http://www.ijwhr.net

doi 10.15296/ijwhr.2023.24

Open Access Original Article

IJWHR

International Journal of Women's Health and Reproduction Sciences
Vol. 11, No. 2, July 2023, 138–144

ISSN 2330-4456

The Regulatory Effect of Eugenol on FSHR, LHCGR, and ER Expression during Follicular Development in Female Rats With Ovarian Torsion

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Abstract

Objectives: This study aimed to investigate the role of Eugenol in regulating the expression of FSH receptor (FSHR), human luteinizing hormone choriogonadotropin receptor (LHCGR), and estrogen receptor (ER) during follicular development in female rat ovarian torsion.

Materials and Methods: In this experimental study, 48 female rats were randomly assigned to 4 groups, including G1 (i.e., sham), G2 (i.e., ovarian torsion/detorsion group), G3 (i.e., ovarian torsion/detorsion group treated with 30 mg/kg of eugenol), G4 (i.e., healthy group treated with 30 mg/kg of eugenol). After covering a treatment period of ten days, the ovarian tissue was collected for the histological analysis, the measurement of ER, FSHR, and LHCGR expression, as well as the assessment of testosterone, LH, FSH, and estrogen levels in blood serum.

Results: Histological evaluation revealed the damage to ovarian tissue, the reduced oocyte, and the granulosa cell diameter in the torsion/detorsion group. However, the treatment with eugenol mitigated this damage. Eugenol administration increased the levels of estrogen, LH, and FSH, but it decreased the testosterone levels in the treated group. Moreover, the expression of ER, FSHR, and LHCGR was upregulated in the treated groups. Administration of eugenol was associated with an enhanced fertility.

Conclusions: It was concluded that eugenol administration may have been effective in protecting the ovarian tissue from the damage caused by torsion/detorsion. Furthermore, eugenol was found to have the potential to modulate hormonal profiles and regulate the expression of ER, FSHR, and LHCGR, thereby contributing to an increased fertility.

Keywords: Ovarian torsion, LHCGR, FSHR, Eugenol

Introduction

Folliculogenesis is a complex and lengthy process occurring in females' bodies, regulated by a coordinated molecular signaling between the ovarian cells and the anterior pituitary gland. Follicle growth occurs during this process, and the dominant follicles are selected for ovulation. The duration of the folliculogenesis process depends on the storage of resting follicles in the ovary, with some primordial follicles remaining in a resting state while others progressing to the developing stage. Once selected, developing follicles undergo growth in size due to the formation of granulosa cells and the growth of the oocyte (1).

Folliculogenesis is divided into two distinct steps. The first step, which is gonadotropin-independent, involves the growth and development of the oocyte. This step is regulated by local growth factors that act through paracrine and autocrine mechanisms. The second step, gonadotropin-dependent, is characterized by an increase in follicle size and is regulated by follicle stimulating hormone (FSH) and luteinizing hormone (LH) along with growth factors. Growth factors play a role in stimulating cell division and modulating the function of gonadotropins (2,3).

Following the growth and development step, the follicular cells continue to grow under the influence of FSH and LH, which bind to their respective receptors. FSH and LH promote the meiosis and differentiation of granulosa cells as well as the production of estradiol in mature follicles. FSH plays a crucial role in the proper functioning of the ovary, and studies have shown that the activation of the FSH receptor leads to ovarian degeneration and infertility (2,4,5).

During the late luteal phase and early follicular phase, the level of FSH hormone increases, serving as the basis for the selection of the dominant follicle. The dominant follicle is not selected in the absence of FSH. Throughout the process of folliculogenesis, some follicles undergo atresia, a degenerative process induced by testosterone hormone in preantral follicles. Studies have demonstrated that both exogenous and endogenous forms of testosterone have the potential to degenerate the granulosa cells and

Received 9 February 2023, **Accepted** 10 July 2023, **Available online** 20 July 2023

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Key Messages

- Ovarian torsion caused tissue damage and reduced the expression of FSHR, LHCGR, and ER genes.
- Eugenol may have improved the ovarian tissue damage.

oocytes in preantral follicles (1,3,4,6).

