Expression and localization of nuclear factor erythroid 2-related factor 2 in the ovarian tissues of mice at different ages

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Abstract. The aim of the present study was to investigate the expression and localization of nuclear factor erythroid 2-related factor 2 (Nrf2) in the ovaries of mice in different age groups, and to explore the association between Nrf2 and premature ovarian aging. The present study identified the localization of Nrf2 protein by performing immunohistochemical assay of ovarian tissues obtained from mice in different age groups. The mRNA expression levels of Nrf2 were detected via reverse transcription-quantitative polymerase chain reaction, while the expression levels of Nrf2 protein and apoptosis-associated proteins, including Caspase3 and B-cell lymphoma 2 (Bcl-2), were evaluated by western blot analysis. The results revealed that Nrf2 protein was mainly localized in granulosa cells, as well as in the secondary follicles and antral follicles of oocytes. Nrf2 expression levels were significantly lower in mice aged 4 days compared with 12-week-old mice ($P<0.05$), and the level of Nrf2 was lower in mice aged 40 weeks compared with those aged 12 weeks ($P<0.05$). In addition, the expression of the apoptosis protein Caspase3 in the ovarian tissue of mice aged 3, 8 and 12 weeks remained markedly greater when compared with those aged 4 days and 40 weeks. Bcl-2, an anti-apoptotic protein, was also significantly expressed in the ovarian tissues of juvenile (4-day-old) mice when compared with mice aged >40 weeks ($P<0.05$). In conclusion, Nrf2 was highly expressed in the ovarian tissues of mice of childbearing age (8-12 weeks old) and may possibly be involved in ovarian regulatory functions. The results indicated that Nrf2 expression and localization may have important implications in the prevention of ovarian aging.

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Introduction

The ovaries are known to age more rapidly in comparison with other organs. Numerous women present premature ovarian failure (POF), with ~1 in 1,000 women considered to suffer from POF by 30 years of age, while this occurrence is reported to increase to ~1 in 100 women by 40 years of age (1). The primary cause of POF remains unknown; however, it is associated with a number of congenital conditions or a gene abnormality (19%), autoimmunity (19%), iatrogenic causes (37%) or unidentified/unknown causes (25%) in patients worldwide (2).

Reactive oxygen intermediates, including oxygen ions, superoxides, singlet oxygen and peroxides, are considered to affect ovarian aging and even cause premature aging (3). Ovarian aging is induced by an escalation in the levels of oxygen radicals, leading to failure of the antioxidant defense system, which in turn initiates and regulates the release of apoptotic factors that cause ovarian granulosa cell death (4). B-cell lymphoma 2 (Bcl-2) is an anti-apoptosis protein that protects cells by preventing a proteolytic cascade dependent on Caspase3, a type of cysteine-aspartic acid protease, which in turn prevents cell death (4). A surge in reactive oxygen species (ROS) not only has detrimental effects, such as protein oxidation, nucleic acid damage and eventual premature cell death, but also negatively impacts the reproductive abilities (5). ROS are formed throughout ovulatory processes and have biological roles during ovulation, such as supporting the ovum release. However, a significantly increased ROS levels can cause oxidative stress (OS) and induce the death of oocyte granulosa cells, as well as oocytes. A number of previous studies investigating OS have reported that enhanced ROS levels caused oocyte destruction, maturation of the ovum and degradation of oocyte superiority (6,7).

Under normal conditions, human cellular systems limit the release of excessive amounts of oxygen intermediates, inhibiting their levels and supporting cell repair (8-11). A woman's ability to reproduce is highly influenced by OS and other events, such as the menopause. It has previously been hypothesized that OS may be responsible for decreasing

fertility in women (12). Furthermore, OS serves a major role throughout the 9-month gestation period (13), in normal vaginal delivery (14,15), as well as in the commencement of normal and preterm labor (16,17). Lipid peroxidation, and reduced ATP and protein formation are some of the effects of excess oxygen radical levels (18). In addition, ROS are involved in the rupture of Graafian follicles, and are known to serve major effects during the maturation of oocytes, steroid hormone production, gamete embedding and also during the formation of an embryo. Furthermore, ROS are known to help maintain the corpus luteum in order to prevent abortion and later support luteolysis (19-23).

