Protective effects of Imedeen on in vitro fertilization potential in cyclophosphamide treated male mice

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Abstract

The objective of the current study was to assess the protective effects of Imedeen (IME) in mice given cyclophosphamide (CP). 24 young, adult male mice were separated into 3 groups for this purpose. The second group received CP (12 mg/kg IP), while the control group merely received normal saline (0.2 mL per day, IP). For 35 days, the third group was given daily oral doses of 222 µg/kg IME in addition to CP. After that, the animals were put to sleep under anesthesia, and the sperm and epididymis were separated. Malondialdehyde (MDA) levels, DNA damage levels, and in vitro fertilty were all assessed. When compared to the control group, the number of sperms with damaged DNA and MDA levels in the CP-treated group increased significantly ($p < 0.05$). When compared to the CP group, the group receiving IME in addition to CP showed a decrease in DNA damage and MDA ($p <0.05$). Also, compared to the control group, the CP-treated group had significantly lower levels of in vitro fertilization and embryonic development, and there was a higher level of embryonic arrest ($p <0.05$). In vitro fertility and embryonic development were higher ($p <0.05$) and arrested embryos exhibited a decrease in the group that got IME in addition to CP. Imedeen may be able to lessen the negative consequences of CP, according to this study.

Keywords: Imedeen, sperm, mice, antioxidant, cp

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Introduction

Many couples struggle with infertility, which adds stress to the situation and creates psychological friction between spouses. [1] 15% of couple's battle with infertility, and men alone or men and

women are at fault 50% of the time, according to recent statistics. Around 10% of cases are primarily caused by sperm shortage in the semen. [2] There are numerous recognized and unrecognized reasons for infertility. Chemotherapy is one of the most common causes of infertility. [3] Cyclophosphamide (CP), an alkylating agent, was first made available as a chemotherapy in 1959. [4] Men treated with cyclophosphamide had azoospermia and infertility, according to earlier investigations. A variety of malignancies and autoimmune diseases are treated using CP. [5] In-depth research has been done to determine the negative effects of CP, and it has been proven to be hazardous to the heart, [6] reproductive organs, [7] liver, [8] immune system, [9] and urinary tract. [10] Moreover, the CP is well-known for its effects on teratogenicity during embryonic development [16, 17], genetic mutations [11], secondary malignancies, particularly bladder cancers [12], suppression of hematopoietic tissue [13], brain oxidative injuries [14], and diabetes [15]. Active CP metabolites include acrolein and phosphorylamide mustard. [18]

Acrolein appears to be the primary culprit in CP poisoning, according to earlier research. [19] Acrolein causes the production of oxygen free radicals, which cause cell mutations in mammals. [20] Male rats treated with atrazine had lower fertility due to reactive oxygen species (ROS), which also lower sperm quality. [21] According to studies, antioxidants have the ability to mitigate the harmful effects that hydrogen peroxide has on developing human embryos. By reducing sperm ROS generation, earlier research has demonstrated the benefit of antioxidants in improving the impairment of embryonic development. [22, 23, 24, 25] Imedeen is a drug that is used as a new method and an antioxidant for skin care. As an antioxidant, this drug prevents skin aging, which has this effect by strengthening different layers of the skin, and plays a significant role in the synthesis of collagen and elastin. This pill has been used in European countries since 1995 [26].

The composition of the drug includes Biomarine marine complex, which is one of its main ingredients and is rich in proteins and polysaccharides similar to the structure of the skin, which is necessary to maintain its natural structure. This substance contains large amounts of amino acids and peptides that are directly involved in the synthesis of collagen and elastin. Another effective ingredient in this pill is lycophenes G-S, which has two types of very strong antioxidants and is obtained from tomato extract and grape seed extract, which has a hundred times stronger antioxidant properties than vitamin E. and plays an important role in defending lipophilic environments such as cell membranes. On the other hand, grape seed extract is 50 times stronger than vitamin C. This substance also has antioxidant properties in hydrophilic environments such as cell plasma and accumulates in environments that are rich in protein and polysaccharides [27]. According to these findings, the present study was designed to determine whether the administration of Imedeen as a strong antioxidant can prevent the occurrence of reproductive toxicity and oxidative stress caused by the administration of cyclophosphamide in male mice.