The proper development of follicles is influenced by the function of estrogen. Animal experiments have shown that testosterone reduces the sensitivity of granulosa cells to estrogen and decreases the expression of estrogen receptors. This disruption in the folliculogenesis pathway ultimately leads to infertility (5,6).

Several studies have shown that a stress oxidative condition and an increased ROS activity elevate the testosterone hormonal level and reduce the estrogen level because the elevation of testosterone level disrupts the folliculogenesis process and reduces the estrogen hormone level. Torsional stress, succeeded by its release, induces the generation of unpaired electrons known as free radicals, triggering an upsurge in reactive oxygen species (ROS) formation, consequently culminating in oxidative stress. This state subsequently leads to a reduction in the conversion of testosterone to estrogen, thus impeding the folliculogenesis process (2, 7).

Eugenol is an antioxidant combination that elevates the antioxidant capacity and prevents the free radical activity. Since oxidative stress is the main cause of the ovarian injuries in torsion/detorsion process, this study aimed to evaluate the role of eugenol antioxidant in regulating the estrogen receptor, LH, and FSH genes.

Materials and Methods

A total of 48 female Wistar rats weighing 180-220 g were included in this study. The rats were randomly selected from the animal house facility at Tabriz University of Medical Sciences and were kept under standard conditions. They had *ad libitum* access to water and food throughout the study. The rats were assigned to four groups using a random allocation method. These groups were:

- Sham group (n=8): A 2.5 cm longitudinal incision was made in the midline of the abdomen without inducing any torsion. The incision was then sutured using 6/0 nylon.
- Torsion/detorsion group (TD) (n=8): Ovarian torsion was induced in the left horn of the uterine tube and ovary. Detorsion was performed after a period of three hours. Normal saline was injected 30 minutes before the detorsion.
- Torsion/detorsion with eugenol group (TDO30) (n=8): Ovarian torsion was induced in the left horn of the uterine tube and ovary. Detorsion was performed after three hours. In this group, the first dose of eugenol (30 mg/kg) was administered 30 minutes prior to detorsion.
- Eugenol 30 group (n=8): This group consisted of eight healthy rats that were treated with a dose of 30 mg/kg of eugenol without undergoing any surgical operation.

Surgical Method

The ovarian torsion induction was explained based on our previous study (8,9). Firstly, all rats were anesthetized using ketamine and xylazine. Then, the abdominal area was shaved, and a 1-inch incision was made along the median part of the abdomen. After locating the uterine tube and ovary, they were exposed. The ovary with its adnexa was rotated 720 degrees around its ligament to induce torsion. To maintain the position of ovarian torsion, the twisted ovary was stabilized to the abdominal wall using a 6/0 nylon suture. This torsion was maintained for a period of three hours before the detorsion process, which was carried out half an hour later. Afterwards, the first dose of eugenol was administered via intraperitoneal injection. Subsequently, the detorsion process was completed, followed by a 10-day reperfusion period during which the treatment groups received different doses of eugenol. All rats were anesthetized at the end of the 10-day treatment period and after the administration of the last doses of eugenol. Ovarian tissues were then extracted for histopathological assessment, evaluation of oxidative stress markers, and gene expression analysis (8,10).

Ovarian Tissue Examination

Ovarian tissue samples were fixed by 10% formalin, and then the tissue passage was executed. At the end of this process, samples staining was performed adopting H&E method and was examined for histopathological changes. To this end, the samples were appraised with a light microscope for measuring the diameter of granulosa cells and follicles.

Evaluation of FSHR and LHCGR and ER Gene Expression in Ovarian Tissue With RT-PCR Method

The left ovaries were used to examine the gene expression of FSHR, LHCGR, and ER. To preserve the RNA integrity, the ovaries were immediately frozen in the liquid nitrogen at a temperature of -196°C. RNA extraction from the ovarian tissue was then performed using a commercially available kit following the protocol provided by Thermo Scientific (Waltham, MA). The extracted RNA was quantified using a NanoDrop spectrophotometer to determine the concentration. After the quantification, complementary DNA (cDNA) synthesis was carried out using the extracted RNA. The RNA concentration used for cDNA synthesis was determined to be 500 ng/mL (8, 11) (Table 1).

