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Moxibustion improves ovary function by suppressing apoptosis events and upregulating antioxidant defenses in natural aging ovary

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ABSTRACT

Aims: Ovarian aging is a natural physiological phenomenon accompanied by follicular atresia as well as the decline of oocyte quality. Moxibustion is a form of traditional Chinese medicine therapy which has been reported to treat many aging-related problems and improve immune defense.

Materials and methods: Moxibustion treatment was applied to the 10-month female rats for 2 or 6 months to evaluate whether moxibustion could delay ovarian aging. The expression levels of NQO-1, HO-1, Bax and Bcl-2 were examined by Western blotting. The serum levels of E_2 and FSH concentration were measured through ELISA. P21, P16, NQO-1, HO-1, Bax and Bcl-2 were measured by qRT-PCR.

Key findings: We demonstrated that moxibustion treatment could attenuate oxidative stress and apoptosis in ovaries, which lead to ovarian aging. The ovary histomorphology, serum FSH, E_2 levels as well as aging markers P21 and P16 expression were compared among the groups, which showed that moxibustion treatment could alleviate the ovary fibrosis, decrease the aging markers expression and increase secretion of ovary functional hormones. The mRNA and protein expression levels of the antioxidative stress-related genes HO-1 and NQO-1 were increased after moxibustion treatment. The antiapoptotic factor Bcl-2 and proapoptotic factor Bax were also detected by qRT-PCR and western blotting, and the results demonstrated that moxibustion significantly downregulated the ratio of Bax/Bcl-2, suggesting that moxibustion could reduce apoptosis in the ovaries of aged rats.

Significance: In conclusion, our research revealed that moxibustion could improve ovary function by suppressing apoptosis events and upregulating antioxidant defenses in the natural aging ovary.

1. Introduction

The ovary is the most sensitive organ to aging. Ovarian aging is a natural physiological phenomenon with a gradual decrease in the quantity and quality of oocytes [1]. An obvious feature of ovarian aging is the gradual declines of ovaries from a vigorous state, which manifests itself as ovarian volume shrink, secretory function decline as well as depletion of the ovarian pool of nongrowing follicles [2]. Meanwhile, decreased estrogen also leads to information transmission and metabolic disorders, triggering a series of physical and physiological changes including hot flushes, irritability, insomnia, osteoporosis, depression and decreased immunity [3–5]. These symptoms seriously affect women's health and the quality of life. Hence, anti-ovarian aging needs more attention and exploration in both biomedical field and the pharmaceutical industry.

It has been reported that oxidative stress is closely linked with the oocyte quality. Oxidative stress, caused by the accumulation of the reactive oxygen species (ROS), can change the ovarian microenvironment and bring about a wide range of oxidative damage [6–9]. ROS accumulation is highly reactive and it can cause severe damage to mitochondrial DNA, promote lipid peroxidation in ovarian follicles as well as increase granulosa cells apoptosis [1,10]. In the meantime, the disrupted balance between ROS generation and scavenging is likely to decrease the levels of antioxidants. The antioxidative-related enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione S-transferase (GSH-ST), thioredoxin and other biological antioxidants [10]. Antioxidant enzymes play an essential role in eliminating excess ROS and maintaining the cellular redox balance. So during the senescence process, the increased ROS and decreased antioxidants lead to the damage of oocytes and granulosa cells within follicles [11–13]. Previous studies demonstrated that the increase of ROS in oocyte is responsible for oocyte apoptosis by increasing mitochondrial membrane permeability and activating the mitochondria-mediated apoptosis pathway [14–16]. Granulosa cells

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apoptosis is a reflection of ovarian aging, which increases in perimenopause women. The balance between proapoptotic and antiapoptotic factor plays an important role in folliculogenesis, ovulation and luteal production [17]. There are many factors associated with apoptosis, such as B cell lymphoma 2 (Bcl-2) family members and caspase family members [18].

Hormone replacement therapy (HRT) is usually a useful method to cure hormone disorders, but it increases the risk of breast cancer, ovarian cancer and cardiovascular diseases [19]. To delay ovarian senescence and cure perimenopausal syndrome, better therapies and further researches are urgently required.

