β-sitosterol), β-carotenes, lutein and selenium [9, 10]. Pumpkin seeds contain L-tryptophan, omega-6 and -3 fatty acids [11] and very high concentration of vitamin E [12]. The antioxidant property of PSO could enhance male fertility [13]. It has been reported that PSO ameliorated the effect of quinine - induced testicular damage in rats [14]. Vitamin E (α- tocopherol) is a fat soluble vitamin which regulates oxidation processes in the body as it acts as a powerful antioxidant. Previous studies showed that vitamin E could normalize the damaging effect of oxidative stress induced by free radicals in rat testis and improve male fertility [15-18].

The present study was therefore designed to investigate the protective effect of pumpkin seed oil and vitamin E against sodium valproate -induced testicular

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injury in male rats. Tissue lipid peroxidation and antioxidant capacity as well as histopathology of testes were also carried out.

MATERIALS AND METHODS

Pumpkin Seed Oil and Chemicals: Pumpkin seed oil (PSO) was purchased from Arab Company for vegetable oils extraction and refining (ARECO), Egypt, as dark green oil backed bottles each containing 100 ml. The human therapeutic dose of PSO is 320 mg /day according to Hong *et al.* [19]. The equivalent rat dose of this oil was calculated using conversion table of Paget and Barnes [20]. Vitamin E (α-tocopherol) was obtained from Pharco Company for Pharmaceuticals, Alexandria, Egypt. It is dispensed in the form of soft gelatinous capsules each containing 1000 mg of alpha tocopherol acetate. Sodium valproate is one of products of Sanofi- Synthelabo Company, Paris, France. It is obtained as oral solution packed in brown bottles each containing 40 ml. It is sold commercially under trade name Depakin® 200 mg/ml solution.

Animals: Forty five mature male rats of Sprague Dawley strain weighing 220-225 g body weight and 14-16 weeks age were purchased from Laboratory Animals Colony, Agricultural Research Center, Giza, Egypt. Rats were housed at a controlled temperature of 23 ± 1°C, 55 % humidity and under 12 hr light/12 hr dark schedules. Animals were fed on basal diet and water was provided *ad libitum*. Rats were allowed to acclimatize to the laboratory environment for 7 days before start of the experiment.

Preparation of Basal Diet: Basal diet was prepared using AIN 93 according to the method of Reeves *et al*. [21]. It is consisted of 20 % casein (≥85%) protein (casein), 10 % sucrose, 5% fat (corn oil), 0.2% choline chloride, 1% vitamin mixture, 3.5% salt mixture and 5% fibers. The remainder was corn starch up to 100 %.

Experimental Design and Grouping of Rats: Forty five adult male rats were randomly distributed into 5 equal groups, of 9 animals each. Group (1) was given orally 1 ml distilled/rat and used as negative control. Group (2) was positive (intoxicated) control and orally given sodium valproate (500 mg/kg/day) during the last week of the experiment period (8 weeks) for induction of testicular injury according to Hamza and Amin [22]. Groups (3), (4) and (5) were pretreated orally with pumpkin seed oil (PSO) at a dose of 40 mg/kg/day, vitamin E (Vit E) at 200 mg/kg/day and PSO coadministered with Vit E, respectively and received SVP during the last week. At the end of the experiment, blood samples were withdrawn via vein puncture of retro-orbital plexus of veins in the inner canthus of eye and blood was collected into dry plastic centrifuge tubes. Blood samples were kept at room temperature for 10 min. to clot and the serum was separated by centrifugation using an Anker centrifuge (Model TGL-16 G, Shanghai, China) at 4000 rpm for 10 minutes. The serum samples were used for estimating testosterone, FSH and LH. Rats were anesthetized using ether anesthetic and a longitudinal incision was made in middle of the scrotum and both testes were exposed. Semen samples were collected from the cuda epididymis by its cutting and squeezing into clean watch glass. The semen samples were used for semen analysis. The testes and accessory sex organs (seminal vesicles and prostate glands) were then dissected out and weighed on digital electric balance. The right testes were rapidly taken on ice bags and frozen at – 18°C till used for assessment of lipid peroxidation and antioxidant activity in testicular tissue. The left testes were preserved in 10% neutral formalin solution till processed for histological examination.