Materials and Methods:

Animals and treatment groups: 24 healthy, young male mice (8 to 12 weeks old; NMRI strain) were employed in this investigation, while female mice were used to gauge the fertility of the male mice. The animals were kept in the first stage under typical conditions for one week, which included a temperature of 22 ± 2°C, a humidity of 30–60%, and circadian lighting cycles of 14°C light and 10°C darkness. Food and drink were freely available. Male mice were weighted and then divided into three groups of eight mice each after one week of consistent environmental conditions and random division.

Control group: The thought-to-be-healthy animals were given saline solution (0.2 mL per day, intraperitoneally).

CP group: Animals received 12mg kg-1 per week of CP (Baxter Oncology GmbH,Halle,Germany),intraperitoneally.[28]

Experimental group (CP + IME]: Animals were treated with IME (222 µg/kg/day, orally) along with CP.

The study period was 35 days for all groups. Sperm were then removed from the epididymis after the animals had been placed to sleep under anesthesia using 25 mg kg-1 of ketamine (Alfasan, Utrecht, and The Netherlands).

DNA strand damage level evaluation: The double strands of DNA in sperm were examined using the acridine orange staining method. 200 spermatozoa per instance were counted in order to ascertain the percentage of stained spermatozoa. Cridine orange aggregates on single-stranded DNA create a yellow to red fluorescence, while the monomeric form of acridine orange coupled to regular double-stranded DNA exhibits a green fluorescence. PBS buffer was used to set the final precipitate volume following the three times that the sperm sample was eluted. After air-drying in the lab for 30 minutes, sperm smears were created from culture medium and transferred to a container with the identical ratios of acetone and ethanol. The slides were then air dried before spending seven minutes immersed in an acridine orange solution. A 100 × fluorescence microscope (Model IX70; Olympus, Tokyo, Japan) was used to analyze the slides after that, and the results were expressed as a percentage. [29, 30]

Timing for injection of gonadotropins for *in vitro* fertilization (IVF): Within 35 days, mice were ready for IVF. After establishing for female mice the light/dark cycles necessary for sexual cycles, which span at least two weeks, they were prepared to stimulate ovulation. This was done by intraperitoneally administering 10 units of PMSG hormone and 10 units of HCG hormone (Folligon; Intervet International BV, Boxmeer, The Netherlands) in 0.1 mL volumes each over a 48-hour period.

Preparation of IVF culture medium: The culture media required for fertilization were ready the day before and were incubated at 37°C with 5% CO2 for 12 hours to achieve stability. Human tubal fluid (HTF; Sigma, St. Louis, USA) medium was added before fertilization dishes were dropped. During fertilization, a 500-liter droplet was deposited in each plate, and many 100 µL droplets were scattered across the dishes for elution. Mineral oil was applied to all drops.

Collecting of oocytes and IVF: The female mice were put to death 13 hours (the following morning) after receiving the HCG injection. After a laparotomy, shaving, and sterilization of the abdominal region, the fallopian tubes were separated and put in a culture medium that was kept at a constant 37 °C. After washing, the oocytes were put into droplets of mineral oil in HTF-BSA medium after being extracted using the "dissecting" procedure. Thereafter, motivated and capable sperm were introduced to the growth medium at a concentration of 1 × 106 total sperm per mL. Three to five hours later, the fertilization process was discovered after injecting sperm and seeing two pronuclei. As a result, zygotes from fertilized oocytes were developed for 120 hours in 100-ml droplets of mineral oil. 23 The number of two-cell embryos was counted about 24 hours after the zygotes were cultivated, and 120 hours later, inversion microscopy was used to evaluate the development of blastocysts and arrested embryos (Model IX70; Olympus, Tokyo, Japan)..

The types of arrested embryos according to their fragmentation and necrosis [31] were as follows:

Type I: Fully cellular lysing, necrotic and/or fragmented embryos.

Type II: Embryos with partially fragmented blastomeres.

Type III: Embryos with some fragmented blastomeres and/or cytoplasmic vesicles.

