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Experimental Research

Histological and biochemical apoptosis changes of female rats′ ovary by Zinc oxide nanoparticles and potential protective effects of l-arginine: An experimental study

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ABSTRACT

Background: This research aims to investigate the adverse effects of ZnO NP on ovarian tissue and the follicular and menstrual cycle and the protective effects of L-arginine on the aforementioned tissues.
Material and methods: 30 rats were divided into five groups. The first group was the control group. The second and fourth groups received 100 mg/kg and 200 mg/kg ZnO NP, respectively. The third and fifth groups received the same doses of ZnO NP as the second and fourth groups, respectively. However, the third and fifth groups received an additional dose of 1.3 gr/kg of LA amino acid. ZnO NP and LA are given intraperitoneal for 21 days. Blood samples from each rat and a part of the ovarium were collected to test for gene expression and histological analysis.
Results: Compared to levels of housekeeping gene β-actine, levels of apoptosis effectors such as Bax, Bcl, Caspase 3, and Caspase 9 were significantly increased in all groups. In groups that received doses of LA (three and five), atretic follicle size was smaller compared to groups that did not receive LA (two and four). In addition, in the third group, the secondary and primordial follicle's generated oocytes were smaller compared with groups two, four, and five. Compared with the control group, all groups experienced morphological degeneration of follicles and tissue.
Conclusion: ZnO NP has inevitable, morphological, and physiological effects on the ovary and can detrimentally impact the tissue. LA can aid in the regeneration of the tissue and block damage induced by stress and toxicity.

1. Introduction

Over the past decade, the ability to engineer and produce nanomaterials has been a driving factor behind the rapid product development and importance of nanotechnology. As a result, the use of nanomaterials has become a significant part of daily life [1]. Nanoparticles (NP) are beneficial for human life due to their interesting properties and easy usability. Nanotechnology has hence started to become rapidly ingrained into everyday life. Nanoparticles and nanomaterials can be found in many materials, such as glass, cement, pigments, plastics, and paints [2]. Compared to the corresponding bulk materials, NP with identical characteristics less than 100 nm in size typically have a large surface area to capacity ratio and distinctive scaling properties [3].

ZnO NP powders have wide usage in everyday products, such as food packaging and additives; cosmetic sunscreens, body oils, and ointments; and in UV absorptive and reflective bioimaging [4]. Different sizes of NP (1 nm–100 nm) have different effects on the human body. NP exposure may occur orally via inhalation, dermal exposure, or gastrointestinal tract absorption. Smaller-sized NP are better able to quickly pass through the skin pores, cell membranes, and barriers [5]. The impact of ZnO NP exposure is relatively rapid. Brunner et al. found that a three-day exposure to ZnO NP 19 nm in size caused mitochondrial and DNA damage in human mesothelioma and rodent fibroblast cells. NP with sizes from 35 to 50 nm may be more easily able to go through cellular uptake, and therefore better able to interact with internal or

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membrane molecules. Consequently, they are able to induce cytotoxic and genotoxic effects on embryonic cells, adenocarcinoma cells, and oocytes [6]. ZnO NP induce apoptosis, change cell morphology, disturb the cell cycle distribution, increase oxidative stress and ROS generation, and decrease mitochondrial membrane potential and function [7]. Landsiedel et al. found that ZnO NP are responsible for inducing inflammatory responses and necrosis [8].

This study focuses on the effects of ZnO NP on the ovary and reproductive organs of the female rat, an area that has not been addressed in the previous literature. Liu et al. found that exposure to ZnO NP (ZnSO4-200 mg/kg and ZnO-NP-200 mg/kg in diet) in hens could increase levels of γ-H2AX and decrease levels of NF-κB, thereby decreasing cell proliferation and increasing apoptosis in embryos [9].

In the present study, the effects of ZnO NP alone and in combination with the supplement L-arginine were examined in each rat's ovary, uterus, vagina, and cervix. The LA supplement was used to examine the effect of nitric oxide (NO) on the ovary. LA was used as it is a precursor to NO, which is formed via the enzymatic activity of nitric oxide synthesis (NOS). NO was examined as it is known to be effective for the regeneration of tissue [10].

