J Kermanshah Univ Med Sci. 2025 March; 29(1): e157822 https://doi.org/10.5812/jkums-157822

Published Online: 2025 February 26 **Research Article**

Protective Effect of Grape Seed Extract on Sperm DNA Integrity and Early Embryonic Development in Male Balb/c Mice Treated with Fluoxetine

Ghodrat Ebadimanas [1,*], Gholamreza Najafi [2]

[1] Department of Biology Education, Farhangian University, Tehran, Iran
[2] Department of Basic Veterinary Sciences, Histology and Embryology Sections, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

[*] ***Corresponding Author:*** Department of Biology Education, Farhangian University, P. O. Box: 14665-889, Tehran, Iran. Email: ebadimanas@gmail.com

Received: 18 November, 2024; **Revised:** 9 February, 2025; **Accepted:** 18 February, 2025

Abstract

Background: Fluoxetine (FLX), an antidepressant, has toxic effects on embryonic stem cell differentiation and fertility.

Objectives: The aim of this study was to investigate the effect of grape seed extract (GSE) on sperm DNA integrity and early embryonic development in Balb/c mice exposed to FLX.

Methods: In this study, 32 male Balb/c mice (6 - 8 WK, 30 ± 2 g) were utilized and randomly divided into four groups: Control, FLX (40 mg/kg B.W), FLX (40 mg/kg B.W) + GSE (150 mg/kg B.W), and GSE (150 mg/kg B.W). In all groups, the caudal epididymis was collected on day 42. Superovulation was induced in adult female mice, and fertilized oocytes were assessed. The amount of two-cell embryos was checked after 24 days of cell culture, and at 96 hours after fertilization, blastocysts and hatched embryos were examined.

Results: The results indicated a significant increase in the percentage of sperm with DNA damage (P = 0.04) and immature sperm in the FLX group (P = 0.02), along with a significant reduction in the percentage of zygotes (P = 0.25), blastocysts (P = 0.01), and hatching embryos (P = 0.03). However, the FLX + GSE group exhibited a remarkable improvement in all of the aforementioned parameters and in reducing embryo toxicity. The percentage of two-cell embryos did not differ significantly between the groups.

Conclusions: This study demonstrated the antioxidant activity of GSE in mitigating FLX-induced embryo toxicity.

Keywords: Embryo, Fertilization, Fluoxetine, Grape Seed Extract, Sperm

1. Background

Fluoxetine is an antidepressant in the selective serotonin reuptake inhibitor class, generally used to treat depression and anxiety disorders (1). Research has demonstrated its toxic effects on the differentiation of embryonic stem cells (2). Moreover, fluoxetine (FLX) is known to traverse the human placental barrier (3) and is excreted in breast milk (4), inhibiting the activity of multidrug transporters like milk phosphoglycoprotein (5). Studies have also suggested that FLX increases cell proliferation and cancer risk (6). Chronic administration of FLX in male rats has been found to inhibit sexual behavior, leading to long-term delayed ejaculation (7).

Grape seed extract (GSE), is a natural extract of Vitis vinifera seeds, exhibits antioxidant activity and the ability to inhibit free radicals. The GSE contains a potent group of plant flavonoids, proanthocyanidin oligomers, known for their strong antioxidant properties and various health benefits (8).

2. Objectives

Recent research has highlighted GSE's role in inhibiting oxidative damage by modulating the expression of antioxidant enzyme systems (9). The GSE has also shown promise in ameliorating hepatic

ischemia-reperfusion injury and reducing infarct size in cardiac ischemia in mice (10). Its extract contributes to improving oxidative stress both in vitro and in vivo (11). Previous studies have revealed that GSE protects liver damage caused by cisplatin in rabbits (12). This study aimed to evaluate the protective effects of GSE on in vitro fertilization and embryo development in adult mice treated with FLX.

3. Methods

3.1. Preparation of Grape Seed Extract

The grapes were sourced from the areas surrounding Urmia, a city in northwestern Iran. Following the collection, the grapes were washed, dried, and ground, and the nuclei were isolated. To the resulting 75 grams of grape powder, 200 mL of 70% ethanol (consisting of 70% ethanol and 30% distilled water) was added. The mixture was centrifuged at 3000 rpm for 20 minutes, and the supernatant was filtered. The solvent was evaporated using a rotary evaporator, and the resulting dried extract was collected.

