Curcumin Delays Oocyte Apoptosis Through Overexpression of *BCL-2* Gene in Young and Middle-Aged Mouse Models

Saeideh Hasani Azami[1], Hamid Nazarian[1], Mohammad-Amin Abdollahifar[1], Mehdi Allahbakhshian-Farsani[2], Seyede Zahra Banihosseini[1], Marefat Ghaffari Novin[3*]

Abstract

Objectives: Oxidative stress can initiate the process of apoptosis which affects the oocyte quality and reduces development competency in the ovarian follicles. Accordingly, the present study determined the effects of curcumin as a well-known antioxidant on the apoptosis prevention of mature oocytes during the natural increasing age in female mice.

Materials and Methods: In this case-control, interventional, and quantitative applied research, 21-day-old NMRI (Naval Medical Research Institute) female mice were used as control, vehicle, and curcumin groups. The mice in the curcumin group received 100 mg/kg/d curcumin intraperitoneally. After initial interventions, the Annexin-V-FLUOS staining was applied to evaluate the oocyte apoptosis rate in the three groups at 6, 12, and 33 weeks of age. The expression of oocytes apoptosis-related genes (*Bcl2* and *Bax*) was also assessed by the real-time polymerase chain reaction (PCR) technique, followed by measuring oxidation-reduction markers in the ovaries.

Results: Our results showed that oocyte apoptosis and necrosis in the curcumin group decreased in comparison with the control and vehicle groups at 12 and 33 weeks ($P < 0.001$). Moreover, the use of curcumin led to the upregulation of *Bcl2* and downregulation of *Bax* genes at 6, 12, and 33 weeks ($P < 0.001$). In addition, the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities increased in the curcumin group compared with control and vehicle groups at 12 and 33 weeks ($P < 0.001$) while malondialdehyde (MDA) decreased in the curcumin group at 12 ($P < 0.001$), in the control at 12 ($P < 0.01$), and in the vehicle at 33 weeks ($P < 0.01$).

Conclusions: In general, curcumin could suppress oocyte apoptosis through upregulating *Bcl2* and the downregulating of *Bax* gene, as well as suppressing oxidative stress pathways involving oocyte apoptosis and necrosis.

Keywords: Aging, Apoptosis, Curcumin, Oocyte, Oxidative stress

Introduction

Fertility is an age-related phenomenon which is characterized by the gradual loss with advancing age until the appearance of menopause. This event is naturally accompanied by a gradual decline in the quality and number of ovarian follicles (1,2). Aging is related to functional organ impairment due to the progressive storage of free radicals in the normal cell metabolism and is examined as one chief mechanism indicating ovarian aging (3)

Free radicals are introduced as remarkable factors which affect oocyte quality. It is shown that aging in a woman is associated with lower antioxidant activity due to lipid peroxidation, enzyme inactivation, protein oxidation, and even DNA distribution (4-6).

In fact, reactive oxygen species (ROS) can potentially disturb the balance of physiological processes in the ovary (7,8) by follicle apoptosis (6,9). Thus, it seems that oxidative stress has an important role in the decrease of fertility by increasing age. In this regard, the use of antioxidant agents may decrease age-related infertility processing (9).

Curcumin is a natural polyphenol in the rhizome of Curcuma longa using as a flavor because of its anti-inflammatory, anti-oxidant, and anti-apoptotic features (10). In addition, curcumin can target and inhibit multiple signaling molecules related to systemic inflammation, degenerative conditions, and stress oxidative (11,12). Based on a recent report, curcumin has beneficial effects and can play a crucial role in keeping ovarian oocytes from oxidative stress by increasing tissue anti-oxidant marker levels while decreasing apoptosis (13).

Considering the above-mentioned explanation, the present study attempted to demonstrate whether curcumin can effectively prevent apoptosis in the ovary during the process of aging in mice until the age of 33-week. To this end, a novel method was used to determine the relationship of the apoptosis with advancing maternal age by subjecting female mice to daily administration of curcumin after prepubertal influences on oocyte quality on the verge of reproductive failure during a 33-week investigation.

