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Investigation of the Apoptotic Effect of Levofloxacin on Ovarian Follicles of NMRI Mice and its Anticancer Effect on Human Ovarian Cancer Cell Line

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Abstract

Background: Levofloxacin is a fluoroquinolone antibiotic with extensive anti-bacterial effects. Levofloxacin is widely used for treatment of urinary and vaginal infections.

Objectives: This research surveyed the cytotoxicity effects of levofloxacin on ovarian follicles of mice in both *in vitro* and *in vivo* conditions, as well as its anticancer effect on human ovarian cancer cell line (SKOV3).

Methods: For *in vitro* study, the ovaries of animals were isolated and treated with levofloxacin at doses of 1, 2, 5, and 10 µg/ml for 6 days. For *in vivo* study, animals were treated with levofloxacin at concentrations of 100, 200, 400, and 800 mg/kg for 24 days. Histopathological and morphological examinations of ovarian tissues were performed. Real-time PCR and Western Blot techniques were performed to analyze apoptosis-related genes and proteins of ovarian tissues.

Results: Levofloxacin at higher concentrations caused morphological changes and remarkably decreased the number of primary, secondary, and adult follicles compared to the control group. The percentage of viable SKOV3 cells was 10.12%, 7.63%, and 2.17% following exposure to levofloxacin (at a concentration of 800 µg/ml) for 24, 48, and 72 h, respectively. The half-maximal inhibitory concentration of levofloxacin against SKOV3 was found to be 181.1, 74.84, and 27.58 µg/ml at 24, 48, and 72 h, respectively. The percentage of SKOV3 cells apoptosis following exposure to levofloxacin after 72 h at 20, 80, and 200 µg/ml doses was 11%, 42%, and 52%, respectively. Real-time PCR revealed up-regulation of *Bax* and *Caspase-3* genes after exposure to levofloxacin in SKOV3 cells, whereas the expression of *Bcl2* was significantly decreased in a concentration-depended manner.

Conclusion: The present study demonstrates that levofloxacin induces apoptosis of both ovarian follicles and human ovarian cancer cell lines.

Keywords: Apoptosis, *Bax, Bcl2, Caspase-3,* Levofloxacin, Ovary, Ovarian cancer

1. Background

Antibiotics have been widely used to prevent bacterial infections in humans and animals (1). However, repeated antibiotic use may be associated with severe adverse effects. Consequently, it is necessary to explore their short and long-lasting side effects in clinical applications. Levofloxacin is a member of fluoroquinolone antibiotic that treats many types of bacterial pathogens in the genitourinary system and lower respiratory tract (2, 3). Studies revealed that levofloxacin acts against a wide range of gram-positive and -negative bacteria by inhibiting DNA Gyrase (topoisomerase II and IV) function and preventing DNA supercoiling (4, 5). In addition, some studies demonstrated that levofloxacin has a strong anticancer effect against different cancer types such as lung cancer cells (6), genitourinary cancer (7), bladder cancer (7), and acute myeloid leukemia (8). It has been shown that levofloxacin remarkably enhances apoptosis and decreases cancer cell formation by inducing cell cycle arrest (5, 9, 10). More recently, Kloskowski *et al.* (7) have demonstrated that levofloxacin caused an increase in late apoptotic cells and an inhibition of bladder and prostate cancer cell cycle at the S phase.

Furthermore, it led to cancer cell apoptosis by inducing the expression of *Bax* in cancer cells (7). These data indicate that levofloxacin not only has an anti-bacterial effect but also promotes cancer cell apoptosis (11). For this reason, it is a suitable candidate for cancer therapy; however, its use should be monitored carefully as its long-term consumption may be associated with some adverse effects (3, 12). Therefore, further in-depth studies are essential to explore its adverse effects on different organs.

The mammalian ovary is a dynamic organ and a rich source of multiple hormones and growth factors that nurture and prepare oocytes for the process of ovulation (13). Identification of risk factors associated with ovarian dysfunction is vital. Recent evidence has reported that levofloxacin use may destroy the structure and function of ovary tissue by affecting the production of follicle-stimulating and luteinizing hormones and progesterone (14).

2. Objectives

These data emphasize that ovarian tissue may be a target for levofloxacin so that this antibiotic can be used to treat ovarian cancer; however, little information is available regarding the effects of

levofloxacin and its underlying mechanisms on ovary tissue. Given that ovary structure can be affected by levofloxacin, we pointed to assess its effects on apoptosis of ovarian normal and cancer cell lines in both in vivo and *in vitro* models.

3. Methods

3.1. Animals

Total of 66 female NMRI mice (age:6-8 weeks, body weight: 25-30 g) were purchased from the animal lab of Qom Islamic Azad University (Qom, Iran). All experiments were conducted following the ethical guideline of animal studies approved by the ethical committee of Qom Islamic Azad University (IR.IAU.SRB.REC.1398.012). After adaptation to the lab conditions (temperature of 22± 2 °C, humidity 50%±5%, and a 12 light/dark cycle), mice were randomly split up into two experimental groups (*in vivo* and *in vitro* groups), containing 36 and 30 mice Respectively per cage (30×15×15 cm) with free access to food and water.

3.2. Treatments

For *in vivo* study, 36 female mice were divided into 6 groups, including control, sham (received standard pellet/water), and four other groups were fed with various doses of levofloxacin (100 mg/kg, 200 mg/kg, 400 mg/kg, and 800 mg/kg) solution (Sigma Aldrich, USA). All the treatments were performed for a period of 4 weeks. One day after the last treatment, mice were anesthetized with chloroform, and ovarian tissues were removed for histopathological examination and gene expression analysis.

