AMLS Archives of Medical Laboratory Sciences

Original Article

The Study of the Protective Effect of Vitamin E and Retinoic Acid on Testicular Tissue in Mice Treated with Cyclophosphamide

Akbar Karimi[1], Vahideh Behmard[2], Shima Toghiani[1], Fatemeh Sadat Moravej[1*]

1. Department of Biology, School of Basic Sciences, Payame Noor University of Isfahan, Isfahan, Iran.
2. Department of Midwifery, School of Medicine, Gonabad University of Medical Sciences, Gonabad, Iran.

Received: August 24, 2020, Accepted: November 16, 2020

Abstract

Background and Aim: One of the side effects of anticancer drugs is spermatogenesis disorder. Hence, the evaluation of sperm production after chemotherapy has been the subject of several studies today.

Methods: Adult male albino mice weighing 20±2 g were randomly divided into 5 groups (n = 4);control, cyclophosphamide (12 mg/kg/day), cyclophosphamide and vitamin E (200 mg/kg/day), cyclophosphamide and retinoic acid (500 µL/72h), cyclophosphamide and retinoic acid (250 µL/72h) and vitamin E (100 mg/day). The treatment was continued for 35 days and at the end of the rats was anesthetized and testicular tissue was isolated, weighed, fixed, and stained with hematoxylin-eosin.

Results: A significant decrease (p-value ≤ 0.0001) in the mean diameter of seminal vesicles, epithelial thickness, and index of tubular differentiation was observed in the testis tissue of mice treated with cyclophosphamide compared to the control group. In cyclophosphamide recipients with retinoic acid or vitamin E, parameters significantly increased to the level of the control group (p-value ≤ 0.0001), whereas cyclophosphamide co-administration with retinoic acid and vitamin A had a less tissue-protective effect.

Conclusion: Vitamin E and retinoic acid can offset the adverse effects of cyclophosphamide on spermatogenesis and may be inducers of spermatogenesis, while the combination of these two antioxidants has fewer efficacies.

Keywords: Cyclophosphamide, Vitamin E, Retinoic acid, Spermatogenesis.

[*]**Corresponding Author:** Fatemeh Sadat Moravej; **Email:** akbar.karimi@es.isfpnu.ac.ir

Please cite this article as: Karimi A, Behmard V, Toghiani S, Moravej FS. The Study of the Protective Effect of Vitamin E and Retinoic Acid on Testicular Tissue in Mice Treated with Cyclophosphamide. Arch Med Lab Sci. 2020;6:1-8 (e9). https://doi.org/10.22037/amls.v6.31520

Introduction

Chemotherapy is a common way to treat various cancers to control and inhibit the proliferation of cancer cells and, albeit, has known side effects on Spermatogenesis. Since reproduction and fertility are essential human biological processes and infertility has always been one of the leading social and medical problems, attention to spermatogenesis recovery after chemotherapy courses is one of the critical and modern aspects of infertility studies (1). Among the side effects of chemotherapy drugs, oxidative stress damages spermatozoa and reduces fertility. Like other chemotherapeutic drugs, cyclophosphamide has a disruptive impact on other tissues in the body. It can upset the redox balance of testicular tissue and cause the accumulation of free radicals and oxidative stress factors in the testis. Therefore, applying antioxidants that prevent the formation of free radicals in cells and the occurrence of chain reactions of oxidation can reduce the tissue damage caused by its consumption (2). Cyclophosphamide is an anti-tumor and immunosuppressive compound that targets fast-growing cells. It is used in high doses to treat various cancers, including malignant lymphoma, multiple myeloma, and leukemia, and in low doses to treat a type of lupus. In hepatocyte cells, the drug is converted to an active metabolite by cytochrome p450 enzymes and returned to the bloodstream (3). Cyclophosphamide causes the accumulation of free radicals and oxidative stress in the testicles. Finally, the accumulation of these compounds leads to DNA fragmentation and loss of normal sperm function

and mitochondrial peroxidative damage, and membrane breakdown.