Materials and Methods

Animals
One hundred fifty female NMRI (Naval Medical Research Institute)mice were provided in the experimental study. The animals were placed in a room with a temperature of 20-24℃ under a standard condition of a 12-hour light/dark cycle and had free availability to food and water. All experiments were verified in matching with the Institutional Animal Welfare Law of Iran (14). Further, all study protocols were confirmed by the internal substitute for Animal Study of Shahid Beheshti University of Medical Sciences and the respective local government committee.

Experimental Design
Based on the aim of the study, 21-day-old mice that completed the infancy period were randomly assigned to control, vehicle, and curcumin groups. In the treatment group, mice received daily intraperitoneal injections of 100 mg/kg curcumin powder (Sigma-Aldrich) (15-17) dissolved in the sesame oil obtained from Sigma, Aldrich Company (18) in the consecutive day for 6, 12, 33 weeks. Mice in the vehicle group were injected daily with the sesame oil for 6, 12, 33 weeks whereas those in the control group received no injections.

Afterward, 6 to 10 mice per group were anaesthetized with ketamine (100 mg/kg) and sacrificed by cervical dislocation. Subsequently, the bodies of the mice were cut to collect the oocytes from the ovaries so that to evaluate apoptosis, gene expression, and ovarian antioxidant.

Oocyte Collection
To gain metaphase II (MII) stage oocytes, mice in all groups were injected with 10 units of pregnant mare's serum gonadotropin (PMSG, Sigma-Aldrich) intraperitoneally. After 48 hours, 10 units of human chorionic gonadotrophin (hCG, Pregnyl, Organon) were injected for ovulation. Furthermore, the mice were sacrificed by the displacement of the cervical vertebrae14 to 16 hours after hCG injection. Cumulus oocyte complexes were separated from the oviducts. Then, normal MII oocytes were selected after denuding (19).

Normal oocyte criteria had a clear round zona pellucida, perivitelline space was small, and cytoplasm was observed with no inclusion bodies (19). Normal oocytes were only selected for examination.

Two methods were used to assess the cellular apoptosis including the investigation of the process of apoptosis in oocytes by measuring the *Bax* and *Bcl2* expressions using the real-time polymerase chain reaction (PCR) and the investigation of apoptosis in oocytes using the Annexin-V kit.

Quantitative Real-time PCR (Q-PCR)
The messenger RNA (mRNA) expression of *Bax* and *Bcl2* genes was examined by using the real-time PCR technique in oocytes. The primer sequences are shown in Table 1. In this regard 50-60 oocytes in the MII phase were isolated for mRNA extraction in each group of mice. After denuding, the oocytes were washed in phosphate-buffered saline solution and then put in triplicate, followed by separating the total RNA by using Qiagen RNeasy Micro kit (20). The total RNA reverse transcripted into cDNA (21) and the expression of *Bcl2* and *Bax* genes was done by the real-time PCR technique utilizing SYBR Green I (Takara, Japan) by ABI real-time PCR system (Applied Biosystems). The PCR was executed with one holding at 95℃ for 10 seconds, followed by 40 amplification cycles at 95℃ for 5 seconds and 60℃ for 30 seconds. The obtained data were analysed by the $\Delta\Delta C_T$-method (22).

Annexin V Staining
Apoptosis was defined using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis finding kit (Roche Diagnostics, Mannheim, Germany). On the membrane of apoptotic cells, Annexin V-FITC has a high relation to phosphatidylserine. Likewise, PI can diffuse and stain the late-apoptotic and necrotic cells. For oocyte staining, the cumulus cells around the oocytes were completely removed by the hyaluronidase enzyme after the completion of the maturity period. The oocytes were then cleaned several times with TCM HEPES containing 10% fetal bovine serum. To prepare the coloring solution, 100 μL of buffer was first taken and then 2 μL of Annexin and 2 μL of PI were added to the kit. Next, the oocytes were placed in this dark compound for 15 to 25 minutes. An apoptosis condition was detected with a microscope equipped with a fluorescent system and a photographic camera (Labomed Lx 400, Labo America). The FITC and PI colors were observed with green and red filters, respectively. The oocytes were defined into three groups based on the presence or absence of a red or green signal. The groups included live and non-apoptotic oocytes with no green signal on their plasma membranes or the red signal in the cytoplasm and nucleus, as well as apoptotic

