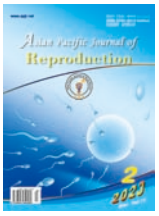

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305−0500.372377

Pumpkin seed ethanolic extract protects against escitalopram−induced reproductive toxicity in male mice

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ABSTRACT

Objective: To investigate the protective role of pumpkin seed ethanolic extract against escitalopram-induced reproductive toxicity in male mice.

Methods: Swiss albino male mice were randomly divided into five groups with six mice in each group. Group Ⅰ received normal water orally, Group Ⅱ, Ⅲ, Ⅳ and Ⅴ received escitalopram oxalate (10 mg/kg), pumpkin seed extract (300 mg/kg) plus escitalopram oxalate (10 mg/kg), escitalopram oxalate (20 mg/kg), and pumpkin seed extract (300 mg/kg) plus escitalopram oxalate (20 mg/kg), respectively. All test doses were continuously administered orally once daily per animal body weight for 30 days and 60 days. Body weight and sexual organ weight were evaluated on day 31 and 61. Effects of pumpkin seed extract on sperm parameters, biochemical parameters and histology of testis were also investigated.

Results: Escitalopram 10 or 20 mg/kg caused reproductive toxicity in male mice after 30 and 60 days of treatment. However, simultaneous administration of escitalopram oxalate (10 or 20 mg/kg) with pumpkin seed extract (300 mg/kg) attenuated escitalopram-induced testicular toxicity. Significant increase in the body weight and relative organ weight was observed. Sperm count, sperm motility and viability significantly increased (P<0.05). The histopathological alterations caused by escitalopram was also ameliorated.

Conclusions: Ethanolic extract of pumpkin seeds (300 mg/kg body weight) protects again reproductive toxicity induced by escitalopram. Therefore, dietary intake of pumpkin seed extract might be useful for male patients who expose to antidepressant drug due to depression.

KEYWORDS: Escitalopram oxalate; Pumpkin seeds; Testicular toxicity; Sperm parameters; Male Swiss albino mice

1. Introduction

Approximately 3%-4% of India's 100 crore plus population and 7%–10% of the world population is affected by depression and anxiety and considered as common public health problems[1,2]. For the treatment of major depressive disorder, antidepressant drugs are frequently prescribed[3]. Among antidepressants, selective serotonin reuptake inhibitors (SSRIs) are the newer antidepressant, first-line drug in treating depression and anxiety and in year 2000, 65% of 20.5 million psychiatric patients are prescribed by SSRIs[4,5]. Escitalopram and other SSRIs act by blocking the uptake of serotonin into pre synaptic neuron and causing increased concentration in the synaptic space, resulting in the stimulation of post synaptic receptors. SSRIs treatment are responsible for adverse effects on the reproductive system due to various hormonal and neurochemical changes in central and peripheral nervous system[6]. Several studies showed that male reproductive toxicity may be caused by treatment with different SSRIs such as Citalopram[7], Trazodone[8]. The results showed decrease in sperm motility, reduction in sperm concentration, increase in abnormal sperm

Significance

No studies have been performed earlier on the effects of ethanolic extract of pumpkin seeds with escitalopram on male reproductive profile. Long term consumption of escitalopram may cause male reproductive toxicity as evident by the present investigation in mice. This study indicated that ethanolic extract of pumpkin seed effectively reduces reproductive toxicity caused by escitalopram. It could be further recommended to patients suffering from depression to increase intake of pumpkin seed in their diet.

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How to cite this article: Karuna A, Lata S, Pradeep B. Pumpkin seed ethanolic extract protects against escitalopram-induced reproductive toxicity in male mice. *Asian Pac J Reprod* 2023; 12(2): 71-80.

Article history: Received: 5 November 2022; Revision: 28 December 2022; Accepted: 26 January 2023; Available online: 30 March 2023

morphology and sperm DNA damage, induced degeneration of testicular structure, increase in levels of follicle stimulating hormone, luteinizing hormone and testosterone in serum levels and oxidative stress which leads to production of reactive oxygen species in the testicular tissue.

For the cure of diseases, herbal remedies are used on an individual basis or together with standard medicines in numerous medical studies. Due to many medicinal properties and the presence of natural edible substances, pumpkin is used as traditional medicine. The pumpkin, *Cucurbita maxima* belongs to family Cucurbitaceae. It has several phyto-constituents such as alkaloids, flavanoids, palmitic, oleic and linoleic acids. Several studies describe the medicinal properties such as anti-diabetic, cardioprotective, anti-depressive, antioxidant, anti-carcinogenic, anti-inflammatory of pumkin seeds and seed oil. Ryan *et al*[9] have found several compounds in pumpkin seeds such as phytosterols, squalene and tocopherols. Kim *et al*[10] reported that stearic, oleic and linoleic acids were the major fatty acids present in pumpkin seeds. However, the effects of pumpkin seed extract on reproductive toxicity induced by the antidepressant drug, escitalopram still remain unclear. So, the objective of this study is to examine the protective effects of pumpkin seed extract against reproductive toxicity induced by subchronic escitalopram oxalate treatment by studying the effect on sperm parameters, biochemical parameters and histology of testis.

2. Materials and methods

2.1. Drug

Escitalopram oxalate (Nexito-10 and Nexito-20 manufactured by Sun Pharma laboratories Ltd., Gangtok- 737135, Sikkim, India) was purchased from the market. Escitalopram oxalate doses were given orally to male mice through intubation tube at a dose of 10 mg/kg and 20 mg/kg body weight (b.w.) per day and the dose was selected for the experiment according to previous studies performed by Waugh and Goa[11].