The human immune system is able to alter the expression levels of cell protective enzymes in response to specific stimuli as a defense mechanism (24,25). One of the roles of nuclear factor erythroid 2-related factor 2 (Nrf2) is to induce the production of cellular defense proteins. Nrf2 stimulates a wide range of cellular defense processes, which lead to the destruction of harmful factors that are detrimental to human health. It is a member of the cap-n-collar subdivision of basic region-leucine zipper-type family of enzymes responsible for regulating the transcription frequency (26). Nrf2 binds to one of the smallest Maf macromolecules (sMaf), and the Nrf2-sMaf macromolecular complex then binds to antioxidant response elements (AREs) or electrophile response elements, which are located in the surveillance sections of DNAs coding for several cell defense-associated proteins (26-29).

A previous study suggested that during OS induced by cell tension or stress, the Nrf2 protein may be involved in chemical sensing, as well as transcription, supporting redox balancing and functioning with Kelch-like ECH-associated protein 1 (30). The stability of cells, primarily when challenged by external stimuli, including toxins or reactive oxygen radicals, is constantly adjusted by the secretion of numerous macromolecules that assist in the response to stimuli by acting as transporters to eliminate harmful stimuli from the host's system (31), and Nrf2 serves a key role in this process. The Nrf2-ARE signaling pathway stimulates a defensive mechanism during cell stress induced by OS (32), and is therefore considered to serve an important role in the process of ovarian aging. During the continuous process of aging, apoptosis is the main cause of follicle loss from the ovaries (33).

The present study evaluated Nrf2 protein localization and release in mice of diverse age groups. The aim of the present study was to determine the association between the release of Nrf2 and apoptosis-associated proteins, including Caspase3 and Bcl-2, in the ovaries of different age groups of mice.

Materials and methods

Laboratory animals. Adult ICR mice (n=32; age, 12 weeks old; weight, 25-30 g) were acquired from the Animal Center of Wenzhou Medical University (Wenzhou, China) and reproduced in the Animal Center of the Second Affiliated Hospital of Wenzhou Medical University. The adult mice (male to female ratio, 1:2) were housed in a plastic cage for 1 week, maintained at a controlled temperature of 21-24˚C and a 12-h light/dark cycle. Food and fresh water were available to the mice *ad libitum*. Pregnant mice were observed daily to determine whether they had delivered. All procedures were performed in accordance with the guidelines for laboratory animal use and welfare of Wenzhou Medical University, and the experimental protocol was approved by the Ethics Committee Board of the Second Affiliated Hospital of Wenzhou Medical University (approval no. wydw2013-0002).

Mouse ovaries of the offspring were obtained at different stages of growth for immunohistochemical procedures. In accordance with the reproductive physiology of female mice, the ovaries were obtained at 4 days (young group; n=12), 3 weeks (prepubertal group; n=10), 8 weeks (sexual maturity group; n=10), 12 weeks (childbearing age group; n=12) and 40 weeks (older age group; n=9) of age for use in subsequent experiments. Briefly, mice were sacrificed and the freshly excised ovaries were fixed with 4% paraformaldehyde for 1 h. The ovaries were then cleaned with water and dehydrated by passing through a series of 70, 80, 95 and 100% alcohol for 5 min each time. Ovarian tissues were then washed in xylene and finally embedded in paraffin.

Immunohistochemical assay. The paraffin-embedded ovaries were sectioned at 4 μm and placed on glass slides. Next, the slides were dipped in xylene in order to remove the paraffin and transferred through a descending ethanol series (100, 95, 70 and 55%) for 3-4 min. Slides were then heated in a solution of citrate buffer in a microwave at a 100˚C for 10 min, in order to perform antigen retrieval. Following cooling to room temperature, slides were washed three times with PBS (5 min each) and then treated with 3% H_2O_2 in methanol for ~10 min to quench endogenous H_2O_2 activity. Subsequently, tissues were blocked using 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) for 20 min, placed in a dark environment, washed with PBS and then incubated with Nrf2 primary antibody (cat. no. ab62352; Abcam, Cambridge, MA, USA) in Primary Antibody Dilution Buffer (1:500 dilution) at 4˚C overnight. For the negative control, PBS was used instead of the antibody. Following overnight incubation, the slides were kept in a warm water bath at 37˚C for at least 30 min, which was followed by washing three time with PBS for 5 min each and then incubation with the secondary antibody (1:20,000; cat. no. 14709; Cell Signaling Technology, Inc., Danvers, MA, USA) for at least 20 min at room temperature. The samples were washed thrice with PBS, stained with 3,3'-diaminobenzidine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and then stained with hematoxylin for 2 min, followed by dehydration in alcohol for 5 min each. Finally, the slides were mounted using a mounting solution. Photomicrographs were captured with a Nikon digital camera (Nikon Corporation, Tokyo, Japan) and SPOT Advanced Plus software (SPOT Imaging; Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Western blot analysis. Mouse ovaries from the different age groups were collected at the aforementioned time points. Protein extraction buffers (radioimmunoprecipitation assay and phenylmethane sulfonyl fluoride; Sigma-Aldrich, Merck KGaA) were added to the tissues, and the protein concentrations were quantified using a bicinchoninic acid kit. The samples were then homogenized, centrifuged at 12,000 x g at 4˚C for 30 min, then subjected to ultra-sonication and the supernatant was aspirated again via centrifugation at 4,000 x g