2. Material and methods

In this experimental research, ZnO NP (10–30 nm) were ordered from Sigma Aldrich. NP were dissolved in distilled water; 1,260 gr/ml in 50 wt % dH2O. The solution was placed on a shaker overnight at room temperature. L-Arginine (99%) powder was also ordered from Sigma Aldrich. The powder was dissolved in a 100 mL mix solution and placed on the shaker overnight at room temperature.

3. Animals and study design

In this study, 30 healthy, female Wistar Albino rats that were, on average, three weeks old and weighed 170–200 g were used. The animals were housed in identical standards. Plastic, transparent, laboratory animal cages were used and were kept at room temperature 25 °C ± 2 °C with 70% relative humidity and ventilation, on a 12-h light/dark cycle, with ad libitum feedings for 21 days.

The animals were divided into five groups, with each group containing six animals:

G1 - bi-distilled water injection intraperitoneally daily for 21 days.
G2 - 100 mg/kg ZnO NP injection intraperitoneally daily for 21 days.
G3 - 100 mg/kg ZnO NP and 1.3 g/kg L-arginine injection intraperitoneally daily for 21 days.
G4 - 200 mg/kg ZnO NP injection intraperitoneally daily for 21 days.
G5 - 200 mg/kg ZnO NP and 1.3 g/kg L-arginine injection intraperitoneally daily for 21 days.

3.1. Sample collection

After the 21 day treatment, all rats were sacrificed during the proestrus-oestrus phase, using Xylacin® 10 mg/kg intramuscular (IM) and Ketamin® 90 mg/kg IM as anesthesia. From each rat, the left ovary and cervix were removed and placed into 10% formaldehyde solution for histological observations. The right ovary was also removed and placed immediately into ice-cold water before being stored at −80 °C until it was used to analyze gene expression.

3.2. Histological analyses

For microscopic observation, fixed tissues in 10% formaldehyde solution were used. Processed tissue standard protocol was employed for the ovary tissues, using the Leica TP1020. Tissue sections were embedded in 5 mm thick paraffin via rotary microtome Leica RM2125 RTS. Hematoxylin-eosin staining was performed using Mayer's Hematoxylin & Eosin staining kit (Labvision: TA-125-MH). Masson's trichrome stains were performed using the Masson-Goldner staining kit (10,048 Sigma Aldrich). Periodic acid–Schiff (PAS) stains were performed using the PAS staining kit (PAS5-100TSigma Aldrich). The tissue sections were viewed under a light microscope (ZEEİSYJ-2009P, Japan).

3.3. Hormonal analysis

For hormonal observation, blood samples (2 mL)were collected from each rat via cardiac puncture after they had been sacrificed. After centrifugation, the serum was removed from each tube and, using the instructions of ELISA Progesterone Assay Kit (Bt-laboratory: YHB0673 China), the levels of the progesterone hormone were measured in each sample.

3.4. RNA extraction and determination of gene expression in ovary using real-time RT-PCR reaction

Total RNA extraction and gene expression from 30 ovarian tissue samples was performed using an ABM Cat. No. G487 ultRNA Column Purification Kit according to the manufacturer's instructors. UV spectroscopy (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific) was used to measure the quality and quantity of isolated mRNA. Each treatment was repeated a minimum of three times (Table 1). Primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). For gene expression analysis, the primers detailed in Table 1 were used.

After extraction of RNA and the synthesis of cDNA with the One Script Plus cDNA Synthesis Kit (Cat. No. G236), a One-Step RT-PCR was performed using RT-PCR Bio-Rad (Bio-Rad CFX96, USA) and a Real-Time First-Strand Kit (ABM Cat. No. G487). Each PCR was performed three times. The RT-PCR reaction system followed a set protocol: 20 μl, reaction conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 60 s. With β-actin as an endogenous control, expression levels of Caspase-3, Bax, and Bcl-2 were calculated using automatic output by RT-PCR.

Comparative CT (Comparative Threshold Cycles – ΔΔCT) analysis was used to quantify the synthesis levels of the target genes. 2- ΔΔCT was calculated using ΔΔCT values, which were obtained by determining the difference in the synthesis levels of target genes and the housekeeping gene β-actin.

3.5. Ethics

The experimental design adhered to the set guidelines and was approved by the Animal Ethics Committee.

3.6. Statistical analysis

The obtained results were evaluated and compared with corresponding statistical parameters, such as the mean ± standard deviation, for each group of rats (n = 6). SPSS program Version 20.0 (SPSS Inc.,

Table 1
The specified primers for qRT PCR.