For *in vitro* study, 30 female mice were anesthetized with chloroform, and ovarian tissues were removed and cultured in DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 10% FBS, BSA (3 mg/ml), 0.23 mM pyruvic acid, and 1% penicillin-streptomycin with pH 7.4. Cultured ovarian tissues were incubated at 37 ± 0.5 ^{o}C with 5% CO_2 for 6 days. During this period, the cultured ovarian tissues were divided into 6 groups, including control (without treatment), ovarian tissues treated with 1 µg/ml, 2 µg/ml, 5 µg/ml, and 10 µg/ml levofloxacin solution every day for 6 days. At the end of the study, cultured ovarian tissues were evaluated for gene expression analysis and histopathological examination. Levofloxacin concentrations for both *in vivo* and *in vitro* studies were selected based on LD50 and the cytotoxicity effect of levofloxacin described by previous studies (2, 6, 15-17).

3.3. Histological examinations

To find histological alterations, ovarian samples were fixed in Bouin solution for 5-6 h. After dehydration and paraffinization, 5 µm sections were prepared using an automatic microtome, stained (H&E), and a light microscope was applied for histological assessment.

3.4. Gene expression analysis

Total RNA of ovarian samples was extracted using the GeneAll Kit (RiboEx, Korea). Briefly, 150 mg of ovarian specimens were homogenized and mixed with 400 µl lysis buffer and 10 µl 2-mercaptoethanol (2-ME) for 10 min. The mixture was then centrifuged at 11,000 rpm/4 °C for 1 min. The quantity and purity of extracted RNAs were assessed with gel electrophoresis and a Nanodrop ND-1000 spectrophotometer (Thermo Sci., Newington, NH). The cDNA was synthesized using HyperScript ™ RT premix kit (GeneAll) according to the manufacturer's protocol. Around 10 µl HyperScript ™ RT premix was mixed with 1 µg RNA and increased to the volume of 20 µl using distilled water. Moreover, cDNA was synthesized in a thermocycler as follows: 5 minutes at 25 °C, 55 minutes at 55 °C, and 5 minutes at 95 °C. Subsequently, 40 cycles of amplification were accomplished using SYBR Green-based real-time PCR. Each reaction included 10 µl master mix, 5 µM primers, 2 µl cDNA, and 2 µl of ddH_2O. Forward and reverse primers are depicted in Table 1. Furthermore, *GAPDH* was adopted as a reference gene, and $2^{-\Delta Ct}$ method was considered for the relative expression of studied genes.

Table 1. Primers sequence used for *in vivo* study of apoptosis-related genes

Genes		Sequence (5'->3')
Bax	Forward	TTCACAGGTTGGCATTAGG
	Reverse	TCAGCCCATCTTCTTCCAG
Bcl2	Forward	CCCTTCGGAGTTTAATCAGA
	Reverse	CCTTCTTCTCGGCAATTTAC
Caspase-3	Forward	CCTCAGAGAGACATTCATGG
	Reverse	GCAGTAGTCGCCTCTGAAGA
GAPDH	Forward	AAGGTCATCCCAGAGCTGAA
	Reverse	CTGCTTCACCACCTTCTTGA

3.5. Effect of levofloxacin on ovarian cancer cells viability

Normal ovarian cell line (GCN-01) and ovarian cancer cell line (SKOV3) were purchased from Pasteur Institute of Iran and cultured in DMEM (Sigma), enriched with FBS 10% and pen strep (100 mg/ml). Cells were grown at 37 ^{o}C, under a 5% CO_2 atmosphere, and at 90% humidity for 24 h. The cytotoxic effects of levofloxacin against ovarian cancer and normal cells were assessed using the MTT test. Cell suspensions (10^4 cells) were loaded in a 96-well tissue culture plate and treated with various doses of levofloxacin (10, 20, 40, 80, 100, 200, 400, and 800 µg/ml) which were prepared in 1% dimethyl sulfoxide (DMSO) solution. The culture media was treated with different concentrations of levofloxacin for 24, 48, and 72 h. Cell viability was then assessed in a triplicate form for each concentration. In short,

the supernatants were removed from the wells, and 20 µl of MTT solution was added to each plate. The plates were incubated for 4 h at 37 °C, and 100 µl of DMSO was added to the wells to dissolve the MTT crystals. The plates were then shaken for 15 min, and the absorbance was read at 570 nm. The percentage of cells viability was calculated using the following formula:

Cells viability = (OD treated cells/OD negative control) ×100

3.6. Effect of levofloxacin on ovarian cancer cells apoptosis

Flow Cytometry method using a fluorescein isothiocyanate annexin V (Annexin V-FITC Apoptosis Detection Kit, Roch, Germany) and propidium iodide (PI) was hired for apoptosis detection. The SKOV3 cells (2×10^6 cells/well) were treated with levofloxacin (20, 80, and 200 µg/ml) and incubated in 12-well plates at 37 °C with 5% CO_2 for 72 h. Cells were then isolated from the plate using 0.25 M Trypsin-EDTA and centrifugation at 1500 ×g for 5 min. The cell pellets were treated with 5 mg/ml Annexin V and 6 mg/ml PI for 20 min in a dark place. FlowJo software (version 9) was adopted to determine the percentage of apoptotic cells.