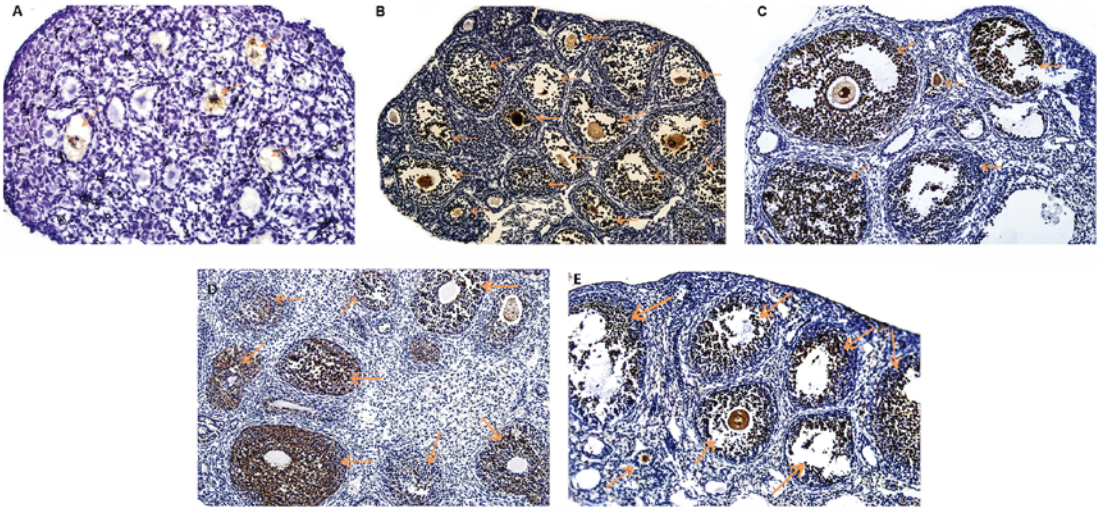

Figure 1. Immunohistochemical staining of ovarian tissues obtained from mice in the different age groups: (A) 4 days old (magnification, x400); (B) 3 weeks old (magnification, x200); (C) 8 weeks old (magnification, x200); (D) 12 weeks old (magnification, x100); and (E) 40 weeks old (magnification, x100). Arrows indicate follicles at each stage.

at 4˚C for 20 min. The sample was used to perform quantification of the protein expression. A total of 40 μg protein samples from the different age groups of mice were subjected to 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes, which were activated with methanol for 1-2 min. Next, the membranes were blocked with 5% skimmed milk at room temperature for 2 h and then incubated at 4˚C overnight with the Nrf2 (cat. no. ab62352; Abcam), Bcl-2 (cat. no. ab692; Abcam) and Caspase3 (cat. no. ab13847; Abcam) primary antibodies at 1:500 dilution, with human β-actin (cat no. 85845; Cell Signaling Technology, Inc.) serving as the internal reference. Membranes were subsequently washed three times with Tris-buffered saline/Tween 20 for 5 min each and then incubated with goat anti-rabbit immunoglobulin G secondary antibody (Alexa Fluor 488; cat. no. ab150077; 1:1,000 dilution; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 2 h in room temperature. The membranes were washed with TBST and the grey bands of proteins were evaluated using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). The intensity of the bands was determined semi-quantitatively through colorimetry detection with AlphaEaseFC software (Genetic Technologies, Inc., Miami, FL, USA), and the changes in protein band intensity were normalized to β-actin.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA isolation was performed using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT was then performed with the ReverTra Ace qPCR RT Kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol, using oligo-dT-primers and 1 μg RNA per reaction as a template. The isolated RNA was measured at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.) to assess RNA quality and quantity. qPCR analyses were run and analyzed using the ABI 7500 quantitative PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the Thunderbird SYBR qPCR Mix (Toyobo Life Science). The qPCR cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec and 60˚C for 1 min. The primer sequences used were as follows: Nrf2 forward, 5'-CAGCAT GATGGACTTGGA-3', and reverse, 5'-TGAGACACTGGT CACACT-3'; GAPDH forward, 5'-GGTCGGAGTCAACGG ATTTG-3', and reverse, 5'-ATGAGCCCCAGCCTTCTC CAT-3'. The relative expression of Nrf2 was normalized to GADPH expression and calculated according to the $2^{-\Delta\Delta Cq}$ method described by Livak and Schmittgen (34).