Table 1. The Sequences of the Considered Primers for the Real-Time PCR

Gene	Primer Sequences (5′-3′)	Accession No.	Length	Temperature (°C)
Bcl2	F: ATGTGTGTGGAGAGCGTCAA R: AGGAGAAATCAAACAGAGGTCG	NM_009741.5	172	61
Bax	F: CCGGCGAATTGGAGATGAACT R: CCAGCCCATGATGGTTCTGAT	NM_011250780.2	229	60

Note. PCR: polymerase chain reaction.

oocytes with a green signal on their cytoplasm membrane and necrotic oocytes with a red signal on their cytoplasm and nucleus (23).

Oxidative Stress Marker Assay
Malondialdehyde
Thiobarbituric acid reactive substances were used to evaluate lipid peroxidation and it was observed by a spectrophotometer. The calibration curve was attained from a Stock TCA-TBA-HCL: 15% w/v trichloroacetic acid (TCA), 0.375% w/v thiobarbituric acid, and hydrochloric acid (0.25 N). The standards and samples were put in a boiling water bath for 15 minutes and then the reaction was finished by placing the samples on the ice. The fluorescence was evaluated by a spectrophotometer at a wavelength of 535 nm.

Antioxidant Marker Assay
Glutathione Peroxidase
Glutathione peroxidase (GPx) activity was evaluated by Paglia and Valentine method. Furthermore, the GPx was catalysed for glutathione by cumene hydroperoxide. In the vicinity of glutathione reductase and NADPH, the oxidized glutathione was transformed NADPH to NADP$^+$. This reaction was measured by a decline in the absorbance at 340 nm (24).

Superoxide Dismutase
The superoxide dismutase (SOD) activity was measured by using the Marklund method. This method explains the activity of the enzyme SOD to prohibit the autoxidation of pyrogallol. The autoxidation elevated by increasing the pH. The reaction was still inhibited at a pH of 9.1 by the SOD, but free O_2^- rapidly replaced at the dominant alkalinity condition. Quantifying pyrogallol autoxidation was at 420 nm. One unit of enzyme is the quantity of the enzyme capable of prohibiting the rate of pyrogallol oxidation by 50% (25).

Statistical Analysis
The analyses were done using SPSS software, version 22 (SPSS, Chicago, IL, USA). Moreover, GraphPad PRISM 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to draw the graphs and the results were elucidated as the mean ± SEM. Finally, the one-way analysis was used to determine significant differences in variance by a Tukey test. Additionally, repeated-measures ANOVA were used for comparing the effect of aging on all parameters in this study. Differences at $P<0.05$ were considered statistically significant.

