Original Article

Protective effect of Vitamin E against Glyphosate-induced Reproductive failure in Female Rats

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Abstract

Background & Objective: The glyphosate-based herbicide has a prominent role in the toxic mechanism in living systems. Previous studies have shown that vitamin E can have a protective effect on cells against oxidative damage. This study aimed to evaluate the protective effect of vitamin E against the toxic potential of glyphosate (Gly) in the reproductive system of female rats.

Materials & Methods: In this experimental study, 30 female Wistar rats were randomly divided into five groups (n=6), including control, glyphosate (50 mg/kg per day), glyphosate (500 mg/kg per day), glyphosate (50 mg/kg per day) + vitamin E (50 mg/kg per day), and glyphosate (500 mg/kg per day) + vitamin E (50 mg/kg per day). Glyphosate was administered orally, and vitamin E was administered by intraperitoneal injection. After 35 days, rats were euthanized, and embryo development, malondialdehyde (MDA) concentration, and catalase (CAT) activity were examined. Moreover, the levels of estrogen and progesterone were measured in the serum. Results were analyzed by SPSS software using one-way ANOVA and Tukey's post hoc test.

Results: The glyphosate significantly decreased the percentages of fertilization, two-cell embryos, blastocysts, estrogen, and progesterone level as well as catalase activity and significantly increased MDA level in glyphosate-treated rats in comparison with controls ($p<0.05$). However, the results showed that vitamin E can reduce the toxicity of glyphosate administration in the mentioned parameters ($p> 0.05$).

Conclusion: These results have shown that vitamin E can provide the antioxidant defense with potent preventive activity against glyphosate-induced reproductive toxicities.

Keywords: Glyphosate, Vitamin E, Ovary, Rat, Reproductive failure

Introduction

Ovulation-related abnormalities are usually the most common cause of infertility in humans, and 31% of infertility in women is caused by these abnormalities (1). On the other hand, human and mammalian populations exposed to industrial and environmental pollutants such as herbicides have raised concerns about this pollution effect on reproductive performance and development (2). Although the use of herbicides is beneficial in some cases, it can cause some abnormalities, such as sperm abnormalities, production of reactive oxygen species (ROS), disruption of the antioxidant defense system, and induction of

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apoptosis in different tissues and cells (3, 4).Glyphosate is one of the components of Roundup herbicide. Presently, the use of this herbicide is increasing in agriculture, gardening, and forestry (5). Glyphosate disrupts the Shikimate enzyme pathway and inhibits aromatic amino acid synthesis (6). Exposure to this herbicide can lead to the overproduction of malonaldehyde and oxidative stress. The production of reactive oxygen species in organisms is determined by the antioxidant defense enzymes, such as catalase, that eliminates hydrogen peroxide (7).

One of the special effects of this herbicide is its effect on ROS production, causing oxidative damage (8). ROS causes several defects, including multiple injuries to the lysosomal membrane, severe damage to the cell membrane through lipid membrane oxidation, proteins, and eventually, cell apoptosis (9). Also, glyphosate disrupts the sex hormone levels due to aromatase enzyme dysfunction, which is involved in converting androgens to the estrogen process (10). Studies have shown that glyphosate in adult rats can affect liver metabolism and disrupts its essential functions by increase certain liver enzymes (11). Other studies have shown that glyphosate can cause severe damage to the gastrointestinal tract (12). Glyphosate effects on the reproductive system are DNA structure changes, sperm abnormalities, and acting as inducers of heat shock proteins (13, 14). Glyphosate reduces the male reproductive function and their fertility through apoptosis-inducing in Sertoli cells (15). Studies in zebrafish have shown that high glyphosate doses can lead to embryo abnormalities, including the short tail, vertebral column curvature, and increased mortality (16). The effect of glyphosate on the amphibian ovaries has shown that glyphosate inhibits 19-beta estradiol. As a result, the disorder in steroidogenesis and follicle growth can happen, which may be due to reactive oxygen species (17).