Statistical analysis. Statistical analysis was performed using IBM SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). The differences between groups were analyzed using Student's t-test or with one-way analysis of variance, followed by the Student-Newman-Keuls post hoc test. The results are expressed as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Nrf2 protein localization in mouse ovaries. The results indicated that Nrf2 protein was expressed in the ovarian tissue of all age groups. Immunohistochemical staining revealed that Nrf2 protein was predominantly expressed in follicular cells (granulosa cells), secondary follicles and antral follicles in the oocytes, while lower levels of expression were also observed in primary and primordial follicles (Fig. 1). The expression of Nrf2 protein in the ovarian tissues of the 4-day-old group was significantly lower when compared with that in the ovarian tissues of 3, 8, 12 and 40-week-old mice.

Expression of Nrf2 in the mouse ovaries. Significant differences in the expression of Nrf2 were observed when comparing the results of the different age groups. As shown in Fig. 2A, the relative expression of Nrf2 mRNA in mouse ovarian tissues was initially increased until ~12 weeks of age and then decreased with the increase in the age of mice. Furthermore, multiple comparisons among the different age

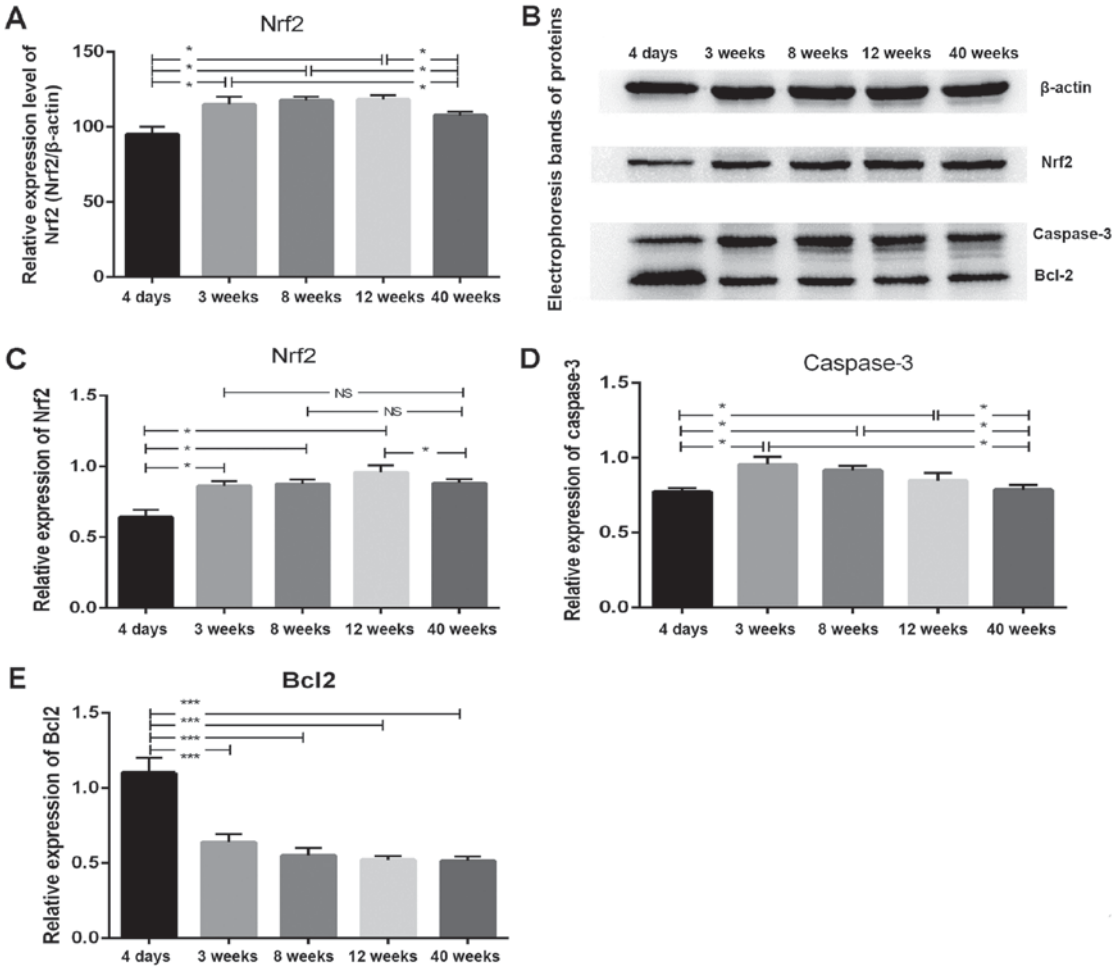

Figure 2. (A) Nrf2 mRNA expression was evaluated in mouse ovaries by reverse transcription-quantitative polymerase chain reaction analysis. (B) Western blot analysis of Nrf2, Caspase3 and Bcl-2 proteins in the ovarian tissues of different age groups. Quantified results of the relative protein expression of (C) Nrf2, and the apoptosis-associated proteins (D) Caspase3 and (E) Bcl-2 are shown. *P<0.05 and ***P<0.001. Nrf2, nuclear factor erythroid 2-related factor 2; Bcl-2, B-cell lymphoma 2; d, days; w, weeks; NS, non-significant.

groups revealed that the expression of Nrf2 protein in the ovaries of 4-day-old mice was significantly lower compared with that observed in the ovaries of the mice aged 3, 8 and 12 weeks (P<0.05; Fig. 2B and C). In addition, Nrf2 protein expression in the ovarian tissues of the 40-week-old group was markedly reduced when compared with that observed in the 12-week-old mice (P<0.05).

