

# Resveratrol improves ovarian function in aged rat by inhibiting oxidative stress and activating the Sirt1

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**Abstract.** Oxidative stress is a leading driver of ovarian aging. Silent mating-type information 2 homolog-1 (Sirt1) plays a role in ovarian function. Resveratrol has numerous effects, including anti-oxidant and Sirt1 activator. The aim of the study was to investigate the effect of resveratrol on aging-induced ovarian change in rats. The female Sprague Dawley rats were randomly divided into three groups: young control (Con), Aged+Res (20 mg/kg/day resveratrol for 45 days), and Aged. Anti-Müllerian hormone (AMH) was detected by ELISA assay. Malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were detected by conventional method. The ovarian structure and follicles were observed by hematoxylin staining, the caspase-3 and Sirt1 were detected by immunohistochemistry and Western blotting. The AMH in the Aged+Res group was elevated, compared to that in Aged group ( $p < 0.05$ ). The MDA was decreased and GSH-Px and SOD were increased in the Aged+Res group ( $p < 0.05$ ). The primordial and primary follicles were increased in the Aged+Res group ( $p < 0.05$ ). The Sirt1 was increased and caspase-3 was decreased in the Aged+Res group ( $p < 0.05$ ). These results indicate that resveratrol can delay ovarian aging, probably by reducing oxidative damage and increasing Sirt1.

**Key words:** Resveratrol — Aging — Ovary — Oxidative stress — Sirt1

## Introduction

Female reproduction is accompanied with depletion of ovarian reserve with aging. Ovarian aging is characterized by gradual decline in ovarian follicle quantity and quality. The ovarian aging process is affected by a number of factors. The free radical theory has been a classical theory, which is generally accepted that oxidative stress is one of the major mechanisms in aging (Meldrum and David 2013). The previous studies have documented that oxidative stress is a leading driver of the ovarian aging process and promotes the ovarian aging (Benkhalifa et al. 2014). Alleviating oxidative stress in ovary is an important mean to delay ovarian aging.

Resveratrol, a natural polyphenol compound, is extracted from a variety of plant species and has wide biological effects including anti-inflammatory, antioxidant, and anti-aging (Oh and Shahidi 2018). Currently, a study has indicated that resveratrol has potentially beneficial effects for ameliorating ovarian function (Pasquariello et al. 2020). In addition, studies have shown that the antioxidant can reduce reactive oxygen species (ROS) in aged rat (Liu et al. 2012). Previous studies indicated that resveratrol has been shown to act as an antioxidant, which can effectively remove and prevent DNA damage caused by ROS (Leonard et al. 2003) and also showed a direct ROS-scavenging property (Bradamante et al. 2004). So far, studies have shown that resveratrol reduced oxidative stress and inhibited apoptosis in granulosa cells in a rat model of primary ovarian insufficiency (POI) (Li and Liu 2018). Resveratrol also directly affects ovarian cell sirtuin, proliferation, apoptosis, hormone release and response to follicle-stimulating hormone (FSH) and insulin-like growth

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factor I (IGF-I) (Sirotkin et al. 2019). Whether resveratrol can change ovarian dysfunction during the aging process is undefined. These findings pushed us to explore the possibility of that resveratrol might also be able to delay the ovarian aging process.

Sirtuins (SIRT1) control ovarian functions at various regulatory levels and can be markers of the pathological state of ovarian cells (Alexander 2016). Silent mating-type information regulation 2 homolog-1 (Sirt1), a member of the SIRT1 family, plays an important role in regulation ovarian function and female fertility (Estienne et al. 2021). The Sirt1 expression in ovaries is decreased with aging (Ma et al. 2015). Previous research has suggested that Sirt1 plays a potential protective role against oocyte aging by controlling ROS generation (Zhang et al. 2016). Otherwise, more recent results provide that the Sirt1 plays an important role in regulating follicular growth and development (Zhou et al. 2015). Sirt1 can increase ovarian reserve and prolong reproductive life in obese mice (Zhou et al. 2014) and can be also involved in human reproduction and may have a role in oocyte maturation and clinical pregnancy (Bódis et al. 2019). Previous studies have shown that resveratrol is a Sirt1 activator (Howitz et al. 2003).