Moxibustion is a form of traditional Chinese medicine therapy by burning compressed powdered herbal material (moxa) over acupuncture points [20]. Through warm and near-infrared stimulation, the effective substances of mugwort penetrate into acupuncture points and transmit into human body meridian improving treatment effect [21,22]. Moxibustion is widely applied to the treatment as well as the precaution of diverse diseases, including knee osteoarthritis, gastrointestinal disorders, stroke rehabilitation and hormone disorders [23]. Over last several decades, clinical and basic researches on moxibustion have been conducted and showed positive effects [24]. However, the molecular mechanism supporting the effectiveness of moxibustion is unrevealed. The objective of the present study was to assess the effect of moxibustion on the ovary apoptosis and antioxidant defenses in the natural aging rat model.

2. Materials and methods

2.1. Animals and treatment

Young female (3 months, n = 6), and perimenopause female (10 months, n = 30) Sprague-Dawley (SD) rats were purchased from Shanghai Jiesijie laboratory animal Co., Ltd. (Shanghai, China). The SD rats were randomly divided into following groups (that were sacrificed at different time points): the young control group at 3 months, the aging control groups at 10, 12, and 16 months, the moxibustion groups at 12, 16 months (n = 6 per group). In the moxibustion groups, the moxa sticks (Qinghai Aiaitie, China) were ignited 1–2 cm above the ST36 (acupuncture point located on the anterior aspect of the lower leg, about one finger-breadth lateral to the tibia, just inferior to the tibial tuberosity [20]) for 15 min every two days, and moxibustion treatment for 2 and 6 months; the control groups were not treated by moxa. All the SD rats were bred under standard conditions, at 21 ± 2 °C in a light/dark cycle as well as free access to water and food. The rats were anesthetized with pentobarbital sodium (50 mg/kg) and then taken blood by orbital vein after moxibustion treatment. After blood collection, the rats were sacrificed by duct execution.

2.2. Histopathological evaluation

Ovary tissues were excised and fixed in 4% paraformaldehyde fixing solution, then embedded in paraffin. Hematoxylin and eosin (H&E) staining and Masson staining were performed according to the standard protocols after the embedded tissue was sectioned at 5 μm. Finally, histological changes were observed under the light microscope at a magnification of 40× and 100×.

2.3. Measurement of serum hormone levels

Blood was collected and coagulated for 2 h then centrifuged at 4 °C, 4000 rpm for 15 min to separate the serum. Then the serum hormone levels (FSH, E_2) were detected using commercial ELISA kits (Meimian, China), according to the manufacturer's instructions. Other serum samples were stored at −80 °C.

Table 1
Primer sequences of qRT-PCR test.

Name	Forward primer (5′-3′)	Reverse primer (5′-3′)
Bax	CCTCCTTTCCTACTTCGG	GGTTTATTGGCACCTCCC
Bcl-2	ACGCGAAGTGCTATTGGTA	TCAGGCTGGAAGGAGAAGA
HO-1	AGCGAAACAAGCAGAACC	CAGCAGCTCAGGATGAGTAC
NQO-1	CCTTGCTTTCCATCACCACCG	TCTCCAGACGCTTCTTCCACC
P21	TGATGTCCGACCTGTTCC	ACGCTCCCAGACGTAGTT
P16	AGCAGCATGGAGTCCTCTG	ACGCTCCCAGACGTAGTT
β-Actin	GTAAAGACCTCTATGCCAACA	GGACTCATCGTACTCCTGCT

2.4. Determination of oxidative stress

Malondialdehyde (MDA) and SOD were measured at serum level. MDA concentration was detected by MDA kits (A003-1, Nanjing Jiancheng, China) and SOD level was detected by SOD kits (A001-3, Nanjing Jiancheng, China) following the manufacturer's instructions.

2.5. Quantitative real-time PCR

Total RNA was extracted from ovary tissues using Trizol (CW0580, CWBIO, China) according to the producer's guidelines and then the cDNA was synthesized using HiFiScript cDNA Synthesis Kit (CW2569, CWBIO, China). The oxidative stress, apoptosis-related gene expression at mRNA level and the sequence of primers were shown in the following table (Table 1). Comparisons of expression levels were measured by delta CT method normalized to β-actin.