3.2. Animal Model

In this study 32 adult male Balb/c mice (6 - 8 WK) weighing 30 ± 2 g were obtained from the animal house of Uremia Veterinary Faculty. They were housed under standard conditions, including a temperature of 25 ± 2°C, and a 12 h light/12 h dark cycle. Animals had access to standard diet and water. Mice were randomly divided into 4 groups: Control (received distilled water), FLX (received 40 mg/kg B.W Fluoxetine), GSE (received 150 mg/kg B.W GSE), FLX + GSE (received 40 mg/kg B.W Fluoxetine and 150 mg/kg B.W GSE). All mice were administered orally via gavage needle. Treatment continued for 42 days.

3.3. Sperm Collection

After completing the treatment period, each mouse was anesthetized using 20 mg/kg of xylazine and 50 mg/kg of ketamine. Euthanasia was performed by cervical dislocation. Sperm were harvested from the caudal epididymis at 20x magnification using a stereo zoom microscope. The caudal epididymides were carefully removed from each mouse and finely minced in 1 mL of pre-warmed (37°C) human tubal fluid (HTF) medium. After incubating the mixture for 60 minutes at 37°C in a 5% CO_2 environment, the sperm were separated from the epididymis tissue.

3.4. Aniline Blue Staining

A sperm smear was prepared, and after drying, it was fixed using a 3% glutaraldehyde solution for 30 minutes. The smears were stained with 5% aniline blue (AB) solution (dissolved in 4% acetic acid) for 5 to 8 minutes. The proportion of mature sperm (with colorless heads) and immature sperm (with blue heads) was examined under a light microscope. For each mouse, five slides were prepared, and 100 sperm per slide were counted using the light microscope.

3.5. Acridine Orange Staining

To evaluate sperm DNA integrity, smears were made from each sample on glass slides and allowed to air dry. The smears were subsequently fixed in Carnoy's solution (75 mL methanol and 25 mL acid acetic). Once dried, the slides were immersed in a tampon solution (80 mmol/L citric acid and 15 mmol/L Na_2HPO_4, pH 2.5) and incubated at 75°C for 5 minutes.

The slides were then stained with acridine orange (AO) (0.19 mg/mL) and rinsed with water to eliminate any excess stain. The stained slides were covered with coverslips and examined with a fluorescence microscope. Sperm with normal DNA show green fluorescence and sperm with abnormal DNA show fluorescence from yellow-green to red. Five slides were prepared for each mouse, and 100 sperm per slide were counted under the fluorescence microscope.

3.6. Collection of Ovulated Oocytes and In Vitro Fertilization

Ovulation was induced in 6 - 7-week-old female mice through an intraperitoneal injection of 10 IU of Pregnant Mare Serum Gonadotropin (PMSG, Sigma G4877). In female mice aged 6 - 7 weeks, ovulation was induced by an injection of 10 IU of PMSG (Pregnant Mare Serum Gonadotropin). After 48 hours, the mice received an injection of 10 IU of human chorionic gonadotropin (HCG). About 12 - 14 hours after the HCG injection, the mice were anesthetized using xylazine and ketamine, and subsequently, euthanasia was carried out by cervical dislocation in all the animals.

The oviducts were carefully removed and the ampoule part of the oviducts was removed and placed in a plastic container containing HTF with 4 mg/mL

bovine serum albumin (BSA). After dissection, the oocytes were extracted from the ampulla of the oviduct and transferred to HTF medium containing 4 mg/mL BSA. Fertilized sperm micro drops were prepared in HTF medium with 4 mg/mL BSA, and 8 - 12 oocytes were placed in each micro drop. The culture medium was incubated for 4 - 6 hours to allow fertilization. Subsequently, fertilized oocytes were transferred to fresh drops of the same medium. All drops, including the fertilized oocytes, were covered with mineral oil. Fertilization was confirmed by observing the pronuclei under an inverted microscope at 200x magnification. After fertilization, the fertilized zygotes were washed three times and transferred to fresh culture medium. They were then incubated for 5 days. The development rate of the embryos was assessed 24 hours post-fertilization under laboratory conditions and monitored daily until day 5 using a phase-contrast microscope.