2.2. Plant material and preparation of ethanolic extract of pumpkin seeds

Pumpkin seeds were used and purchased from True Elements, Amazon, India. For the preparation of 80% ethanolic extract of pumpkin seeds, they were washed, dried and grained into the fine powder mechanically and then subjected to Soxhlet apparatus for extraction with 80% ethanol. Under Soxhlet extraction procedure, 100 g of powdered seeds was taken in 500 mL of 80% ethanol. It was heated for 5-6 h at 60℃. The extract obtained was filtered using Whatman No.1 filter paper and then dried under reduced pressure. The dried extract was stored at 4℃. The extract was prepared according to WHO protocol CG-04. The ethanolic extract of seeds was orally administered to mice at a dose of 300 mg/kg b.w. and this dose was selected according to the Aghaei *et al*[12].

2.3. Experimental animals

A total of sixty healthy adult male Swiss albino mice (*Mus musculus*) 7-8 weeks old (approximately 25-30 g b.w. were procured and kept in IIS (deemed to be University) animal house approved by CPCSEA (Registration No: 1689/PO/Re/S/13/CPCSEA). They were kept in poly-propylene cages in the animal house. The animals were feed with food pellets and water *ad libitum*. The temperature in the room was maintained at (22±2)℃ and humidity (60±10)%. The cages had wooden shavings to provide bedding for animals. The animals were acclimatized for 1 week and then used for experimentation.

2.4. Experimental design and treatments

The mice were divided into five groups. Group Ⅰ received normal water orally through intubation tube for continuous 30 days (n=6) and 60 days (n=6) and served as the normal control group and was used as benchmark for comparing the treated group which was studied. Group Ⅱ received escitalopram oxalate (10 mg/kg b.w./d)[11] dissolved in water orally through intubation tube for continuous 30 days (n=6) and 60 days (n=6). Group Ⅲ received simultaneously administration of escitalopram oxalate 10 mg/kg b.w./d and pumpkin seed extract 300 mg/kg b.w./d[12] orally through intubation tube for continuous 30 days (n=6) and 60 days (n=6). Group Ⅳ received escitalopram oxalate (20 mg/kg b.w./d)[11] dissolved in water orally through intubation tube for continuous 30 days (n=6) and 60 days (n=6). Group Ⅴ received simultaneously administration of escitalopram oxalate (20 mg /kg b.w./d) and pumpkin seed extract 300 mg/kg b.w./d[12] orally through intubation tube for continuous 30 days (n=6) and 60 days (n=6).

On 31st day (n=30) and 61st day (n=30), mice were weighed and sacrificed by cervical dislocation and then testis, cauda epididymis was collected, and used for estimation of sperm parameters, biochemical parameters and histology of testicular tissue.

2.5. Determination of body weight and organ weight

The change in the body weight of all the groups was recorded throughout the experimental period. The animals were weighed using the standard weighing balance at the beginning and at the end of the experiment. The weight was recorded and analysed by using the Statistical Software Programme (SPSS.20).

The testis and cauda epididymis of the control and treated animal groups was weighed by using the standard weighing balance immediately after autopsy. The weight was recorded and analyzed by using the Statistical Software Programme (SPSS.20).

2.6. Assessment of sperm parameters

2.6.1. Sperm count

The sperm count (in %) in cauda epididymis was done according to the procedure of Prasad *et al*[13]. In 2 mL of physiological saline, 100 mg of cauda epididymal tissue was taken. Tissue was teased gently to release the sperms. Suspension of sperm was diluted in 1:20 ratio with $NaHCO_3$ in WBC micropipette. The sample was diluted thoroughly and the drop was placed in hemocytometer Neubauer chamber and covered with coverslip.

Counting of spermatozoa was done in 64 sub-squares of WBC Chambers.

$$\text{Sperm concentration} = \frac{\text{No. of sperms} \times \text{dilution (20)} \times 1000}{\text{Volume of 64 subsquares (0.4 cu.mm)}}$$

The unit for sperm count is million sperm per mL.

2.6.2. Sperm motility

Prasad *et al*[13] protocol was used for calculating the sperm motility (%) in cauda epididymis. In 2 mL of physiological saline, 100 mg of cauda epididymal tissue was taken, then the tissue was teased gently to release the sperms. Suspension drop was kept on the Neubauer chamber. In each field, motile spermatozoa and the total number of spermatozoa were counted. Scoring of minimum 10-12 separate field was done.

$$\text{Sperm motility (\%)} = \frac{\text{No. of motile sperms}}{\text{Total number of sperms}} \times 100$$

2.6.3. Sperm viability

Sperm viability was done using guideline by the WHO Laboratory Manual (WHO, 1999). In 2 mL of physiological saline, 100 mg of cauda epididymal tissue was taken. 50 μL of sperm suspension was taken and mixed with 100 mL of eosin stain. After 30 s, an equal volume of Nigrosin stain was added. Approximately 200 sperms were observed and percentage of dead and live cells was recorded. Live cells appear colourless and dead cells appear as red or pink in colour.

$$\text{Sperm viability (\%)} = \frac{\text{Total viable sperms observed}}{\text{Total number of sperms observed}} \times 100$$

2.7. Determination of testis biochemical parameters

2.7.1. Total protein

The total protein content was determined by the method of Lowry *et al*[14] using bovine serum albumin as the standard. During colorimetrical estimation at 540 nm, the blue colour which is produced is quantitatively proportional to the total protein. The protein levels in the tissue were expressed as mg/g fresh tissue weight.