2.6. Western blotting analysis

The protein expression levels of Bax, Bcl-2, HO-1 and NQO-1 were assessed via western blotting analysis. The total protein of ovary tissue was extracted through RIPA method, and protein content was determined using the BCA method (P0010S, Beyotime, China). Protein samples were separated by SDS-polyacrylamide gels and then transferred to PVDF membranes. The membranes were incubated in 5% skim milk saline solution for 2 h at room temperature and then incubated in the primary antibodies against Bax, Bcl-2, HO-1, NQO-1 or Tubulin overnight at 4 °C, followed by HRP-conjugated secondary antibodies incubated for 2 h at room temperature. Finally, the protein bands were visualized using the ECL solution (Tanon, China). The relative density of bands was assessed by gel pro-analyzer and the Tubulin was used as the loading control.

2.7. Statistical analysis

All data were represented as mean ± standard error of the mean (SEM). Statistically significant differences were determined by one-way ANOVA in multiple groups and student's *t*-test between two groups with Graph Pad Prism Software (version 5.0). $p < 0.05$ was considered as statistically significance.

3. Results

3.1. Effect of moxibustion on the ovarian morphologic change in natural aging ovaries

Ovarian sections were stained with H&E and analyzed under a light microscope for differential follicle counts as previously described. The process of ovary aging is accompanied by reduced primary follicles and increased atretic follicles [25,26]. In the Fig. 1A, the 3-month group ovary was integrated by primary follicles and corpora lutea. They then developed into secondary and atretic follicles in the 10-month group. The numbers and volume of atretic follicles increased significantly and the ovary began to shrink in the 12-month and 16-month group. After