3.7. Statistical Analysis

The data obtained from the study were analyzed using one-way ANOVA, followed by Bonferroni post hoc tests, with SPSS software (version 19). Results are presented as mean ± SE, and a P-value of less than 0.05 was considered statistically significant.

4. Results

4.1. DNA Damage of Sperm

In the FLX-treated group, AO staining revealed a significantly higher percentage of sperm with DNA damage compared to the control and GSE groups after 42 days($P = 0.03$). In contrast, the FLX + GSE group showed a important reduction in sperm DNA damage compared to the FLX-only group ($P = 0.04$), (Table 1 and Figure 1).

4.2. Immature Sperm

In the FLX-treated group, AB staining revealed a significant increase in the percentage of immature sperm compared to the control and GSE groups after 42 days ($P = 0.02$). Additionally, there was an important reduction in the percentage of immature sperm in the FLX + GSE group when compared with the FLX-only group ($P = 0.04$), (Table 1).

4.3. Early Embryo Assay

In the FLX-treated group, in vitro fertilization results revealed a significant reduction in the zygote percentage compared to the control and GSE groups ($P = 0.00$). Conversely, the FLX + GSE group demonstrated a significant increase in the average zygote percentage at day 42 compared to the FLX group alone ($P = 0.04$), (Table 1 and Figure 2).

The comparison of the average percentage of two-cell embryos across different groups revealed no significant differences after 42 days ($P = 0.25$), (Table 1 and Figure 2).

In the FLX-treated group, the percentage of embryos reaching the blastocyst stage was significantly lower than in both the control and GSE groups ($P = 0.02$). Moreover, the FLX + GSE group showed a significant increase in the percentage of blastocyst-stage embryos compared to just the FLX group. ($P = 0.01$), (Table 1 and Figure 2).

After 42 days, the FLX-treated group exhibited a significant reduction in the percentage of hatched embryos compared to both the control group and the GSE group ($P = 0.02$), (Table 1 and Figure 2).

5. Discussion

This study found that FLX significantly increased the percentage of immature sperms and sperms with damaged DNA compared to the control and GSE treated groups. A reduction in immature sperms and sperms with damaged DNA was observed in the (FLX + GSE) group compared with the FLX group alone. Many researchers believe that disruptions in DNA packaging can cause damage to the two strands of sperm DNA, potentially leading to sperm apoptosis (13). Physiological changes and transition may mitigate the detrimental effects of oxidative stress (14). Some of these physiological processes include the protamination of sperm. Protamines also appear to play a role in silencing sperm DNA and reprogramming the imprinting patterns of sperm cells (15). Therefore, it can be inferred that the use of FLX may damage the packaging of sperm DNA. The potent antioxidant properties of GSE could potentially mitigate the effects of FLX.

The results of this study revealed a distinguished decrease in the percentages of zygote cells, blastocysts, and hatched embryos in the FLX group compared to the control. It is postulated that FLX prompts the generation

Table 1. Average Percentage of Sperm DNA Damage, Immature Sperm, Fertilized Oocyte, Percentage of Two-Cell, Blastocysts and Hatched Embryos [a]

Groups	Sperms DNA Damage (%)	Immature Sperm (%)	Zygote (%)	2-Cell Embryos (%)	Blastocysts (%)	Hatched Embryos (%)
Control	4.46 ± 0.57	7.50 ± 0.26	90.3 ± 2.9	89.90 ± 0.55	88.79 ± 0.62	79.50 ± 0.53
GSE	4.54 ± 0.44	8.00 ± 0.25	89.80 ± 0.8	90.50 ± 0.54	89.1 ± 0.63	79.90 ± 0.73
FLX	5.30 ± 0.80 [b]	8.60 ± 0.22 [b]	79.77 ± 0.6 [b]	88.4 ± 1.49	86.2 ± 0.31 [b]	76.30 ± 0.76 [b]
FLX + GSE	4.50 ± 0.71 [c]	7.61 ± 0.22 [c]	83.78 ± 1.11 [c]	89.50 ± 7.1	89.27 ± 0.82 [c]	79.30 ± 0.71 [b]
P-value	0.045	0.021	0.47	0.251	0.016	0.037

Abbreviation: FLX, fluoxetine; GSE, grape seed extract.