2.7.2. Sialic acid

The sialic acid concentration in testis was estimated by the method of Jourdian *et al*[15]. The concentration of sialic acid was expressed as μg/mg tissue weight.

2.7.3. Total cholesterol

The cholesterol content in testis was determined by the method of Zlatkis *et al*[16]. The concentration of cholesterol was expressed as mg cholesterol/100 mg fresh tissue weight.

2.7.4. Glycogen

The glycogen content was determined by the method of Montgomery[17]. Total glycogen content was expressed as μg/100mg tissue.

2.8. Histological evaluation

For histological observations, cervical dislocation was done to sacrifice the control and experimental animals. Testis was dissected out and washed in normal saline and was fixed in 10% formalin, dehydrated in graded series of alcohol, and washed in xylene. Tissue was embedded in paraffin wax, sectioned at 5 μm in microtome, and stained with hematoxylin and eosin (H & E). Tissue sections were examined for histological effects under phase contrast microscope with Nikon digital light camera at ×400 magnification.

2.9. Statistical analysis

The statistical analysis was performed by using IBM® SPSS® Analytic Server version 2.0. Statistical analysis was performed by using one-way analysis of variance followed by *post hoc* Tukey's test multiple comparison tests and data were expressed as mean±standard deviation (mean±SD). $P<0.05$ was considered to be statistically significant.

2.10. Ethical statement

This study was approved by "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA) with Registration No: 1689/PO/Re/S/13/CPCSEA.

3. Results

3.1. Effect on body weight and relative sex organs weight

3.1.1. Body weight

Mice continuously treated with escitalopram oxalate (10 mg/kg or 20 mg/kg b.w.) showed a significant decline in the body weight as compared to their control group after 60 days ($P<0.05$). However, no significant change in body weight was observed after 30 days of treatment. Simultaneous administration of pumpkin seed extract 300 mg/kg b.w. with escitalopram oxalate for 60 days (group Ⅲ and group Ⅴ) resulted in significant elevation in the body weight

as compared to their individual treatment group Ⅱ and group Ⅳ, respectively (P<0.05; P<0.01) (Table 1).

3.1.2. Testis weight and cauda epididymis weight

Escitalopram oxalate (20 mg/kg b.w./d) treated group for 30 days resulted in significant decrease in the testes and cauda epididymis weight compared to the control group (P<0.05). But when the treatment was continued for 60 days, significant decrease was observed in both escitalopram oxalate (10 mg/kg b.w. and 20 mg/kg b.w.) (Group Ⅱ, Group Ⅳ) treated mice as compared to the control mice (Group Ⅰ) (P<0.01). Simultaneous administration of pumpkin seed extract 300 mg/kg b.w. with escitalopram oxalate for 60 days (group Ⅲ and group Ⅴ) resulted in significant elevation in the organ weight as compared to their individual treatment group Ⅱ and group Ⅳ, respectively (P<0.05; P<0.01) (Table 1).

3.2. Effect on sperm parameters

The treatment of mice with escitalopram oxalate at both doses resulted in a significant decrease (P<0.05) after 30 days and highly significant decrease P<0.01) after 60 days in the sperm count, motility and viability in cauda epididymis as compared to the control group. When the mice was treated with escitalopram oxalate and pumpkin seed extract for 30 days, significant increase (P<0.05, P<0.05) and for 60 days, highly significant increase (P<0.01, P<0.01) in the sperm count, motility and viability was observed as compared to their individual treated group (Table 2).

3.3. Effect on biochemical parameters

3.3.1. Protein and sialic acid

A significant decrease (P<0.05) in protein content and sialic acid content in both escitalopram oxalate (10 mg and 20 mg/kg b.w.) treated mice for 30 days was observed and after 60 days of treatment, highly significant decrease (P<0.01) in protein and sialic acid content at both dose levels (10 mg/kg and 20 mg/kg b.w.) was observed when compared with the control group.

Pumpkin seed extract treatment with escitalopram oxalate for 30 days resulted in a significant increase (P<0.05, P<0.05) in protein content in both group Ⅲ and group Ⅴ. No significant changes were observed in sialic acid content after 30 days. But after 60 days of treatment, highly significant increase (P<0.01, P<0.01) in protein and sialic acid content was observed in both group Ⅲ and group Ⅴ when compared with group Ⅱ and group Ⅳ, respectively (Table 3).

Table 1. Effects of simultaneous administration of escitalopram oxalate and pumpkin seed extract on body weight and relative organ weight of mice.

Parameters	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Body weight, g					
30 days	31.05±1.19	30.36±0.67	30.73±0.57	28.38±1.59	26.55±0.76
60 days	32.30±0.70	29.62±0.702*	31.62±0.53#	26.12±0.69*	30.72±0.51^^
Testes weight, mg					
30 days	125.29±0.87	123.12±0.33	124.52±0.12	120.48±0.63*	121.05±0.57
60 days	124.29±0.33	118.01±0.48**	123.69±0.97#	112.09±0.11**	116.18±0.43^^
Cauda epididymis weight, mg					
30 days	15.39±0.51	12.14±0.39	14.40±1.38	13.05±0.63*	15.19±0.61
60 days	15.50±0.29	10.95±0.48**	14.93±0.65	9.18±0.11**	12.76±0.61^^

Data are expressed as mean±SD; n=6 in each group for 30 days and 60 days treatment, respectively. Group Ⅰ receives normal water orally and serves as the normal control group; Group Ⅱ is administered with escitalopram oxalate (10 mg/kg); GroupⅢ is administered with pumpkin seed extract (300 mg/kg) plus escitalopram oxalate (10 mg/kg); Group Ⅳ is administered with escitalopram oxalate (20 mg/kg); Group Ⅴ is administered with pumpkin seed extract (300 mg/kg) plus escitalopram oxalate (20 mg/kg). All test doses are continuously administered orally once a day as per animal body weight for 30 days and 60 days. *P<0.05: compared to group Ⅰ; #P<0.05: compared to group Ⅱ; ^P<0.05: compared to group Ⅳ.