On the basis of the background, we aimed to investigate the effect of resveratrol on aged rats - whether addition of resveratrol could prevent aging-induced ovarian insufficiency.

## Materials and Methods

### Animals

A total of 18 Sprague Dawley female rats were used. The rats were provided by the Experimental Animal Centre of Hebei Medical University. The rats were kept in room temperature (22°C) and were fed *ad libitum*. Animal experiments were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals.

### Experimental groups and design

The female Sprague Dawley rats were randomly divided into three groups: Con, Aged+Res, and Aged. Con group: 2-month-old rats were given equal volume saline for 45 days,  $n = 6$ ; Aged+Res group: 15-month-old rats were given resveratrol 20 mg/kg/day for 45 days,  $n = 6$ ; Aged group: 15-month-old rats were given equal volume saline for 45 days,  $n = 6$ . All drugs were administered *intraperitoneally* (*i.p.*). The dose of resveratrol was chosen according to the previous study that resveratrol may inhibit primordial follicle assembly to increase the size of primordial follicle pool (Kong et al. 2011) and our studies (Nie et al. 2021). Resveratrol (900386, Vetec, St.Louis, USA) was dissolved

in 96% ethanol to a concentration of 50 mg/ml as stock solution and was diluted in saline immediately before using.

At the end of the period, rats were sacrificed using high-dose anesthetic (50 mg/kg of thiopental *i.p.*). Their blood samples were collected by cardiac puncture using a 5 ml syringe. The ovarian samples were removed and weighed. The right ovaries were carefully dissected from the surrounding tissues and kept at  $-80^{\circ}\text{C}$  for Western blotting. The left ovaries were fixed in 4% paraformaldehyde (PFA) for histological follicle assessment.

### Enzyme immunoassay for AMH

Blood samples were collected when rats were sacrificed. Levels of serum AMH (Anti-Mullerian hormone) were measured by enzyme-linked immune sorbent assay (ELISA) according to the manufacturer's instructions (CSB-E11162r, KeyGEN BioTECH, Nan Jing, China). The detection range of AMH was 0.2–15 ng/ml. The microplate reader wavelength used for measurement was 450 nm. Each biological sample was performed with two technical repetitions, and the analysis of the data was carried out using six independent biological replicates.

### Ovarian histology and follicular evaluation

For histological examination, the ovaries were fixed in 4% PFA for 12 h and then dehydrated, and 5- $\mu\text{m}$  thick sections were prepared. The paraffin-embedded sections were stained with haematoxylin for 5 min and eosin for 2 min. The follicles were counted as defined previously (Luo et al. 2008). The number of follicles *per ovary* was calculated by five fields throughout the whole ovaries. Follicles were classified as primordial and primary surrounded by a single layer of squamous or cuboidal granulosa cells. Secondary follicles were identified with more than one layer of granulosa cells and no visible antrum. Maturing and mature or antral follicles were identified with small areas of follicular fluid (antrum) or a single large antral space.

### Measurement of oxidative stress parameters

The samples from each rat were collected and were measured according to the manufacturer's instructions. The malondialdehyde (MDA; KGT004-1, KeyGEN BioTECH, NanJing, China) was determined according to the thiobarbituric acid (TBA) method. A 100  $\mu\text{l}$  test sample and 1000  $\mu\text{l}$  working reagent were mixed and the standard tube and blank tube were set. The solutions were incubated in boiling water for 40 min. After cooling, the solution was centrifuged at 4000 rpm for 10 min to remove the precipitate and then the absorbance was measured at 532 nm with a microplate reader. The results were expressed as nmol/ml.

The superoxide dismutase (SOD; A001-3, Jiancheng Institute of Biological Engineering, NanJing, China) activity was determined according to the WST-1 method. 20  $\mu$ l test sample, 20  $\mu$ l enzyme diluent and 100  $\mu$ l substrate application solution were mixed at 37°C for 20 min. The optical density (OD) values were measured by a microplate reader at 450 nm. The GSH-Px (KGT014, KeyGEN BioTECH, NanJing, China) activity was determined according to the colorimetric method. A 100  $\mu$ l test sample and 200  $\mu$ l GSH were mixed at 37°C for 5 min, and the working fluid was added at 37°C for 5 min. Then the color reaction was performed. The OD values were measured by a microplate reader at 412 nm. Each biological sample was performed with two technical repetitions, and data analysis carried out using six independent biological replicates.