3.7. Analysis of apoptotic genes expression in ovarian cancer cells

The SKOV3 cells (2×10^6 cells/well) were treated with levofloxacin at doses of 20, 80, and 200 µg/ml in 6-well plates in a Co_2 incubator for 72 h to analyze apoptosis-related genes. Total RNAs were extracted using a nucleic acid extraction kit (Yekta Tajhiz Nika, Iran), and cDNA was synthesized using the One Step M-MULV RT-PCR Kit (Bio Basic, Canada). For cDNA synthesis, 5 µg RNA was mixed with 1 µl random hexamer, 6.5 µl cDNA Synthesis Mix and met a total extent of 20 µl using ddH_2O. Subsequently, a SYBR green-based real-time PCR assay in 124 cycles was applied for amplification (1 cycle 5 min at 95 °C; 40 cycles 15 sec at 95 °C; 40 cycles 30 sec at 60 °C; 40 cycles 30 sec at 72 °C; 1 cycle 1 min at 95 °C; 1 cycle 1 min at 60 °C; 1 cycle 1 min at 95 °C). Each reaction contained 10 µl master mix, 2 µl primers (5 µM), and 2 µl cDNA to reach a total volume of 20 µl with ddH_2O. Primer sequences are shown in Table 2. In addition, *β-Actin* was used as the reference gene, and $2^{-\Delta Ct}$ method was used for the calculation of gene expressions.

3.8. Analysis of expression of apoptosis-related proteins in ovarian cancer cells

The Western Blot method was applied to analyze apoptosis-related proteins in ovarian cancer cells. The SKOV3 cells (2×10^6 cells/well) were exposed to different quantities of levofloxacin (20, 80, and 200 µg/ml) in 6-well plates and incubated at 37 °C with 5% CO_2 for 3 days. Furthermore, proteins were extracted using RIPA buffer. The presence of proteins was analyzed using SDS-PAGE electrophoresis and then verified by the Western Blotting technique. Western Blotting (Bio-Rad, USA) was run at a constant 75 V voltage for 90 min. The PVDF sheet was blocked overnight with 3% BSA (Sigma, USA). Each primary monoclonal (BioLegend USA) and secondary polyclonal antibodies [IgG-HRP, (Sigma Aldrich, USA)] were applied for 60 min and washed out with PBS-Tween 20. DAB chromogen (Roch, German) was used for staining. Dot Blotting was carried out on diluted (1:20) samples with the same staining protocol as Western Blotting.

3.9. Statistical analysis

All experimental tests were carried out with three replicates. All quantitative data were reported as means ± SD. The one-way ANOVA test was selected to compare means on SPSS software (IBM, Version 20). A *P*-value < 0.05 was considered statistically significant.

4. Results

4.1. Histopathological changes of levofloxacin in vitro

Histopathological examination of ovarian tissue showed no abnormalities in the no-treated group (Figure 1A). Normal germinal epithelium was seen in the ovary. The thickness of tunica albuginea was normal. The ovarian parenchyma is composed of loose connective tissue, highly cellular, and numerous ovarian follicles. Follicles were observed in all stages, and primitive follicles were witnessed in both cortical and medullary regions. There were no degenerated oocytes and disrupted granulosa cells in the ovary. Additionally, there was no pyknotic nucleus in granulosa cells. The corpus luteum was not observed due to the short treatment time of ovaries with levofloxacin in a culture medium (6 days). No bleeding was seen in the ovaries (Figure 1A). Histopathological examination of ovarian tissue treated with 1 µg/ml levofloxacin showed a decrease in different forms of follicles compared with normal mice, and the most significant reduction was seen in mature follicles. The size of the theca and granulosa layers did not change (Figure 1B). Ovarian tissue treated with 2 µg/ml levofloxacin showed the highest reduction of primary follicles compared to other groups. While the decrease in the number of secondary follicles in this group was higher than in ovarian tissue treated with 1 µg/ml levofloxacin, the number of adult follicles was higher in this group. No changes were observed in the size of the theca and granulosa layers. Moreover, the blood vessels were seen scattered (Figure 1C). In ovarian tissue treated with 5 µg/ml levofloxacin, the number of primary follicles remained unchanged while secondary follicles were significantly reduced.

Additionally, mature follicles recognizably surged

in comparison with mice treated with 2 µg/ml levofloxacin. The size of theca and granulosa layers showed no significant change (Figure 1D). In ovarian tissue treated with 10 µg/ml levofloxacin, the follicular assembly was completely disrupted. In some cases, disrupted granulosa cells were seen. Blood vessels were increased along with the number of mature and primary follicles, while secondary follicles had decreased. The size of theca and granulosa layers did not change (Figure 1E).

Table 2. Primers sequence used for *in vitro* study of apoptotic genes

Genes		Sequence (5'->3')
Bax	Forward	TGCTTCAGGGTTTCATCCA
	Reverse	GACACTCGCTCAGCTTCTTG
Bcl2	Forward	TGTGGATGAGTGACTACCTGAACC
	Reverse	CAGCCAGGAGAAATCAAACAGAGG
Caspase-3	Forward	GGAAGCGAATCAATGGACTCTGG
	Reverse	GCATCGACATCTGTACCAGACC
Actin	Forward	AAGATCCTCACCCAGTGTGG
	Reverse	CAGCACTGTGTTGGTGTACAGG

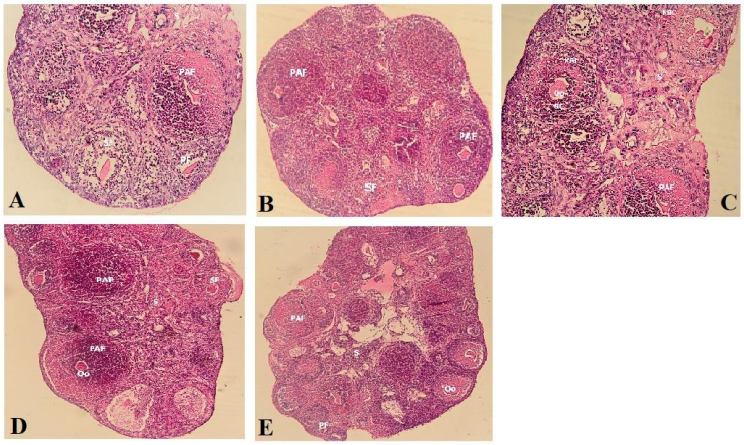

Figure 1. Histopathological alterations of ovarian tissue after *in vitro* exposure to levofloxacin. (A) Control group, (B) group treated with 1 µg/ml, (C) group received 2 µg/ml, (D) group administrated with 5 µg/ml, and (E) group exposed to 10 µg/ml levofloxacin.; PrF: Primordial Follicle, PF: Primary Follicle; SF: Secondary Follicle, PAF: Preantral Follicle; S: stroma, Oo: oocyte; AF: Antral Follicle. 100 X and 400 X magnifications