Table 2. Effects of simultaneous administration of escitalopram oxalate and pumpkin seed extract on sperm parameters of mice.

Parameters	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Sperm count, million/mL					
30 days	53.24±1.31	47.85±0.98*	50.53±1.09#	43.69±1.16*	46.12±1.07^
60 days	52.66±2.75	30.90±0.23**	45.37±2.31##	28.62±1.70**	41.07±1.39^^
Sperm motility, %					
30 days	80.24±0.90	52.28±1.83*	59.53±2.56#	50.03±0.80*	55.22±1.69^
60 days	82.93±3.64	40.90±2.62**	54.63±1.07##	38.62±2.70**	51.12±1.90^^
Sperm viability, %					
30 days	72.65±3.96	60.60±0.65*	67.29±1.59#	40.69±1.19*	52.49±4.25^
60 days	71.10±1.45	45.51±3.19**	65.17±2.07##	30.15±1.90**	38.24±1.35^^

Data are expressed as mean±SD; n=6 in each group for 30 days and 60 days treatment, respectively. *P<0.05: compared to group Ⅰ; #P<0.05: compared to group Ⅱ; ^P<0.05: compared to group Ⅳ.

Table 3. Effects of simultaneous administration of escitalopram oxalate and pumpkin seed extract on protein and sialic acid levels of the testis in mice.

Parameters	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Protein level, mg/g					
30 days	32.26±3.03	28.50±0.97*	30.06±3.32#	25.20±1.49*	28.29±0.58^
60 days	33.94±0.31	24.85±1.22**	29.02±2.95##	19.11±0.82**	26.59±1.20^^
Sialic acid level, μg/mg					
30 days	17.66±0.51	13.12±0.67*	14.82±0.43	10.74±1.38*	10.11±1.59
60 days	17.67±0.29	7.83±0.70**	14.58±1.27##	6.80±0.65**	12.47±0.39^^

Data are expressed as mean±SD; n=6 in each group for 30 days and 60 days treatment, respectively. *P<0.05: compared to group Ⅰ; #P<0.05: compared to group Ⅱ; ^P<0.05: compared to group Ⅳ.

Table 4. Effects of simultaneous administration of escitalopram oxalate and pumpkin seed extract on cholesterol and glycogen levels of the testis in mice.

Parameters	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ
Cholesterol, mg/100 mg					
30 days	72.32±2.60	75.48±3.86	75.26±3.91	86.73±3.67*	84.19±2.46
60 days	69.23±3.81	100.27±10.37*	81.60±1.29#	135.25±5.18**	104.47±5.48^
Glycogen, μg/100 mg					
30 days	372.49±2.14	380.24±0.94*	375.40±1.80	385.75±0.70*	373.18±1.45
60 days	374.88±0.78	396.58±0.28**	385.33±1.75#	408.73±1.53**	396.79±1.13^

Data are expressed as mean±SD; n=6 in each group for 30 days and 60 days treatment, respectively. *P<0.05: compared to group Ⅰ; #P<0.05: compared to group Ⅱ; ^P<0.05: compared to group Ⅳ.

3.3.2. Cholesterol and glycogen

Cholesterol content increased significantly only in escitalopram oxalate (20 mg/kg b.w.) treated mice for 30 days (P<0.05). But, after 60 days of treatment significant increase (P<0.05) in escitalopram oxalate (10 mg/kg b.w.) treated mice and highly significant increase (P<0.01) in escitalopram oxalate (20 mg/kg b.w.) treated mice were observed when compared with the control group. Glycogen content increased significantly (P<0.05, P<0.01) at both dose levels after 30 days and 60 days of escitalopram treatment when compared with their respective control group.

No significant changes were observed in cholesterol and glycogen levels when mice were treated with pumpkin seed extract treatment and escitalopram oxalate for 30 days. After 60 days of treatment, significant decrease (both P<0.05) in cholesterol and glycogen levels in both group Ⅲ and group Ⅴ was observed when compared with group Ⅱ and group Ⅳ, respectively (Table 4).

3.4. Effect on histology of testis

The histological picture of the testis in the control mice (30 and 60 days) displayed normal histological structure of seminiferous tubules, Leydig cells, lumen filled with spermatozoa and Sertoli cells in seminiferous tubules was seen (Figure 1A and 2 A). After 30 days of treatment duration, the histoarchitecture of testis in mice treated with low dose of escitalopram oxalate (10 mg/kg b.w./day) showed mild focal degenerative changes in germinal epithelium (Figure 1B) whereas, the high dose group (20 mg/kg b.w./day) displayed relatively more degenerative and atrophic changes in seminiferous epithelium, seminiferous tubules with less number of spermatids and spermatozoa in the tubular lumen. The Leydig cells also showed degenerative and atrophic changes (Figure 1C).