[a] Values are expressed as mean ± SE.

[b] Significant differences were observed compared to the control.

[c] Significant differences were observed compared to the FLX group.

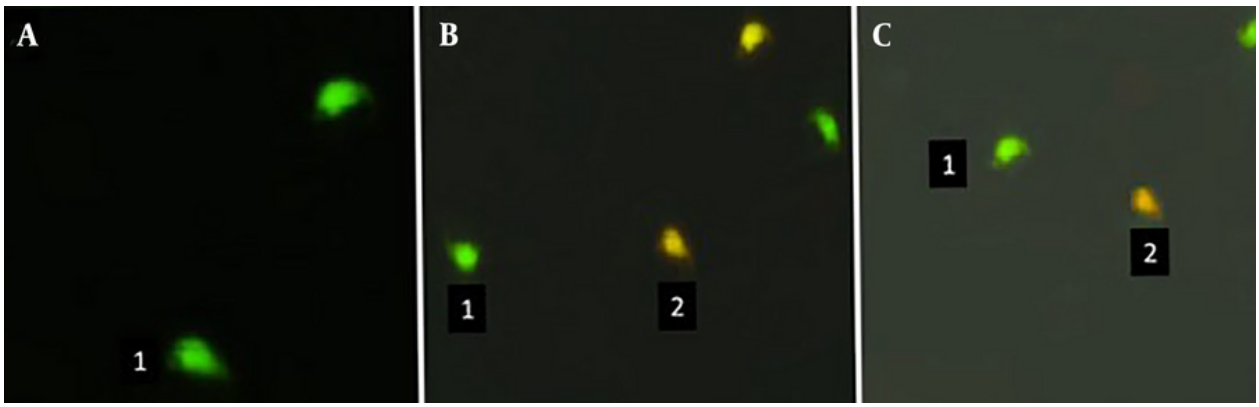

Figure 1. A, Control; B, fluoxetine (FLX); and C, FLX + GSX: (1) Sperm with normal DNA (green nucleus), (2) sperm with DNA damaged (orange nucleus) (acridine orange, ×1000)

of reactive oxygen species (ROS), thereby inducing damage to the sperm membrane through lipid peroxidation.

This damage impedes fatty acid double bonds from participating in membrane penetration reactions, thus preventing damaged sperm from effectively interacting with ovules (16), consequently leading to diminished sperm binding to the ovules, reduced fertilization rates, and fewer zygote cells (17). These findings are supported by a previous study which demonstrated that FLX exacerbates toxicity in embryonic stem cells and inhibits the growth of embryonic mesoderm (2). Similarly, Sanchez et al. reported that antidepressants such as FLX during pregnancy cause craniofacial defects due to serotonin-mediated deregulation of stem/progenitor cells (18). Grapes are renowned for their significant anti-cancer and antioxidant properties (19). Due to the presence of potent antioxidant agents such as phenolic compounds and proanthocyanidins, GSE is considered a bioactive substance that modulates physiological and cellular activities, potentially offering health benefits (20). The current study demonstrated that co-administration of FLX with GSE markedly increased the percentage of zygote cells, blastocysts, and hatched embryos compared to FLX treatment alone. The antioxidants present in GSE likely counteract the free radicals generated by FLX, thus alleviating oxidative stress in testicular tissue. This reduction in oxidative stress decreases the toxicity of FLX on spermatogenic cells and promotes sperm production. Consequently, this enhances sperm's fertilization capacity with ovules, ultimately improving fertility in mice. Consistent with the present findings, Hajizadeh et al. reported that black