Caspase-3	F: 5′-GTGGAACTGACGATGATATGGC-3′ R: 5′-CGCAAAGTGACTGGATGAACC-3′
Bax	F: 5′-CGGCGAATTGGAGATGAACTGG-3′ R: 5′-CTAGCAAAGTAGAAGAGGGCAACC-3′
Bcl-2	F: 5′-TGTGGATGACTGACTACCTGAACC-3′ R: 5′-CAGCCAGGAGAAATCAAACAGAGG-3′
Caspase-9	F:5′-GAATTCCAGCAATCCGCTAGCCATGGAGG-3′ R: 5′-GAATTCAACTCATGAAGTTTAAAGAACAG-3′
β-actin housekeeping gene	F: 5′-AAGATCCTGACCGAGCGTGG-3′ R: 5′-CAGCACTGTGTTGGCATAGAGG-3

Chicago, IL, USA) was used for statistical analysis. Biochemical P levels and histological follicle enumeration and proliferation of the 30 rats were calculated using a one-way ANOVA with Bonferroni correction. In addition, a Pearson's correlation (two-tailed) test was performed to analyze the correlation between parameters. A confidence interval above 95% was accepted, and values with different superscripts were accepted as being significantly different ($P < 0.05$ or 0.01).

4. Results

4.1. Histological observation

The results of the Hematoxylin-eosin (H&E), Masson's trichrome (MT), and Periodic acid–Schiff (PAS) stainings in the control group show normal development of the tissue, with normal presentation of the cortex and medulla on the peripheral region and normal follicular sizes and numbers, at diverse maturation stages. Toward the periphery of the tissue, an easy and fine-lined germinal epithelial layer was observed (Fig. 1). Fig. 1d shows a secondary follicle with a smooth, bright basal lamina, along with the zona pellucida, theca interna, and theca externa. Germ cells within the antrum space are also visible within the inner layer of the cell. The number of the atretic follicles was considered acceptable.

Group 2 was treated with intraperitoneal injections of 100 mg/kg ZnO NP, resulting in an increasing number of corpus luteum and fibrosis observed after MT staining (Fig. 2a). A large number of atretic follicles with pyknotic cells in the cumulus oophersus was observed. The number of healthy follicles decreased as the nucleolus became increasingly damaged. Degeneration of the primary oocyte can be seen in Fig. 2c. Compared with the control group, a significant increase in epithelial disruptions was observed in group 4, with several epithelial disruptions and hyperplasia of the endometrial glands visible. A decrease in the size of the corpus luteum, development of follicular cysts, and an increase in follicular atresia were observed in group 4. Additionally, granulosa cells in group 4 were not cuboidal, instead displaying an interesting spherical shape. Compared with the control group, the granulosa cells were pale and contracted, with many apoptotic cells visible in the granulosa cell layer (Fig. 2d). An absence of the corpus luteum and degeneration of the follicular unit was observed in group 4 after H&E staining was performed. Fibrous tissue proliferation was not observed. The absence of large follicles, such as secondary and Graafian follicles, was significantly increased in group 4. Tissue necrosis was also observed in group 4. Layers of theca interna and theca externa were not possible to identify (Fig. 2e and f).

PAS staining of tissue from group 3 shows several atretic cells and pyknotic cells with decreased fibrosis (Fig. 3a). Disturbance of follicular development was present, and compared to the control group, the zona pellucida was thinner. Follicular degradation was significant. The contracture of fibrosis was similar to that seen in the control group. Theca cell layer, with fine formation of cuboidal and polygonal granulosa cells, was observed in the developed follicle (Fig. 3b and c). Staining of tissue from group 5 displayed similar results to those observed in group 4. Follulicar degeneration was observed, as was zona pellucida separation from the oocyte. However, follicular diversity within the cortex was not significantly different compared with group 4 (Fig. 3d and e). There were not many small follicles as primordial and