Vitamin E is an essential factor for rat fertility due to its potent antioxidant compound and important protective agent for cell membranes against ROS damage (18). This Vitamin acts as the first line of defense against ROS and then reduces oxidative stress. It is also essential for oocyte maturation and oocyte quality due to its antioxidant properties (19). At a molecular level, vitamin E and some of its metabolites are shown to regulate cell signaling capacity, and modulating gene transcription (20). Previous studies have shown that antioxidant properties of vitamin E, reduces the toxicity effect of free radicals on the reproductive system (18). Therefore, this study aimed to determine the protective effect of vitamin E on glyphosate-induced ovariotoxicity and embryotoxicity.

Materials & Methods

Animals. Thirty adult female Wistar rats weighing 180 ± 20 g were located in standard conditions (12:12 h light/dark cycle, the temperature of 22±2 °C, and relative humidity of 50±10 %) with ad libitum access to food and water. Working with animals was performed by the Principles of Laboratory Animal Care (NIH publication #85−23, revised in 1985) and was approved by the Urmia University Animal Care Committee.

Treatment. Following two weeks, the rats were randomly assigned into five groups (n=6), including control, glyphosate (50 mg/kg per day), glyphosate (500 mg/kg per day), glyphosate (50 mg/kg per day) + vitamin E (50 mg/kg per day), glyphosate (500 mg/kg per day) + vitamin E (50 mg/kg per day). The experimental period was 35 days. Glyphosate was administered orally, and vitamin E was administered by intraperitoneal injection. The glyphosate (Merck, Darmstadt, Germany) and vitamin E (Karen, Tehran, Iran) were dissolved in normal saline and olive oil (Mazo Light, Tehran, Iran), respectively (21, 22).

Sampling. At the end of the study, rats were anesthetized by intraperitoneal injection of ketamine (75.00 mg/kg) and xylazine (10.00 mg/kg; Alfasan, Woerden, Netherlands). Under sterile conditions, ovaries were collected, the left ovaries were transferred to -70 °C for Malondialdehyde and Catalase analyses, and right ovaries were used for in vitro fertilization process.

Oocyte collection and embryo development analyses. To induce superovulation, female rats were injected intraperitoneally with 25.00 IU of PMSG (pregnant mare serum gonadotropin; Folligon, Boxmeer, Netherlands) hormone and 15.00 IU of hCG (human chorionic gonadotropin; Folligon, Boxmeer, Netherlands) hormone and 15.00 IU of hCG (human chorionic gonadotropin; Folligon, Boxmeer, The Netherlands) hormone, 48 hr, and 12 hr before experimenting to all rats, respectively (23). The female rats were euthanized, their oviducts were removed, and the ovulated oocytes were collected from the ampulla portions using a dissecting technique under a stereo zoom microscope. Rats' sperm was collected from the caudal epididymis of one rat and incubated for 1 hr in mR1ECM medium. The sperm that had a concentration of 1×106 total sperm/ml was added to a 500 µl fertilization drop of oocyte containing mR1ECM medium. For each animal, we divided a total of 20 oocytes into ten drops. After 6 hr of incubation at 37˚C under 5% CO2, the cumulus cell-free fertilized oocytes were transferred to fresh drops of mR1ECM medium for embryo culture. All drops were covered by mineral oil. Fertilized oocytes (zygote) were evaluated by the pronuclei and polar bodies' appearance under an inverted microscope with ×200 magnification. After 24 hours following the zygote culture, we assessed the two-cell embryo formation rate and morula formation rate. Blastulation and hatching rate were calculated by determining the number of embryos that had reached the blastocyst development stage and the number of fully hatched blastocysts after 72 hr incubation, respectively (24).

Malondialdehyde (MDA) assays. For each animal, a sample of ovarian tissue homogenized in KCl, and 300 µL of 10.00% H3PO4 was added to150 µL homogenate, then centrifuged at 1000 rpm for 10 min at 4 ˚C. The solution was heated for 25 min in 300 µL of 67.00% thiobarbituric acid at 100 ˚C. After cooling, its optical density was determined by a spectrophotometer at 535 nm absorption wavelength (25).