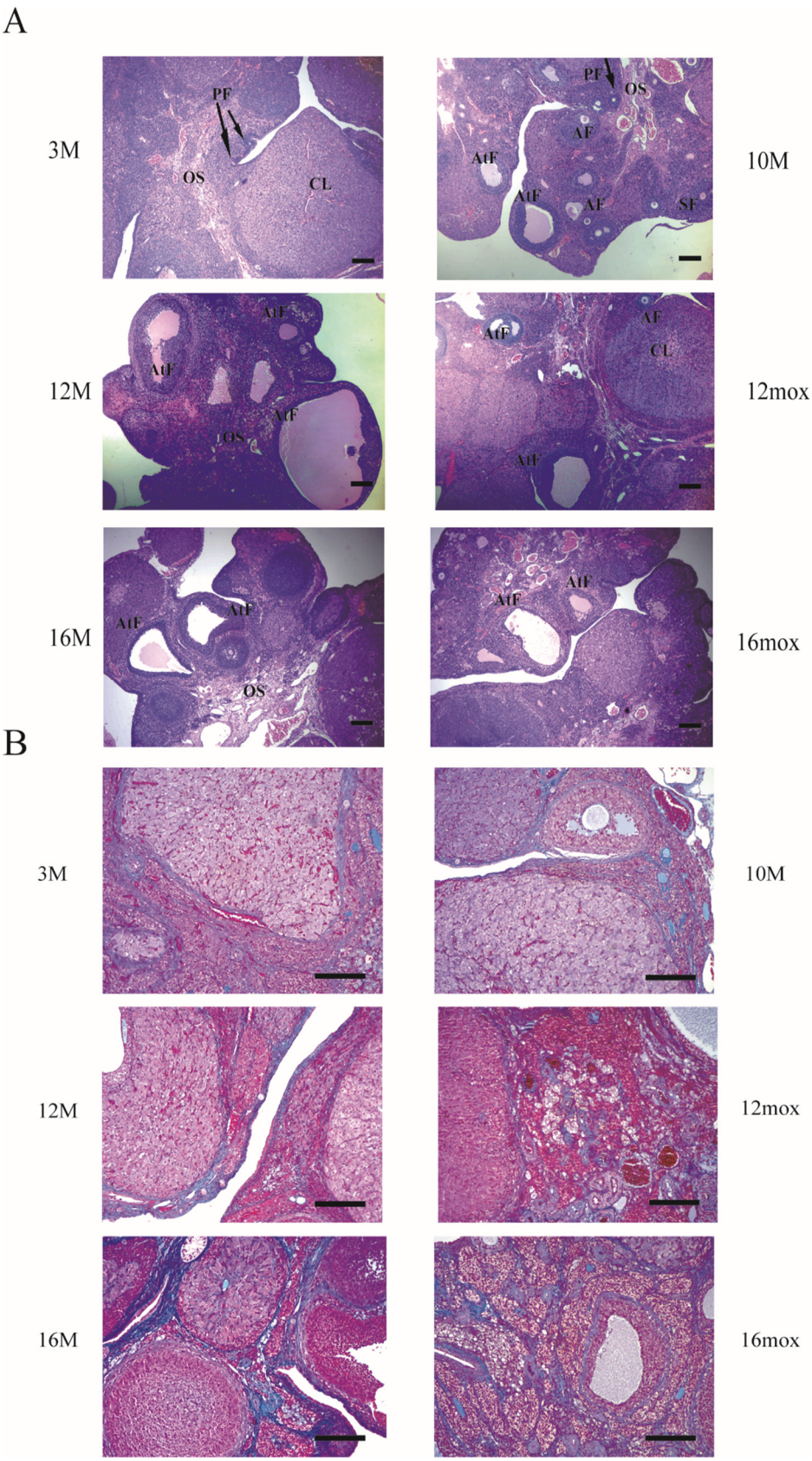

Fig. 1. Photomicrographs of HE-stained and Masson-stained histological sections of ovaries. (A) The histopathological changes in ovary tissues determined by HE staining, PF: primordial follicle; SF: secondary follicle; AF: antral follicle; AtF: atretic follicle; CL: corpora lutea; OS: ovarian stroma. (B) Ovarian fibrosis degree determined by Masson staining. The scale bar in the figure is 100 μm.