Results
The Effects of Curcumin on *Bax* and *Bcl2* Gene Expression
The comparison of the relative transcripts of *Bax* and *Bcl2* genes (Figure 1) showed a significant difference
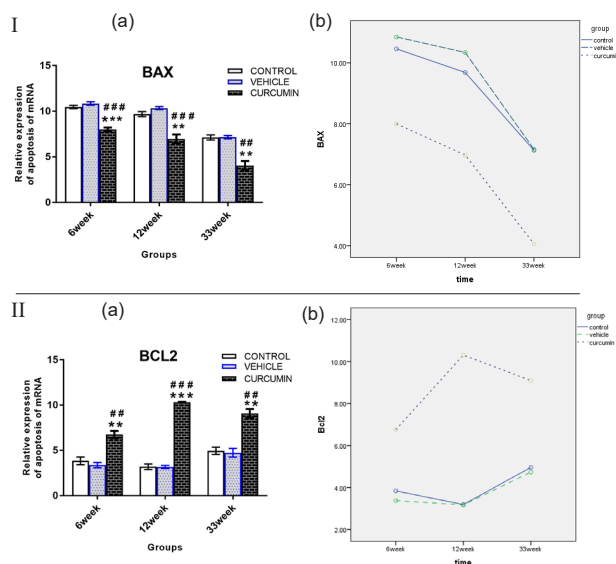
Figure 1. Effect of Curcumin on Bax and Bcl2 Gene Expression. **(Ia)** Decreased MII oocyte mRNA expression of Bax and **(IIa)** increased MII oocyte mRNA expression of Bcl2 from curcumin-treated mice versus control and vehicle groups, as determined by real-time polymerase chain reaction (PCR). Data are presented as mean±s.e.m.**$P<0.01$, ***$P<0.001$ versus control; ##$P<0.01$, ###$P<0.001$ versus vehicle. **(Ib, IIb)** Comparison of Bax and Bcl2 gene expression between the groups during aging by repeated-measures ANOVA.
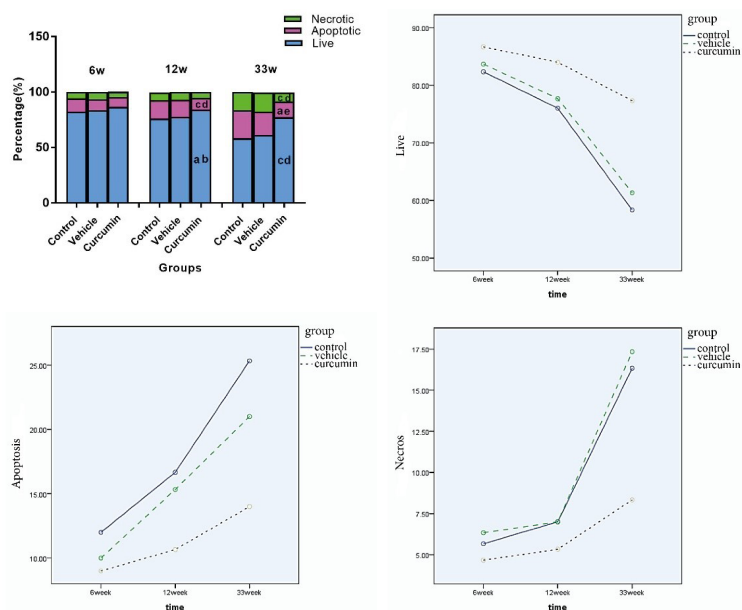
between the oocytes of the curcumin, control, and vehicle groups. The mean transcription rate of the *Bax* gene was significantly lower in the curcumin group as compared to control and vehicle groups ($P<0.001$) at six weeks of intervention. Similar results were obtained at 12 ($P<0.001$) and 33 ($P<0.001$) weeks. The use of curcumin also led to an increase in the transcription rate of the *Bcl2* gene as compared to the other groups at 6 ($P<0.001$), 12 ($P<0.001$), and 33 ($P<0.001$) weeks.

The Effects of Curcumin on Oocyte Apoptosis/Necrosis/Health
After the Annexin V-FITC staining, the histogram results (Figure 2) demonstrated lower apoptosis in the oocytes of the group receiving curcumin compared to control and vehicle groups at 12 ($P<0.001$) and 33 ($P<0.001$) weeks. Similarly, the rate of healthy oocytes was significantly higher in the curcumin group in comparison to control and vehicle groups at 12 ($P<0.001$) and 33 ($P<0.001$) weeks. Moreover, the use of curcumin decreases the rate of necrosis in the curcumin group when compared to control and vehicle groups at 33 weeks ($P<0.001$) after the intervention. However, no difference was revealed across the groups at 6 weeks of intervention. The image of the oocyte staining is illustrated in Figure 3.