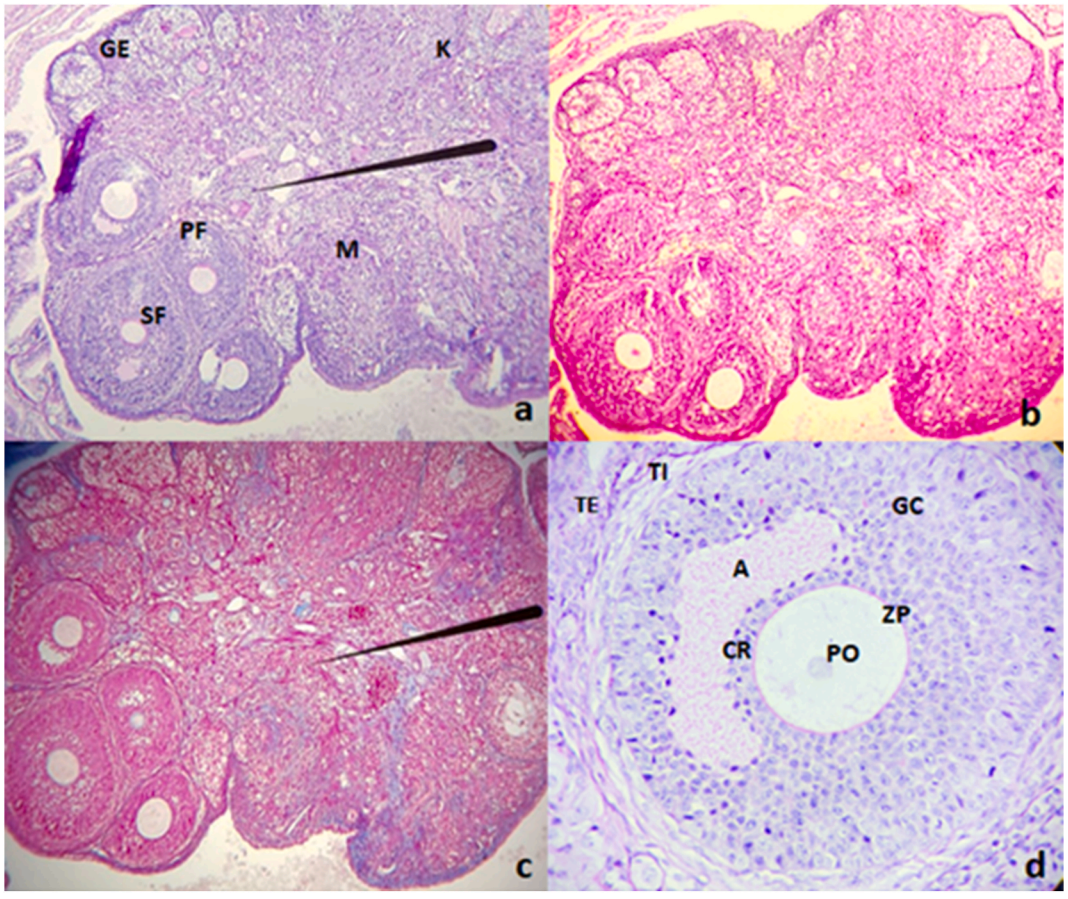

Fig. 1. Four different stainings of control group tissues: a) PAS staining under 10X, the germinal epithelial layer (GE), cortex (K), medulla (M), secondary follicle (SF), and primary follicle (PF) are visible; b) H&E staining under 10X; c) MT staining under 10X, the middle medulla, cortex layer, secondary and primary follicles are visible, and the tissue is covered with germinal epithelial layer; d) PAS staining under 40X, the secondary follicle with primary oocyte (PO), zona pellucida (ZP), antrum space (A), inner corona radiata (CR) and with granulosa cells (GC) surrounded by the theca interna (TI) and externa (TE) are visible.