Expression of apoptosis-associated proteins Caspase3 and Bcl-2 in the mouse ovaries. Western blot analysis revealed significant differences in the expression levels of Caspase3, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein, among different age groups. As shown in Fig. 2D, with increasing age, the expression of the pro-apoptotic protein Caspase3 was initially increased, reached a peak level at 3 weeks and then decreased. The expression of Caspase3 in the ovaries of 4-day-old mice was markedly lower when compared with that in the 3, 8 and 12-week groups (P<0.05). In addition, the expression of Caspase3 in the ovarian tissue of 40-week-old mice was significantly reduced when compared with that in 3, 8 and 12-week groups (P<0.05). However, no significant differences in Caspase3 expression were identified among the 3, 8 and 12-week-old mouse groups (P=0.162). As presented in Fig. 2E, the expression of the anti-apoptotic protein Bcl-2 in the ovarian tissues of 4-day-old mice was significantly higher in comparison with that observed in the 3, 8, 12 and 40-week-old mice (P<0.001). However, no significant differences in Bcl-2 protein expression were identified among the 3, 8, 12 and 40-week-old groups (P=0.55).

Discussion

The ovaries are important for the production of sex steroidal hormones, such as progesterone and estrogen, and in reproduction. In women, the functional ability of the ovaries is gradually reduced with increasing age. Currently, failure of ovarian function due to the continuous aging process is unpreventable. Advancing age is a major factor in the release of excess ROS; coupled with aging, the defense mechanism is impaired at the cellular level, which initiates oxidative destruction (35). An excess output of free oxygen ions and intermediates causes cellular damage, which cannot be attenuated by the impaired defense system of the host. Such disequilibrium has a severe effect, stimulating the production of elements associated with cell death and the release of other enzymes, such as cytochrome

c (36), which are accountable for the decline in ovarian function observed during ovarian aging. Nrf2 is an important protein that adjusts the production of proteins associated with cytoprotection in response to harmful stressors from external sources (30). Nrf2 protein functions as a detector whose main action is to induce the inhibition of oxidation in various organs in the body, including the ovaries (37,38).