### Effect of Curcumin on Antioxidant Enzyme Activity in Ovaries
The effect of curcumin on ovarian antioxidant enzyme activity was analyzed by assessing GPX, SOD (Figure 4)

Figure 2. The Frequency of the Live, Apoptotic, and Necrotic Oocytes (percentages) in Control, Vehicle, and Curcumin-treated Mice at 6, 12, 33 Weeks.
Note. There were no significant differences between the groups at 6 weeks. Data are presented as the mean ± SEM.
a: A significant difference with the control group (*P*<0.01). b: A significant difference with the vehicle group (*P*<0.01). c: A significant difference with the control group (*P*<0.001). d: A significant difference with the vehicle group (*P*<0.001). e: A significant difference with the vehicle group (*P*<0.05).
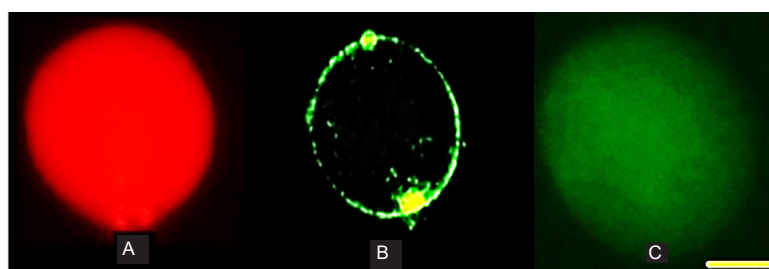
Figure 3. The Image of the Oocyte Staining With Propidium Iodide and Annexin V-FITC
Note. The images show A. Necrotic oocytes observed with a red filter (wavelength 540 nm), B. Apoptotic oocytes observed with a green filter (wavelength 488 nm), and C, Non-apoptotic oocytes observed with a green filter (wavelength 488 nm). 10 μm scale. Magnification X20.

and oxidative marker (MDA) (Figure 5) in the mice of curcumin, control, and vehicle groups. As shown, the activity of GPX in the curcumin group increased more than the control and vehicle groups at 12 weeks (*P* < 0.001) and 33 weeks (*P* < 0.05). In addition, the activity of SOD in the curcumin groups was significantly higher than the control and vehicle groups at 12 weeks (*P* < 0.001) and also at 33 weeks (*P* < 0.001). Moreover, the use of curcumin led to decrease MDA in curcumin group when compared to control group (*P* < 0.001) and vehicle group (*P* < 0.01) at 12 weeks after intervention and 33 weeks later control group (*P* < 0.01) and vehicle group (*P* < 0.05). However, no difference was revealed across the groups at 6 weeks of intervention.

Discussion

Based on the findings, curcumin could protect the oocytes against apoptosis and recover antioxidants in the ovarian tissue with aging in female mice. Apoptosis is a physiological process that results in eliminating about 99% of germ cells in the ovary through atresia of the ovary. About 1% of germ cells may undergo apoptosis within the last phase of oogenesis leading to ovarian reserve deletion (26).

Several factors have been identified for inducing oocyte-related apoptosis such as the premature loss of granulosa cells from immature oocytes, the balance disturbance levels of calcium (Ca(2+)) and oxidants, the decreased level of maturation-promoting factor, and the increased levels of proapoptotic factors that lead to oocyte apoptosis (27). It is highly important that the occurrence of apoptosis in the ovary can reverse the ability of ovulation and therefore fertilization (28).