Malondialdehyde (MDA) assaying: To determine the rate of lipid peroxidation, the MDA content of the collected testis samples was assessed using the previously described thiobarbituric acid (TBA) technique. In a nutshell, 0.3 to 0.4 g of the testis samples were homogenized in 150 mM KCL, which was ice-cold, before the mixture was centrifuged at 3000 g for 10 min. The samples were then treated with 2 mL of 6.7 g L-1 TBA after being vortexed with 0.5 mL of the supernatant and 3 mL of phosphoric acid (1% v/v). The samples were heated at 100 °C for 45 minutes before being cooled on ice. Finally, 3 mL of N-butanol was added, and the samples underwent a second, 10-minute centrifugation at 3000 g. A spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, UK) was used to detect the supernatant's absorbance at 532 nm, and calibration curves created using MDA standards were used to simultaneously estimate the supernatant's MDA content. The amount of MDA in the samples was calculated at nmol per mg protein. [32] The protein content of the sample was measured using the Lowry method. [33]

Statistical analyses: One-way ANOVA and the Bonferroni test were used to analyze the data in SPSS (Version 20; SPSS Inc., Chicago, USA), while Minitab was used to evaluate the data from

IVF (Version 16; Minitab, State College, USA). A p-value of 0.05 or less was regarded as significant.

Results:

Malondialdehyde changes in testicular tissue: According to the results of the MDA assay, the CP group's average MDA levels (37.02 ± 2.12) increased considerably in contrast to the other groups ($p < 0.05$). However, there was no discernible difference between the control group and the CP + IME group (table. 1).

DNA damage assessment: According to damage level, sperms with orange-to-red nuclei were regarded as having DNA damage, while sperms with green nuclei were considered to be normal. This method was used to calculate the average percentage of sperm with damaged DNA. In the CP group, the average proportion of sperms with DNA damage (37.5 ± 3.44) increased significantly more than in the other groups ($p < 0.05$). However, there was no discernible difference between the control group and group CP + IME in terms of the average percentage of sperms with damaged DNA (Fig. 1 and Table 1).

In vitro fertilization and embryonic development: The percentage of fertilization was found to be substantially lower in the CP group (75.86%) than in the control group (91.8), according to the most recent research ($p < 0.05$). In the CP + IME group, however, the difference was not statistically significant. When compared to the control group (83.93), the proportion of two cell embryos that were produced in the CP group (74.55) exhibited a significant decline ($p < 0.05$). Group CP + IME, however, did not demonstrate a significant difference. (Fig 2)

In comparison to the control group [67.27], the overall percentage of blastocysts in the CP group (18.18) indicated a significant decline ($p < 0.05$). However, there was no discernible difference between the CP+IME group and the control group. When compared to the control group, the total percentage (81.92) of arrested embryos in various developmental stages prior to blastocyst has increased in the CP group (p 0.05). However, there was no discernible distinction between the CP+IME group and the control group. The number of arrested embryos was found to be significantly higher in the CP group compared to the control group ($p < 0.05$) based on observation of the embryos' developmental quality and consideration of fragmentation. When compared to the CP group, IME with CP was able to reduce the level of fragmentation of embryos, resulting in the majority of embryos being in the type III category ($p < 0.05$). (Table 2)

Discussion

The majority of chemotherapeutic drugs have harmful side effects on various bodily systems. [34, 35] Previous research has indicated that testicular toxicity is a significant CP adverse effect. [36, 37, 38] A growing focus is on defending the gonadal cells from chemotherapeutic substances. The preventive impact of IME on CP-induced derangements was looked at in the current study. When compared to the control group, the number of sperms with DNA damage and the MDA level in

the CP-treated group significantly increased ($p < 0.05$), whereas in the IME and CP group, DNA damage level and MDA amount significantly decreased ($p < 0.05$). In vitro fertilization and embryonic development were considerably lower in the CP-treated group than in the control group, and there was more embryonic arrest ($p < 0.05$). In vitro fertility and embryonic development were higher in the IME group than in the CP group ($p < 0.05$), and the number of arrested embryos decreased. According to this study, Imedeen may be able to lessen the negative consequences of CP. Reactive oxygen species (ROS) and the antioxidant reserve system become unbalanced, which leads to oxidative stress. The regular metabolic processes of cells result in the production of ROS. Free radicals of oxygen are also produced by sperm, which is the outcome of testicular function. Low levels of generation of reactive oxygen radicals are associated with sperm capacitation, the acrosome reaction, and sperm attachment to the zona pellucida. [39] On the other hand, high levels of ROS cause sperm abnormalities and infertility. The polyunsaturated fatty acids in the sperm membrane are abundant. Due to the rise in ROS, this causes membrane lipid peroxidation. [40] Hence, a key contributing factor to decreased sperm function is peroxidative damage. [41]