### Immunohistochemistry

The 5  $\mu$ m-thick sections from paraffin-embedded ovaries were deparaffinized and rehydrated using xylene and ethanol and were immersed in a 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase. The sections were boiled for 30 min in citrate with buffer solution (pH 6.0) for antigen retrieval. The slides were incubated with 10% goat serum for 30 min, and the Sirt1 (Cat. ab189494, Abcam, London, UK) and caspase-3 (Cat. ab184787, Abcam, London, UK) antibody were added (1:400) at 4°C for 16 h. The secondary antibody working fluid was added for 1 h and horseradish peroxidase was incubated for 30 min at room temperature. Counter staining was performed using haematoxylin. Immunohistochemical quantification was carried out by measuring the optical density of 5 fields using image analysis software (Image J).

### Western blotting

The ovaries were homogenized by ultrasound and the protein was extracted by RIPA lysis buffer with protease inhibitor. The protein concentration was analyzed by Nucleic acid protein concentration meter (Nanodrop ND-1000, Thermo Scientific, MA, USA). 30  $\mu$ g protein samples were separated by polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred into nitrocellulose membranes, which were blocked in 5% milk with tris-buffered saline with Tween-20 and were incubated overnight at 4°C with primary antibodies, including caspase-3 (Cat. ab184787, Abcam, London, UK) and Sirt1 (Cat. ab189494, Abcam, London, UK) antibody. Next day, the membranes were washed and 1:5000 biotinylated anti-rabbit IgG (H+L) antibody (474-1612, KPL, USA) was added at 37°C for 2 h. The protein bands were visualized using chemiluminescence reagents. The  $\beta$ -actin (AC026, ABclonal, USA) was added as the internal control. Three biological repeats were performed for each sample. The

results were scanned using an Amersham Imager 600 and the bands were quantified using Image J software.

### Statistical analysis

SPSS 21.0 software was used to conduct the statistical analyses. Descriptive statistics for each variable were determined. Normality of the data distribution was assessed with the Kolmogorov-Smirnov test. Results for continuous variables were demonstrated as mean  $\pm$  SD. Significance of differences between the groups was determined using oneway ANOVA test followed by Fisher's *post-hoc* LSD (least significant differences) analysis.  $p < 0.05$  was considered statistically significant.