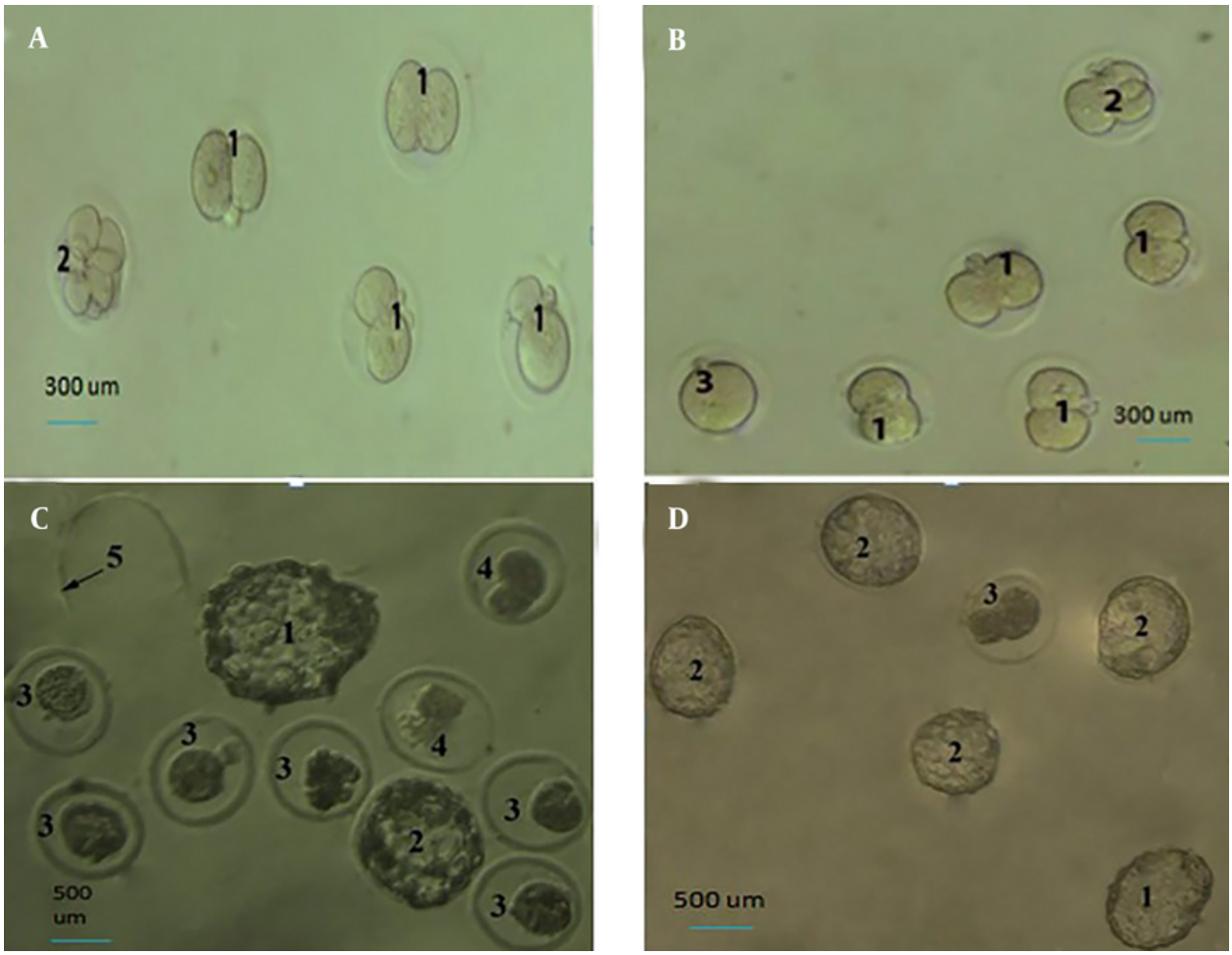

Figure 2. A, Control; and B, GSX: (1) Two cell stage, (2) one embryo with three cell, (3) Unfertilized oocytes; C, fluoxetine (FLX); and D, FLX + GSX: (1) Hatched embryo, (2) blastocyst stage, (3) unfertilized oocytes, (4) arrested embryo, (5) zonapellucida

GSE mitigates FLX-induced oxidative stress in the mice testis (21).

The use of FLX alone causes disturbances in sperm parameters and decreases fertility in mice. However, the use of GSE along with FLX improves sperm parameters and fertility.