Because CP increased the MDA level in the current investigation, lipid peroxidation caused by CP is evident. In line with earlier findings, this conclusion was made. [36,37] Sperm need to be protected from ROS by the antioxidative defense mechanisms in the testis. Resveratrol, an antioxidant, is said to reduce lipid peroxidation caused by CP, according to earlier studies. [37,38] The fact that the levels of MDA were considerably reduced in groups receiving CP+IME ($p < 0.05$) in accordance with this confirms the findings of the current investigation. As free radicals rise, oxidative damage, lipid peroxidation, and DNA and protein damage all follow. [38] The pathophysiology of CP-induced testicular damage has been demonstrated to include oxidative stress significantly. [37] The results of staining with acridine orange to support previous researchers' findings suggested that CP damages DNA and elevates oxidative stress. As a result, compared to groups receiving CP+IME, DNA damage has dramatically increased in groups treated with CP. ($p < 0.05$).

Also, in the current study, mice treated with CP had lower levels of fertilization and embryonic development than the control group and other groups receiving CP+IME. According to research, in vivo and in vitro embryonic development and sperm viability and quality are positively correlated. [38,42.43,44] Further proving the strong correlation between sperm quality and successful IVF results, administration of IME along with CP dramatically increased the rate of fertilized oocytes, two-cell zygotes, and blastocysts while significantly reducing the percentage of arrested embryos. There are some indications that CP damages the DNA, membrane, and mitochondria of sperm as well as causing azoospermia, decreased sperm count, abnormal sperm morphology, and oxidative stress. [45, 46] All of these situations have an impact on sperm quality. This led to a reduction in fertilization and embryo viability in the CP-treated group. Another study supported the antioxidant function of IME and found that it improved sperm implantation and

pregnancy rates while reducing sperm damage and apoptosis. [47] Another study on the benefits of antioxidants has revealed that they reduce sperm damage and apoptosis, which benefits DNA, implantation, and pregnancy rates. [48] In line with the positive effects of IME on sperm parameters and their connection to successful fertilization, the percentages of fertilized oocytes and blastocysts decreased and the number of blocked embryos increased in the CP+IME group.

In conclusion, our investigation demonstrated that the predominant adverse effect of CP that caused testicular injury and decreased sperm quality was oxidative stress. However, the treatment of both IME and CP together reduced oxidative stress, sustained spermatogenesis, and increased in vitro fertilization rates while reducing embryonic arrest and fragmentation rates. The results of this study suggest that these protective benefits may be brought about by IME's antioxidant characteristics.