Hormonal Assay: Serum testosterone concentration was determined using radioimmunoassay (RIA) which is intended for the quantitative determination of total testosterone in the serum. The RIA of testosterone is based on the competitive binding principal according to Wilke and Utley [23]. Serum levels of FSH and LH were determined by enzyme linked immunosorbent assay (ELISA) using specific commercial kits (Amersham, Buckinghamshire, UK) according to Loraine and Bell [24].

Semen Analysis: The semen was obtained by cutting of cuda epididymis using razor blades and squeezed into clean watch glass. The obtained semen content was diluted 10 times with 2.9% sodium citrate solution and rapidly examined to estimate the percentage of sperm progressive motility and sperm cell count as described by Bearden and Fluquary [25]. Thereafter, one drop of semen suspension was withdrawn, smeared on a glass slide, stained by Nigrosin and Eosin (N&E) stain and examined microscopically to determine sperm vitality (alive/dead ratio). Other seminal smears were prepared and stained with Sperm Blue dye (Biodiagnostic Company for Diagnostic and Research Reagents, Dokki, Egypt) for examining sperm morphology [26].

Assessment of Oxidant/Antioxidant Activity: After thawing of the right testes, one gram of the testicular tissue was homogenized using soft tissue homogenizer (Omni International, USA) in 9 volumes of ice cooled 0.9% buffer saline solution. The homogenate was then centrifuged at 4000 x g for 10 min. at 4°C and the supernatant was used for estimating tissue lipid peroxidation and activities of antioxidant enzymes. In testicular homogenate, the product of lipid peroxidation malondialdehyde (MDA) was determined according to Placer *et al.* [27] and the content of reduced glutathione (GSH) was estimated according to Rajesh and Latha [28]. Activities of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were determined as described by Nishikimi *et al.* [29], Paglia and Valentine [30] and Sinha [31], respectively.

Histological Procedure: The preserved specimens of left testes were trimmed, washed and dehydrated in ascending grades of alcohol. These specimens were cleared in xylene, embedded in paraffin, sectioned at 4-6 microns thickness using microtome and stained with Hematoxylin and Eosin (H&E) then examined microscopically according to Bancroft *et al.* [32].

Statistical Analysis: Data were presented as means ± SEM. Statistical analysis of data were tested for significance using a one way analysis of variance (ANOVA) followed by Duncan's multiple range test using computerized SPSS program [33]. Values were considered significant at $P < 0.05$.

RESULTS

In the current study, oral administration of sodium valproate (SVP) in a dose of 500 mg/kg/day for 7 consecutive days to male rats induced a significant ($P<0.05$) decrease in the weight of testes when compared with the negative control group. Oral pretreatments with pumpkin seed oil (PSO), vitamin E (Vit E) and their combination significantly ($P<0.05$) increased the weight of testes when compared with the positive control group. Non significant changes were found in weights of seminal vesicles and prostate glands between the pretreated groups and the control groups as shown in Table 1. Data in Table 2 showed that sodium valproate (SVP) when given to male rats (500 mg/kg) for 7 consecutive days caused significant ($P< 0.05$) decreases in serum levels of testosterone, FSH and LH when compared with the negative control group. Oral pretreatments with PSO, Vit E and their combination significantly ($P<0.05$) normalized serum levels of testosterone, FSH and LH when compared with the positive control group. Oral administration of sodium valproate (SVP) to male rats (500 mg/kg) for 7 consecutive days led to significant decreases in sperm count, progressive motility and vitality and an increase in sperm morphological abnormality when compared with the negative control group. Oral pretreatments with pumpkin seed oil (PSO), vitamin E (Vit E) and their combination significantly ($P<0.05$) increased sperm count, progressive motility and vitality and decreased sperm cell abnormality when compared with the positive control group as depicted in Table 3. Oral pretreatments with PSO, Vit E and their combination decreased percents of sperm morphological abnormality to 10.5, 6.5 and 4.4 %, respectively, versus to 18.6% in the positive control group. The most frequently seen sperm morphological abnormalities in positive control rats were double head (Fig. 1B) (3.4%), detached head (Fig. 1C) (7.2%), large oval head (Fig. 1D) (3.0 %) and coiled tail (Fig. 1E) (5.0 %) as compared to the normal sperm in the negative control group (Fig. 1A). Sodium valproate (SVP) when orally given to male rats for 7 consecutive days significantly ($P<0.05$) decreased tissue reduced glutathione (GSH) and increased malondialdehyde (MDA) contents in testes when compared with the negative control group. Oral pretreatments with PSO, Vit E and their combination significantly ($P<0.05$) increased tissue GSH and decreased MDA when compared with the positive control group as recorded in Table 4.