Catalase activity assays. Catalase activity was evaluated in ovarian homogeneity by the Aebi method (26). Ovarian tissue was homogenized with cold phosphate buffer and centrifuged at 5000 rpm for 5 min, then 100 µL of the centrifuged supernatant fluid was added to 2.8 µL of phosphate buffer. Finally, 100 µL of hydrogen peroxide solution was added, and the reaction was initiated by the addition of hydrogen peroxide. Absorbance changes were measured at 240 nm (25 °C) for 30 s. The results were expressed as U/g ovarian tissue.

Biochemical analysis. To measure estrogen and progesterone level changes in the serum, blood samples were directly drawn from hearts and centrifuged at 12000 x g in 10 minutes. Then, serum was placed at –80.00 °C until testing. The estrogen and progesterone level were measured by an ELISA method using a commercial kit (Ideal Tashkhis Atieh, Tehran, Iran).

Statistical analysis. All statistical analyses were performed using SPSS software (version 24.0). Differences between groups were determined by one-way ANOVA and Tukey's multiple comparison post-hoc test. The values expressed in mean ± SE, and significance were set at $p<0.05$.

Results

In vitro fertilization (IVF) outcome. Table 1

shows the IVF results in different experimental groups. According to the results, the zygote percentage in rats receiving low and high glyphosate doses was 62.86% and 52.39%, respectively, which was a significant decrease compared to the 82.90% of the control group ($p < 0.05$). While, in the glyphosate 50 + Vitamin E receiving rats, the zygote percentage was 77.89% that was not significantly different from the control rats ($p > 0.05$). However, the zygote percentage in the glyphosate 500 + Vitamin E receiving group was 52.37% which was not significantly different from the glyphosate 500 receiving group ($p > 0.05$).

The mean of two-cell embryo percentage in rats receiving low and high doses of glyphosate was 61.53% and 51.72%, respectively, which was a significant decrease compared to the 78.23% of the control group ($p < 0.05$). The two-cell embryos percentage in the glyphosate 50 + vitamin E group was 78.56%, indicating no significant difference compared to the control group ($p>0.05$).

The morula percentage in the low and high dose of glyphosate receiving groups was 45.17% and 38.57%, respectively, which showed a significant decrease compared to 56.17% of the control group ($p <0.05$). However, the morula percentage in the glyphosate 50 + vitamin E receiving group was 54.90%, which, compared to the control, showed no significant change observed ($p > 0.05$).

The mean blastocysts percentage in the low and high dose of glyphosate receiving rats was 30.58% and 15.65%, respectively, which showed a significant decrease compared to 45.10% of the control rats ($p < 0.05$). However, there was no significant difference in the 44.28% of glyphosate 50 + vitamin E receiving rats compared to control ($p > 0.05$).

The rate of completely hatched blastocysts in the low and high dose of glyphosate receiving rats was 21.00% and 11.70%, respectively, which showed a significant reduction compared to 29.77% of the control ($p < 0.05$), while this ratio in the glyphosate 50 + vitamin E receiving rats was 28.79%, which was nearly restored to the control rats ($p > 0.05$; Table 1 and Figure1).

Table 1. The glyphosate (Gly) and vitamin E (Vit E) effect on in vitro fertilization outcomes in different experimental groups (Mean ± SEM)