Cyclophosphamide also reduces the thickness of the epithelial layers of seminiferous tubules, and histological complications associated with this drug include the presence of irregular, fibrotic, and perivascular seminiferous tubules in the testis (4). In addition to investigating the effects of cyclophosphamide in this study, two potent antioxidant agents were used separately and together to support the spermatogenesis of small rats in the laboratory while receiving chemotherapy. The purpose of the experiments was to investigate the role of each factor in preventing damage caused by oxidative stress in testicular tissue and also to investigate the simultaneous administration of vitamin E and retinoic acid as a combined antioxidant treatment. Vitamin E is a well-known antioxidant, and a necessary factor in relieving oxidative stress, and retinoic acid is an imperative and useful factor in the development and regulation of male reproductive system activity. Vitamin E inhibits fat peroxidation in mitochondria and testicular microsomes and reduces the destructive effects of oxidative stress caused by stressors (5-7). Low doses of retinoic acid since 1987 (discovery of nuclear receptors) have been identified as required factors in stimulating growth, cartilage formation, and cell proliferation. Researchers have done extensive research on the regulation of intracellular retinoid concentrations and their role in the cell. This antioxidant is useful in many vital activities of the body, such as growth and immune system, and reproductive system, and prevents lipid peroxidation in the testicles. It improves Spermatogenesis and the structural differentiation of epididymal epithelial cells during embryonic development and testicular tissue development (8-10). Therefore, this study aims to find a logical answer for prescribing the therapeutic use of these vitamins separately and together during chemotherapy courses. The results will promote molecular studies and signaling pathways related to cell proliferation and preservation.

Methods

To investigate the effects of retinoic acid and vitamin E on cyclophosphamide-induced spermatogenesis disorder, a total of twenty adult male albino mice (4 to 6 weeks old; 25±2 g body weight) were housed at 23±2°C on a 12 h light/dark cycle). After one-week adaptation period, mice were categorized into 5 groups as follows:

1. Control group: no drug treatment;
2. Treatment group: cyclophosphamide;
3. Co-treatment group: cyclophosphamide (12 mg/kg/day) and vitamin E (200mg/kg/day);
4. Co-treatment group: cyclophosphamide (12 mg/kg/day) and retinoic acid (500 µl/72 h);
5. Co-treatment group: cyclophosphamide (12mg/kg/day) with retinoic acid (250 µl/72 h) and vitamin E (100 mg/kg/day).

It should be noted that all treatments were injected intraperitoneal

Cyclophosphamide powder was prepared by dissolving in saline at the mentioned concentration, and the volume of each injection was 0.3 ml intraperitoneally. Retinoic acid is also dissolved in a mixture of Corn oil and absolute ethanol and is administered as 0.3 ml intraperitoneally. Vitamin E was also administered intraperitoneally 0.1 ml in each injection.

Mice were humanely killed after the treatment was completed, and the testicular tissues were isolated and stabilized in a 10% formalin solution. Subsequently, the incisions were made by microtome and stained by hematoxylin and eosin (H&E).

Statistical Analysis

The microscopic examination results of tissues were analyzed by SPSS 22 statistical software. For calculating the tubular differentiation index, the percentage of sperm-forming tubes containing three or more rows of spermatogenic cells differentiated from spermatogonia was calculated. For assessing the RI index, the ratio of active spermatogonia cells to inactive spermatogonia cells in seminiferous tubules was calculated. For obtaining the spermiogenesis coefficient, the ratio of semen-producing tubes containing sperm to sperm fewer tubes was calculated. The comparison of the TDI

(Tubular differentiation Index) in different groups showed that in rats treated with methylphenidate, this index was decreased.

Results

Evaluated parameters in this study were as follows: measuring the small and large diameter of Seminiferous tubules in H&E testicular tissue laminae, measuring the thickness of seminiferous epithelium in central and peripheral tubes of H&E testicular tissue laminae, evaluating the density of seminiferous tubules per testicular tissue unit. The TDI index or tubular differentiation index and the qualitative study of the changes in the histopathological changes of the testicular tissue were performed by a pathologist and based on the results of statistical analyzes, a significant data coefficient (p-value≤0.0001) was recorded. The results are comparable in the following diagrams and illustrations. In the charts, the groups from A to E, respectively, include:

Control group, cyclophosphamide receiving group, cyclophosphamide and vitamin E receiving group, cyclophosphamide and retinoic acid receiving group, cyclophosphamide, and both antioxidants receiving group.