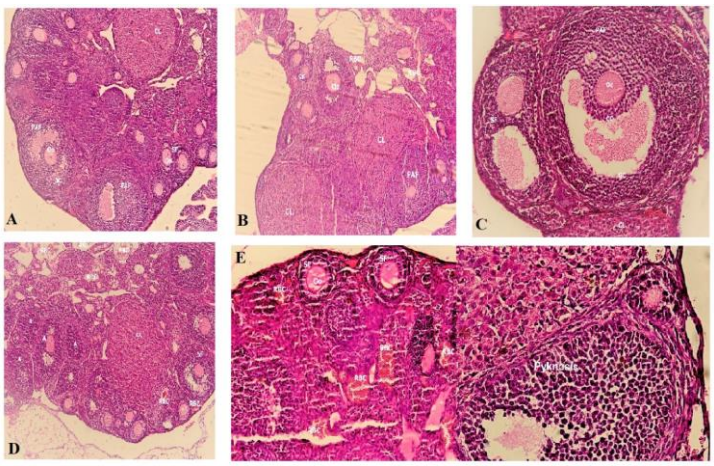

Figure 2. Ovarian sections of mice exposed to levofloxacin *in vivo*. (A) Control (B) treated with 100 mg/kg, (C) 200 mg/kg, (D) 400 mg/kg, and (E) 800 mg/kg of levofloxacin. PrF: Primordial Follicle, PF: Primary Follicle; SF: Secondary Follicle, PAF: Preantral Follicle; S: stroma, Oo: oocyte; AF: Antral Follicle; CL: Corpus Luteum; RBC: Red Blood Cell; A: Atretic Follicle. 100 X and 400 X magnifications;

4.2. Histological alterations by different doses of levofloxacin in vivo

A comparison of the histopathological examination of ovarian tissue *in vivo* condition is presented in Figure 2. The ovarian tissue of healthy mice had normal germinal epithelium. The thickness of tunica albuginea was normal, and the ovarian parenchyma had loose connective tissue with highly

cellular and numerous ovarian follicles. Follicles were observed in all stages. Normal blood supply was observed, and the ovaries and primitive follicles were found in the ovarian cortex. Follicular assembly was normal; there were no degenerated oocytes and disrupted granulosa cells in the ovary. The corpus luteum was observed without bleeding in the ovaries (Figure 2A). The ovarian tissue of rats treated with 100 mg/kg levofloxacin showed a decrease in the number of primary, secondary, and adult follicles compared to the control group. The corpus luteum and follicular atresia were observed in the ovary. The size of the theca and granulosa layers was minimally decreased. The ovarian parenchyma had loose connective tissue (Figure 2B). In contrast to the normal figure of adult follicles, in mice treated with 200 mg/kg of levofloxacin, the number of primary and secondary follicles was noticeably decreased. While an increase was found in follicular atresia, a decrease was observed in the size of the theca and granulosa layers, as well as the number of corpus luteum (Figure 2C). The ovarian tissue of animals treated with 400 mg/kg levofloxacin revealed a significant decrease in the number of primary, secondary, and mature follicles compared to those in control and received 200 mg/kg levofloxacin.

Follicular atresia was climbed dose-dependently; however, the number of corpus luteum was decreased. While no change was found in the size of the theca layer, the size of the granulosa layer was decreased (Figure 2D). In mice that received 800 mg/kg of levofloxacin all types of follicles dramatically declined, contrary to the control group. Follicular assembly was completely disrupted, and disrupted granulosa cells were found in some animals. An increase was observed in blood vessels and follicular atresia. The number of corpus luteum and the size of the granulosa layer were significantly decreased (Figure 2E).

4.3. Quantitative analysis of morphological parameters following in vitro and in vivo exposure to levofloxacin

A comparison of the morphological parameters of ovarian tissue between different groups after *in vitro* and *in vivo* levofloxacin exposure is summarized in tables 2 and 3, respectively. Levofloxacin showed a significant effect on morphological parameters of ovarian tissue in a dose-depended manner. *In vitro* and *in vivo* exposures to levofloxacin significantly decreased all types of follicles in a dose-depended manner compared with the normal group. Furthermore, the thickness of theca and granulosa layers was significant following *in vitro* and *in vivo* exposure to levofloxacin, especially at higher concentrations. A significant decrease was observed in the number of corpus luteum following *in vivo* exposure to levofloxacin at concentrations of 200-800 mg/kg when opposed to the control group. On the other hand, *in vivo* exposure to levofloxacin caused a significant increase in follicular atresia at concentrations of 100-800 mg/kg compared to the control group (Table 2).