In 60 days treatment groups, the histopathological study of the testis in the low dose group (10 mg/kg b.w./day) showed degenerative and atrophic changes in the seminiferous tubules with reduced number of germ cell populations and spermatozoa in the lumen of seminiferous tubules (Figure 2B). In the high dose group (20 mg/kg b.w./day), the histology of the testis showed severe disintegration of spermatogenic cells, intraepithelial vacuolization, sloughing of germ cells, shrinkage of seminiferous tubules as well as presence of sperm debris and almost absence of spermatozoa in the lumen. The Leydig cells were reduced in size showing degenerative and atrophic changes (Figure 2C).

Co-treatment of ethanolic pumpkin seed extract (300 mg/kg b.w.) with escitalopram oxalate (10 mg/kg b.w. and 20 mg/kg b.w.) for 30 and 60 days in mice showed approximately normal histological structure of the testis indicating ameliorative effect of pumpkin seeds. Testicular histoarchitecture of pumpkin seed extract (300 mg/kg b.w.) plus escitalopram oxalate (10 mg/kg b.w. and 20 mg/kg b.w.) treatment for 30 days showed mild recovery in the seminiferous tubules with almost normal morphology of Leydig cells. The germ cells count and spermatozoa number in seminiferous tubules significantly increased as compared to the escitalopram oxalate alone treated group (Figure 1D, E).

Long-term exposure (60 days) of pumpkin seed extract (300 mg/kg b.w.) along with escitalopram oxalate (10 mg/kg b.w.) showed marked recovery in the germinal epithelium as compared to their individual treated group. Increase in the number of sperms in the lumen and germ cells in the epithelium was seen. Decline in the sloughing in the epithelium was observed (Figure 2D). The histoarchitecture of testis of mice treated escitalopram oxalate (20 mg/kg b.w.) with along with pumpkin seed extract (300 mg/kg b.w.) showed marked restoration along with reduction in the intertubular spaces, decrease in vacuolization and increase in the germ cells of epithelium as compared to the individually treated group (Figure 2E).

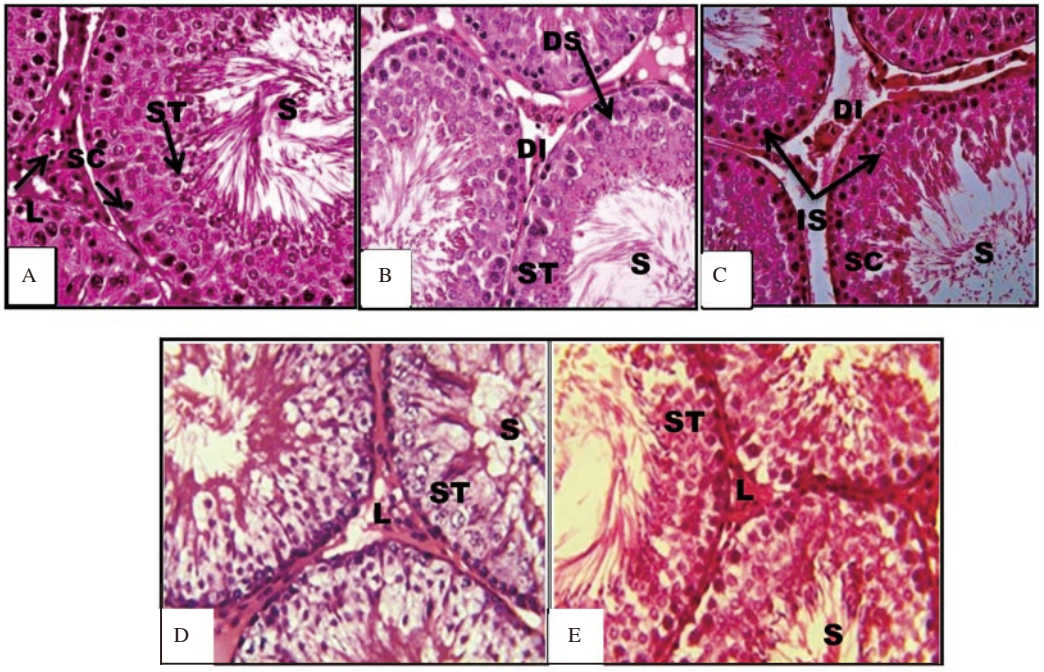

Figure 1. Transverse section of the testis of mice treated with escitalopram oxalate for 30 days alone and along with pumpkin seed extract (H & E; 400×). A: Sections of the testes of the control mice show normal arrangement of germ cells, spermatids (ST) and normal morphology of the Leydig cells (L) in interstitial area between the tubules, lumen filled with spermatozoa (S) and Sertoli cells (SC) in seminiferous tubule. B: Low dose (10 mg/kg b.w.) of escitalopram oxalate treated mice show moderate degenerative and atrophic changes in the seminiferous tubules (DS) and interstitial tissue (DI), diminished germ cell population and impairment of spermatogenesis. C: High dose (20 mg/kg b.w.) of escitalopram oxalate treated mice show increase in the inter tubular space (IS), interstitial cell degeneration (DI) and Sertoli cells (SC), slight decline in the number of round spermatid (ST) and spermatozoa (S) in the lumen. D & E: Low dose (10 mg/kg b.w.) & high dose (20 mg/kg b.w.) of escitalopram oxalate plus pumpkin seed extract (300 mg/kg b.w.) treated mice group show nearly normal structure of seminiferous tubule with spermatids (ST) in the lumen of the tubules, all the developing stages of spermatogenic cells with presence of Leydig cell (L), Sertoli cells (SC) and decrease in the intertubular space (IS).