Oral administration of sodium valproate (SVP) to male rats for 7 consecutive days significantly ($P<0.05$) decreased the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) enzymes in testes when compared with the negative control group. Pretreatments with PSO, Vit E and their combination significantly ($P<0.05$) increased the activity of tissue SOD, GPx and CAT enzymes when compared with positive control group (Table 5). Histopathological examination of the testes showed that rats pretreated with vitamin E (Vit E) alone had intact and functioning seminiferous tubules containing mature spermatozoa in the lumen and complete spermatogenic germ cell series (Fig. 2.1). In rats pretreated with pumpkin seed oil (PSO) alone, the examination of testes revealed relatively intact seminiferous tubules with few spermatozoa in the lumen, mild interstitial edema and leukocytes infiltration as demonstrated in Fig. 2.2. The testes of rats pretreated with combination of PSO and Vit E showed normal intact seminiferous tubules completely filled with mature

Table 1: Effect of pumpkin seed oil (PSO), vitamin E (Vit E) and their combination on weights of male sex organs of rats with testicular damage induced by sodium valproate (SVP)

	Relative weight of sex organs (g/100 g b.wt.)		
Groups	Testes	Seminal vesicles	Prostate glands
Group (1):Negative control	2.80 ± 0.03[a]	0.88 ± 0.02[a]	0.58 ± 0.01[a]
Group (2): Positive (SVP) control	1.24 ± 0.02[d]	0.82 ± 0.03[a]	0.56 ± 0.02[a]
Group (3):PSO (40 mg/kg)	1.48 ± 0.05[c]	0.83 ± 0.01[a]	0.55 ± 0.02[a]
Group (4):Vit E (200 mg/kg)	1.57 ± 0.03[c]	0.85 ± 0.03[a]	0.57 ± 0.01[a]
Group (5):PSO (40 mg/kg)+Vit E (200 mg/kg)	2.25 ± 0.04[b]	0.81 ± 0.02[a]	0.56 ± 0.02[a]

Means ± SEM with different superscript letters in the same column are significant at $P<0.05$ using one way ANOVA test and those with the same superscript letters are not significant. n = 9 rats.

The positive control group was compared with the negative control.

The pretreated groups were compared with the positive control group.

Table 2: Effect of pumpkin seed oil (PSO), vitamin E (Vit E) and their combination on serum levels of total testosterone (TT), FSH and LH in rats with testicular damage induced by sodium valproate (SVP)

Groups	TT(ng/ml)	FSH(ng/ml)	LH(ng/ml)
Group (1):Negative control	4.30 ± 0.03[a]	9.7 ± 0.3[a]	1.8 ± 0.02[a]
Group (2): Positive (SVP) control	4.30 ± 0.03[a]	5.5 ± 0.1[d]	0.9 ± 0.02[d]
Group (3):PSO (40 mg/kg)	1.32 ± 0.04[d]	6.3 ± 0.4[c]	1.2 ± 0.04[c]
Group (4):Vit E (200 mg/kg)	2.96 ± 0.02[b]	7.7 ± 0.3[c]	1.4 ± 0.03[c]
Group (5):PSO (40 mg/kg)+Vit E (200 mg/kg)	3.28 ± 0.02[b]	8.4 ± 02[b]	1.6 ± 0.01[c]

Means ± SEM with different superscripts in the same column are significant at $P<0.05$ using one way ANOVA test. n= 9 rats.

The positive control group was compared with the negative control.

The pretreated groups were compared with the positive control group.