4.4. Gene expression analysis

A marked difference was found in the expression figure of studied apoptotic genes, *Bax*, *Bcl2,* and *Caspase-3*, between groups (Figure 3). Overall, *in vitro* exposure to levofloxacin caused a notable increase in *Bax* and *Caspase-3* expression. However, a remarkable decrease in expression of *Bcl2* genes when compared to the control group (Figure 3A). Levofloxacin exposure at concentrations of 5µg/ml and 10µg/ml caused a sensational surge in the expression of *Bax* by 8.33-fold and 20-fold, respectively (Figure 3A; $P<0.001$). Similarly, exposure to levofloxacin at concentrations of 5µg/ml and 10µg/ml caused upregulation of *Caspase-3* by 9.33-fold and 16.33-fold, respectively (Figure. 3A; $P<0.001$). In contrast, a considerable drop was observed in the expression of *Bcl2* by 1.5-fold and 3-fold at concentrations of 5µg/ml and 10µg/ml, respectively (Figure 2A; $P<0.001$). Similarly, i*n vivo* exposure to levofloxacin, especially at concentrations of 400 mg/kg and 800 mg/kg, led to a noticeable rise in *Bax* and *Caspase-3* expression; however, dropped the expression of *Bcl2* genes when compared to normal mice (Figure 3B). *In vivo* treatment with levofloxacin at concentrations of 5µg/ml and 10µg/ml caused a significant increase in expression of *Bax* by 4.08-fold and 7.8-fold, respectively (Figure 3B; $P<0.001$). Additionally, a significant increase was observed in the expression of *Caspase-3* by 4.61-fold and 6.64-fold following *in vivo* exposure to levofloxacin at concentrations of 5µg/ml and 10µg/ml, respectively (Figure 3B; $P<0.001$). In contrast, a significant reduction was found in the expression of *Bcl2* by 2.89-fold and 9.45-fold at concentrations of 5µg/ml and 10µg/ml, respectively (Figure 3B; $P<0.001$).

Table 3. Comparison of the mean of morphological parameters of ovarian tissue between different groups following *in vitro* levofloxacin exposure

	Control	1 µg/ml	2 µg/ml	5 µg/ml	10 µg/ml
Primary follicles (n)	15.92 ± 2.23	11.04 ± 2.14**	9.17 ± 2.33*	6.76 ± 2.65*	4.67 ± 2.03*
Secondary follicles (n)	18.48 ± 3.17	14.29 ± 2.95***	12.64 ± 2.57**	7.33 ± 1.89*	5.27 ± 2.86*
Mature follicles (n)	8.96 ± 1.77	5.32 ± 1.27**	4.79 ± 0.86*	3.02 ± 0.74*	2.13 ± 0.62*
Theca layer (µm)	25.02 ± 1.11	24.32 ± 0.79	20.02 ± 1.56**	20.06 ± 1.72**	15.03 ± 0.68*
Granulosa layer (µm)	49.08 ± 0.81	48.1 ± 0.72	47.69 ± 1.02	47.65 ± 0.94	41.02 ± 0.82*

One-Way ANOVA: Post-hoc Tukey test was applied to compare mean value of parameters between all groups. *$P<0.001$; **$P<0.01$; ***$P<0.05$ compared to control group.

Table 4. Comparison of the mean of morphological parameters of ovarian tissue between different groups following *in vivo* levofloxacin exposure

	Control	Sham	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Primary follicles (n)	16.82 ± 2.13	16.78 ± 2.21	11.37 ± 1.02**	9.63 ± 0.84*	9.27 ± 0.85*	8.02 ± 0.69*
Secondary follicles (n)	18.45 ± 1.17	18.22 ± 1.14	11.85 ± 1.22**	8.93 ± 0.62*	8.47 ± 0.92*	7.85 ± 0.94*
Mature follicles (n)	6.02 ± 0.57	5.82 ± 0.62	4.93 ± 0.72***	4.41 ± 0.63**	3.92 ± 0.58*	3.08 ± 0.55*
Follicular atresia (n)	1.03 ± 0.47	1.12 ± 0.29	2.58 ± 0.28***	5.96 ± 0.43*	6.71 ± 0.51*	7.94 ± 0.39*
Corpus luteum (n)	6.29 ± 0.38	6.14 ± 0.35	6.01 ± 0.33	4.86 ± 0.59***	4.43 ± 0.51***	3.61 ± 0.56*
Theca layer (µm)	24.33 ± 0.73	23.89 ± 0.67	23.79 ± 0.71	20.82 ± 0.55**	17.04 ± 0.88*	14.39 ± 1.02*
Granulosa layer (µm)	48.22 ± 0.37	47.93 ± 0.77	45.22 ± 0.88***	39.14 ± 0.52*	37.55 ± 0.67*	35.12 ± 0.44*

One-Way ANOVA: Post-hoc Tukey test was applied to compare the mean value of parameters between all groups. *P<0.001; **P<0.01; ***P<0.05 compared to control group.