Real-Rime RT-PCR

The quantitative polymerase chain reaction (qPCR) experiments were conducted using the Applied Biosystems

Table 1. Primer Sequence Used for Expression Analysis of Genes

Genes	Primer
FSHR	Forward: 5'-ATGCTGACGATGCTGTGAAC-3' Reverse: 5'-TCAGGTGCCAGTTTCCATGT-3'
LHCGR	Forward: 5'-TGGTCCTGTGGGTTACATCG-3' Reverse: 5'-GGCAGGCTGTTGAAGTGTAG-3'
ER	Forward: 5'-GGCTGCTTTCTCTCCAGTCT-3' Reverse: 5'-GGTGACACAGAGGTCCTGGA-3'
GADPH	Forward: ATGGAGAAGGCTGGGGCTCACCT Reverse: GCCCTTCCACGATGCCAAAGTTGT

7500 Fast Real-Time PCR System. Each reaction was performed in a 48-well plate with a total volume of 20 µL. The reaction mixture consisted of 1 µL of complementary DNA (cDNA), 2 µL of a primer mix containing forward and reverse primers, 7 µL of deionized water, and 10 µL of a SYBR Green PCR Master Mix (12).

The cycling and melting conditions for the qPCR were as follows:

- Initial denaturation: 1 cycle at 95 °C for 10 minutes;
- Amplification: 40 cycles at 95 °C for 15 seconds, 58°C for 30 seconds, and 72 °C for 30 seconds;
- Melting curve analysis: 1 cycle at 95 °C for 15 seconds, 60 °C for 60 seconds, and 95 °C for 15 seconds.

At the end of the qPCR, the quantitative analysis was performed using the Pfaffl method. This method calculates the expression ratios as $2^{-\Delta\Delta Ct}$, ΔΔCt = ΔCt Sample-ΔCt Control, where ΔCT represents the difference in threshold cycle (CT) values between the target gene and the reference gene. This calculation allows for the relative quantification of the gene expression levels of the target gene compared to the reference gene (13-15).

Measurement of Testosterone, LH, and FSH Levels in the Serum Samples

Plasma hormone levels of testosterone and estrogen were assessed using an enzyme-linked immunosorbent assay (ELISA) kit from Demeditec Diagnostics, Germany. Additionally, the ELISA kit from ZellBio, Italy, was utilized to analyze the plasma concentrations of LH and FSH. ELISA is a widely employed scientific technique involving the application of specific antibodies to detect and quantify target hormones or proteins in biological samples. The ELISA kits used in this investigation utilize antibodies that specifically bind to the hormones of interest. This binding interaction facilitates the detection and quantification of the hormones by employing colorimetric or fluorescent detection techniques. These ELISA assays provide sensitive and reliable measurements of the hormone levels, and are extensively utilized in both research and clinical settings for hormone analysis.

Results

Histological Result

The diameter of preantral follicles was significantly declined in TD group compared to other groups ($P<0.05$). Also, the diameter of follicle was enhanced in therapeutic groups after the treatment with eugenol ($P<0.05$). A reduction was observed in TD group, compared to other groups, regarding the measurement of diameter of Graafian follicles ($P<0.05$). Furthermore, an increase was detected in groups treated with eugenol regarding the diameter of Graafian follicles (Figure 1).

The Expression of FSHR, LHCGR and ER Genes

The expression of FSH receptor genes was declined in TD group in contrast to sham group. On the other hand, the groups receiving eugenol exhibited a higher ratio of follicle-stimulating hormone receptor (FSHR) gene expression than the control (TD) group ($P<0.05$). The expression of LHCGR genes associated with the LH hormone receptor was significantly decreased in torsion/detorsion group, while it was upregulated in groups treated with 30 mg/kg of eugenol in contrast to TD group ($P<0.05$). The torsion/detorsion caused downregulation of the ER gene expression in TD group in contrast to sham group ($P<0.05$), whereas it resulted in upregulation of ER gene expression in groups treated with eugenol (Table 2).