Groups	Zygote (%)	Two-cell embryos (%)	Morula (%)	Blastocysts (%)	Hatching embryos (%)
Control	82.90 ± 0.90	78.23 ± 2.17	56.17 ± 1.52	45.10 ± 1.89 [a]	29.77 ± 1.08
Gly50	62.86 ± 1.49 *	61.53 ± 1.68 *	45.17 ± 1.39 *	30.58 ± 1.26 *	21.00 ± 0.86 *
Gly500	52.39 ± 2.17 *	51.72 ± 1.83 *	38.57 ± 1.11 *	15.65 ± 1.19 *	11.70 ± 0.59 *
Gly50+Vit E	77.89 ± 2.47	78.56 ± 1.47	54.90 ± 1.39	44.28 ± 1.90	28.79 ± 1.05
Gly500+Vit E	52.37 ± 2.41 *	54.04 ± 2.31 *	38.95 ± 0.88 *	20.40 ± 1.62 *	13.42 ± 0.97 *

* Significant differences as compared with the control group at $P <0.05$

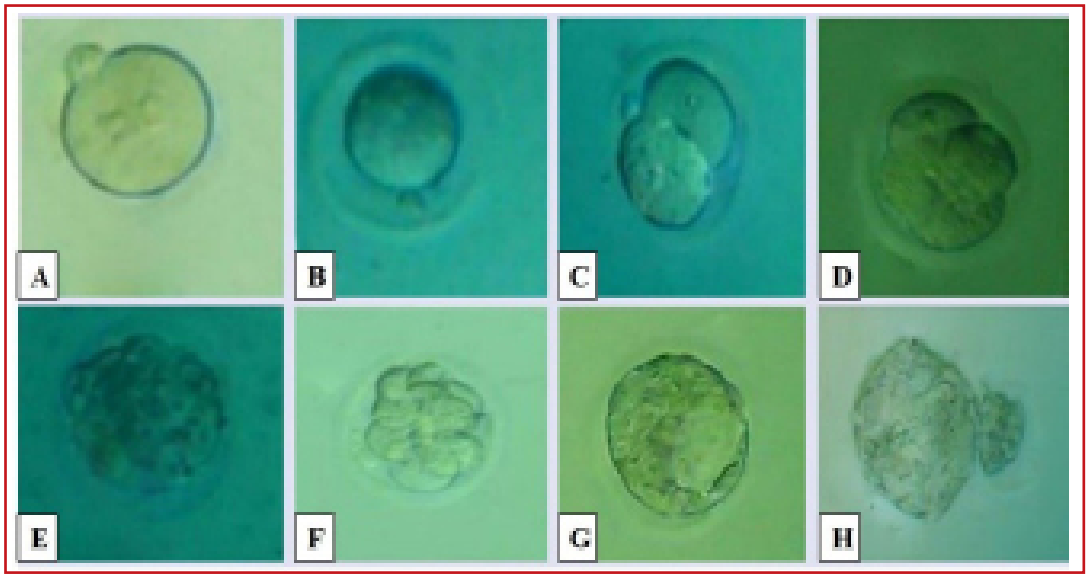
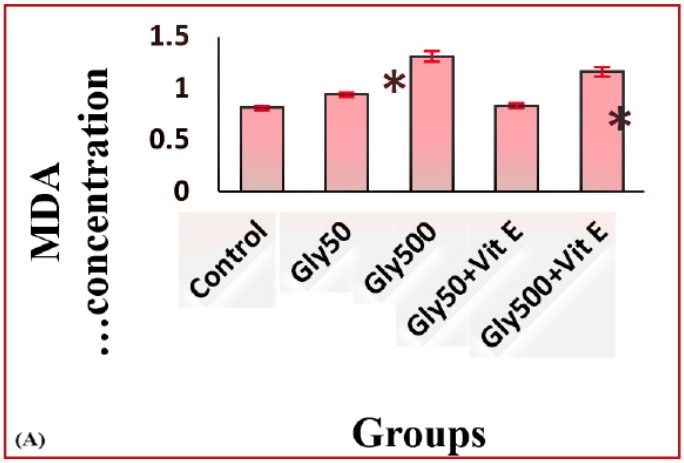
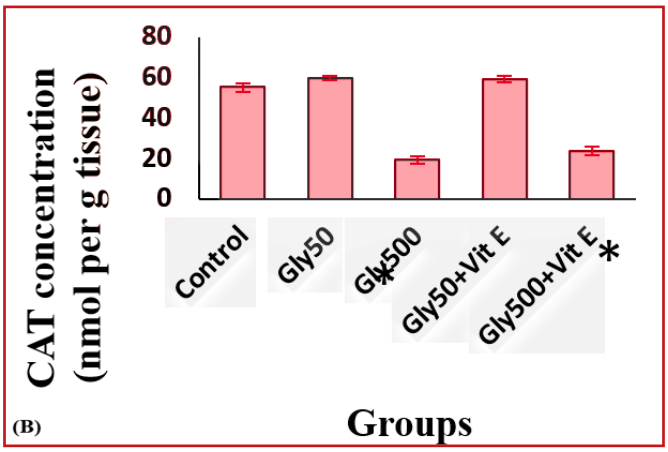