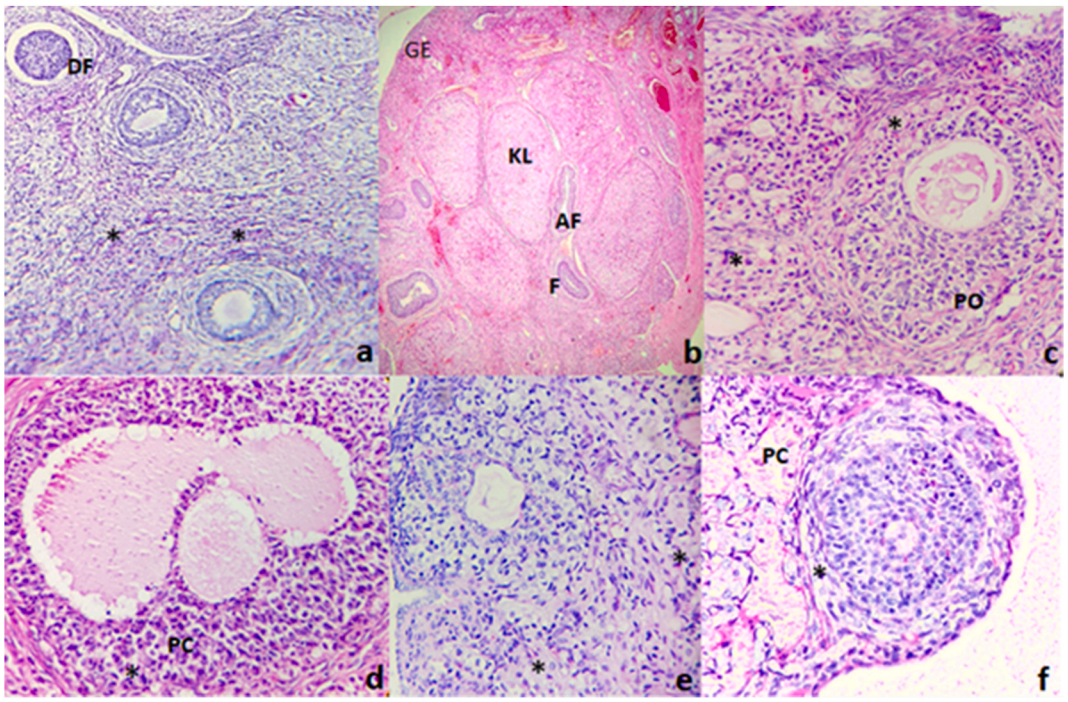

Fig. 2. Stainings of group two tissues: a) PAS staining under 40X, degeneration of interstitial cells and (DF) and degeneration of the primary follicle with numerous apoptotic cells (*) in medulla layer are visible; b) H&E staining under 20X, disrupted germinal epithelia (GE), multiple corpus luteum follicles (KL), and several atretic follicles (AF) are visible; c) H&E staining under 40X, a degenerative primary follicle (PO) and pyknotic cells (*) are visible. Stainings of group four tissues: d) H&E staining under 40X, vacuoles in secondary follicles with pyknotic cells are visible; e-f) PAS staining, vacuolization in ooplasm, large pyknotic cells, necrotic nuclei, and uncharacteristic layers of zona pellucida are visible.