Recently, several studies have described the critical role of the inflammatory and oxidative process on triggering cell apoptosis. It is emphasized that the ROS initiates apoptosis in antral follicles caused by multiple chemical and physical factors (26,29,30). To further describe the
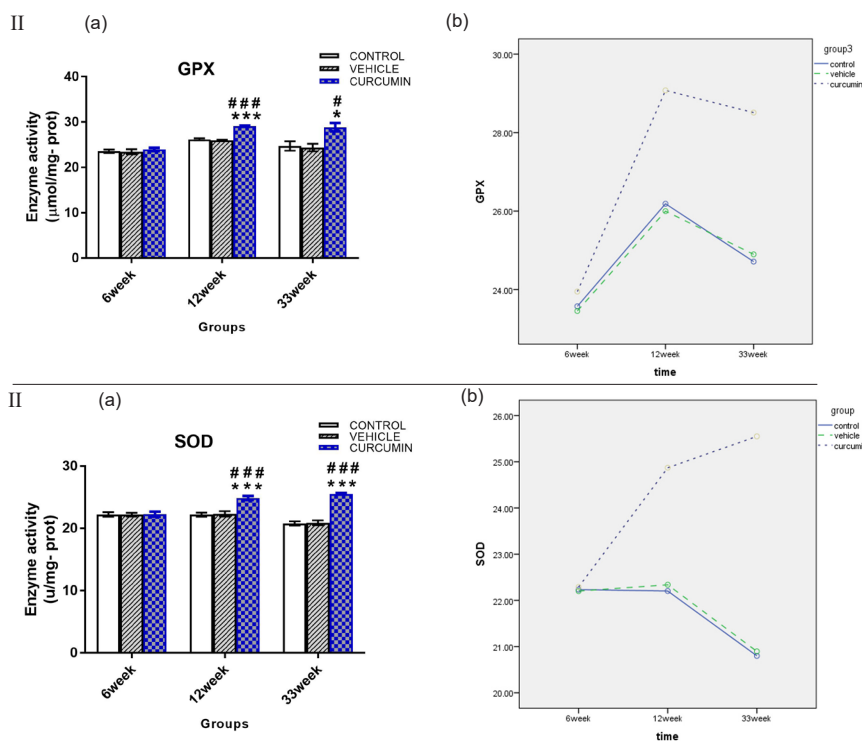
Figure 4. Effect of Curcumin on Antioxidant Enzyme Activity in Ovaries.
Note. (*Ia*) In curcumin group at 12 and 33 weeks, GPX and *(IIa)* SOD activity significantly enhanced compared to control and vehicle groups. There were no significant differences between the groups at six weeks. Data are presented as the mean ± SEM from at least three separate experiments; *$P < 0.05$; ***$P < 0.001$ versus control; #$P < 0.05$; ###$P < 0.001$ versus vehicle. (*Ib, IIb*) Comparison of GPX and SOD activity between the groups during aging by repeated-measures ANOVA.

association between the effects of anti-oxidant and anti-inflammatory agents on oocyte apoptosis in ovulatory phases, curcumin was considered as an effective antioxidant that could decrease mature oocyte apoptosis in the current study. In this regard, the results revealed that using curcumin could reduce the rate of apoptosis in mature oocytes while increasing the rate of healthy oocytes about 12 and 33 weeks after administration.

The overexpression of *Bcl2* gene and the downregulation of *Bax* gene in oocytes were considered as other important

findings of this study. Regarding the effects of curcumin on reducing apoptosis, some recent experimental studies have reported similar effects. Based on previous evidence, curcumin could decrease ROS formation in ESC-B5 cells and blastocysts after the rescue from apoptosis (30). Thus, it seems that the anti-apoptotic effect of curcumin may be directly associated with its inhibitory effect on stress oxidative activation. As another finding, upregulating the *Bcl2* gene and downregulating the *Bax* gene were also shown to be associated with ovulation processing. It is now
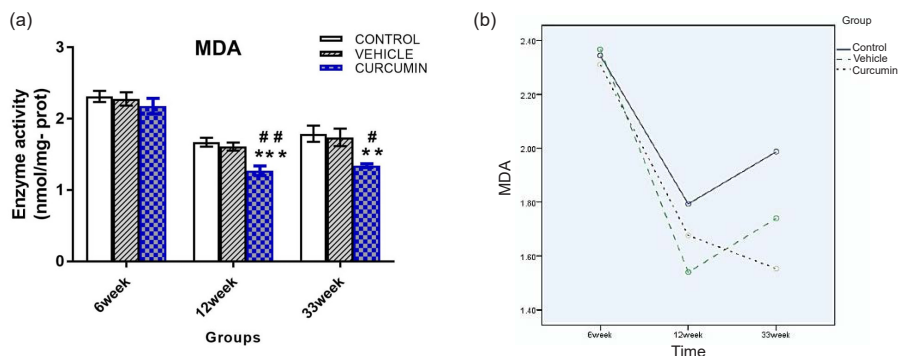