Figure 1. Photomicrograph of pre-implantation embryo development (×1000). A) Fertilized oocytes with specific Pre-nucleus (control group); B) Unfertilized oocytes (glyphosate 50 group); C) 2-cell embryo stage (glyphosate 50 + vitamin E group); D) 4-cell embryo stage (glyphosate 500 + vitamin E group); E) Compacted morula (glyphosate 50 + vitamin E group); F) Non-compact and stopped morula (glyphosate 500 group); G) Blastocyst stage (control group); H) Hatching embryo (glyphosate 50 + vitamin E group)

Oxidative stress factors outcome. The results of MDA concentration measurement in Chart 1A show that MDA concentration in the high-dose of glyphosate receiving group was 1.31 nmol per g tissue, indicating a significant increase compared to 0.81 nmol per g tissue of the control group ($p < 0.05$). However, MDA concentration in the glyphosate 50 and glyphosate 50 + vitamin E receiving rats was 0.94 and 0.83 nmol per g tissue, respectively, indicating no significant differences from the control rats ($p > 0.05$; Chart 1A).

Catalase (CAT) activity in high doses of glyphosate and glyphosate 500 + vitamin E receiving rats was 19.15 and 23.64 nmol per g tissue, respectively, which is a significant decrease compared to 55.2 nmol per g tissue in the control rats ($p<0.05$). However, catalase (CAT) activity in the low-dose of glyphosate and glyphosate 50 + vitamin E receiving groups was 59.65 and 59.32 nmol per g tissue, respectively, which was not a significant change compared to the control rats ($p>0.05$; Chart 1B).