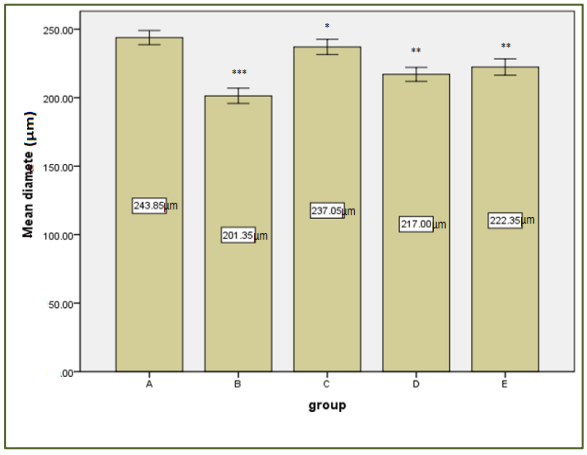


Figure 1. Comparison of the large diameter of testicular seminiferous tubules in terms of the micrometer in different groups whit control group.
*p-value=0.085; **p-value ≤0.0001; ***p-value ≤0.0001
Each bar represents mean ± SD
A: Control group;
B: Cyclophosphamide receiving group;
C: Cyclophosphamide and vitamin receiving group;
D: Cyclophosphamide and retinoic acid receiving group;
E: Cyclophosphamide, and both antioxidants receiving group.

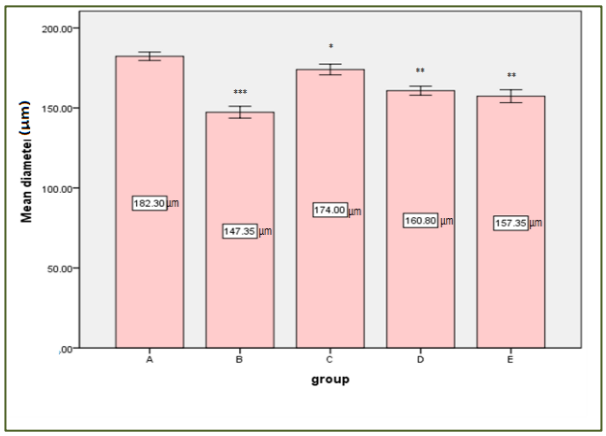


Figure 2. Comparison of the small diameter of testicular seminiferous tubules in terms of the micrometer in different groups with the control group
*p-value≤0.0001); **p-value≤0.0001); ***p-value≤0.0001
Each bar represents mean ± SD
A: Control group; B: Cyclophosphamide receiving group;
C: Cyclophosphamide and vitamin receiving group;
D: Cyclophosphamide and retinoic acid receiving group;
E: Cyclophosphamide, and both antioxidants receiving group;

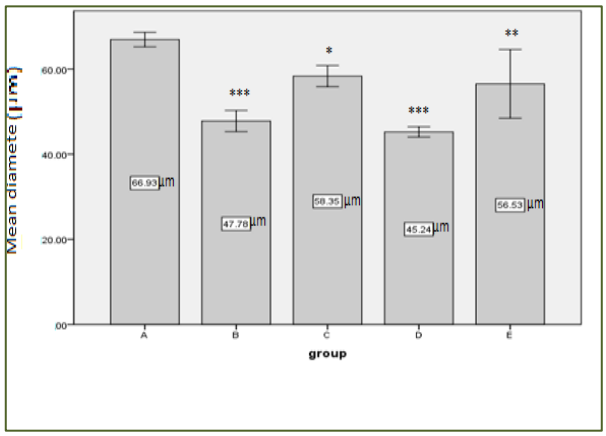


Figure 3. Comparison of the epithelial thickness of peripheral testicular seminiferous tubules in terms of micrometers in different groups whit control group whit control group
*p-value=0.003; **p-value≤0.0001; ***p-value≤0.0001
Each bar represents a mean ± SD
A: Control group;
B: Cyclophosphamide receiving group;
C: Cyclophosphamide and vitamin receiving group;
D: Cyclophosphamide and retinoic acid receiving group;
E: Cyclophosphamide, and both antioxidants receiving group.