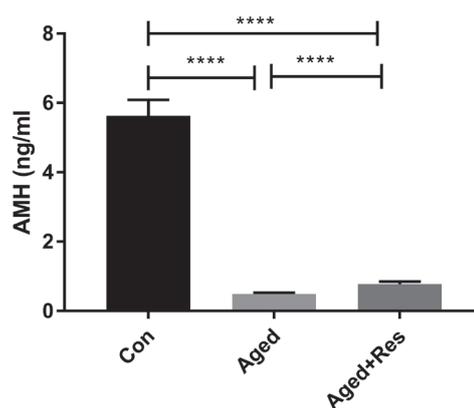
## Results

### Resveratrol increased AMH of aged rats

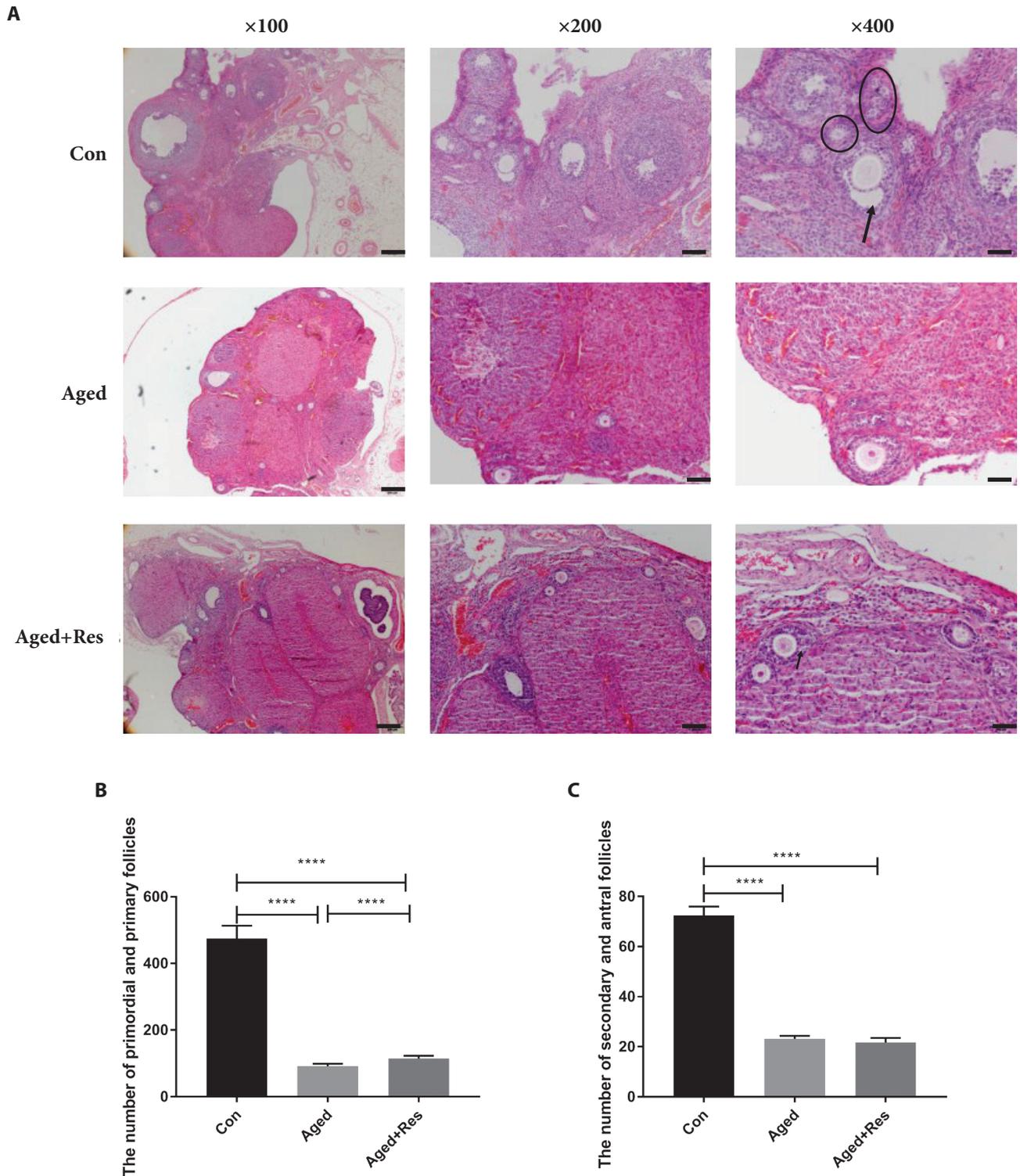
The level of AMH was significantly lower in the Aged and Aged+Res groups than that in the Con group; however, it was significantly increased in the Aged+Res group then that in Aged group (Fig. 1). The result indicates that resveratrol can improve rat ovarian endocrine functions.

### Resveratrol increases the number of primordial and primary follicles

We found that the control rats exhibited a large number of primordial and primary follicles, as well as growing and mature follicles (Fig. 2A). The rats of Aged group showed



**Figure 1.** The effects of resveratrol on AMH (Anti-Mullerian hormone) in Con, Aged+Res, and Aged groups. Con, 2-month-old rats were given equal volume saline; Aged+Res, 15-month-old rats were given resveratrol 20 mg/kg/day for 45 days; Aged, 15-month-old rats were given equal volume saline. Each treatment group,  $n = 6$ . Values of  $p < 0.05$  was considered to be statistically significant. \*\*\*\*  $p < 0.0001$ .