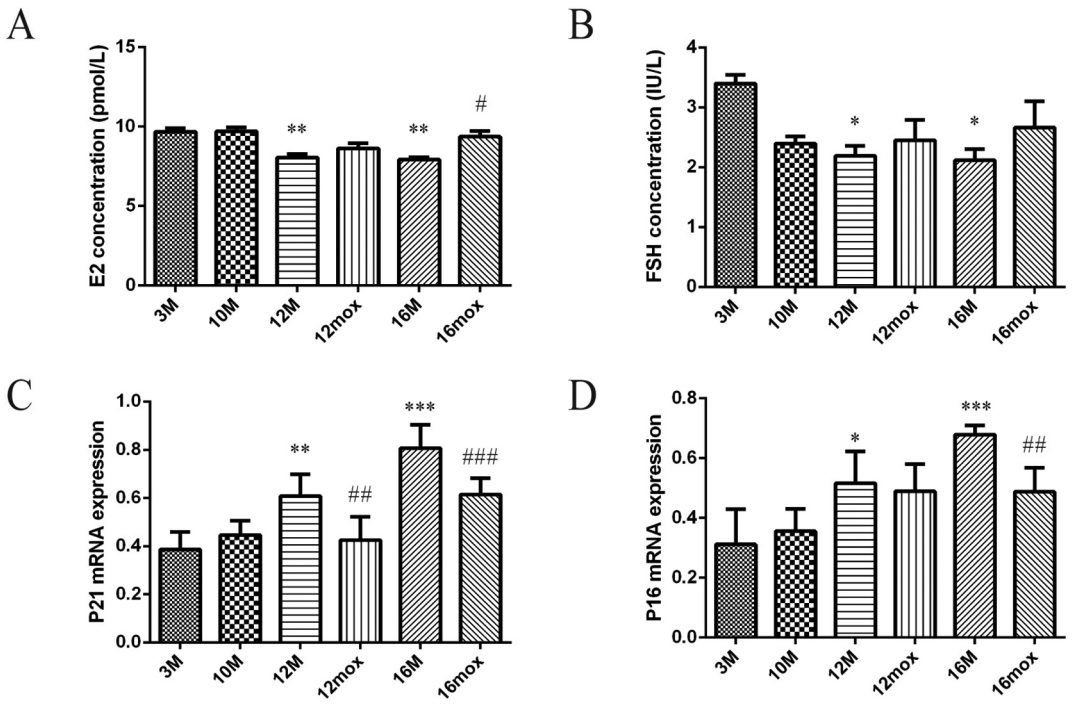

Fig. 2. Moxibustion increased the hormones levels and reduced the aging markers expression. (A and B) The levels of E_2 and FSH concentration were measured by ELISA kits. (C and D) The P21 and P16 mRNA expressions were assayed by qRT-PCR. Data were represented as the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 or ***p < 0.001 vs. 3 M (3-month) control group, #p < 0.05, ##p < 0.01 or p < 0.001 vs. 12 M (12-month) or 16 M (16-month) control groups.

treatment of moxibustion, the number of atretic follicles reduced while the number of antral follicles increased. Fibrosis is the accumulation of ECM (extracellular matrix) which replaces parenchymal tissue. Shawn's study [27] showed that fibrosis increased in the ovarian stroma with advanced reproductive age. In the Fig. 1B, ovarian fibrosis increased with age, and the degree of fibrosis was more severe in 16-month group compared to 3-month group. And the ovarian fibrosis decreased after moxibustion treatment in 12-month and 16-month groups compared to those control groups (12-month and 16-month without moxibustion treatment.)

3.2. Moxibustion increases serum hormone levels and suppress aging markers expression in natural aging ovary

The hormone levels of E2 and FSH decrease when the ovary ages. In the Fig. 2A, compared to the 3-month old rats, the E2 levels in 12-month and 16-month old rats have significantly decreased. The E2 levels were significantly increased by moxibustion compared to the 16-month control group but had no significant differences between 12-month moxibustion group and 12-month control group. In the Fig. 2B, FSH level of 3-month rats was much higher than 12-month and 16-month groups. But moxibustion did not significantly affect the serum levels of FSH at both 12-month and 16-month groups.

P21 and P16 are aging markers related to cellular senescence and contribute to organ dysfunction. To confirm the effect of moxibustion on the prevention of cellular senescence, the transcriptional expression of P21 and P16 were detected. In Fig. 2(C and D), P21 and P16 mRNA expression were significantly increased in 12-month and 16-month groups compared to the 3-month group. After treatment of moxibustion, P21 expression was decreased in 12-month and 16-month group compared to those control groups. The P16 expression was also decreased in 16-month moxibustion group but had no significant difference in 12-month groups. It indicated that moxibustion had effects on the serum hormone levels as well as aging genes expression.

3.3. Moxibustion upregulated serum antioxidant factor levels and protein expression in natural aging ovary

MDA is an indicator for lipid peroxidation to estimate the levels of oxidative stress and SOD is a classical antioxidant index. To determine whether moxibustion played a protective role by affecting the antioxidant products, the serum levels of SOD and MDA were determined. As showed in the Fig. 3(A and B), the SOD activity decreased in 12-month and 16-month groups, compared with the 3-month group. However, moxibustion had no effect on increasing SOD activity. But the MDA concentration was decreased at the 16-month moxibustion group, comparing to the 16-month control group.

The anti-oxidative enzymes heme oxygenase 1 (HO-1) and NAD(P)H quinoneoxidoreductase 1 (NQO1), are produced to provide protection by regulating and maintaining intracellular redox states. So, the HO-1 and NQO-1 expression were detected both at transcriptional and protein levels. In the Fig. 3C, the NQO-1 mRNA expression level at 16-month group slightly decreased, comparing to the 3-month old rats, and increased after the treatment of moxibustion but did not change at 12-month groups. Interestingly in the Fig. 3D, no considerable differences of HO-1 mRNA expression were observed between the control groups and the moxibustion groups. In the Fig. 4E, we examined the protein expression of HO-1 and NQO-1. The NQO-1 protein expression trend was the same as mRNA expression, which decreased at 16-month group and increased after moxibustion treatment. Furthermore, with the treatment of moxibustion, the HO-1 protein expression was much higher than the control group. There was an interesting phenomenon that HO-1 expression was inconsistent at mRNA and protein level as Liang has reported [28]. It indicated that moxibustion may have affected HO-1 expression at post-translational level or decrease the rate of HO-1 protein degradation. However, the NQO-1 expression at 10-month group and HO-1 expression at 12–month group were much higher than 3-month group. Based on the above observation, we concluded that the antioxidant capacity of ovarian tissues increased after moxibustion treatment.