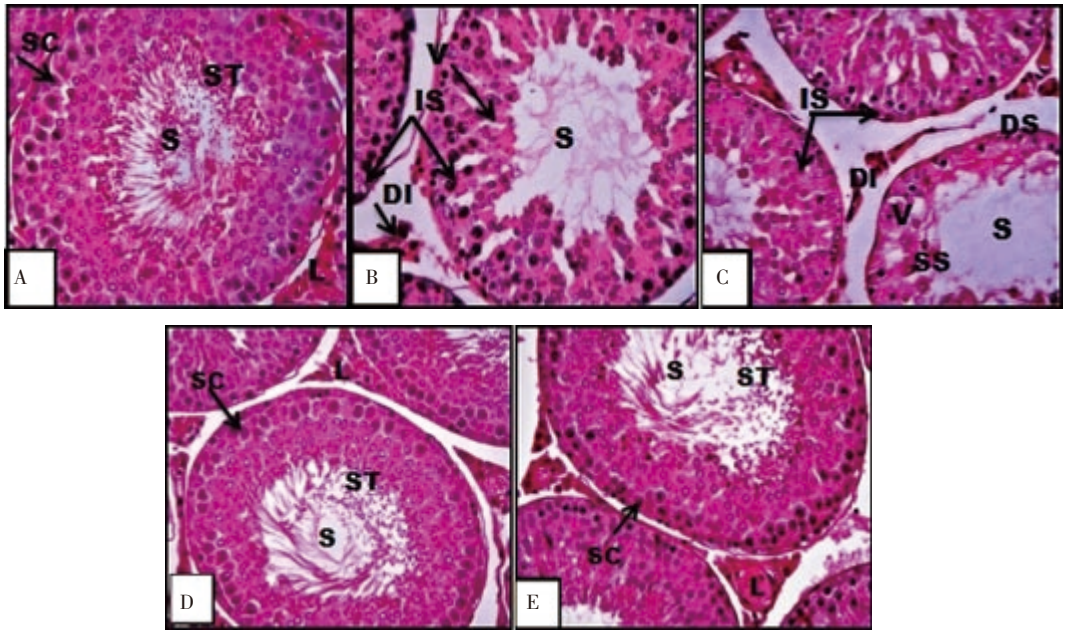

Figure 2. Transverse section of the testis of mice treated with escitalopram oxalate for 60 days alone and along with pumpkin seed extract (H & E; 400×). A: Sections of the testes of the control mice show normal normal arrangement of germ cells, spermatids (ST) and normal morphology of the Leydig cells (L) in interstitial area between the tubules, lumen filled with spermatozoa (S) and Sertoli cells (SC) in seminiferous tubule. B: Low dose (10 mg/kg b.w.) of escitalopram oxalate treated mice show marked degeneration in the seminiferous tubules, destruction in the interstitial cells (DI), increased intertubular space (IS), reduced germ cell population and scanty spermatozoa (S) in the lumen and vacuolization (V) in germinal epithelium. C: High dose (20 mg/kg b.w.) of escitalopram oxalate treated mice display severely damaged seminiferous tubules (DS) and degenerating interstitial cells (DI) depicting disrupted and vacuolated (V) germinal epithelium increase in the inter tubular space (IS) and sloughing of germ cells. Note marked decline of spermatogenic cells and almost absence of spermatozoa in the lumen of seminiferous tubule. No definite arrangement of seminiferous tubules is observed. D & E: Low dose (10 mg/kg b.w.) & high dose (20 mg/kg b.w.) of escitalopram oxalate plus pumpkin seed extract (300 mg/kg b.w.) treated mice group show marked recovery and improvement of histoarchitecture with reduction in the intertubular space, increase in the number of spermatozoa (S) in the lumen and germ cells population in the epithelium, all the developing stages of spermatogenic cells, presence of Leydig cell (L), Sertoli cells (SC), decline in the sloughing in the epithelium and presence of all stages of spermatogenesis.

4. Discussion

The current study was designed to evaluate the ameliorative effect of ethanolic extract of pumpkin seed extract against escitalopram oxalate treatment on the physical parameters, sperm parameters, biochemical parameters and histology of testis of adult male Swiss albino mice (*Mus musculus*).

Oral administration of escitalopram oxalate was selected as it is one of the most prescribed antidepressant drug. Pumpkin seeds are used as traditional medicinal plant due to its rich nutritional value and are an excellent source of iron, magnesium, copper, phosphorus, manganese and zinc which is required for maintenance of healthy male reproductive system[18].

In this study, simultaneously administration of ethanolic seed extract of pumpkin (300 mg/kg b.w.) with escitalopram oxalate (10 or 20 mg/kg b.w.) to mice provided an evidence for a protective role of pumpkin seeds by significantly increasing the body weight, organ weight, while improving the sperm quantity, quality in cauda epididymis, significantly decreasing the cholesterol and glycogen content in testis, significantly increasing the level of protein, sialic acid and improving the histology of testicular tissue thus decreasing the toxicity in testis and cauda epididymis of mice which was altered by treatment of escitalopram oxalate.