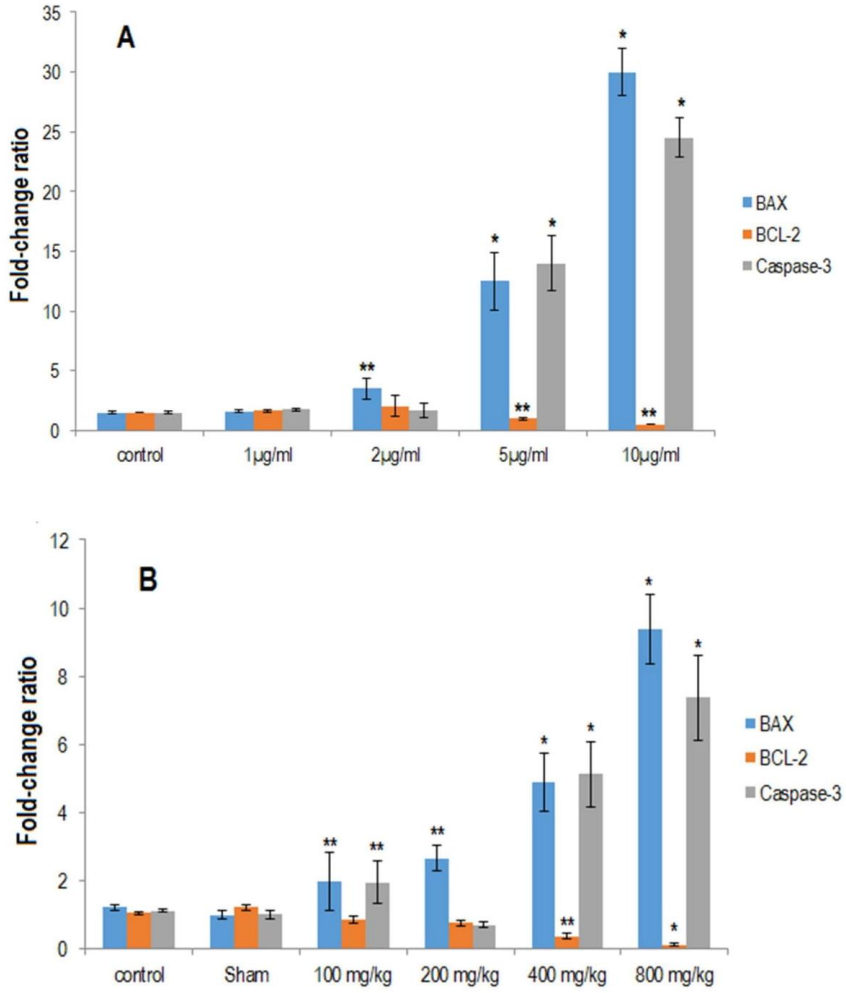

Figure 3. Comparison of the expression pattern of studied genes in (A) *in vitro* and (B) *in vivo* conditions. One-Way ANOVA test was adopted for comparison of findings between groups (*P<0.001 and **P<0.01).

4.5. Effect of levofloxacin on ovarian cancer cells viability

While levofloxacin showed no significant cytotoxicity effect on normal ovarian cells (GCN-01) at different times (Figure 4A), it significantly decreased the percentage of ovarian cancer cells (SKOV3) in a dose-related manner (Figure 4B). Levofloxacin exhibited poor anticancer activity at a concentration of 10 µg/ml, whereas it showed a strong anticancer effect at a concentration of 800 µg/ml. The percentage of SKOV3 cells was 10.12%, 7.63%, and 2.17% following exposure to a higher concentration of levofloxacin (800 µg/ml) after 24, 48, and 72 h, respectively (Figure 4B). The IC50 value of levofloxacin against SKOV3 was found to be 181.1 µg/ml, 74.84 µg/ml, and 27.58 µg/ml at 24, 48, and 72 h, respectively (Figure 4C).

4.6. Effect of levofloxacin on ovarian cancer cells apoptosis

Annexin V-FITC test analysis showed that the percentage of SKOV3 cells apoptosis following exposure to levofloxacin after 72 h at concentrations of 20 µg/ml, 80 µg/ml, and 200 µg/ml was 11%, 42%, and 52%, respectively (Figure 5A). The percentage of early and late apoptosis was considerably higher in levofloxacin-treated SKOV3 cells than healthy mice (Figure 5B).

Real-time PCR test analysis revealed increased expression of *Bax* and *Caspase-3* genes after exposure to levofloxacin in SKOV3 cells. In contrast, the expression of *Bcl2* was significantly decreased in a concentration-depended manner (Figure 6A). Immunoblotting test results showed lower expression of *Bcl2* following treatment with levofloxacin, whereas over-expression of *Bax* and *Caspase-3* in SKOV3 cells in a dose-depended manner (Figure 6B).