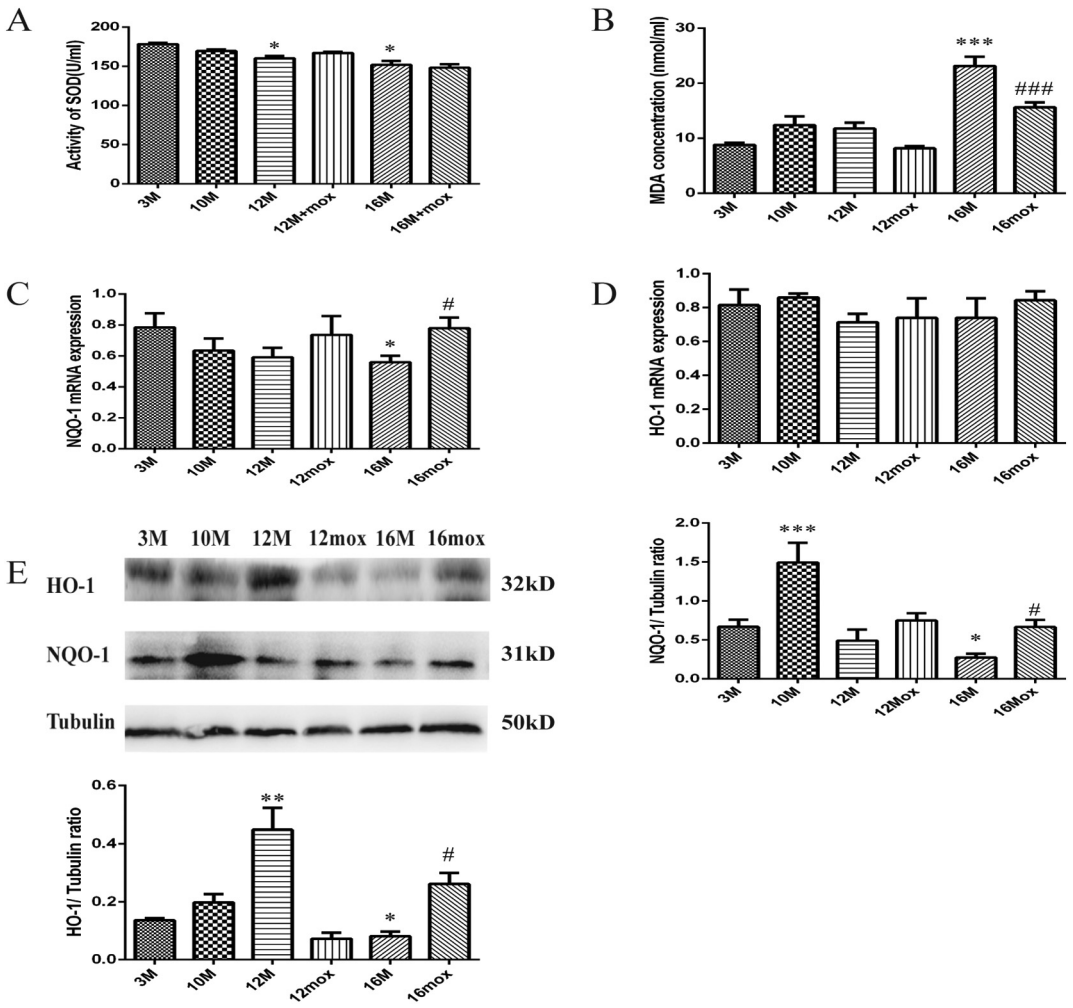

Fig. 3. Moxibustion treatment suppressed oxidative stress. (A) Serum SOD activity. (B) MDA levels in serum. (C and D) NQO-1 and HO-1 mRNA expressions were measured by qRT-PCR. (E) The western blotting image and analysis of protein levels of NQO-1 and HO-1. Data were represented as the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 or ***p < 0.001 vs. 3 M (3-month) control group, #p < 0.05, ##p < 0.01 or ###p < 0.001 vs. 12 M (12-month) or 16 M (16-month) control groups.