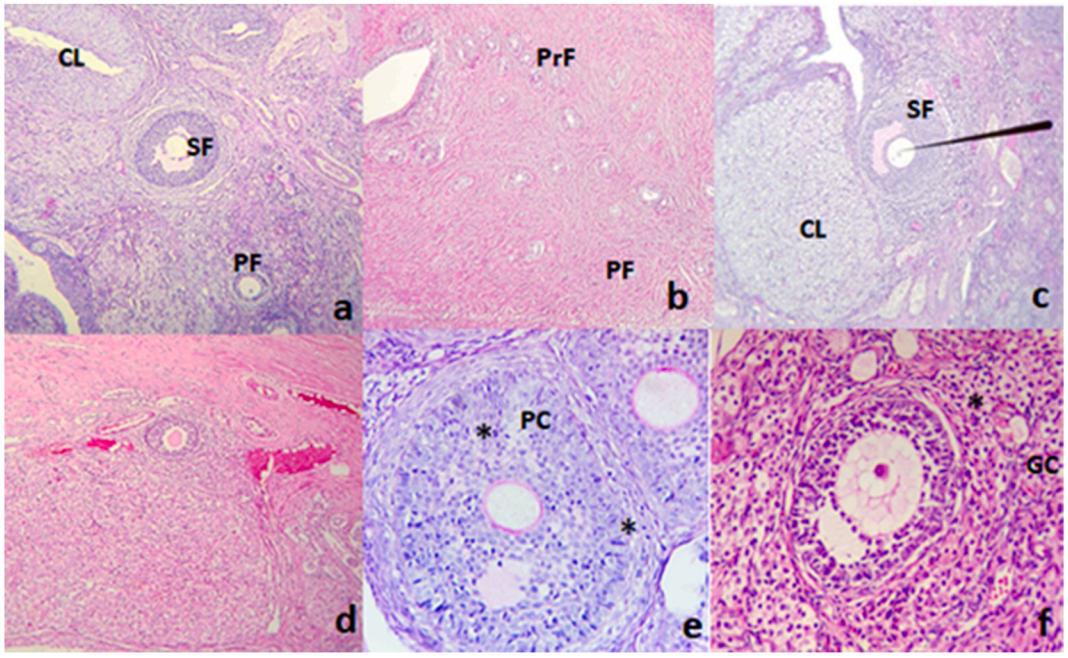

Fig. 3. Stainings of group three tissues: a) PAS staining under 20X, the corpus lutea (CL), secondary (SF) and primary follicles (PF) are visible; b) H&E staining, regenerated tissue with an increased number of primordial follicles (PrF) is visible; c) A secondary follicle (SF) with large corpus lutea (CL) surrounded by epithelia is visible. Stainings of group five tissues: d) H&E staining, several primary and primordial follicles are visible; e) A secondary follicle with fine-lined theca interna and theca externa, and several pyknotic cells within the granulosa cell area are visible; f) MT staining, a secondary follicle with a significant amount of granulosa autophagy is visible.

primer follicles. As in group 4, the absence of corpus luteum was observed in group 5. An increase in the presence of vacuoles and apoptotic cells was observed in the secondary follicles (Fig. 3f).

4.2. Hormonal analysis

Comparing the ELISA results of hormonal progesterone levels of the

control group with groups 2 and 4, who were injected with ZnO NP, a significant decrease in the level of progesterone during the proestrus oestrus phase can be seen (Table 2). In groups 3 and 5, which received injections of ZnO NP and injections of LA, a decreased level of progesterone was also seen compared to the control group. However, groups 3 and 5 had higher levels of progesterone compared to groups 2 and 4 that did not receive the LA supplement. Therefore, in comparing groups 2 and 4 to groups 3 and 5, a *P*-value of $p < 0.05$ is accepted as statistically significant. In comparing the control group to and groups 2, 3, 4, and 5, a *P*-value of $p < 0.01$ is accepted as statistically significant.

4.3. Real-time RT-PCR results

This study examined the expression levels of apoptosis-related genes in rat ovarian tissue. Using a one-way ANOVA with Bonferroni correction, a significant difference ($p < 0.02$) was observed between the expression levels of the apoptosis genes and the expression level of the β -actin housekeeping gene across Groups 2, 3, 4, and 5. The largest expression level difference between the apoptosis genes and the housekeeping gene was seen in the Bax gene ($p < 0.03$). The largest increase in expression of the Bax genes was seen in group 4.

The Bcl-2 gene had the next largest difference in expression level when compared to β-actin levels. Results demonstrated that the expression of caspase-9, caspase-3, Bcl, and Bax mRNA was significantly increased in group 4. Conversely, in the groups that received LA injections [3, 5], expression levels of the target genes decreased compared to groups 2 and 4, which only received ZnO NP injections (Fig. 4).

5. Discussion

Research on the toxicity of ZnO NP using animal experiments as a research model has shown that ZnO NP have various effects on different organs [11]. Additionally, research on the specific effects of ZnO NP exposure on chick oocytes has found that such exposure can lead to several deficits during the developmental stage of chicks [12]. In vitro research has found that ZnO NP penetration in the ovary creates cytotoxicity within cells. Induced DNA damage resulting from high protein expression may also trigger apoptosis in fetal [13]. NP have been found to cause damage during the oogenesis cycle, leading to a depletion of the follicle pool. Smaller sized NP cause more severe damage, as it is easier for them to enter and be absorbed into the cell. Small ZnO NP have displayed characteristics such as smooth binding abilities and the ability to cross [14]. The current study selected small size NP, which were delivered to the animal subjects via intraperitoneal injections, enabling fast results. LA is an α-amino acid that is used in the biosynthesis of proteins. LA is classified as a semi-essential amino acid, depending on an individual's developmental stage and health status. LA plays critical roles in cell division, immune function, wound healing, ammonia removal from the body, and hormone release. It is a precursor for the synthesis of nitric oxide (NO). Additionally, LA may play a crucial role in ovarium blood fluidity regulation, ovarian maturity, follicle development, and protection against damage. LA is also a potential agent to remedy the damage of ZnO NP on ovaries.