Hormonal Assessment

The level of FSH and LH as gonadotropin hormones in plasma was decreased in TD group in contrast to sham group, while the level of these hormones in groups

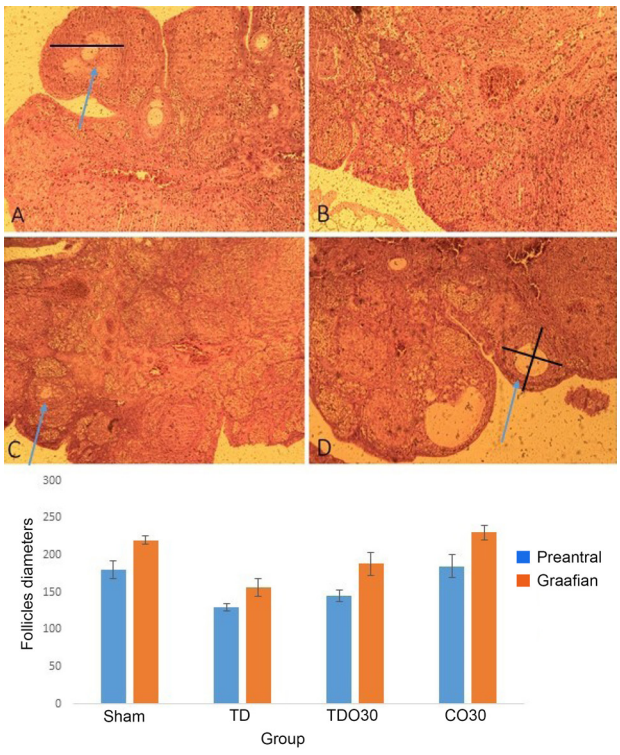

Figure 1. Histological Finding and Follicles Diameters. A: Sham group; B: Torsion/detorsion group; C: Torsion/detorsion+ eugenol group; D: healthy group treated with eugenol. The blue arrow indicates the follicles. The black line shows the diameter of the follicles.

Table 2. Expression of FSHR, LHCGR, and ER Genes

Group	FSHR Mean ± SD	LHGR Mean ± SD ± SD	ER Mean ± SD ± SD
Sham	1.00±0.09	1.02±0.06	1.00±0.09
TD	0.34±0.012	0.38±0.05*	0.32±0.07*
TDO30	0.58±0.06[b,a]	0.62±0.05 #*	0.68±0.06 #*
CO30	1.05±0.07	0.98±0.01	1.13±0.08

[a] Indicates significance compared to the sham group; [b]Indicates significance compared to the TD group.

Table 3. Results of Hormone Profile

Group	Estrogen Mean ± SD	Testosterone Mean ± SD	FSH Mean ± SD	LH Mean ± SD
Sham	45.98±2.09	1.02±0.6	4.80±0.10	3.09±0.09
TD	24.55±2.12	1.88±0.5	1.85±0.73	1.030±0.07
TDO30	33.06±1.95	1.30±0.5	3.12±0.11	2.18±0.06
CO30	47.43±0.75	0.98±0.01	5.38±0.18	3.23±0.08

treated with eugenol was higher than that in TD group. The serum concentration of estrogen hormone in TD group was significantly lower than that in sham group. The concentration of estrogen hormone was significantly higher in the therapeutic group than that in the control (TD) group. On the other hand, the level of testosterone hormone was significantly elevated in TD group in contrast to sham group. Furthermore, the concentration of testosterone was significantly declined in group treated with dose of eugenol (30 mg/kg) in contrast to TD group (Table 3).