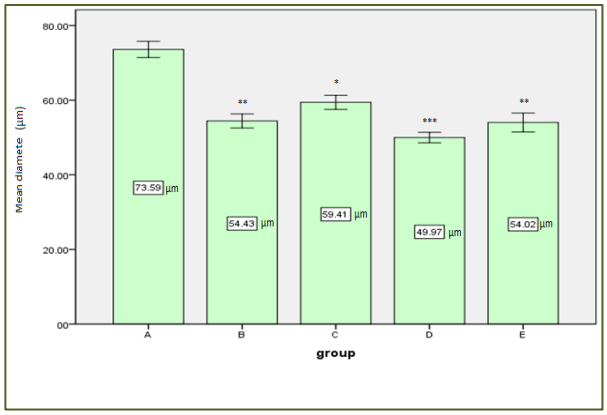


Figure 4. Comparison of the epithelial thickness of central testicular seminiferous tubules in terms of the micrometer in different groups whit control group
*p-value≤0.0001); **p-value≤0.0001; ***p-value≤0.0001.
Each bar represents the mean ± SD
A: Control group;
B: Cyclophosphamide receiving group;
C: Cyclophosphamide and vitamin receiving group;
D: Cyclophosphamide and retinoic acid receiving group;
E: Cyclophosphamide, and both antioxidants receiving group.

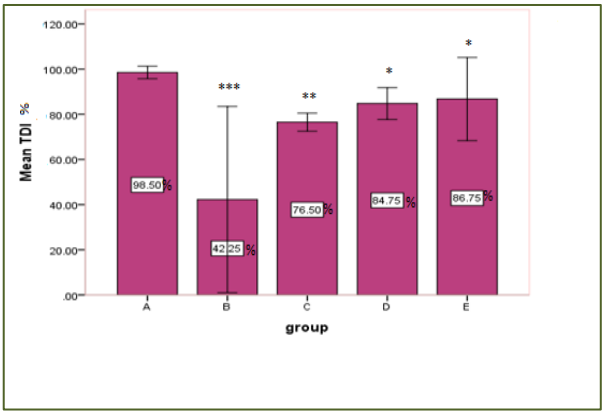


Figure 5. Comparison of TDI index by percentage in testicular seminiferous tubules in different groups whit control group
*p-value=0.218; **p-value=0.029; ***p-value≤0.0001
Each bar represents mean ± SD
A: Control group;
B: Cyclophosphamide receiving group;
C: Cyclophosphamide and vitamin receiving group;
D: Cyclophosphamide and retinoic acid receiving group;
E: Cyclophosphamide, and both antioxidants receiving group.

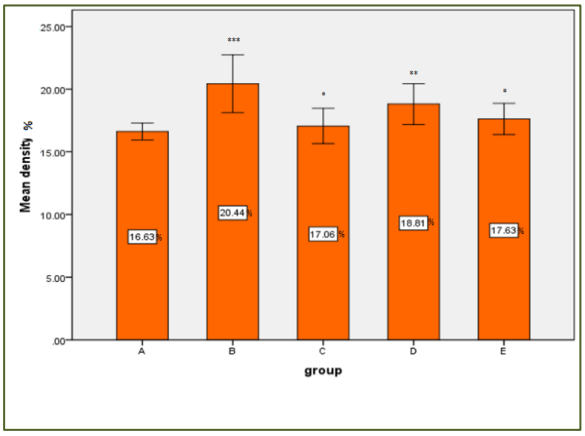
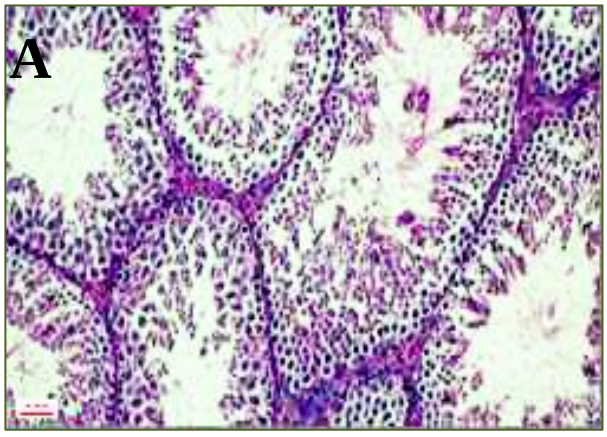