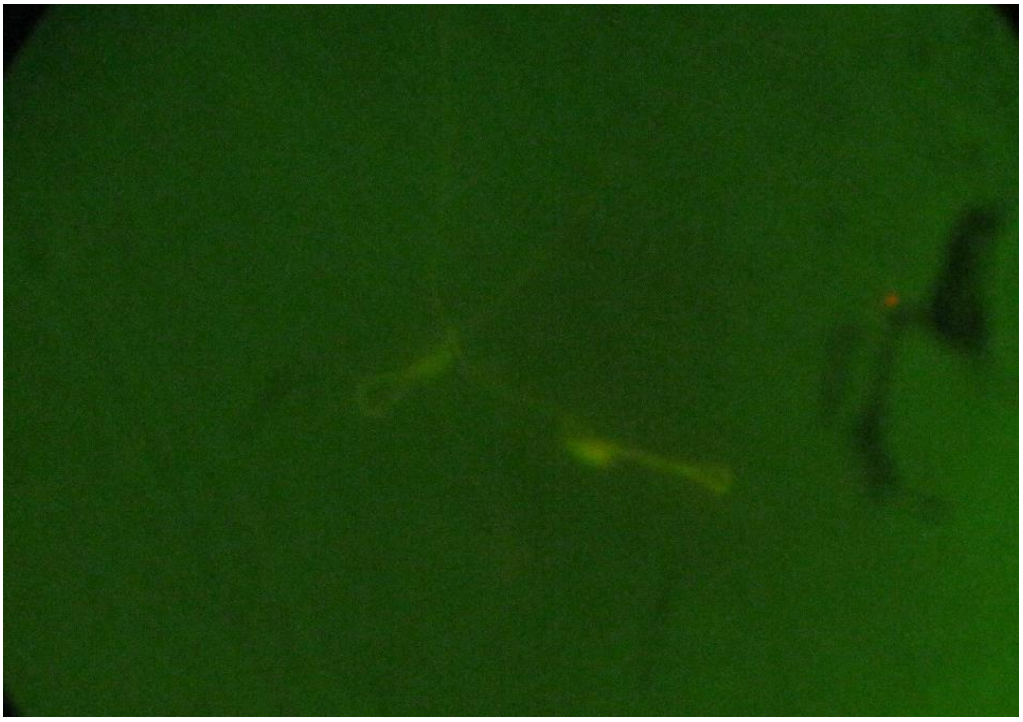

Fig. 1 Micrograph of sperm .Green colored in head of sperm indicate DNA intact. Yellow colored indicate DNA damage in sperms. (Acridine orange staining , 1000×).

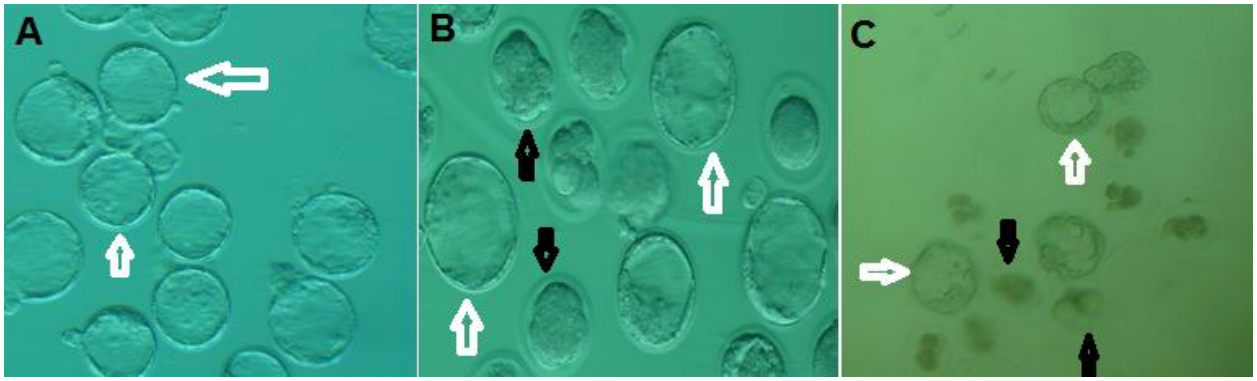

Fig. 2. Photomicrograph from pre-implantation embryos. A) Control group: Normal embryos that some of them are at the blastocyst stage (white arrows)

B) CP group: Most of the embryos are in arrested and lysed conditions (black arrows).

C) CP+IME group: A low number of embryos is arrested and lysed.

Table.1Effects of CP and IME on the sperm DNA integrity and MDA

Groups	**Positive Acridine Orange staining (%)**	MDA nmol/mg
Control	8.75 ± 2.01a	11.5± 2.46a
CP	37.5 ± 3.44b	37.02 ± 2.12b
CP+IME	9.37± 0.31a	18.72± 1.23c

abc Different letters indicate the significant difference between control and the experimental groups, respectively

Table 2: In vitro fertilization parameters in different experimental groups .

Groups	Oocyte count	Fertilized oocytes (%)	Two-cell embryos (%)	Blastocysts (%)	Arrested embryos (%)	Type I arrested embryos (%)	Type II arrested embryos (%)	Type III arrested embryos (%)
Control	183	91.8%	83.93%	67.27%	32.73%	2.4%	4.16%	26.17%
CP	145	75.86% a	74.55% a	18.18%a	81.92%a	10.91% a	14.55% a	56.37% a
CP+IME	152	87.50% b	80.45	37.59%ab	62.40%ab	0.00b	0.00ab	62.4%a

ab Different letters indicate the significant difference between control and the experimental groups, respectively