Discussion

One of the critical factors influencing female fertility is the process of folliculogenesis, which involves the transformation of primordial follicles into Graafian follicles culminating in ovulation. The folliculogenesis process can be described as follows: the size of the follicles increases after the selection of follicles for growth, leading to the formation of granulosa cells and the growth of oocytes. When the follicles consist of 3-6 granulosa cells, the connective tissue surrounding the follicle differentiates into two layers: the external layer known as the ovarian stroma or external theca, and the internal layer known as the internal theca. The cells in the internal theca differentiate into epithelioid cells. Prior to the preantral stage of folliculogenesis, a significant number of developing follicles undergo atresia, a process associated with apoptosis. Therefore, folliculogenesis can be divided into two steps: an independent gonadotropin preantral step that starts with oocyte growth and differentiation, and a gonadotropin-dependent step involving follicle development. The gonadotropin-dependent step is regulated by FSH and LH, along with growth factors that stimulate cell division and modulate the activity of gonadotropins (16,17).

Following the growth and development step of the follicles, influenced by FSH and LH, the resumption of follicle growth occurs through the interaction of FSH and LH with their specific receptors located on the surface of granulosa cells. These hormones stimulate mitosis and the resumption of granulosa cell differentiation. LH and FSH also specifically stimulate the production of estradiol in mature follicles. Moreover, the increase in FSH secretion towards the end of the luteal phase results in the selection of the Graafian follicle, while the absence of FSH causes non-ovulation (18,19).

One of the crucial factors during the folliculogenesis process is follicular atresia. Several studies have demonstrated that atresia of preantral follicles is induced by testosterone hormone. Testosterone, based on histological assessments, disrupts the granulosa cells and oocytes in preantral follicles. Although maintaining the developmental and growth processes relies on estrogen, the functional mechanisms of testosterone involve reducing the estrogen receptor in granulosa cells. This phenomenon is likely related to the response of the theca cells and second intermediate cells surrounding the preantral follicles to low levels of LH, leading to an increase in the testosterone secretion. Testosterone binds to its specific receptors, resulting in a reduction of estrogen receptors and causing preantral granulosa cells to be unresponsive to estrogen stimulation and ultimately leading to follicular atresia (20,21).

After the initial growth and development phase of the follicles, which is influenced by FSH and LH, the resumption of follicle growth occurs due to the interaction of FSH and LH with their respective receptors located on the surface of granulosa cells. These hormones stimulate mitosis and prompt granulosa cell differentiation to resume. Furthermore, LH and FSH play a specific role in stimulating the production of estradiol in mature follicles.

Towards the end of the luteal phase, there is an increase in FSH secretion, leading to the selection of the

Graafian follicle. Conversely, the absence of FSH results in the non-occurrence of ovulation (22-24). Indeed, the underlying mechanism of follicular atresia is closely tied to LH-induced alterations in both the theca and granulosa cells, coupled with the production of androgens. The key function of these androgens is modulated by LH's interactions with the cells, thereby playing a significant role in promoting the process of follicular atresia (25-27).

Gonadotrophin hormones (LH, FSH) act as the most important hormones in folliculogenesis process. In effect, FSH is known as initiator of folliculogenesis process. This pathway is: FSH stimulated by gonadotropin-releasing hormone (GnRH) is released from anterior pituitary gland. Upon binding of FSH to its specific receptor on growing follicles, the receptor forms a complex with the target cell membrane. This interaction triggers the activation of adenylate cyclase, an enzyme responsible for converting adenosine triphosphate (ATP) into cAMP. As a consequence, the cellular cAMP levels increase, leading to an upregulation of protein kinase activity. Protein kinases are crucial enzymes involved in various cellular signaling pathways, and their heightened activity plays a significant role in regulating the growth and development of follicles during the folliculogenesis process. Afterward, it facilitates the steroidogenesis activation at the end create luteal phase. The activities of FSH and estrogen are synergic, and they have a simulative effect on mitosis of granulose cells. The development of follicles is influenced by gonadotropin hormones, which affects the granulosa cells and modifies the function of their receptors. This coincides with the development of follicles and leads to an increase in hormone receptors. FSH, in particular, has a specific receptor on granulosa cells and can convert low levels of androgens into estrogens within the preantral follicles, creating an estrogenic environment. The elevated levels of FSH and estrogen contribute to an increase in FSH receptor expression. The early secretion of estrogen within the follicle enables it to respond to low concentrations of FSH through an autocrine response. It is noteworthy that not all granulosa cells have the required receptors for gonadotropins. Stimulation of the cells is transferred intracellularly via gap junctions, resulting in the activation of protein kinases. Additionally, FSH facilitates the aromatization of androgens (27,28). The increase in estrogen secretion triggers the secretion of LH. LH binds to its specific receptor and affects theca cells, ultimately leading to ovulation. Moreover, the increased secretion of LH results in a shift in the performance of theca and intermediate cells, transitioning from the production of androgens to progesterone-secreting cells. This transition is necessary for the subsequent processes involved in fertility (19,28).