**Figure 2. A.** The effects of resveratrol on follicles observed by H&E staining. The ovary showed different stages of follicles in the Con group and a reduced number of primordial and primary follicles, and few growing follicles in the Aged group. In the Aged+Res group the ovary showed a relatively reduced number of primordial and primary follicles, and growing follicles. Scale bars, 50  $\mu$ m (left), 100  $\mu$ m (middle), 200  $\mu$ m (right). The ellipse shows primordial follicles, the circle indicates a primary follicle, short arrow indicates a secondary follicle, and long arrow indicates an antral follicle. **B.** The effects on the primordial and primary follicles. **C.** The effects on secondary and antral follicles. Values are expressed as mean  $\pm$  SEM,  $n = 6$  (in each treatment group). \*\*\*\*  $p < 0.0001$ . For abbreviations, see Fig. 1.

a markedly reduced number of primordial and primary follicles, and a few growing and mature follicles. The Aged+Res group had significantly more primordial and primary follicles compared with Aged group.

As shown in Figure 2B and 2C, the follicle counts showed that follicles were reduced in the Aged group compared to that in Con group ( $p < 0.05$ ). In the Aged+Res group, the counts of the primordial and primary follicles were increased compared to that in Aged group ( $p < 0.05$ ). The number of secondary and antral follicles showed no significant difference between the Aged group and Aged+Res group ( $p > 0.05$ ). These results demonstrate that resveratrol can increase ovarian reserve significantly.

#### Resveratrol attenuated oxidative stress of aged rats

As shown in Figure 3, the level of MDA was significantly higher in the Aged group ( $p < 0.05$ ), whereas GSH-Px and SOD were significantly lower in the Aged group than Con group ( $p < 0.05$ ). The MDA was significantly lower in the Aged+Res group, whereas GSH-Px and SOD were higher in the Aged+Res group than that in Aged group ( $p < 0.05$ ). These results demonstrate that resveratrol decreases oxidative damage in ovaries.

#### Resveratrol reduced caspase-3 and increased Sirt1 expression

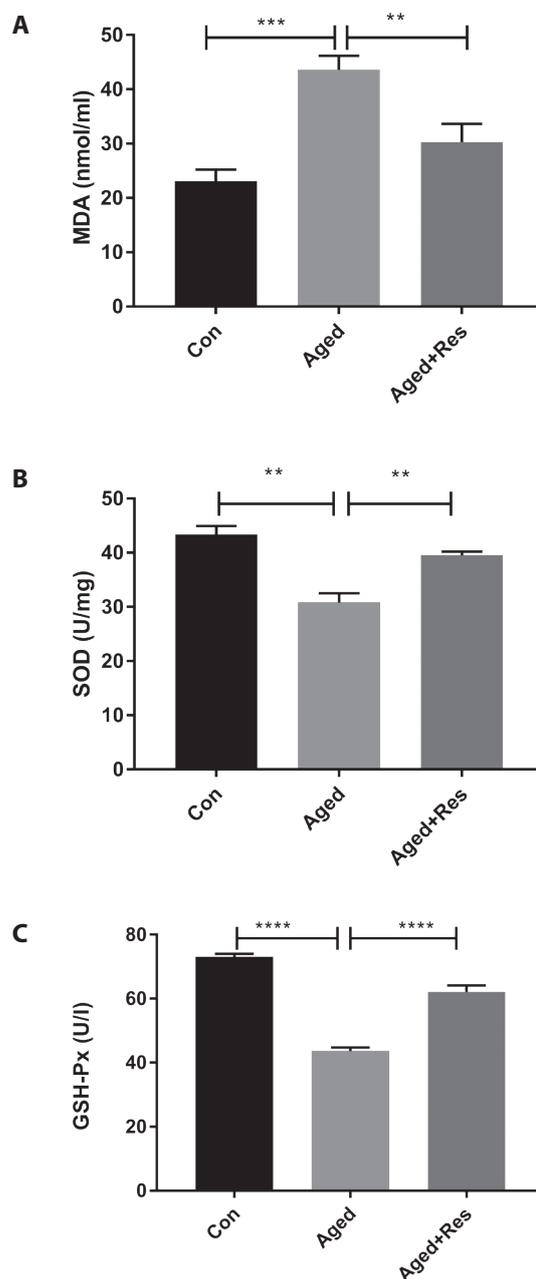
We also tested the levels of caspase-3 and Sirt1 by immunohistochemistry. As shown in Figure 4A, the caspase-3 was located in the nucleus and cytoplasm of granulosa cells, theca cells as well as luteal cells. The caspase-3 expression was significantly lower in the Aged+Res group than that in Aged group (Fig. 4B). The Sirt1 was located in the nucleus and cytoplasm of granulosa cells, theca cells and luteal cells (Fig. 4A). Immunohistochemical staining revealed that ovarian sections from the Con group and Aged+Res group showed a strong immunostaining for Sirt1. The Aged group showed a decreasing in Sirt1 immunopositive cells (Fig. 4C).