REFERENCES

1. Freeman EW, Boxer A, Rickels K, et al. Psychological evaluation and support in a program of *in vitro* fertilization and embryo transfer. Fertil Steril 1985; 43(1):48-53.
2. Matzuk MM, Lamb DJ.Genetic dissection of mammalian fertility pathways.Nat Cell Biol 2002; 4: 41- 49.
3. Peters G, Van der Wilt C, Van Moorsel C, et al. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther 2000; 87(2):227-253.
4. Richard L, Schilsky MD, Brian J, et al. Gonadal dysfunction in patients receiving chemotherapy for cancer. Ann Intern Med 1980; 93(Part 1): 109-114.
5. Selvakumar E, Prahalathan C, Mythili Y, et al. Protective
1. effect of DL-alpha-lipoic acid in cyclophosphamide induced oxidative injury in rat testis. Reprod Toxicol 2004; 19(2): 163-167.
6. Santos GW, Sensenbrenner LL, Burke PJ, et al. Marrow transplantation in man following cyclophosphamide. Transplant Proc 1971; 3(1): 400-404.
7. Hosseini A, Ghaderi Pakdel F, Ahmadi A, et al. Beneficial effects of American ginseng on epididymal sperm analyses in cyclophosphamide treated rats. Cell J 2012; 14(2): 116-121.
8. DeLeve LD. Cellular target of cyclophosphamide toxicity in the murine liver: Role of glutathione and site of metabolic activation. Hepatology 1996; 24(4): 830 837.
9. Bin-Hafeez B, Ahmad I, Haque R, et al. Protective effect of *Cassia occidentalis* L. on cyclophosphamide-induced suppression of humoral immunity in mice. J Ethnopharmacol 2001; 75(1): 13-18.
10. Korkmaz A, Topal T, Oter S. Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; Implication of reactive oxygen and nitrogen species as well as PARP activation. Cell Biol Toxicol 2007; 23(5): 303-312.
11. Stahlmann R, Bluth U, Neubert D. Effects of the cyclophosphamide metabolite acrolein in mammalian limb bud cultures. Arch Toxicol 1985; 57(3): 163-167.
12. Levine LA, Richie JP.Urological complications of cyclophosphamide. J Urol 1989; 141(5): 1063-1069.
13. Baumann F, Preiss R. Cyclophosphamide, and related anticancer drugs. J Chromatogr B Biomed Sci Appl 2001; 764(1-2): 173-192.
14. Oboh G, Ogunruku OO. Cyclophosphamide-induced oxidative stress in brain: Protective effect of hot short pepper (*Capsicum frutescens* L. var. *abbreviatum*). Exp Toxicol Pathol 2010; 62(3): 227-233.

15. Faust A, Burkart V, Ulrich H, et al. Effect of lipoic acid on cyclophosphamide-induced diabetes and insulitis in non-obese diabetic mice. Int J Immunopharmacol 1994; 16(1): 61-66.
16. Gilani SH, Chatzinoff M. Embryopathic effects of cyclophosphamide. Environ Res 1983; 31(2): 296-301.
17. Mirkes PE. Cyclophosphamide teratogenesis: A review. Teratog Carcinog Mutagen 1985; 5(2): 75-88.
18. Chinnaswamy G, Errington J, Foot A, et al. Pharmacokinetics of cyclophosphamide and its metabolites in paediatric patients receiving high-dose myeloablative therapy. Eur J Cancer 2011; 47(10): 1556-1563.
19. Kern JC, Kehrer JP. Acrolein-induced cell death: A caspase-influenced decision between apoptosis and oncosis/necrosis. Chem Biol Interac 2002; 139(1): 79-95.
20. Arumugam N, Sivakumar V, Thanislass J, et al. Effects of acrolein on rat liver antioxidant defense system. Indian J Exp Biol 1997; 35(12): 1373-1374.
21. Rezaei Agdam H, Razi M, Amniattalab A, et al. Coadministration of vitamin E and testosterone attenuates the atrazine-induced toxic effects on sperm quality and testes in rats. Cell J 2017; 19(2): 292-305.
22. Hughes CM, Lewis S, McKelvey-Martin VJ, et al.The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Hum Reprod 1998; 13(5): 1240-1247.
23. Zarei L, Shahrooz R, Sadrkhanlou R, et al. Protective effects of *Cornus mas* extract on *in vitro* fertilization potential in methotrexate treated male mice. Vet Res Forum 2015; 6(1): 55-61.
24. Zhang X, Sharma RK, Agarwal A, et al. Effect of pentoxifylline in reducing oxidative stress-induced embryotoxicity. J Assist Reprod Genet. 2005; 22(11- 12): 415-417
25. Esfandiari N, Falcone T, Agarwal A, et al. Protein supplementation and the incidence of apoptosis and oxidative stress in mouse embryos. Obstet Gynecol 2005; 105(3): 653-660.
26. Kieffer M, Esfen J. Imedeen in the treatment of photoaged skin: an efficacy and safety trial over 12 months. J Eu Acad Dermatol Venereol 11. 1998; 129-136
27. Lassus A, et al. Imedeen for the treatment of degeneratedskin in females. JInt Med Res. 1991;19: 145-157.
28. Niakani A, Farrokhi F, Hasanzadeh S. Decapeptyl ameliorates cyclophosphamide-induced reproductive toxicity in male Balb/C mice: Histomorphometric, stereologic and hormonal evidences. Iran J Reprod Med 2013; 11(10): 791-800.
29. Hodjat M, Akhondi M, Amirjanati N, et al.The comparison of four different sperm chromatin assays and their correlation with semen parameters. Tehran Univ Med J 2008; 65(Suppl 3): 33-40.
30. Talebi AR, Sarcheshmeh AA, Khalili MA, et al. Effects of ethanol consumption on chromatin condensation and DNA integrity of epididymal spermatozoa in rat. Alcohol 2011; 45(4): 403-409.