Normal body weight is a sign of good health and efficient metabolic homeostasis. Alterations in body weight and organ weight reflect the disruption in the structural and functional state of the organ and provide important knowledge about the toxicity of any substance and possible inference for the general health of the animal[19]. Results of this study revealed that oral administration of escitalopram oxalate (10 or 20 mg/kg b.w.) to male mice for 30 and 60 days induced reproductive toxicity. Mice that were continuously treated with escitalopram oxalate (10 or 20 mg/kg b.w.) showed a significant decline in the body weight, testes weight and cauda epididymis weight. Comparable results were observed by Soliman *et al*[20] in treated mice with different doses of fluoxetine (antidepressant drug) which resulted in a decrease in body weight and reproductive organs weight (testes, epididymis, ventral prostate). The observed loss in body weight of escitalopram oxalate reated rats in the present investigation might be due to direct cytotoxic effect of the antidepressant on somatic cells and regulation of endocrine functions, food and water intake by central nervous system which is affected by the antidepressants[21]. Testicular weight and size are normally regulated by differentiated spermatogenic cell mass and the fluid secretion from the Sertoli cells[22]. The decline in the relative weight of testis and cauda epididymis in escitalopram oxalate exposed mice might be due to atrophic and degenerative changes in seminiferous tubules, loss of the spermatids and spermatozoa, disruption of spermatogenesis, reduction in sperm count in lumen of epididymis, and epididymal tubules degeneration[23].

Significant elevation in the body weight and organ weight was observed after administration of 300 mg/kg b.w. dose of pumpkin seed extract for 60 days. This significant increase in body weight gain and organ weight might be due to high protein and vitamin levels in pumpkin seeds[24] and due to the presence of antioxidants and beneficial nutritional components such as essential fatty acids like oleic acid, linoleic acid and linolenic acid, and the high concentration of zinc and iodine present in pumpkin seeds[25].

Semen analyses like sperm count, motility, viability and morphology are key indices of spermatogenesis in testis and epididymal sperm maturation and could be considered as markers for evaluating the fertilizing capacity of men. The present study showed decrease in sperm count, viability, and motility in mice after escitalopram oxalate treatment in dose dependent manner. Our results are in agreement with those obtained by Abbas *et al*[26] who observed that continuous exposure to antidepressants (escitalopram (0.4 mg/kg *p.o*) and citalopram (0.8 mg/kg *p.o*) bring reproductive damage with significant decrease in semen parameters (count, motility, morphology) in Wistar albino rats. Ilgin *et al*[7] concluded that the citalopram when given for 4 weeks at the dose of 5, 10 and 20 mg/kg b.w. exerts decline in the motile sperms and decrease in number of sperms. Serotonin receptors are present in the cauda epididymis, testis, vas deferens, sertoli cells, Leydig cells, and sperm cells. As SSRIs bind to serotonin transporters by interacting with the sulfhydryl group, it has been hypothesized that SSRIs could cause deleterious effects on spermatozoa, because binding to sulfhydryl groups of sperm membrane is associated with spermicidal action[27] which supports the hypothesis that SSRIs affect fertility. The observed alteration in sperm parameters such as concentration, motility, functionality, and morphology of spermatozoa in escitalopram oxalate-exposed mice in the present study might be due to disturbance in blood-testicular barrier[28] which disturbs spermatogenesis and results in declination of sperm count[29].

Pumpkin seed extract (300 mg/kg b.w.) continuously given with escitalopram oxalate for 30 and 60 days of treatment to mice resulted in significant increase in the number of sperms, motile sperms and viable sperms as compared to escitalopram oxalate treated group. Pumpkin seeds have abundant antioxidant and free radical scavenging ability due to the fact that it shows protective effect and stimulatory effect of adenosine triphosphate (ATP) production[30]. Carbohydrate-rich pumpkin seed extract could increase sperm motility and viability by increasing glucose metabolism, leading to the production of pyruvate and ATP[31]. The fertilising capacity of spermatozoa can be increased by the use of pumpkin seeds as it leads to increase in sperm parameters. Omega-3, an essential polyunsaturated fatty acids are derived from pumpkin seed, flax seed and some nuts. Bansal and Bilaspuri[32] reported that Omega-3 had protective role on spermatogenesis against antidepressants-induced cell damage, which is in accordance with the present study.

Testicular proteins are androgen dependent proteins also called androgen binding proteins. These proteins are crucial for proper development of testes, normal spermatogenesis through coordination with Sertoli cells and maturation of spermatozoa. The mice treated

with escitalopram oxalate resulted in a significant decrease in protein level in mice after 30 and 60 days of treatment. In the testis, the protein is considered as a marker of injury in the tissue, damage of cells and healing and a decrease value indicates toxicity in the animal[33]. Our results are in conformity with those of Unnikrishnan *et al*[34] in which fluoxetine (an antidepressant drug) treatment leads to a decrease in testicular protein.

Sialic acid (*N*-acetylneuraminic acid) is a sialo mucopolysaccharide, a prime component of glycoproteins and glycolipids and functions as a lubricant and immunoprotectant and helps in downward movement of sperms by reducing the friction among spermatozoa in the testis[35]. In the present study, the testicular sialic acid value was decreased significantly in dose and the duration dependent fashion in escitalopram oxalate-treated mice. Decrease in sialic acid content in the testis in mice suggests the deficiency of androgens and gonadotropins leading to inhibition of spermatogenesis and decrease in spermatozoal mobility and fertilizing ability[36].

Cholesterol is synthesized actively by testis and other tissues. In the testis, the precursor for androgen synthesis is cholesterol and any change in the cholesterol level in testis leads to inhibition\stimulation of spermatogenesis[37]. The mice treated with escitalopram oxalate resulted in a significant increase in cholesterol level in mice. Accumulation of testicular cholesterol was interrelated with decline in the activities of steroidic enzymes (3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase) which disturb the testicular steroidogenesis[38].