Figure 6. Comparison of the percentage of the density of seminiferous tubules per unit area of testicular tissue in different groups whit Control group
*p-value=0.033; **p-value=0.036; ***p-value ≤0.0001
Each bar represents mean ± SD
A: Control group;
B: Cyclophosphamide receiving group;
C: Cyclophosphamide and vitamin receiving group;
D: Cyclophosphamide and retinoic acid receiving group;
E: Cyclophosphamide, and both antioxidants receiving group;



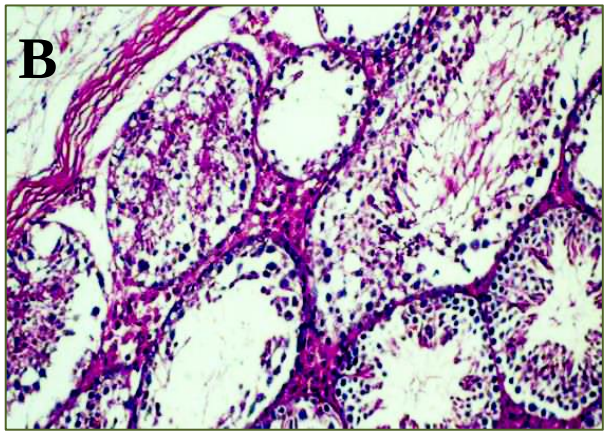


Figure 7. A, transverse section of the seminiferous tube in the control group; The seminiferous tubules were normal, and the tubular epithelium was thick [H&E staining (magnification 40 x)] B, transverse section of the seminiferous tube of the cyclophosphamide-receiving group. The epithelial thickness and the magnitude decreased [H&E staining (magnification 40 x)].

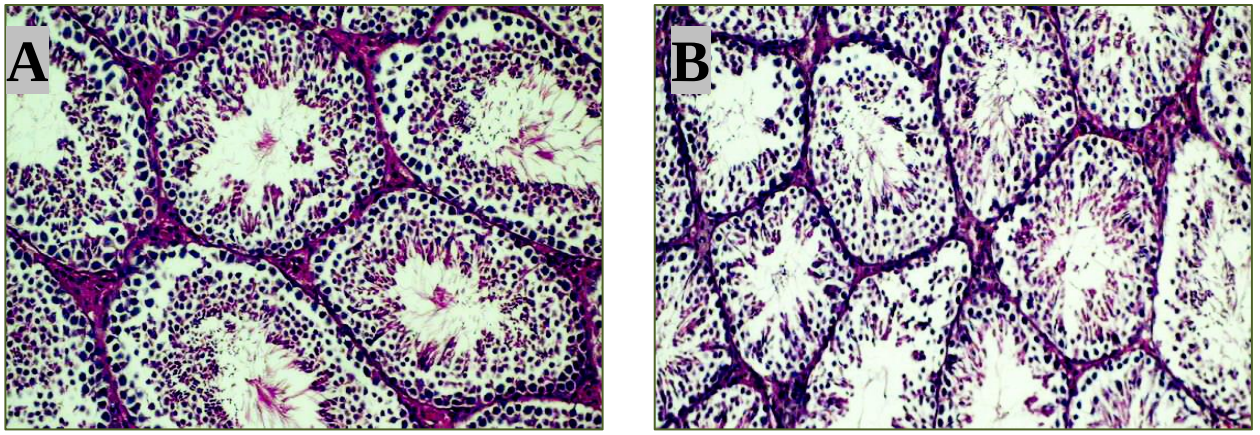


Figure 8. A, the transverse section of the seminiferous tube of the Cyclophosphamide and vitamin E receiving group; the regular arrangement of different cell categories are observed. [H&E staining (magnification 40 x)] B, transverse section of the seminiferous tube of the Cyclophosphamide and retinoic acid receiving group; Disorders of the Leidig cells, atrophy, and tubal deformation [H&E staining (magnification 40 x)].