3.4. Moxibustion suppressed ovary cell apoptosis in natural aging rats

In order to explore the effect of moxibustion on cell apoptosis associated with aging, the antiapoptotic protein Bcl-2 and the proapoptotic proteins Bax expression were detected. The ratio of Bax/Bcl-2 can represent the apoptosis degree [29]. If the value is higher, the cell apoptosis is more severe. Western blotting analysis in Fig. 4A showed that the ratio of Bax/Bcl-2 significantly decreased in 16-month moxibustion group compared to the control group. In the Fig. 4B, the mRNA expression ratio of Bax/Bcl-2 at 12-month and 16-month groups was much higher than 3-month group. And moxibustion on rats for 6 months upregulated the ratio of Bax/Bcl-2 mRNA expression compared to the 16-month control group, suggesting that moxibustion reduced apoptosis in the ovaries of aged rats.

4. Discussion

Ovarian aging and reproductive capacity decreased with age is common and natural in almost all mammals including humans [30]. Chinese traditional moxibustion has been reported to treat many aging-related problems and improve the immune defense as well as physiological functions [31]. Previous studies suggested that age-associated oxidative stress and apoptosis could result in follicular atresia and cause a decline in the number of follicles.

In the present study, moxibustion showed a strong protective effect on naturally aged ovaries. The follicle is an important readout of reproductive function for the production of endocrine hormones [32]. The H&E and Masson staining in the Fig. 1 showed that moxibustion treatment reduced the number of antral follicles and alleviated the ovarian fibrosis. E_2 and FSH are regarded as important indicators of functional ovarian reserve, which can act on the hypothalamus and pituitary, and regulate ovarian folliculogenesis and oogenesis [33,34]. The process of ovarian aging is accompanied by a remarkable decrease of estrogen. Therefore, E_2 and FSH serum levels were detected by ELISA. The results suggested that moxibustion could significantly increase the E_2 concentration but had no effect on FSH levels. P21(Cdkn1a/Cip1) and P16(Cdkn2a) are cell cycle regulatory proteins, which are closely related to cellular senescence [35]. Cellular senescence is a fundamental aging mechanism that contributes to irreversible proliferation and results in age-related organ dysfunction or lost organisms activity [36]. Our study showed that moxibustion could greatly reduce the mRNA expression of the aging markers.

Oxidative stress refers to the imbalance between oxidative agents and antioxidative factors [37]. It is reported that a remarkable decline in the activity of the antioxidative scavenger system happens in the ovary with age [38]. In this study, we explored the effect of moxibustion on SOD, MDA levels and the antioxidative enzymes activities. MDA is a product of ROS-mediated lipid peroxide degradation and SOD is an