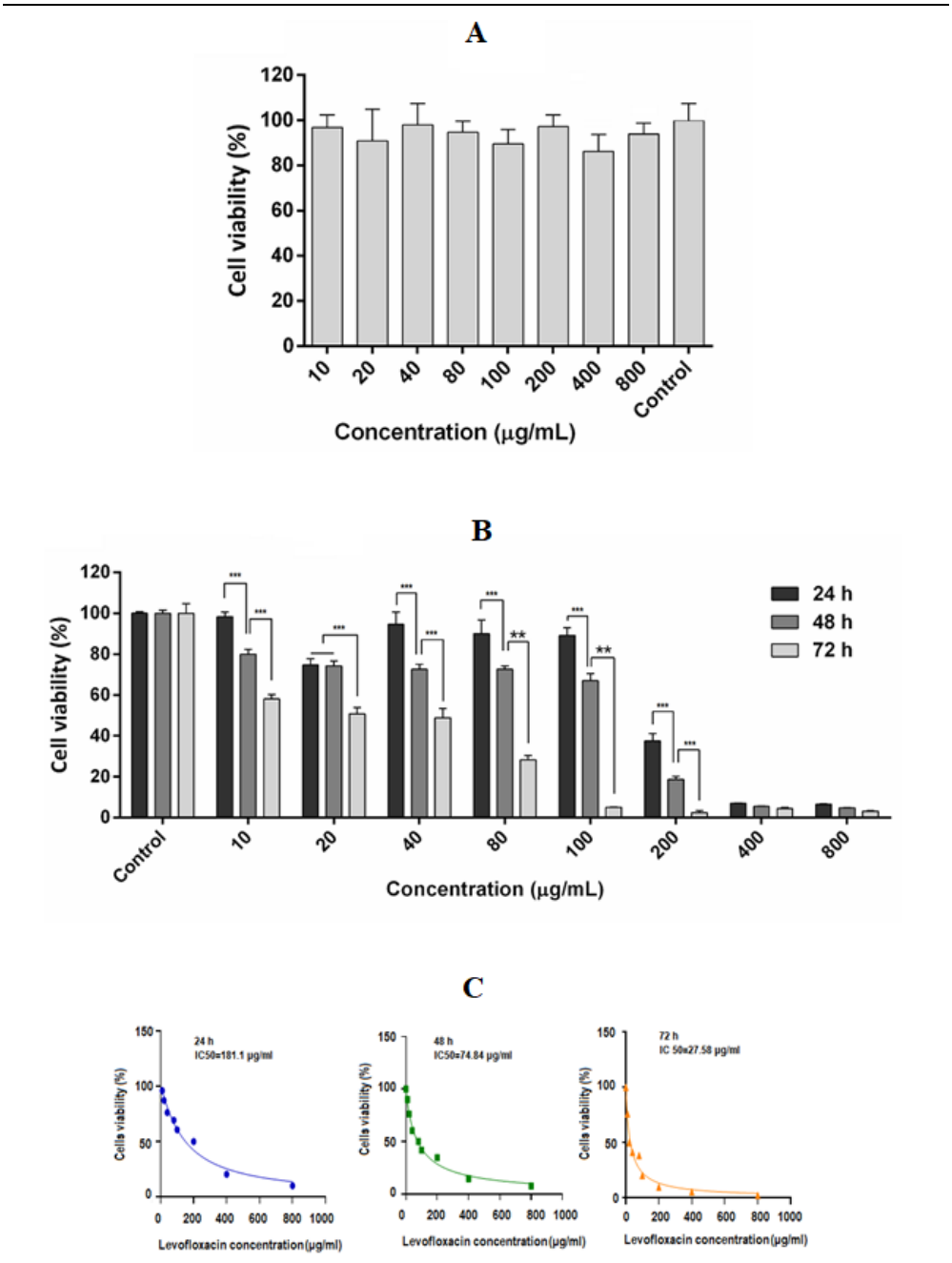

Figure 4. Comparison of the cells viability after exposure to different concentrations of levofloxacin. (A) normal cells and (B) SKOV3 cells and (C) IC50 value of levofloxacin against SKOV3 cell lines at 24, 48, and 72 h. Mean values were compared with the One-Way ANOVA test (*P<0.001, **P<0.01, and ***P<0.05).

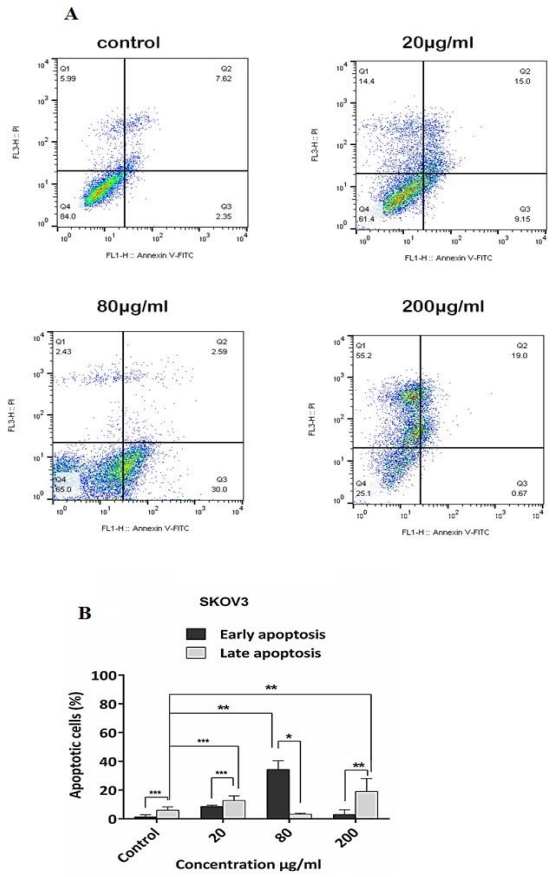

Figure 5. Effect of levofloxacin on ovarian cancer cells apoptosis. (A) Flow cytometry analysis of Annexin V-FITC test following exposure to levofloxacin, Q1: rate of necrosis, Q2: rate of late apoptosis, Q3: rate of early apoptosis, and Q4: rate of viable cells (B) the percentage of early and late apoptosis. One-Way ANOVA: Post-hoc Tukey test was applied to compare the mean value of parameters between all groups. $^{*}P<0.001$ and $^{**}P<0.01$ compared to the control group.

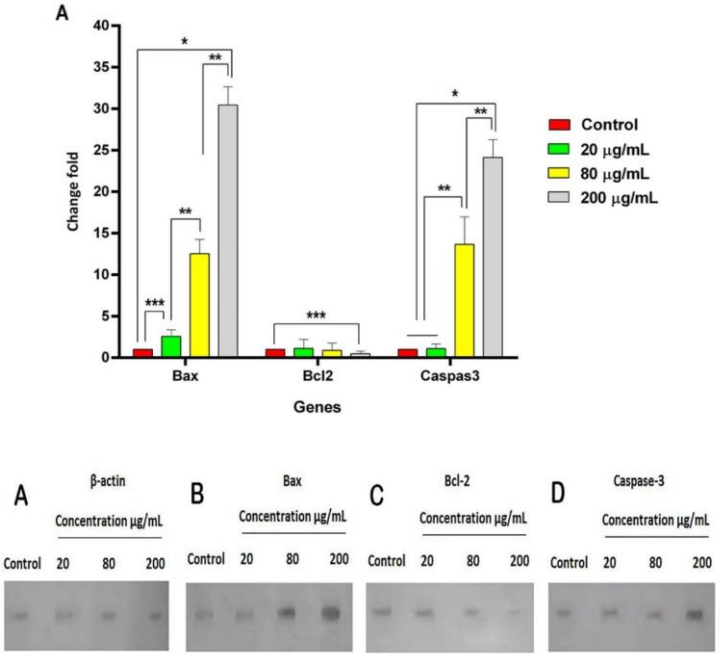

Figure 6. Effect of levofloxacin on apoptosis-related genes in ovarian cancer cells. (A) Gene expression analysis by Real-Time PCR, (B) Immunoblotting analysis of apoptosis-related proteins. One-Way ANOVA: Post-hoc Tukey test was applied to compare the mean value of parameters between all groups. $^{*}P<0.001$ and $^{**}P<0.01$ compared to the control group.