Figure 5. Effect of Curcumin on the Oxidative Stress Marker Level of MDA in Ovaries.
Note. (*a*) MDA level decreased after exposure to curcumin compared to control and vehicle groups at 12 and 33 weeks. No significant differences were observed between the groups at six weeks. Data are demonstrated as the mean ± SEM from at least three separate experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control; #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ versus vehicle. (*b*) Comparison of MDA activity between the groups during aging by repeated-measures ANOVA.

accepted that the elimination of *Bcl2* gene expression can decrease the quantity of oocytes and primordial follicles leading to oocyte apoptosis (31). However, Chen evaluated the cytotoxic effects of curcumin on the blastocyst of mouse embryos and concluded that curcumin can induce apoptosis and disturb the development of mice embryo through ROS generation. The doses of curcumin were 6, 12, 24 μM for blastocyst incubation and apoptosis occurred by 24 μM (32). Therefore, the hazard effects of curcumin are probably dose-dependent but, in this study, the dose for injection was selected according to the LD50 index. In line with our survey, the targeted expression of the *Bcl2* gene in oocytes can be accomplished by the resistance of the female germline to apoptosis/induced apoptosis in chemotherapy and inhibited ovarian follicle atresia can prevent oocyte apoptosis (33). It seems that curcumin can reduce free radicals and improve the antioxidant state in the mouse ovary. In addition, it can regulate the gene expression balance between *Bcl2* and *Bax* genes leading to the close regulation of oocyte apoptosis. Accordingly, curcumin can regulate and inhibit oocyte apoptosis and thus increase the number of mature oocytes through the upregulation of *Bcl2* and the downregulation of *Bax* genes by suppressing oxidative stress pathways involving cell apoptosis.

Likewise, the results of the present study revealed that curcumin could reduce the MDA concentration and increase the SOD and GPx levels to rescue the ovaries from oxidative stress at 12 and 33 weeks compared with control and vehicle groups. One possible explanation for this improvement is that oxidative stress can be caused by an imbalance between the accumulation of ROS and many enzymatic and non-enzymatic antioxidants (34). Aging is related to the increases in the levels of ROS and decreases in antioxidant defenses leading to damage to the DNA and cell structures, along with the inactivation of the enzymes (35, 36). The ovary is a metabolically active tissue and physiologically creates ROS (37). Further, excessive ROS production causes an inappropriate environment for normal female reproduction (38). These results are in agreement with the results of Li et al indicating that curcumin decreases the ROS level in the mouse ovary and can protect it by increasing the level of antioxidant enzyme activity (30). Previous study showed that proanthocyanidins as phenolic compounds display antioxidant activity and protect oxidative stress in the tissue by free radical scavengers (39). Furthermore, Li et al found that curcumin could increase the levels of SOD and GPx while decreasing the concentration of ROS and MDA in the mouse ovary by accumulating the antioxidant enzymes (30).

Nowadays, by women exposure to various kinds of oxidants during their life and considering the results of this study, curcumin can be used as a supplement drug in the reproductive period in females to prevent oocyte apoptosis and increase fertility ability.

However, more studies are needed to illuminate the advantages and disadvantages of using curcumin in other organs, especially the liver and gallbladder and in some chronic reproductive organ diseases like endometriosis in long-term usage.

Conclusions
In general, the findings showed that curcumin could protect the ovaries from oxidative injuries by the accumulation of antioxidant enzymes. In addition, apoptosis results demonstrated that curcumin could protect the oocytes of the apoptosis in the curcumin-treated group compared with control and vehicle groups, similar to the results of oxidation markers. Thus, it seems that curcumin as traditional medicine might have a potential utilization in the treatment of ovarian oxidative stress.

Conflict of Interests
Authors declare that they have no conflict of interests.

Ethical Issues
The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study (No. IR.SBMU.MSP.REC.1395.319).

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