The expressions of caspase-3 and Sirt1 were also assessed by immunoblotting analysis (Fig. 5A). Our results showed that the level of caspase-3 protein was significantly decreased in the Aged +Res group, compared to Aged group (Fig. 5B). The expression of Sirt1 protein was significantly increased in the Aged+Res group compared to Aged group (Fig. 5C).

## Discussion

The current research provides a novel finding that resveratrol probably prevents rat ovarian aging. Our results demon-

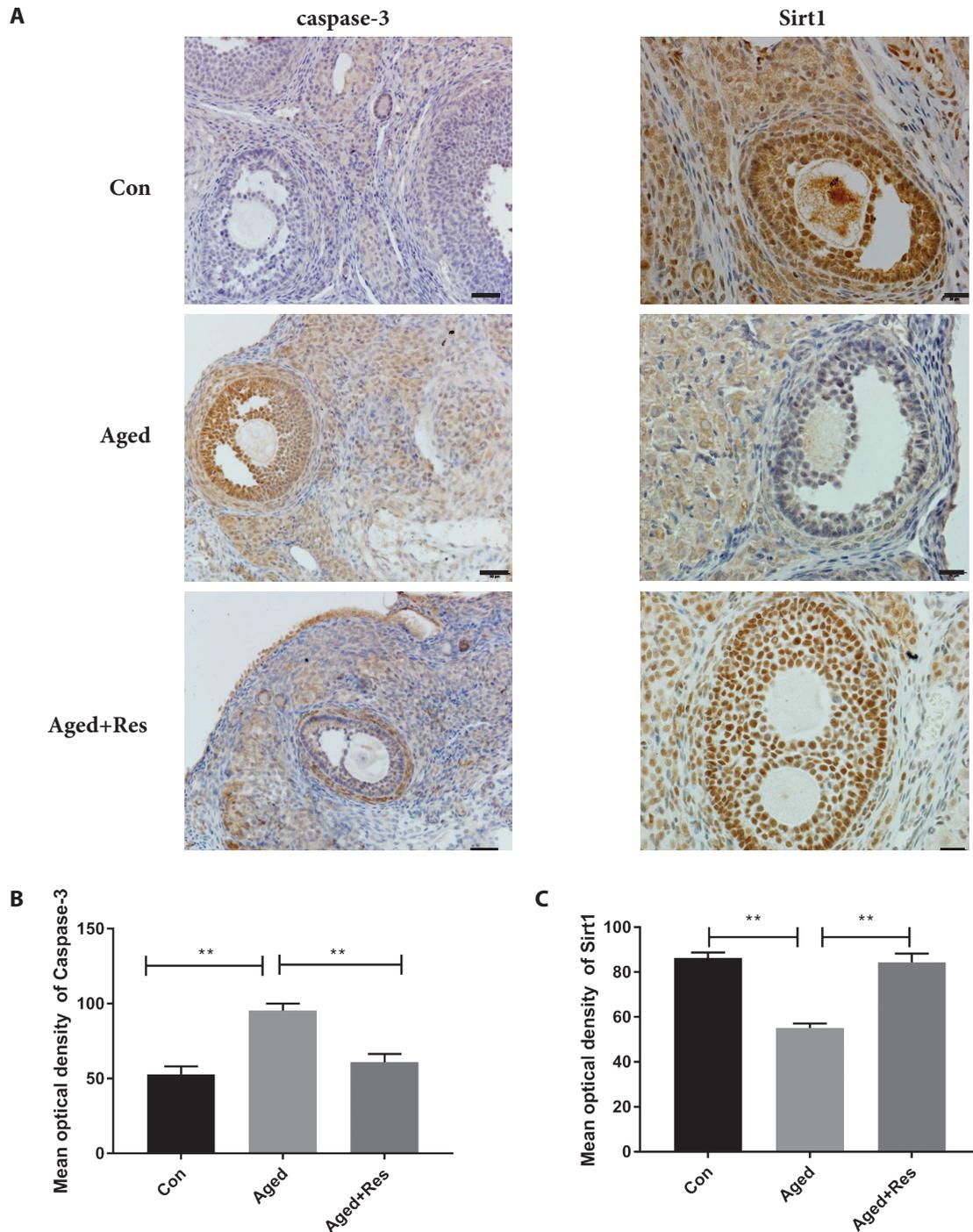
strated that resveratrol increased AMH levels and number of primordial follicles and decreased aging-induced oxidative stress and follicle apoptosis. The study also found the Sirt1 maybe was participated in the process.