In this study, damage in the germinal layer was observed in all experimental groups. This was observed during the histological examination of the ovary with three different morphological staining techniques. Degenerated zona pellucida, pyknotic cells on the basal membrane layer, and uncharacteristic theca interna and theca externa were the most frequently observed types of damage. A significant number of apoptotic cells were recognized at the antral part of the follicle, and follicular atresia was seen in all four groups that received doses of ZnO NP, while primordial and primary follicles were rarely seen. Many cells stayed stable, even as increased ZnO NP exposure increased the cytotoxicity. However, granulosa cells did not stay stable; changes in their deposition within the cytoplasm were observed. Previous research finding indicates that ZnO NP can induce DNA damage in the A431 cell line (human epidermal cells) [15], in human SH-SY5Y neuronal cells, specifically the induction of micronuclei, and disrupt H2AX phosphorylation, which in turn, results in DNA damage and genotoxicity in multiple different cells [16].

In the present study, groups injected with LA only suffered minor damage, with apoptotic cells not being overly present in the ovary tissue. Moreover, deformations on zona pellucida, germinal epithelium and basal lamina were also minimal. However, degeneration of granulosa cells and pyknotic cells increased. In the groups injected with 200 mg/kg of ZnO NP, the largest amount of apoptotic granulosa cells were found in Graafian and secondary follicles. However, following treatment with LA, the tissue damage decreased by 20% overall, including in the more commonly seen secondary follicles [17].

Sacrificing rats in the proestrus oestrus phase meant that the progesterone levels in the rats that received doses of ZnO NP were significantly lower than levels in the control group. The deficiency of activity in granulosa cells was associated with the damage caused by the ZnO NP. This is due to NP ability to cross the blood-brain barrier and enter cells in the hypothalamus and pituitary, where both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are synthesized. This, in turn, inhibits the positive and negative feedback loop of the hypothalamic-pituitary-gonadal axis, thereby affecting the secretion of estradiol and progesterone either partially or completely [18,19]. Previous research has found that prenatal and postnatal ZnO exposure may affect the oogenesis cycle, increase DNA damage, and decrease follicle reserves prior to pregnancy [20]. During the proestrus and oestrus phases, levels of progesterone are at their highest. However, this was not the case in the present study, where the groups injected with ZnO NP showed decreased levels of progesterone compared to the control group. The effect of LA on progesterone levels is mediated by nitric oxide (NO), which is synthesized from LA. The function of NO is stimulated by the pre-pituitary hormones and is regulated in the ovary. NO also plays a role in the regulation of gonadotropin synthesis and plays the primary role in its secretion. The same signal molecule that controls progesterone and estrogen levels is also controlled by NO [21]. These results lend support to the current theory and research, as increased progesterone levels in groups treated with LA were observed.

Histological observation and hormonal examination confirmed the presence of disrupted ovary tissue and increased levels of atretic follicles lacking corpus lutea for increased progesterone circulation. These findings may explain the high level of apoptosis seen in ovary tissues after RT-PRC, where increased levels of apoptotic genes were seen in all experimental groups compared to levels of a housekeeping gene. Levels of apoptotic gene expression were the highest in group 4, followed by group 2, explaining the high level of follicle atresia observed in both groups. Follicle atresia is associated with an imbalance among apoptotic genes such as Bcl 2, Bax, caspase 3, and caspase 9. These proteins play critical roles in ovarian cell death under normal conditions. The expression of Bcl and Bax genes is primarily localized in granulosa cells, which is the primary reason for the frequent absence of pyknotic cells in the granulosa cell area. Tilly et al. found that gonadotropin treatment inhibits apoptosis in granulosa cells and increase follicle atresia by mediating the decreased expression of Bax and Bcl genes. Caspase-3 and caspase-9 have been identified as critical for apoptosis in the oocyte during fetal development, granulosa cell death, and anticancer drug efficacy. Unfortunately, the current research did not determine why the

Table 2
Progesterone levels in rat ovary in proestrus oestrus phases (*$p < 0.05$).