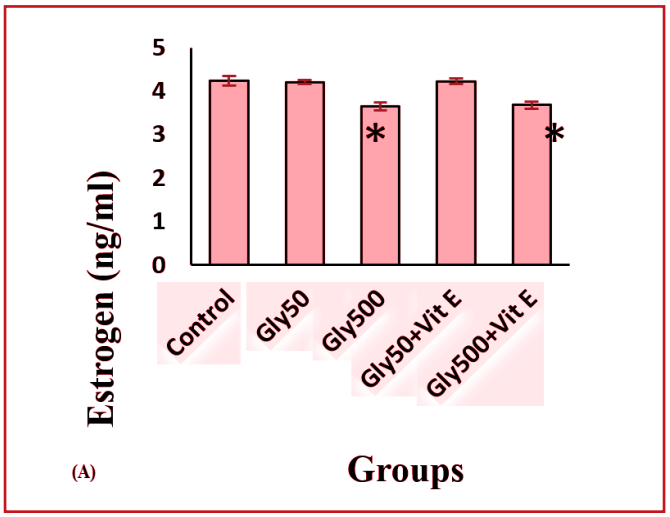


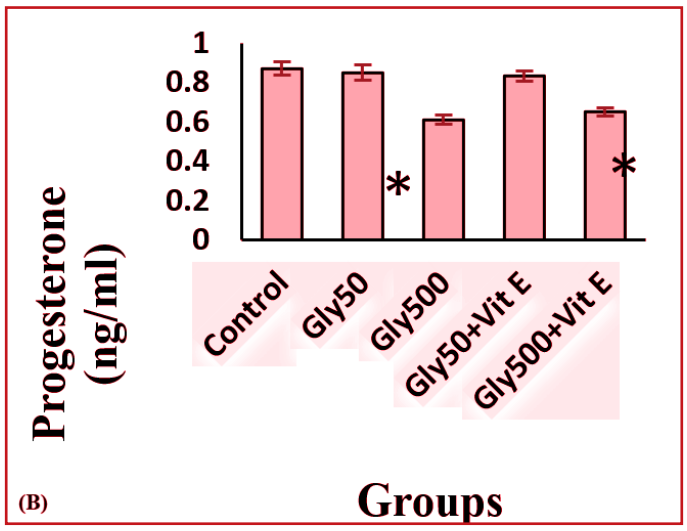
* Significant differences as compared with the control group at P <0.05

Chart 1. Glyphosate (Gly) and vitamin E (Vit E) effect on MDA concentration (A) and catalase activity (B) in ovary tissue of different groups (results are expressed as Mean±SEM (n=6))

Hormone profile outcome. Evaluation of serum estrogen levels showed that the levels of this hormone in high doses of glyphosate and glyphosate 500 + vitamin E receiving rats were 3.65 and 3.68 ng/ml, respectively, which is a significant decrease compared to the estrogen level of 4.24 in control rats ($p<0.05$). The administration of low-dose of glyphosate reduced estrogen levels to 4.21 ng/ml in the low-dose glyphosate group and 4.23 ng/ml in the glyphosate 50 + vitamin E group, but this decrease was not statistically significant ($p>0.05$; Chart 2A).

Administration of glyphosate in high dose glyphosate receiving and glyphosate 500 + vitamin E groups reduced progesterone levels to 0.61 and 0.65 ng/ml, which was a significant decrease compared to 0.87 ng/ml in the control group ($p<0.05$). However, the progesterone levels in the low-dose of glyphosate and glyphosate 50 + vitamin E receiving groups were 0.85 and 0.83 ng/ml, respectively, which was not a significant change compared to the control rats ($p>0.05$; Chart 2B).





* Significant differences as with the control group at P <0.05

Chart 2. The bar chart shows the vitamin E (Vit E) and glyphosate (Gly) effects on serum estrogen (A) and progesterone (B) in the treated rats (results are expressed as Mean±SEM (n=6))

Discussion

Based on the results of the Food and Drug Administration (FDA) study of glyphosate residues in animal products such as milk and eggs, as well as the detection of glyphosate residues in human urine, indicate that glyphosate can enter the human body via the food chain and ultimately affect the organism's metabolism (27). Various studies have shown that Glyphosate, as a type of endocrine-disrupting chemical and causing oxidative stress, has a toxic effect on the reproductive system. Glyphosate in pregnant mice, at the hypothalamic-pituitary-ovarian axis, by altering the expression of GnRH, LHR, FSHR, 3β-HSD, and Cyp19a1 genes, causes histopathological changes in the ovary (28). In rats, glyphosate exposure has also been shown to lead to pregnancy loss through apoptosis and change in ovarian steroid levels, uterine morphology, and endometrial cell proliferation (29).

Few studies have tested the effects of glyphosate on female fertility and the reproductive tract. Thus, our study which investigated the protective effect of vitamin E against glyphosate toxicity can be interesting. In this study, zygote, 2-cell embryo, morula, blastocyst, and hatching embryo percentages were reduced in glyphosate receiving rats. The ovarian follicles maturation and development are a physiological process under the influence of granulosa cells and oocytes.