5. Discussion

Given that levofloxacin antibiotic has been widely used for treating bacterial pathogens worldwide, this study assessed levofloxacin effects on ovarian tissue in both *in vitro* and *in vivo* models, as well as its impact on ovarian cancer cells apoptosis. Our findings revealed that levofloxacin administration destroys the structure of ovary tissue in a dose-depended manner in both *in vitro* and *in viv*o conditions. Levofloxacin dramatically decreased various types of follicles and the size of theca and granulosa layers. Quite the opposite, the number of follicular ranging from primary to mature, atresia, and corpus luteum was significantly exacerbated after treatment with levofloxacin. These data indicate that levofloxacin administration, particularly at higher concentrations, not only causes morphological changes in the ovarian tissue but also decreases the number of oocytes and, consequently, the chance of fertilization in females.

The exact mechanism in which levofloxacin antibiotic affects the function of ovarian tissue and results in ovarian lesions has remained unclear. Previously, Ahmadifar *et al*. (14) reported that levofloxacin destroys ovary tissue and causes an imbalance in the serum levels of female sex hormones. In the current study, we proposed levofloxacin induces ovarian tissue failure through apoptosis of ovarian follicles. To support this hypothesis, we considered the expression of apoptosis-related genes in levofloxacin-exposed ovary tissues *in vivo* and *in vitro*. Furthermore, we found that although fluoroquinolone levofloxacin induced the expression of *Bax* and *Caspase-3* genes, it reduced the expression of *Bcl2* in both *in vivo* and *in vitro* models in a concentration-depended manner. These data emphasize that apoptosis of ovarian follicles is likely the primary mechanism of levofloxacin toxicity on ovarian tissue and decreased number of follicle cells. Several studies reported levofloxacin stimulates apoptosis of various cells. For example, Serebryakova *et al*. (18) reported apoptotic activity of levofloxacin in patients with infiltrative pulmonary tuberculosis. Wang *et al*. (19) found that levofloxacin caused cytotoxic effects on rabbit meniscus cells via apoptosis induction. A previous study reported that levofloxacin administration is associated with higher levels of FSH and LH, a significant decrease in sperm counts, primary and secondary spermatogonia, and spermatocyte cells in the seminiferous tubules of male rats (20).

Given the potential effect of levofloxacin on apoptosis of follicle cells, we evaluated its toxicity on ovarian cancer cell apoptosis to introduce it as a new drug for cancer therapy. Our findings indicate that levofloxacin treatments, especially at higher concentrations, significantly decreased the viability of SKOV3 cells. This finding was confirmed by flow cytometric analysis of Annexin V-FITC test. Real-time PCR and Western Blotting revealed upregulation of *Bax* and *Caspase-3*; however, down-expression of *Bcl2* in SKOV3 cells following exposure to levofloxacin. These data indicate that levofloxacin exerted potent anticancer activity. To support this hypothesis, many studies reported levofloxacin's anti-cancer effect on various cancer cells. For example, Song *et al*. (6) evaluated the impact of antibiotic levofloxacin on lung cancer treatment in both *in vivo* and *in vitro* models. They revealed that levofloxacin suppressed the expansion of cancer cells and induced their apoptosis by inhibiting the activities of the mitochondrial electron transport chain complex. Inhibition of mitochondrial respiration was associated with depletion of cellular ATP, massive production of reactive oxygen species, and cancer cell apoptosis. Therefore, these researchers concluded that levofloxacin is a helpful addition to treating lung cancer by targeting mitochondria (6). In another study, Sun *et al*. (21) reported that levofloxacin treatment arrested human hepatocarcinoma cells growth and induced cancer cells apoptosis by inducing *Bax* and *Caspases* expressions and decreasing the *Bcl2* expression. Similarly, levofloxacin *et al*. (22) found that levofloxacin exhibited a strong cytotoxicity effect against human hepatocarcinoma cells through the mitochondrial-dependent pathways.

6. Conclusion

Taken together, we conclude that levofloxacin destroys the structure and function of ovarian tissues and decreases the number of follicle cells at higher concentrations. The reproductive toxicity effect of levofloxacin is likely mediated through the induction of apoptosis-related factors such as *Bax* and *Caspases.* Furthermore, levofloxacin exhibited a strong anticancer effect on cancerous ovarian cells. It suppressed cancerous cell proliferation and induced their apoptosis via stimulating *Bax* and *Caspase-3* and decreasing *Bcl2* expression. The subsequent activation of *Bax* and *Caspase-3* cascades leads to levofloxacin-induced apoptosis in human SKOV3 cells. Levofloxacin may, therefore, have therapeutic effects for treating ovarian cancer or other types of solid cancer.

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Footnotes

Conflicts of Interest: All authors declare that they have no conflict of interest.
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Ethical considerations: This study was approved by the ethical committee of the Islamic Azad University (IR.IAU.SRB.REC.1398.02).
Authors' contributions: S.S.B was involved in doing laboratory experiments and data collection. N.H.R came up up with the primary idea of research. N.H was contributed to data analysis and interpretation of the results. The primary draft was written by S.S.B and N.H.R. The manuscript was revised by K.R.

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