Glycogen metabolism is a potential source of glucose to both testicular somatic (namely Sertoli and Leydig cells) and germ cells of various mammalian species, which provides carbohydrate reserves as energy source for spermatogenic cells in seminiferous tubules for normal spermatogenesis and capacitation and be an apoptosis regulator in germ cells[39]. The mice treated with escitalopram oxalate resulted in a significant increase in glycogen level in mice. Interfernce of escitalopram oxalate with carbohydrate metabolism and accumulation of nonutilizable sugars in the Sertoli cells due to an arrested spermatogenesis might lead to increased level of glycogen in the testes, which correlates with the present study.

The results obtained showed that there was a significant increase in the protein, sialic acid levels and a significant reduction in the cholesterol, glycogen level in the testis of mice that received co-administration of escitalopram oxalate and 300 mg/kg b.w. dose ethanolic extract of pumpkin seed when compared with escitalopram oxalate treated group. This might be due to phenolic compounds such as polyphenols flavonoids as well as unsaturated fatty acids such as omega 3, 6 and 9, vitamins, *L*-tryptophan, protein and very high concentration of zinc[9]. Zinc found in pumpkin seeds acts as an antioxidant by neutralizing free radical generation and helps in maintaining the structure of proteins and cell membranes and protects cells against damage, so it plays important roles in growth and development of the immune response, neurological function and reproduction[12]. Zinc could exert a direct antioxidant action by occupying the iron or copper binding sites of lipids, proteins, and DNA[40]. El-Din and Abd-El Aty[41] also reported that omega 3 found in pumpkin seed when administered orally after treatment with duloxetine (an antidepressant) leads to biochemical and immunocytochemical changes in the testes of adult rat. Kumar *et al*[42] concluded protective role of omega-3 might be due to anti-inflammatory and powerful antioxidant effects and recommended to use omega-3 whenever antidepressant drugs are used.

Histopathological examinations of the testis are considered as an important biomarker in toxicological researches for detecting male reproductive toxicity. Histopathological changes in the testes can accompany decrease in sperm quantity and quality[43]. The histopathological study of the testis in the present study revealed that exposure of escitalopram oxalate 20 mg/kg b.w. induces more testicular damage in comparison to escitalopram oxalate 10 mg/kg b.w. Histopathological examination of the testis of mice orally administered with escitalopram oxalate (10 or 20 mg/kg b.w.) observed changes in the testis which include shrinked seminiferous tubules, sloughing of germinal epithelium, marked disintegration in spermatogenic cells and depletion of spermatozoa from lumen. Increase in the intertubular space, destruction in the interstitial cells and degeneration of Sertoli cells were also observed in escitalopram-treated mice. Escitalopram oxalate treatment leads to production of reactive oxygen species that results in oxidative stress which cause deteriorations in testicular structure and spermatogenesis processes and testicular atrophy and degeneration of seminiferous tubules by disrupting membrane integrity[44].

Our results were in agreement with Erdemir *et al*[45] where sertraline, paroxetine, fluoxetine and escitalopram treatment resulted in distortion of seminiferous tubules, sloughing, vacuolization and degeneration of germ cells, decrease in number of Sertoli cells and reduction in thickness of germinal epithelium. Histopathological observation revealed decreased spermatogenesis and Sertoli cell hyperplasia in escitalopram (0.4 mg/kg *p.o*) and citalopram (0.8 mg/kg *p.o*) treated rats[26].

The results obtained showed that there was an improvement in the architecture of testicular tissue of mice treated with co-administration of escitalopram oxalate and 300 mg/kg b.w. dose of ethanolic extract of pumpkin seed. This might be due to presence of tannins and vitamin A content in the seed and oil; spermatogenesis could be promoted and improvement in histology of testis was observed[46]. Our results were in agreement with Minisy *et al*[47] who found the histological alterations produced by tramadol were improved when ethanolic extract of pumpkin seed 40 mg/kg treatment was given which might be due to its rich content of polyphenols and flavonoids[30]. Studies reported that omega-3, found in pumpkin seeds helps in moderate regeneration and improvement in the histopathological examination of the seminiferous tubules and germ cell apoptosis in the testes against antidepressant drugs fluoxetine[20], duloxetine[41] induced toxicity in adult male albino rats.

For the study's limitation, it is to note that the research was done on male reproductive toxicity. But various antioxidant parameters along with serum hormonal levels which are also an important factor for determining the reproductive toxicity remain undone. The results on female reproductive parameters can also provide support to our data which was not possible to carry out.

In conclusion, escitalopram oxalate (10 or 20 mg/kg b.w.) induces biochemical, histological and sperm parameters alterations in male mice after 30 and 60 days of treatment. Pumpkin seed extract appears to have abundant beneficial properties. The content of compounds such as polyunsaturated fatty acids, omega-3, zinc, essential amino acids, vitamin E, selenium and polyphenols makes pumpkin seed extract an effective choice which can have beneficial effects by altering the effects of prolonged use of antidepressant drug on male reproductive toxicity.

Conflict of interest statement

The authors declare no conflicts of interest.

Funding

This study received no extramural funding.

Authors' contributions

Lata Shahani is Principal Investigator and Pradeep Bhatnagar is Co-Principal Investigator in the study. Karuna Agrawal performed the experiment and submitted the data for analysis. Karuna Agrawal and Lata Shahni were involved in the designing and interpretation of animal studies. All authors were involved in the preparation of manuscript.

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