Low levels of Nrf2 may impede the inhibition of oxidation and may even cause reduced antioxidant support (39,40). Previous studies have revealed that ovarian aging is associated with decreased ovarian antioxidant defense capacity and increased oxidative damage (41-45). When activated, the Nrf2-ARE signaling pathway may have an important effect on the progression of aging (46). Glutamyl cysteine synthetase and heme oxygenase-1 are the macromolecules that assist in the restriction of oxidation and are induced by Nrf2 activation (47,48). In atrazine (ATR)-induced ovarian aging in rats, the number of ovarian atretic follicles increased with an increase in ATR concentration, while Nrf2 protein content and its expression in the nucleus were also significantly increased (49). In addition, in a 4-vinyl cyclohexene diepoxide (VCD)-induced ovarian failure in a rat model, it was demonstrated that the decrease in Nrf2 markedly increased OS and increased ovarian toxicity (50). It was also revealed that an increase in Nrf2 protein expression protected mouse ovarian cells from VCD ovarian toxicity (49). Therefore, the protein expression level of Nrf2 in ovarian tissues and during ovarian aging has certain relevance. The upregulation of Nrf2 protein in ovarian cells improves the antioxidant capacity, reduces OS and protects ovarian function. Zhao *et al* (49) reported that a severe oxidative reaction was influenced by ATR in the ovaries of rats. Nrf2, as a transcription factor, regulates the enzymes necessary to hinder oxidation and has a major role in protecting cells. When rat ovaries were continually exposed to ATR, Nrf2 action increased, thus, it may be an important antioxidant factor for future curative strategies (48).

In the present study, mice of different ages were employed to simulate the process of natural aging. Nrf2 protein was expressed in the ovarian tissues of mice at all ages, specifically in the follicular cells and secondary follicles, as well as in the antral follicles. In the mice aged 3, 8 and 12 weeks old, the expression of Nrf2 protein was higher when compared with that observed in mice aged 4 days and 40 weeks old; however, no significant difference in the protein expression of Nrf2 was observed between the 3, 8 and 12-week age groups. Thus, it is speculated that there may be a correlation between Nrf2 protein expression and ovarian reproductive function, and that Nrf2 protein may be highly associated with the protection of ovarian reproductive function. During the young (4 days old), growth (3-12 weeks old) and older (40 weeks old) periods of aging, as the reproductive function of the ovaries increased, the Nrf2 protein content in the ovarian tissue also exhibited an increasing and then a decreasing trend.

Detection of the apoptosis-associated proteins Caspase3 and Bcl-2 in the ovarian tissues of mice in the different age groups was also performed in the current study. The results demonstrated that the expression of the apoptosis inhibitor Bcl-2 decreased with increasing age, while the expression of the pro-apoptosis protein Caspase3 was affected by the age of mice. The Bcl-2 family mediates programmed cell death and is comprised of proteins that are pro- or anti-apoptotic in nature, thereby either preventing or inducing cell death (51). Depending upon the external or internal stimuli, the process of cell death is triggered by a series of actions, including the activation of important proteins known as Caspases and activation of numerous mediators from mitochondria, which are responsible for the destiny of a cell (51).

In conclusion, the results of the present study supported and confirmed the existence of a correlation between Nrf2 protein and ovarian function. Upregulated expression of Nrf2 protein in ovarian tissue can protect ovarian function and may delay ovarian aging. The results raised the possibility that Nrf2 protein signaling may be required for anti-OS processes during ovarian aging. Furthermore, the results of the present study, which used mice of different ages to represent the natural aging process, provided support for the hypothesis that increased ovarian OS may be responsible for premature ovarian senescence. However, the current study did have certain limitations, including a small group size, the use of mouse tissues and cells, which may not be representative of the condition in humans; the specific mechanism remains unclear. Thus, further research is required in order to resolve these issues and elucidate the underlying mechanism.

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Availability of data and materials

The data sets supporting the conclusions of the present study are included in this published article. The raw data stored on the main electronic data storage system of the Second Affiliated Hospital of Wenzhou Medical University are available from the corresponding author on reasonable request.

Authors' contributions

NS analyzed the raw data and wrote the manuscript. NS and AB performed the main experiment. YZ and XL collected the raw data. JL conceptualized and designed the study, and provided supervision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee Board of The Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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