Glyphosate intervention can change this physiological process (30). Glyphosate can alter cell function by affecting the structure of DNA and cellular proteins. When the follicle growth stops at the small antrum stage, it causes stopped follicular growth and follicular atresia (29). Glyphosate changes the steroidogenesis and increases the ROS levels of the oocyte. Also, it has been suggested that glyphosates effect on follicles can be related to embryo quality, implantation rate, and IVF results (31). Previous research has ascribed the toxicity effect of glyphosate to cellular apoptosis induced by ROS overproduction. Ultimately, cellular apoptosis causes several types of abnormalities in the embryo organisms (32). Also, recently reported

exposure to glyphosate in adult mouse oocyte causes spindle fiber demolition, disorderliness in chromosomal alignment, and ROS aggregation. Similar results have been shown in glyphosate-exposed mouse embryos during embryo culture, confirming our findings (33).

The granulosa and theca cells in antral follicles by aromatization of androgens convert them to estrogen, and preantral follicles are also slightly involved in the aromatization process (34). The steroidogenesis process requires both theca and granulosa cells. When FSH hormone connects to its specific receptors in the granulosa cells membrane, it causes estrogen levels to increase by adenylate cyclase stimulating and cAMP synthesis. In the estrogen production pathway in granulosa cells, cholesterol by P450 side-chain cleavage enzyme is converted to pregnenolone. Finally, in theca cells, androstenedione is produced by 17 kinds of α-hydroxylase, and androstenedione in granule cells is converted into estrogen by the activation of 17 kinds of β-hydroxysteroid dehydrogenase and aromatase (35). This enzyme also interferes in the production of the progesterone hormone by disrupting estrogen production (36). In rats, it has been reported that glyphosate reduces the production of aromatase mRNA and its protein, thereby disrupting the production of the aromatase enzyme (CYP19). A decreased aromatase enzyme causes a hormonal imbalance that leads to folliculogenesis changes and atretic follicles increase (37). In this study, the results of serum progesterone and estrogen levels are similar to previous studies that reported a decrease in progesterone and estrogen levels after treatment with glyphosate (38).

In the body's metabolism, ROS can react with essential macromolecules such as nucleic acids and lipids. In vitro studies have shown that glyphosate plays a significant role in ROS increase (39). In the present study, both doses of glyphosate increased MDA concentration and reduced CAT activity in ovarian tissues, which cause considerable induce of oxidant/antioxidant imbalance in ovaries. In the body, free radicals cause MDA production by reacting with biomolecule. MDA increasing as an oxidative stress marker indicates increased lipid oxidation and damage to cell membranes. Catalase is the most abundant antioxidant enzyme in peroxisomes that breaks down H2O2 into water and oxygen. Decreased catalase function leads to cell damage by increasing the concentration of H2O2 in tissues (40). Previous studies have shown that glyphosate causes oxidant/antioxidant imbalances and DNA damage, cell apoptosis, and reduces the antioxidant defense system, which proves our findings (41). Generally, glyphosate is considered to stimulate the apoptosis route, and these disorders can be prevented by antioxidant mechanisms (42).

Vitamin E improves oocyte maturity, oocyte quality, and embryo development. Some reports have shown that vitamin E administration in female rats, increases the number of healthy follicles and reduces the apoptosis effects of glyphosate (43). Considering the previous studies, it seems that vitamin E, due to its rich enzymatic and non-enzymatic antioxidants, neutralizes injury patterns of the ovary's integrity and regulates anti-apoptotic patterns. Therefore, considering the mentioned evidence, the vitamin E protection effect through its antioxidant functions provides a marked protective effect against glyphosate-induced follicular impairment and embryotoxicity.

Also, vitamin E, through its anti-inflammatory and antioxidant properties, can positively affect oocyte and embryo development by enhancing the steroid hormone level (44).

Conclusion

Considering the results of our study, it can be concluded that the undesirable effects of glyphosate on the rat's hormone imbalance and disruption of the reproductive system are mainly the result of the induction of oxidative stress by this herbicide, and the effects of glyphosate are dose-dependent, and high doses have more destructive effects on the female reproductive system. Antioxidant properties of Vitamin E can counter the undesirable effects of glyphosate on hormonal balance and the female reproductive system.

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Conflict of Interests

All the authors express that there is no conflict of interest regarding the publication of this article.

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