Acknowledgements

The authors would like to thank the respected laboratory expert of the Faculty of Veterinary Medicine, Uremia University.

Footnotes

Authors' Contribution: Gh. N.: Conceptualization, methodology, software; Gh. E.: Data curation, writing-original draft preparation, and supervision; Gh. E.: Visualization, investigation; Gh. E: Software, validation; Gh. N., Gh. E.: Writing-reviewing and editing. All authors read and approved the final manuscript.

Conflict of Interests Statement: The authors declare that there is no conflict of interests.

Data Availability: Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Ethical Approval: This research was confirmed by the Ethics Committee of Department of Basic Sciences, Faculty of Veterinary Medicine, Uremia University, Iran (IR-UU-AEC.3/48-17.09.2024).

Funding/Support: The authors declared that no funding was received for this study.

References

1. Hoertel N. Do the Selective Serotonin Reuptake Inhibitor Antidepressants Fluoxetine and Fluvoxamine Reduce Mortality Among Patients With COVID-19? *JAMA Netw Open*. 2021;**4**(11). e2136510. [PubMed ID: 34779851]. https://doi.org/10.1001/jamanetworkopen.2021.36510.
2. de Leeuw VC, Hessel EVS, Pennings JLA, Hodemaekers HM, Wackers PFK, van Oostrom CTM, et al. Differential effects of fluoxetine and venlafaxine in the neural embryonic stem cell test (ESTn) revealed by a cell lineage map. *Neurotoxicology*. 2020;**76**:1-9. [PubMed ID: 31593710]. https://doi.org/10.1016/j.neuro.2019.09.014.
3. Arumugasaamy N, Gudelsky A, Hurley-Novatny A, Kim PCW, Fisher JP. Model Placental Barrier Phenotypic Response to Fluoxetine and Sertraline: A Comparative Study. *Adv Healthc Mater*. 2019;**8**(18). e1900476. [PubMed ID: 31407872]. [PubMed Central ID: PMC6752965]. https://doi.org/10.1002/adhm.201900476.
4. Schoretsanitis G, Augustin M, Sassmannshausen H, Franz C, Grunder G, Paulzen M. Antidepressants in breast milk; comparative analysis of excretion ratios. *Arch Womens Ment Health*. 2019;**22**(3):383-90. [PubMed ID: 30116895]. https://doi.org/10.1007/s00737-018-0905-3.
5. Heikkinen T, Ekblad U, Palo P, Laine K. Pharmacokinetics of fluoxetine and norfluoxetine in pregnancy and lactation. *Clin Pharmacol Ther*. 2003;**73**(4):330-7. [PubMed ID: 12709723]. https://doi.org/10.1016/s0009-9236(02)17634-x.
6. Lei B, Xu L, Zhang X, Peng W, Tang Q, Feng C. The proliferation effects of fluoxetine and amitriptyline on human breast cancer cells and the underlying molecular mechanisms. *Environ Toxicol Pharmacol*. 2021;**83**:103586. [PubMed ID: 33460806]. https://doi.org/10.1016/j.etap.2021.103586.
7. Cantor JM, Binik YM, Pfaus JG. Chronic fluoxetine inhibits sexual behavior in the male rat: reversal with oxytocin. *Psychopharmacology (Berl)*. 1999;**144**(4):355-62. [PubMed ID: 10435408]. https://doi.org/10.1007/s002130051018.
8. Habib HM, El-Fakharany EM, Kheadr E, Ibrahim WH. Grape seed proanthocyanidin extract inhibits DNA and protein damage and labile iron, enzyme, and cancer cell activities. *Sci Rep*. 2022;**12**(1):12393. [PubMed ID: 35859159]. [PubMed Central ID: PMC9300616]. https://doi.org/10.1038/s41598-022-16608-2.
9. Salmabadi Z, Mohseni Kouchesfahani H, Parivar K, Karimzadeh L. Effect of grape seed extract on lipid profile and expression of interleukin-6 in polycystic ovarian syndrome wistar rat model. *Int J Fertility Sterility*. 2017;**11**(3):176.