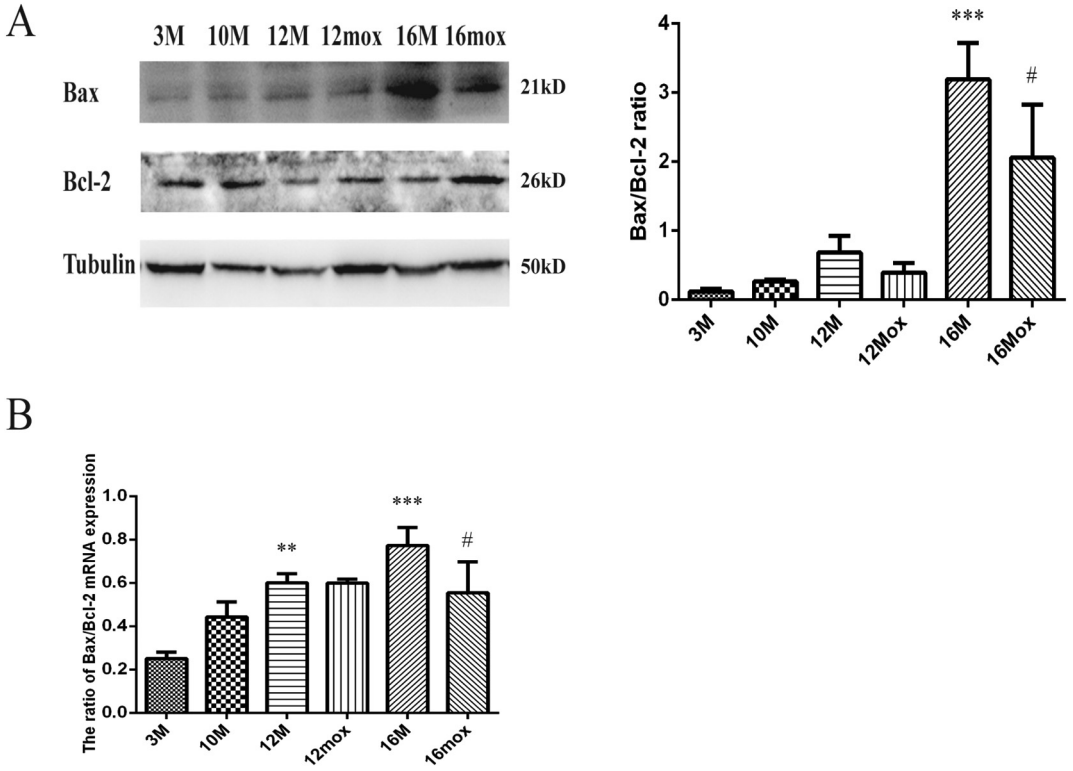

Fig. 4. Effects of moxibustion on apoptosis in aged ovaries. (A) The western blotting image and analysis of protein level of Bax/Bcl-2. (B) The ratio of Bax/Bcl-2 mRNA expression, assayed by qRT-PCR. Data were represented as the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ vs. 3 M (3-month) control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$ vs. 12 M (12-month) or 16 M (16-month) control groups.

important antioxidant enzyme in vivo [39]. Nuclear factor-E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) pathway is a powerful defense against oxidative stress, and the HO-1 and NQO-1 belong to its downstream targeted genes. As shown in Fig. 3, the SOD activity increased and MDA level decreased after moxibustion treatment. Moxibustion upregulated the expression levels of HO-1 and NQO-1 at both transcription and protein level.

Follicular atresia is accompanied by granulosa cell apoptosis. Therefore, granulosa cell apoptosis is a reflection of ovarian aging and reserve [17]. But excessive apoptosis of granulosa cells can damage the oocyte quality and decrease hormone production. Granulosa cells apoptosis depends on apoptosis-related family including paf-1, Bcl and caspase family. Two Bcl family members: Bcl-2 as an antiapoptotic factor and Bax as a proapoptotic factor have been reported as cell death regulators, playing vital roles in regulating ovary apoptosis [34,40]. The Bax and Bcl-2 expressions were examined by qRT-PCR and western blotting. Our results in the Fig. 4, revealed that moxibustion treatment suppressed the ovary granulosa cells apoptosis due to the decreased ratio of Bax/Bcl-2 compared to those control groups. Our research showed that the aging index at 16-month moxibustion group were significantly alleviated compare to 12-month moxibustion group. On the one hand, the effect of moxibustion was not significant in that the ovarian aging at 12-month group was not enough. On the other hand, moxibustion is a gradual process, and accordingly six months treatment may notably improve ovary function than 2 months.

In conclusion, our research revealed that moxibustion improved ovary function by suppressing apoptosis events and upregulating antioxidant defenses in the natural aging ovary. However, the current studies had certain limitations. Thus, further studies are necessary to explain the pathways influenced by moxibustion in regard to downregulated apoptosis and upregulated antioxidative capacity, which would shed light on the mechanisms underlying the beneficial effects of moxibustion in aging ovaries.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.040.

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