**Figure 3.** The effects of resveratrol on MDA (malondialdehyde) level (A), on the activity of SOD (superoxide dismutase; B), and on the activity of GSH-Px (glutathione peroxidase; C). Values are expressed as mean  $\pm$  SEM,  $n = 6$  (in each treatment group).  $p < 0.05$  was considered to be statistically significant. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . For more abbreviations, see Fig. 1.

Ovarian function decreases with age. This was indeed the case in the aging rats with reduced AMH and primordial and primary follicles. By contrast, resveratrol treatment remarkably increased the AMH and amounts

of the primordial and primary follicles in the Aged+Res group compared to those in Aged group, indicating that resveratrol could maintain the ovarian reserve. Kong et al. (2011) reported that resveratrol may inhibit primor-



**Figure 4.** The cellular location of the caspase-3 and Sirt1 in the rat ovary observed using immunohistochemistry. **A.** The cellular location of the caspase-3 and Sirt1 in the rat ovary. **B.** The mean optical density of caspase-3. **C.** The mean optical density of Sirt1. Scale bars, 50  $\mu$ m. Values are expressed as mean  $\pm$  SEM. \*\*  $p < 0.01$ . For abbreviations, see Fig. 1.

dial follicle assembly to increase the primordial follicle pool. Liu et al. (2013) provided evidence that resveratrol protects against the reduction of fertility with aging. We have confirmed previously that resveratrol can prevent the primordial follicle activation (Nie et al. 2021). This study also held the opinions that resveratrol presumably inhibited primordial follicle development and delayed the depletion of primordial follicle.

At present, numerous studies have documented that oxidative stress is a leading driver of ovarian aging. To date, several studies have shown that ROS accumulation in the ovaries deteriorates oocyte quality and induces granulosa cells apoptosis (Wang et al. 2017; Gong et al. 2020; Park et al. 2020). As a powerful antioxidant, resveratrol effectively removes and prevents DNA damage and lipid peroxidation caused by ROS (Leonard et al. 2003). In the present study, we noted that aging leads to the increase of MDA and the decrease of antioxidant enzymes. Resveratrol improved antioxidant enzymes activity and reduced MDA level in the Aged group. The antioxidant enzymes play important roles in follicular development and/or survival (Yant et al. 2003). The cumulus oophorus cells produce antioxidants and protect the oocyte from oxidative damage (Matos et al. 2009). The ovarian immunohistochemistry and Western blotting analysis confirmed there was high caspase-3 expression in the Aged group and low caspase-3 expression in the Aged+Res group. So, we presumed resveratrol possibly enhanced antioxidant capacity and prevented the ovarian apoptosis caused by oxidative stress with aging.