Table 3: Effect of pumpkin seed oil (PSO), vitamin E (Vit E) and their combination on sperm parameters of rats with testicular damage induced by sodium valproate (SVP)

Abnormality	Sperm parameters			
Groups	Count (10^6/ml)	Motility (%)	Vitality (%)	%
Group (1):Negative control	76.54 ± 1.4[a]	90.0 ± 0.1[a]	90.0 ± 3.2[a]	1.5 ± 0.2[d]
Group (2): Positive (SVP) control	54.30 ± 1.2[d]	65.2 ± 2.1[d]	45.6 ± 3.6[d]	18.6 ± 0.3[a]
Group (3):PSO (40 mg/kg)	60.35 ± 1.4[c]	73.3 ± 2.2[c]	55.6 ± 2.6[c]	10.5 ± 0.3[b]
Group (4):Vit E (200 mg/kg)	66.25 ± 3.3[c]	75.5 ± 2.1[c]	60.6 ± 2.8[c]	6.5 ± 0.1[c]
Group (5):PSO (40 mg/kg)+Vit E (200 mg/kg)	72.24 ± 3.1[b]	80.0 ± 4.2[b]	75.6 ± 3.8[b]	4.4 ± 0.3[c]

Means ± SEM with different superscripts in the same column are significant at $P<0.05$ using one way ANOVA test. n= 9 rats.

The positive control group was compared with the negative control.

The pretreated groups were compared with the positive control group.

Table 4: Effect of pumpkin seed oil (PSO), vitamin E (Vit E) and their combination on testes reduced glutathione (GSH) and malondialdehyde (MDA) in rats with testicular damage induced by sodium valproate (SVP)

Groups	GSH (nmol/min/mg protein)	MDA (nmol/min/mg protein)
Group (1):Negative control	27.4 ± 1.3[a]	0.35 ± 0.05[c]
Group (2): Positive (SVP) control	12.5 ± 2.2[d]	0.95 ± 0.03[a]
Group (3):PSO (40 mg/kg)	18.7 ± 2.4[b]	0.65 ± 0.04[b]
Group (4):Vit E (200 mg/kg)	20.9 ± 1.4[c]	0.55 ± 0.07[b]
Group (5):PSO (40 mg/kg)+Vit E (200 mg/kg)	24.2 ± 2.3[c]	0.50 ±0.02[b]

Means ± SEM with different superscripts in the same column are significant at $P<0.05$ using one way ANOVA test. n= 9 rats.

The positive control group was compared to the normal control.

The pretreated groups were compared to the positive control group.

Table 5: Effect of pumpkin seed oil (PSO), vitamin E (Vit E) and their combination on activities of testicular superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in rats with testicular damage induced by sodium valproate (SVP)

Groups	SOD (U/mg protein)	GPx (nmol/min/mg protein)	CAT (nmol/min/mg protein)
Group (1):Negative control	25.10 ± 0.3[a]	241.4 ± 5.2[a]	369.9 ± 6.4[a]
Group (2): Positive (SVP) control	14.20 ± 0.4[d]	133.5 ± 3.8[d]	285.6 ± 3.8[d]
Group (3):PSO (40 mg/kg)	16.15 ± 0.8[c]	145.6 ± 4.6[c]	295.2 ± 6.2[c]
Group (4):Vit E (200 mg/kg)	17.25 ± 0.7[c]	150.2 ± 5.6[c]	300.5 ± 8.2[c]
Group (5):PSO (40 mg/kg)+Vit E (200 mg/kg)	22.25 ± 0.2[b]	197.5 ± 4.2[b]	325.2 ± 7.5[b]

Means ± SEM with different superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test. n= 9 rats.

GPx unit = nmol of GSH utilized/min/mg protein.

CAT unit = nmol of H_2O_2 utilized/min/mg protein.

The positive control group was compared to the normal control.

The pretreated groups were compared to the positive control group.