10. Ruan Y, Jin Q, Zeng J, Ren F, Xie Z, Ji K, et al. Grape Seed Proanthocyanidin Extract Ameliorates Cardiac Remodelling After Myocardial Infarction Through PI3K/AKT Pathway in Mice. *Front Pharmacol*. 2020;**11**:585984. [PubMed ID: 33343353]. [PubMed Central ID: PMC7747856]. https://doi.org/10.3389/fphar.2020.585984.
11. Kadri S, El Ayed M, Limam F, Aouani E, Mokni M. Preventive and curative effects of grape seed powder on stroke using in vitro and in vivo models of cerebral ischemia/reperfusion. *Biomed Pharmacother*. 2020;**125**:109990. [PubMed ID: 32070874]. https://doi.org/10.1016/j.biopha.2020.109990.
12. Khatal AA, Saeid JA, Eljrieby LR, Arhoma AA, Mansur HS, Sati AI. Protective Effects of Grape Seed Extract (Vitis Vinifera) against Cisplatin-Induced Liver Damage in Rabbits. (Histological and Histopathological Study). *Int J Med Sci Dental Health*. 2023;**9**(12):15-36. https://doi.org/10.55640/ijmsdh-09-12-07.
13. Timermans A, Vazquez R, Otero F, Gosalvez J, Johnston S, Fernandez JL. DNA fragmentation of human spermatozoa: Simple assessment of single- and double-strand DNA breaks and their respective dynamic behavioral response. *Andrology*. 2020;**8**(5):1287-303. [PubMed ID: 32416007]. https://doi.org/10.1111/andr.12819.
14. Pisoschi AM, Pop A, Iordache F, Stanca L, Predoi G, Serban AI. Oxidative stress mitigation by antioxidants - An overview on their chemistry and influences on health status. *Eur J Med Chem*. 2021;**209**:112891. [PubMed ID: 33032084]. https://doi.org/10.1016/j.ejmech.2020.112891.
15. Le Blevec E, Muronova J, Ray PF, Arnoult C. Paternal epigenetics: Mammalian sperm provide much more than DNA at fertilization. *Mol Cell Endocrinol*. 2020;**518**:110964. [PubMed ID: 32738444]. https://doi.org/10.1016/j.mce.2020.110964.
16. Jelinek R. *Membranes: From Biological Functions to Therapeutic Applications*. Berlin, Boston: De Gruyter; 2018. https://doi.org/10.1515/9783110453690.
17. Tung CK, Suarez SS. Co-Adaptation of Physical Attributes of the Mammalian Female Reproductive Tract and Sperm to Facilitate Fertilization. *Cells*. 2021;**10**(6). [PubMed ID: 34073739]. [PubMed Central ID: PMC8225031]. https://doi.org/10.3390/cells10061297.
18. Sanchez N, Juarez-Balarezo J, Olhaberry M, Gonzalez-Oneto H, Muzard A, Mardonez MJ, et al. Depression and Antidepressants During Pregnancy: Craniofacial Defects Due to Stem/Progenitor Cell Deregulation Mediated by Serotonin. *Front Cell Dev Biol*. 2021;**9**:632766. [PubMed ID: 34476233]. [PubMed Central ID: PMC8406697]. https://doi.org/10.3389/fcell.2021.632766.
19. Quero J, Jimenez-Moreno N, Esparza I, Osada J, Cerrada E, Ancin-Azpilicueta C, et al. Grape Stem Extracts with Potential Anticancer and Antioxidant Properties. *Antioxidants (Basel)*. 2021;**10**(2). [PubMed ID: 33562442]. [PubMed Central ID: PMC7915920]. https://doi.org/10.3390/antiox10020243.
20. Unusan N. Proanthocyanidins in grape seeds: An updated review of their health benefits and potential uses in the food industry. *J Functional Foods*. 2020;**67**:103861. https://doi.org/10.1016/j.jff.2020.103861.
21. Hajizadeh Z, Soleimani Mehranjani M, Najafi G, Shariatzadeh SMA, Shalizar Jalali A. Black Grape Seed Extract Modulates Fluoxetine-Induced Oxidative Stress and Cytotoxicity in the Mouse Testis. *Jundishapur J Natural Pharmaceutical Products*. 2016;**11**(2). https://doi.org/10.17795/jjnpp-27512.