Disturbances in the folliculogenesis pathway can have detrimental effects on fertility (e.g., ovarian torsion and subsequent detorsion), which can cause injury to the follicles and disrupt hormonal pathways and follicular development. One of the primary factors contributing to the development of follicular atresia is the influence of LH on the theca and intermediate cells, leading to the production of androgens. The gonadotropin hormones LH and FSH play crucial roles in the folliculogenesis process. FSH is particularly important as it initiates the process. The pathway begins with the release of GnRH from the anterior pituitary gland, which stimulates the secretion of FSH. FSH then binds to specific receptors on growing follicles, activating adenylate cyclase and increasing intracellular levels of cyclic adenosine monophosphate (cAMP). This, in turn, triggers steroidogenesis and establishes the luteal phase. FSH and estrogen exhibit synergistic effects, promoting the mitosis of granulosa cells. During follicle development, the gonadotropin hormones influence granulosa cells, leading to changes in receptor function that align with follicle development and an increase in hormone receptors. Granulosa cells possess a specific receptor for FSH, enabling them to convert low concentrations of androgens in preantral follicles into estrogens, thereby creating an estrogenic environment. Elevated levels of FSH and estrogen further enhance the expression of FSH receptors. Early secretion of estrogen within the follicle allows for an autocrine response to low concentrations of FSH. However, not all granulosa cells possess receptors for gonadotropins. Nevertheless, cell stimulation is communicated intracellularly through gap junctions, resulting in the activation of protein kinases. Additionally, FSH plays a role in the aromatization of androgens. Overall, disruptions in the folliculogenesis pathway can lead to fertility issues, and understanding the intricate interactions and influences of hormones is crucial for reproductive health (18,6,29).

The secretion of estrogen increases LH hormone secretion, and the binding of LH to its specific receptor affects theca cells, ultimately leading to ovulation. Furthermore, the increased secretion of LH causes theca and intermediate cells to transition from androgen-producing cells to progesterone-secreting cells, which is necessary for subsequent fertility processes.

Disturbances in the folliculogenesis pathway, such as those caused by ovarian torsion followed by detorsion, can result in follicular injury, disruptions in the hormonal pathway, and impaired follicular development. These disruptions have significant implications for fertility.

The results of the current study indicated a significant decrease in the number of follicles, as well as a marked reduction in follicular diameter, granulosa cell thickness, and oocyte diameter in the TD group in contrast to the sham group. This decline in follicular parameters may have been attributed to the ovary ischemia and a reduced blood supply. The process of reperfusion can activate the free radicals and increase the ROS, leading to histological damage, granulosa cell destruction, and follicular degeneration.

Evaluation of hormone levels in the TD group revealed

a considerable decrease in LH, FSH, and estrogen levels in contrast to the sham group. Conversely, the level of testosterone hormone increased in the TD group. This elevation in testosterone may have been due to the increased ROS activity, which induces oxidative stress and leads to a decline in estrogen levels. The increase in testosterone may also be associated with a decrease in estrogen receptors. Measurement of the serum aromatase levels demonstrated a decline in TD group in contrast to the sham group, which contributed to the attenuation of estrogen hormone levels. This decrease in aromatase levels resulted in a reduction in the conversion of androgens (e.g., testosterone to estradiol), decreasing the estradiol levels and increasing testosterone levels. Furthermore, the lower level of aromatase may have been associated with the apoptosis in granulosa cells, leading to a decreased aromatase secretion and an impaired aromatase function (19,24,28).