Progesterone	$\bar{x} \mp SD$	F	P
Control Group	29,8767 ∓ 2,10,948	50,131	<0.001*
2. Group	11,8133 ∓ 2,67,448		
3. Group	18,37 ∓ 3,34,123		
4. Group	7,804 ∓ 0,61,113		
5. Group	17,2883 ∓ 3,86,409		

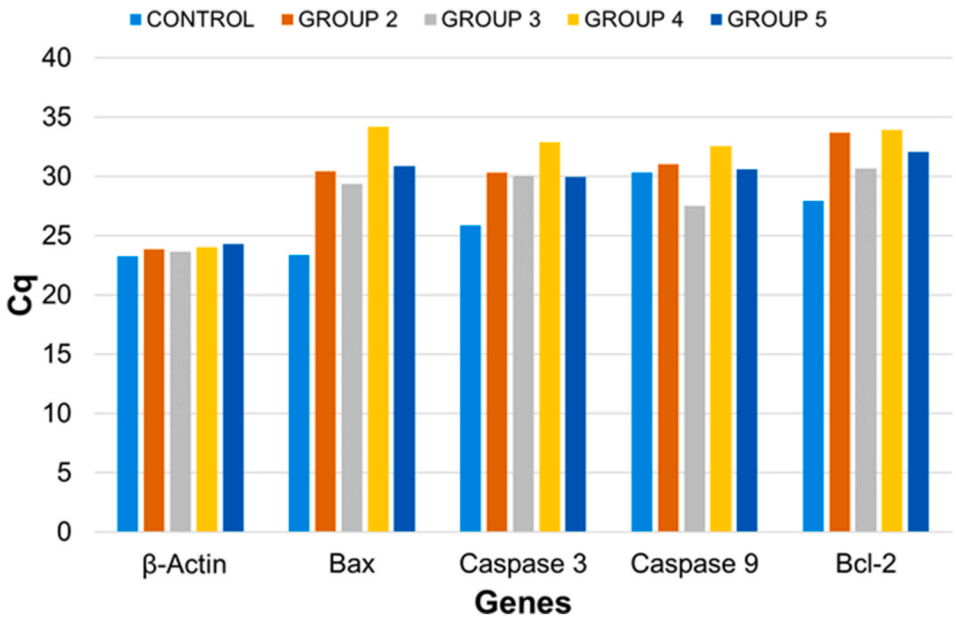

Fig. 4. Expression levels of β-aktin housekeeping gene and Bax, Caspase 3, Caspase 9, and Bcl-2 apoptosis-related genes.

gene expression level of both caspase-3 and caspase-9 was not found to have increased like the other examined apoptotic genes. In all groups treated with LA, the tissue damage and level of apoptosis were decreased. In addition, it was shown that the presence of Bcl, Bax, caspase-3, and caspase-9 are key indicators of damage from ZnO NP in the ovary. The present research has investigated specific pathways of ovarian damage from ZnO NP exposure, explaining programmed and induced cell death by stimulus [22].

ZnO NP can induce significant cytotoxicity, apoptosis, and autophagy in human ovarian cells through reactive oxygen species generation and oxidative stress. In addition, metal NPs can induce oxidative stress within cells, resulting in DNA damage. Therefore, ROS formation and DNA damage in treated human ovarian cancer cells were analyzed to investigate their possible involvement in the induction of DNA damage [23–26].

In some cell types, NO can promote apoptosis, whereas, in other cells, NO inhibits apoptosis. Long-term exposure to nitric oxide in certain conditions like chronic inflammatory states may predispose cells to tumorigenesis through DNA damage, inhibition of DNA repair, alteration in programmed cell death, or activation of proliferative signalling pathways. NO is involved in several aspects of female reproduction, including ovarian follicular development, ovulation, steroidogenesis, and oocyte meiotic maturation [27–29].

6. Conclusion

The current study has demonstrated that ZnO NP can enter multiple cells, including those found in the ovary, and accumulate there. Comparing all observed results, it can be concluded that ZnO NP induce oxidative stress, toxicity, and genotoxicity in ovary tissues. ZnO NP are responsible for decreasing follicular development and follicle atresia and increasing damage to the ovary, apoptosis and apoptotic signalling activation. Using ZnO in daily life may cause a reduction of the follicles within the follicle pool, decrease chances of ovulation, and lead to infertility. LA may be a beneficial daily supplement to prevent damage induced by ZnO and any hormonal imbalance. Further studies are necessary to investigate possible clinical usage in humans.

Ethical Approval

University of Health Science, Committee for research with animal models.

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Author contributions

Fatima Efendic, study design, experiments
Tansel Sapmaz writing data collections
Halime Tuba Canbaz consultation
Halime Hanım Pence consultation
Oktay Irkorucu consultation, data analysis

Trial registry number

1. Name of the registry:
2. Unique Identifying number or registration ID:
3. Hyperlink to your specific registration (must be publicly accessible and will be checked):

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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