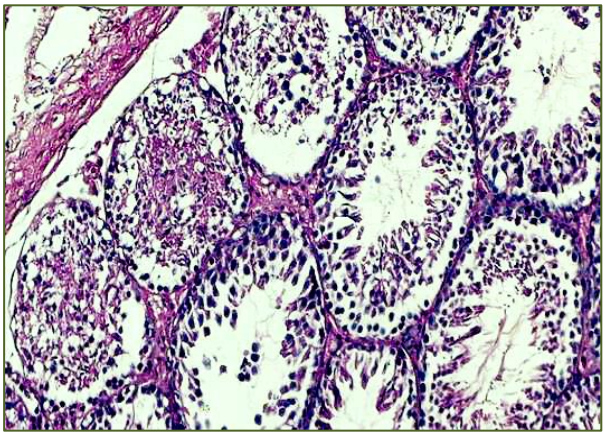


Figure 9. Transverse section of the seminiferous tube of the Cyclophosphamide- and the combination of vitamin E and retinoic acid receiving group. Decreased epithelial thickness and irregularity and increased diameter are observed [H&E staining (magnification 40 x)].

Discussion

In the vitamin E receiving group, the most significant effect of tissue support was observed (237.05 microns). This effect is such that the diameter is close to the control group (243.85 microns). In the retinoic acid (217.00 microns) and the combination of vitamin E and retinoic acid (222.35 microns) receiving groups, the increase in diameter has been significant, and these two treatments have almost given the same and close effect. In the case of small diameters of seminiferous tubules, the data showed a meaningful reduction in the group receiving Cyclophosphamide (147.35 microns), and the most excellent result of diameter increasing was monitored in the group receiving vitamin E (174 microns), which is closer to the control group (182.30 microns). This increase was in the retinoic acid (160.80 microns)-receiving group, while in the retinoic acid and vitamin E co-treatment group, the supportive effect was slightly milder (157.35 microns). The epithelial thickness average of peripheral seminiferous tubules in the CP receiving group decreased significantly. It dropped to 47.78 microns compared with the control group (66.93 microns), indicating a decrease in testicular spermatogenesis activity during chemotherapy treatment. The epithelial thickness reduction and cell density reduction of the germinal epithelial tissue of tubules can also be seen in relevant tissue images. In the co-treatment vitamin E receiving group, a significant increase in the thickness of the germinal epithelium up to 58.35 microns was observed, and it is essential to note that such an increase is seen in the retinoic acid and vitamin E co-treatment groups. In the co-treatment group, this parameter reached 56.53 microns.

In contrast, in the group that received retinoic acid just with cyclophosphamide, the increase in epithelial thickness was less and was 45.24 microns, which is less than the control group, and this could point to the more prominent role of vitamin E in protecting sexual epithelium. A similar pattern has been observed in central seminiferous tubules.

The mean diameter of seminiferous tubules in the control groups is cyclophosphamide, cyclophosphamide and vitamin E, cyclophosphamide, and retinoic acid, and cyclophosphamide, and combined treatment with vitamin E and retinoic acid was 73.59, 54.43, 59.41, 49.79, and 54.02 microns, respectively. Comparing the average tissue density in the transverse section of testicular tissue in different groups shows a decrease in the diameter of the tubules in the cyclophosphamide receiving group. As shown in the tissue density diagram in this group, the highest number of seminiferous tubules per surface unit has been recorded, which means that the sperm-producing tubules' diameter has decreased (20.44). In the control and vitamin E groups, the lowest density was recorded with relatively equal values, which means that vitamin E was able to restore the testicular tissue conditions to the control range in terms of the seminiferous tubules' diameter and density per surface unit. The retinoic acid-receiving group was unable to restore conditions to normal situations as much as vitamin E, and the retinoic acid and vitamin E-receiving group had a slightly lower positive effect than the vitamin E group alone.