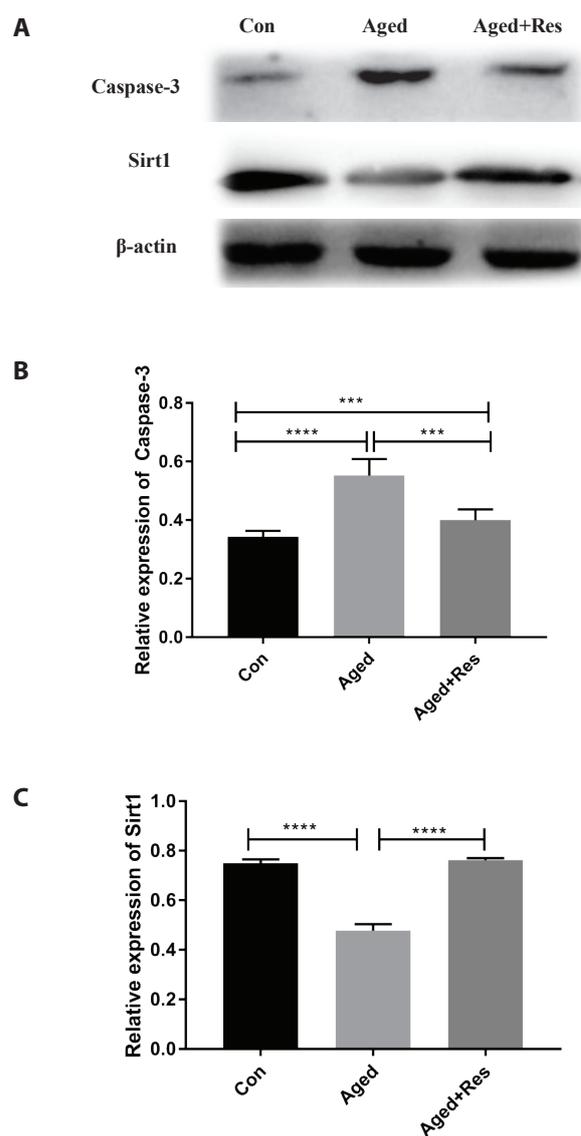
The previous study showed that the expression of Sirt1 declines with age, and ovarian reserve is positively correlated with the increase of Sirt1 in mice (Zhang et al. 2015). The Sirt1, a member of the SIRT family, has recently emerged as a vital molecule in controlling ovarian function (Michan and Sinclair 2007). Sirt1 is markedly decreased in granulosa cells (GCs) from premature ovarian failure (POR) patients relative to normal and polycystic ovary syndrome patients (Ying et al. 2017). Our study demonstrated that Sirt1 was located in the nucleus and cytoplasm of granulosa cells, theca cells as well as luteal cells. We found that Sirt1 in the Con group and Aged+Res group showed a strong immunostaining, while the Aged group showed a decreased in Sirt1 immunopositive cells. The studies suggested that resveratrol improved ovarian functions, which possibly related to activate Sirt1. In summary, these results suggested that resveratrol could increase ovarian reserve and delayed ovarian aging by activating Sirt1.

Our work has some limitations. First, the results were performed on rats and may not be assigned to human. Therefore, our study needs to be verified in human in future studies. In the absence of further research or human studies, the results should be carefully applied to human and clinical applications. In addition, there are many factors involved

in ovarian aging, only a small number of indicators were investigated in the study.

## Conclusion

In conclusion, these results indicate resveratrol staves off ovarian aging by (i) inhibiting the decrease of primordial follicle number, (ii) increasing antioxidants (SOD and



**Figure 5.** The effects of resveratrol on caspase-3 and Sirt1 protein expression. **A.** The caspase-3 and Sirt1 protein levels were observed with Western blotting,  $\beta$ -actin was used as the internal control. **B.** Quantitative analysis of relative expression of caspase-3. **C.** Quantitative analysis of relative expression of Sirt1. Values are expressed as mean  $\pm$  SEM. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . For abbreviations, see Fig. 1.

GSH-Px), (iii) and upregulating Sirt1 and downregulating caspased-3 in the aged rats. Resveratrol increases primordial and primary follicles and decreases apoptosis and Sirt1 probably plays an important role in regulating follicular growth and development in aged rat ovary *in vivo*. However, there is a need for further studies, including human trials, before making a definitive statement about the potential benefit of resveratrol addition to aging ovarian dysfunction.

**Conflict of interest.** The authors declare that they have no conflict of interest.

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