Assessment of LHCGR, FSHR, and ER gene expression, which are related to LH, FSH, and estrogen receptors, revealed a significant decrease in their expression in the TD group in contrast to the sham group. This reduction in receptor gene expression corresponds to a decrease in the number of hormone receptors on granulosa and theca cells. Consequently, the impaired hormone receptor function prevents proper hormonal performance and hampers the growth and development of follicles. The disturbance in hormonal performance, combined with elevated testosterone levels, reduced aromatase activity, and increased oxidative stress leads to estrogen receptor occupancy and inhibition of hormonal effects on follicles. As a result, follicles fail to grow and undergo degeneration, ultimately leading to fertility issues. Furthermore, the reduced expression of LH and FSH receptors, along with the destruction of their receptors on granulosa cells, is associated with a decreased hormone binding and a subsequent decrease in hormone secretion from the pituitary gland due to the negative feedback. Previous studies have shown that ovarian torsion followed by detorsion decreases the estrogen, FSH, and LH levels as well as increases the testosterone levels. Similarly, other studies have demonstrated that an increased oxidative stress results in decreased levels of aromatase, estrogen, LH, and FSH hormones, accompanied by elevated testosterone levels. These findings were in line with the results of our study, highlighting the impact of oxidative stress and hormone disruption on follicular development and fertility (10).

Conclusions

In sum, the ovarian torsion and detorsion had detrimental effects on folliculogenesis and fertility in female rats. The ischemic conditions during torsion and the subsequent reperfusion phase decreased the number of follicles, histological damage, and disrupted hormonal profiles. The decrease in LH, FSH, and estrogen levels as well as the increase in testosterone levels may have been attributed to the oxidative stress, reduced aromatase activity, and altered expression of hormone receptors. These factors were found to contribute to the inhibition of follicular growth, granulosa cell destruction, and follicular degeneration. Our study findings highlighted the importance of maintaining proper hormonal balance and minimizing oxidative stress for optimal folliculogenesis and female reproductive health. It was recommended that further studies should be conducted in order to explore the potential interventions aimed at mitigating the adverse effects of ovarian torsion on fertility outcomes.

Limitations of the study

- Failure to investigate the expression of proteins by immunohistochemistry and western blot.
- Failure to check fertility by IUI method due to budget limitations.

Authors' Contribution

Conceptualization: Ramesh Baradaran Bagheri.
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Formal analysis: Ramesh Baradaran Bagheri, Seyedeh Sara Salami, Linda Mohammadzadeh Boukani and Amir Afshin Khaki.
Funding acquisition: Amir Afshin Khaki.
Investigation: Amir Afshin Khaki and Seyedeh Sara Salami.
Methodology: Ramesh Baradaran Bagheri, Seyedeh Sara Salami, Linda Mohammadzadeh Boukani and Amir Afshin Khaki.
Project administration: Amir Afshin Khaki.
Resources: Amir Afshin Khaki.
Software: Ramesh Baradaran Bagheri.
Supervision: Amir Afshin Khaki.
Validation: Ramesh Baradaran Bagheri.
Visualization: Ramesh Baradaran Bagheri.
Writing–original draft: Ramesh Baradaran Bagheri.
Writing–review & editing: Ramesh Baradaran Bagheri, Seyedeh Sara Salami, Linda Mohammadzadeh Boukani and Amir Afshin Khaki.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

This experimental study was approved by the Ethical Committee of Tabriz University of Medical Science under the ethical code IR.TBZMED.VCR.REC.1398.095.

Financial Support

This study was funded by Tabriz University of Medical Sciences.

Acknowledgments

The authors would like to thank the staff in Tabriz University of Medical Sciences and Clinical Research Development Unit of Tabriz Valiasr Hospital, Tabriz University of Medical Sciences, Tabriz, Iran for their assistance in this research.

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