31. Behringer R, Gertsenstein M, Nagy K. Manipulating the mouse embryo: A laboratory manual. New York, USA: Cold Spring Harbor Laboratory Press 2003: 127-155.
32. Niehaus W, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 1968; 6(1): 126-130.
33. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the folin (1-5) phenol reagent. J Biol Chem 1951; 193(1): 265-275.
34. Barrera G. Oxidative stress and lipid peroxidation products in cancer progression and therapy. ISRN Oncol 2012; 2012: 137289. doi: 10.5402/2012/137289.
35. Gonzalez EJ, Peterson A, Malley S, et al.The effects of Tempol on cyclophosphamide-induced oxidative stress in rat micturition reflexes. Sci World J 2015; 2015: 545048. doi: 10.1155/2015/545048.
36. Bakhtiary Z, Shahrooz R, Ahmadi A, et al. Ethyl pyruvate ameliorates the damage induced by cyclophosphamide on adult mice testes. Int J Fertil Steril 2016; 10(1):79-86.
37. Manda K, Bhatia AL. Prophylactic action of melatonin against cyclophosphamide-induced oxidative stress in mice. Cell Biol Toxicol 2003; 19(6): 367-372.
38. Atashfaraz E, Farokhi F, and Najafi G. Protective effect of ethyl pyruvate on epididymal sperm characteristics, oxidative stress and testosterone level in methotrexate treated mice. J Reprod Infertil 2013; 14(4): 190-196.
39. de Lamirande E, Jiang H, Zini A, et al. Reactive oxygen species and sperm physiology. Rev Reprod 1997; 2(1): 48-54.
40. Sikka SC. Relative impact of oxidative stress on male reproductive function. Curr Med Chem 2001; 8(7): 851-862.
41. Moustafa MH, Sharma RK, Thornton J, et al. Relation-ship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod 2004; 19(1): 129-138.
42. Bakhtiary Z, Shahrooz R, Ahmadi A, et al. Protective effects of ethyl pyruvate on sperm quality in cyclophosphamide treated mice. Iran J Reprod Med 2015; 13(5): 291-296.
43. Cadet J, Wagner JR. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harb Perspect Biol 2013; 5(2): a012559. doi: 10.1101/cshperspect.a012559.
44. Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 2005; 322(1): 33-41.
45. Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. Reproduction 2001; 122(4): 497-506.
46. Bakhtiary Z, Shahrooz R, Ahmadi A, et al. Evaluation of antioxidant effects of crocin on sperm quality in cyclophosphamide treated adult mice. Vet Res Forum 2014; 5(3): 213-218.
47. Sadeghi MR, Hodjat M, Lakpour N, Arefi S, Amirjannati N, Modarresi T, et al. Effects of sperm chromatin integrity on fertilization rate and embryo quality following intracytoplasmic sperm injection. Avicenna journal of medical biotechnology.2009; 1(3):173-4.

48. Hughes CM, Lewis S, McKelvey-Martin VJ, et al.The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Hum Reprod 1998; 13(5): 1240-1247.