The TDI index means the percentage of seminiferous tubules that have more than three differentiated cell layers. In transverse sections, the higher the TDI index, the higher the normal progression of spermatogenesis, and the higher the spermatogenesis level of the testicle. In the cyclophosphamide receiving group, the TDI index decreased to less than half of the control value (42.25), and the vitamin E receiving group increased it only 76.50%, while in the control group, the tubular differentiation index was 98.50%, and in the retinoic acid receiving group, the index was 84.75%, higher than the vitamin E group, and in the vitamin E and retinoic acid combined treatment group, the index was 86.75%. Still, vitamin A has not been able to prevent oxidative stress due to cell aging. In this study, vitamin E resulted in a reduction in the immune response due to aging in peripheral blood lymphocytes. This process was mediated by reducing the effects of natural oxidative stress on aged cells. A similar study by Hosseini et al. shows that cyclophosphamide treatment leads to toxicity in the male reproductive system. Low doses of cyclophosphamide for 50 days cause severe tissue damage and a significant reduction in the rate of tubal differentiation coefficient and spermiogenesis. According to these studies, the number of disrupted tubules and interstitial space in the cyclophosphamide-receiving testicular tissue increases. The oxidative stress induction in the tissue is this compound and its metabolites (11). According to a study by Rezaei and Ahmadi in 2015, this drug significantly reduced sperm motility and viability, and Imidin co-treatment improved many parameters and removed cyclophosphamide side effects in the reproductive system of Syrian mice (12). Reviews of vitamin E effects on spermatogenesis also suggest that while taking vitamin E supplementation can prevent the severe adverse effects of oxidative stress on spermatogenesis (13), by reducing oxidative stress at the same time, this vitamin improves sperm concentration and motility and male fertility (14). In a study of the spermatogenesis process of phenol-treated rats, it was found that vitamin E was able to significantly improve testicular weight loss and tissue changes due to this substance and reach it to the control group. Also, the mean thickness of seminiferous epithelium in the vitamin E group showed a significant increase compared to the control group (15). Vitamin E has been shown to reduce the weight of the testicles produced by these toxins, as in studies of the protective effect of vitamin E against oxidative damage caused by formaldehyde (7) and sodium valproate (16) on the testicles. Vitamin E has been shown to reduce the weight loss of the testicles caused by these toxins and return the testicular weight to the control group range, as in studies of the protective effect of vitamin E against oxidative damage caused by formaldehyde (7) and sodium valproate (16) on mature rats testicles. It also increased antioxidant enzyme activity and reduced lipid peroxidation in the testicles, compensating for the changes caused by these toxins on testicular tissue, including reducing the diameter and atrophy of seminiferous tubules and returning them to normal and

improving Spermatogenesis. In addition to reducing lipid peroxidation and improving lead-induced histopathological changes, vitamin E has been shown to increase serum testosterone levels in lead-treated rats (17). Other research has shown that laboratory mice with mutations in the retinoic acid receptor show seminiferous epithelial degeneration, which is very similar to the treated models for vitamin A deficiency (18), so the protective role of retinoic acid in the process of differentiating germinal cells is more pronounced.

Conclusion

In this study, cyclophosphamide has adversely affected spermatogenesis and testicular morphometric parameters, while vitamin E and retinoic acid as potent antioxidant agents have improved the destructive effects of this drug on testicular tissue. Vitamin E further increases the diameter of seminiferous tubes and maintains their epithelial thickness, and its protective role in these parameters is sufficiently valuable due to the small decrease in the combined treatment group. On the other hand, retinoic acid has a direct effect on increasing the TDI index in the seminiferous tubules, forcing the germinal cells to accelerate the cell cycle and the developmental process. Therefore, the use of vitamin E in support of testicular tissue during cyclophosphamide chemotherapy has been evaluated with a positive effect, and the use of retinoic acid requires additional studies to investigate its effects after completion of the chemotherapy and antioxidant co-treatment course and suggested during a spermatogenesis cycle to accelerate the differentiation induction. This study's findings are a primary step in clarifying the existence of an unforeseen effect similar to synergism in the combined administration of these two vitamins. Based on the results of this study, corn oil had a small negative effect on motility, viability, morphology, testicular weight, and mouse weight which was not statistically significant.

Conflict of Interest

The authors declared that they have no conflict of interest.

Acknowledgment

The authors are highly thankful to Dr. Mojtaba Abbasi for providing insightful comments to conduct this investigation.

Funding/Support

The present research was not supported by any funding organization of the commercial, nonprofit or public sectors.