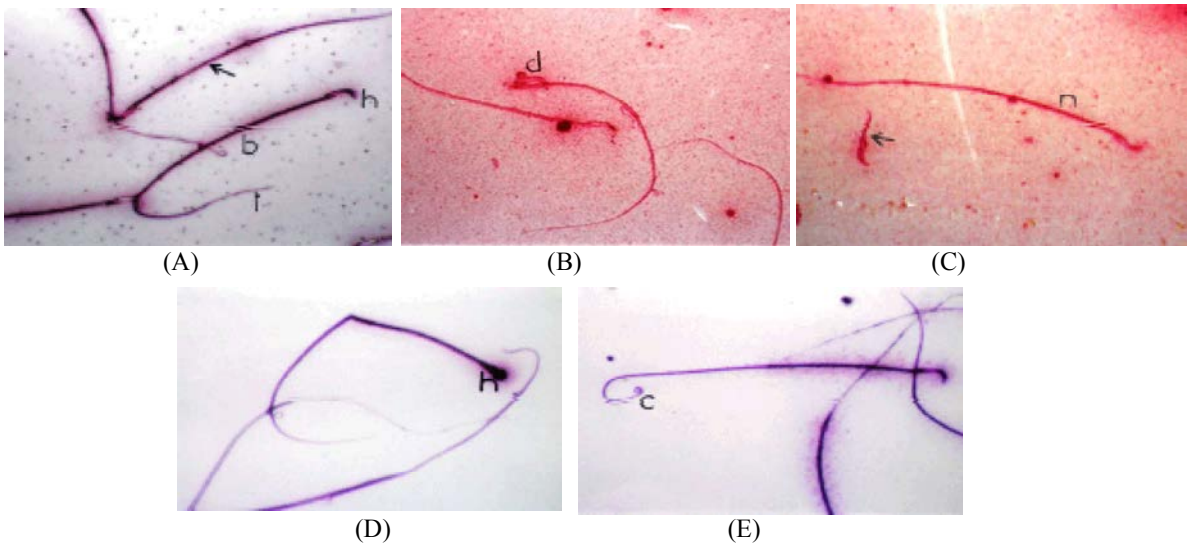

Fig. 1: Photomicrograph of seminal smears of a rat showing:
(A) Normal sperm with head (h), body (b) and tail (t). (N&E x160)
(B) Sperm with double head (d). (N&E x160)
(C) Detached head of sperm (arrow). (N&E x160)
(D) Large oval head of sperm (h). (N&E x160)
(E) Sperm with coiled tail (c). (N&E x160)

spermatozoa and complete spermatogenic germ cell series (Fig. 2.3). The rats orally given SVP (control positive group) showed testicular degenerative changes of spermatogenic germ cell series with diffuse interstitial edema and inflammatory leukocytes infiltration (Fig. 2.4).

DISSCUSION

The current study was designed to investigate the protective effect of pumpkin seed oil (PSO) and vitamin E (Vit E) against sodium valproate-induced testicular injury in male rats. Results of this study revealed that oral administration of sodium valproate (SVP) to male rats (500 mg/kg) for consecutive 7 days induced reproductive toxicity. The toxic effect of SVP was manifested by decreased weight of testes, lowered semen quantity and quality, decreased serum levels of testosterone, FSH and LH as well as testicular histopathological degenerative changes (edema and leukocytes infiltration). These results correlate with those reported by Hamza and Amin [22], Wilke and Utley [23], Walker *et al.* [34], Soliman and Abdel Meguid [35] and Bairy *et al.* [36]. They found that oral administration of SVP to rats decreased relative weights of testes and epididymis and reduced sperm numbers and viability. Serum testosterone, FSH and LH levels were also dropped and severe testicular histopathological lesions were seen. The present results also showed that SVP increased lipid peroxidation and

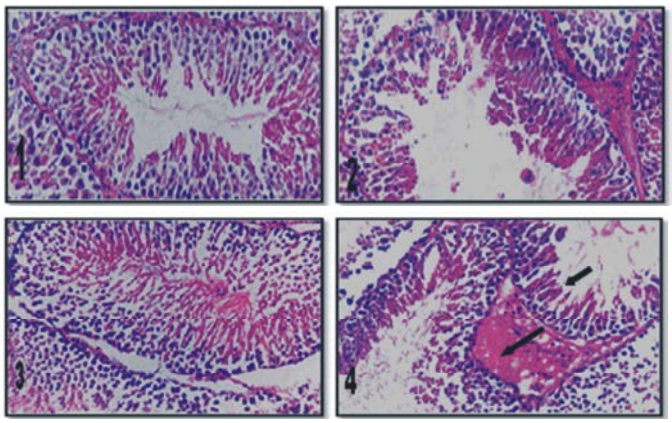

Fig. 2: Photomicrograph of sections of testes of rats pretreated with vitamin E alone (1), pumpkin seed oil alone (2), pumpkin seed oil and vitamin E (3) and sodium valproate alone (4) showing:

- Intact seminiferous tubules with normal spermatozoa in the lumen and complete spermatogenic germ cell series.
- Relatively intact seminiferous tubules with few spermatozoa in the lumen, mild interstitial edema and leukocytes infiltration.
- Normal intact seminiferous tubules completely filled with mature spermatozoa and complete spermatogenic germ cell series.
- Degenerative changes of spermatogenic series (arrow) with diffuse interstitial edema (arrow) and inflammatory leukocytes infiltration. (Control positive group). (H&E x 200)

decreased activities of antioxidant enzymes in the testes. The mechanism of the toxic effect of SVP was attributed to its direct cytotoxic effect on the testis and/or indirectly by decreasing serum testosterone level as concluded by Bairy *et al.* [36]. The oxidative stress in testes induced by SVP in rats was evident by decreases in activities of antioxidant (SOD, GPx and CAT) enzymes. In this study, the reported oxidative stress caused by SVP was similar to that previously demonstrated that SVP induces oxidative stress and reproductive toxicity in male rats [37].

Oral pretreatments of rats with pumpkin seed oil (PSO), Vit E (Vit E) and their combination significantly increased the weight of testes, normalized serum levels of testosterone, FSH and LH, improved semen quality and quantity and ameliorated the testicular degenerative damage induced by SVP. There were also a significant decrease in lipid peroxidation and an increase in activities of antioxidant enzymes in the testicular tissue. Concerning pumpkin seed oil (PSO), the previous studies reported that PSO is rich in many antioxidants and beneficial nutritional components such as essential fatty acids, amino acids, phytosterols, β-carotenes, lutein and selenium [9, 10]. PSO also contain very high concentration of vitamin E which acts as a powerful antioxidant. Essential fatty acids are required constituents of health of cell membrane as they maintain the fluidity of cell membranes [12]. The presence of selenium in PSO helps induce and maintain the glutathione antioxidant system. Selenium is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes. It has been reported that selenium can improve male fertility due to its antioxidant properties [37]. The protective effect of PSO against SVP-induced testicular injury in male rats, reported in this study agree with that reported by Murkovic *et al.* [13], who concluded that the antioxidant property of PSO could enhance male fertility in rats. In addition, Nwangwa *et al.* [14] reported that PSO ameliorated the effect of quinine - induced testicular damage in rats. The reported antioxidant activity of PSO was in agreement with that previously reported by Nkosi *et al.* [38], Akang *et al.* [39], Abd El-Ghany *et al.* [40] and El-Boghdady [41].

The increase of serum testosterone, FSH and LH hormones caused by coadministration of PSO and Vit E to rats, in this study, may be responsible for improving semen quality and quantity as it has been established that testosterone is essential for spermatogenesis. Moreover, FSH and LH play an important role in germ cell progression and improved fertility in animal models [42]. Moreover, the improvement of male reproductive efficiency by oral coadministration of PSO and Vit E could be also attributed to their antioxidant activity in the rat testes. The ameliorative effect of PSO and Vit E against testicular degenerative lesions caused by SVP in rats was

partially similar to that reported by Nkosi *et al.* [38] and Abd El-Ghany *et al.* [40]. Pumpkin seed oil was reported in this study to decrease lipid peroxidation in and enhance activities of antioxidant enzymes (SOD, GPx and CAT) in rat testes. These effects were previously reported by Nkosi *et al.* [38], Abd El-Ghany *et al.* [40] and Makni *et al.* [43]. Regarding vitamin E, the reported improving effect on male fertility by vitamin E in this study was in agreement with that demonstrated by that vitamin E can improve reproductive efficiency of male rats [15, 16, 17,19]. Thy concluded that the improvement of reproductive efficiency of male rat by vitamin E is attributed to its powerful antioxidant property.

CONCLUSION

Combination of PSO and Vit E exhibits protective and antioxidant activities against SVP-induced testicular damage in rats. The improvement of male reproductive efficiency by PSO and Vit E could be due to their antioxidant activity and to the increase in serum testosterone, FSH and LH which are necessary hormones for normal spermatogenesis and production of normal sperms. The study suggested that dietary intake of PSO and Vit E may be beneficial for patients who suffer from male infertility due to oxidative stress.

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