Ethics

This study has been approved by the ethics committee of Payame Noor University.
Registration Code: IR.PNU.REC.1398.068;
URL:
https://ethics.research.ac.ir/EthicsProposalView.php?id=96500

References

1. Meistrich ML, Finch M, da Cunha MF, Hacker U, Au WW. Damaging effects of fourteen chemotherapeutic drugs on mouse testis cells. Cancer research. 1982;42(1):122-31.

2. Meistrich M, Wilson G, Brown B, Da Cunha M, Lipshultz L. Impact of cyclophosphamide on long-term reduction in sperm count in men treated with combination chemotherapy for Ewing and soft tissue sarcomas. Cancer. 1992;70(11):2703-12.

3. Friedman O, OM F. Cyclophosphamide and related phosphoramide mustards. Current status and future prospects. 1979.1:143

4. Fraiser LH, Kanekal S, Kehrer JP. Cyclophosphamide toxicity. Drugs. 1991;42(5):781-95.

5. Lombardo F, Sansone A, Romanelli F, Paoli D, Gandini L, Lenzi A. The role of antioxidant therapy in the treatment of male infertility: an overview. Asian Journal of andrology. 2011;13(5):7-690.

6. Momeni HR, Eskandari N. Effect of vitamin E on sperm parameters and DNA integrity in sodium arsenite-treated rats. Iranian Journal of Reproductive Medicine. 2012;10(3):249.

7. Zhou DX, Qiu SD, Zhang J, Tian H, Wang HX. The protective effect of vitamin E against oxidative damage caused by formaldehyde in the testes of adult rats. Asian journal of andrology. 2006;8(5):584-8.

8. Jauregui EJ, Mitchell D, Topping T, Hogarth CA, Griswold MD. Retinoic acid receptor signaling is necessary in steroidogenic cells for normal spermatogenesis and epididymal function. Development. 2018;145(13):160-465.

9. Fu Z, Kubo T, Sugahara K, Noguchi T, Kato H. Retinoid nutritional status differently affects the expression of Japanese quail retinoic acid receptor-beta isoform transcripts in a tissue-specific manner. Journal of endocrinology. 2001;169(2):281-90.

10. VAN PELT AM, DE ROOIJ DG. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology. 1991;128(2):697-704.

11. Moore H, Akhondi MA. In vitro maturation of mammalian spermatozoa. Reviews of Reproduction. 1996;1(1):54-60.

12. Rezvanfar M, Sadrkhanlou R, Ahmadi A, Shojaei-Sadee H, Rezvanfar M, Mohammadirad A, et al. Protection of cyclophosphamide-induced toxicity in reproductive tract histology, sperm characteristics, and DNA damage by an herbal source; evidence for role of free-radical toxic stress. Human & experimental toxicology. 2008;27(12):901-10.

13. Ghojaie M, Barzegar A, Asadzadeh R. The survey of IRFI005 function about removing of intra and extracellular free radicals. scientific journal of ilam university of medical sciences. 2014;22(2):158-66.

14. Krishnamoorthy G, Venkataraman P, Arunkumar A, Vignesh R, Aruldhas M, Arunakaran J. Ameliorative effect of vitamins (α-tocopherol and ascorbic acid) on PCB (Aroclor 1254) induced oxidative stress in rat epididymal sperm. Reproductive Toxicology. 2007;23(2):239-45.

15. Tamilselvan P, Bharathiraja K, Vijayaprakash S. Protective role of lycopene on bisphenol A induced changes in sperm characteristics, testicular damage and oxidative stress in rats. 2013.

16. Shalaby M, El-Zorba H. Protective effect of celery oil, vitamin E and their combination against testicular toxicity in male rats. Global veterinaria. 2010;5(2):122-8.

17. Ayinde OC, Ogunnowo S, Ogedegbe RA. Influence of Vitamin C and Vitamin E on testicular zinc content and testicular toxicity in lead-exposed albino rats. BMC pharmacology and toxicology. 2012;13(1):1-8.

18. Livera G, Rouiller-Fabre V, Pairault C, Levacher C, Habert R. Regulation and perturbation of testicular functions by vitamin A. Reproduction-cambridge-. 2002;124(2):173-80.