Yifuning postpones ovarian aging through antioxidant mechanisms and suppression of the Rb/p53 signal transduction pathway

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Abstract. Yifuning is a traditional Chinese medicine recipe that has been used for many years in China for its effects on treating climacteric syndrome in women. The present study aimed to demonstrate the effects and underlying molecular mechanism of Yifuning on the ovaries of aging rats. Selected aging rats were administered different doses of Yifuning (1.0 or 2.0 g/kg by lavage), and after 6 weeks the rats were sacrificed. The activit of indicators of oxidative stress in the serum were measured. The expression levels of 8-oxo-2'-deoxyguanosine (8-OHDG) and p53 in the ovaries were examined using immunohistochemistry. The expression levels of the corresponding genes and proteins were detected by reverse transcription-quantitative polymerase chain reaction and western blotting analyses, respectively. The results indicated that Yifuning significantly prevented ovarian failure, as indicated by improvements in estrous cycling, reproductive organ weights and sex hormone serum levels. Yifuning significantly increased the levels of superoxide dismutase, glutathione peroxidase, catalase and reduced malondialdehyde and hydrogen peroxide levels. Yifuning reduced DNA damage in the ovaries by reducing the expression of 8-OHDG and p53. Treatment with Yifuning significantly reduced the age-induced p19, p53, p21 and Rb activity in the ovaries. The present study demonstrates that Yifuning prevents ovarian failure and the mechanism involved is partly associated with antioxidants and suppression of the Rb/p53 signal transduction pathway.

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Introduction

Perimenopause, or menopause transition, is the stage of women's reproductive life which begins several years prior to menopause, and one of the characteristics of this is ovarian aging (OA). In this period, women's ovarian function and estrogen secretion declines, and the body enters the menopausal transition, during which a number of clinical symptoms appear (1-3). Increasing evidence indicates that a number of environmental and dietary factors may contribute to OA through oxidative stress-related mechanisms (4-6).

Oxidative stress has been implicated to serve an important role in the perimenopause. Women who are undergoing perimenopause have a reduced ability to repair DNA (2). Oxidative damage may also be responsible for the declines in ovarian function and oocyte quality with age (7,8). Previous studies have proposed that reduced ovarian antioxidant gene expression is a factor in the oxidative damage to ovarian lipids, proteins and DNA (9,10). p53 is vital in regulating the $p19^{ARF}$-p53-p21-retinoblastoma (Rb) pathway, which serves a critical role in the regulation of cell proliferation, differentiation and aging (11,12). When p53 is activated, the pathway becomes activated, inhibiting cell proliferation and thus promoting cellular aging. The response of p53 to oxidative stress depends on the type and extent of stress signals that activate p53, which may promote or prevent aging (13). High level of oxidative stress can activate p53 and lead to apoptosis and senescence (14,15).

Millions of women suffer from the discomforts of the perimenopause which is characterized by ovarian aging. The search for effective anti-aging medicines is may be key to avoiding numerous diseases associated with aging. Previous studies indicated that certain native medicines may alter ovarian follicular development and affect ovarian follicle reserves,

thereby affecting female reproductive aging (16-19). Yifuning (YFN), a traditional Chinese medicine recipe, is composed of Ranae Oviductus (*Rana temporaria chensinensis David*) and Zedoary Turmeric Oil (oil from *Curcuma wenuyjin* Y. H. Chen et C. Ling). They are common ingredients in prescriptions of anti-aging medication and for treating climacteric syndrome in women, as shown in Chinese Pharmacopoeia (2010 edition) (20) and some modern pharmacological studies (21). In a previous study, it was reported that Yifuning was able to improve perimenopausal symptoms in women (22-24), and YFN was indicated to improve symptoms in ovariectomized rats (25). Ovarian aging is closely associated with the perimenopause, however, the ability of YFN to affect ovarian function remains unclear. In the present study, the effect of YFN on OA in aging female rats and the underlying molecular mechanisms were investigated.

Materials and methods

Reagents. YFN was obtained from the College of Traditional Chinese Medicine of the Southern Medical University (Guangzhou, China) and was prepared in vegetable oil at different concentrations. Diethylstilbestrol (lot no. 20100829) was obtained from Hefei Jiulian Pharmaceutical Company Ltd., (Hefei, China). The rabbit monoclonal p21 (cat. no. 9313) and Rb (cat. no. 9313) antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). The rabbit polyclonal p19 (cat. no. sc-1066) and the 8-hydroxydeoxyguanosine (8-OHDG; cat. no. sc-139586) antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). The mouse monoclonal p53 antibody (cat. no. PB0076, 2524) was purchased from Bioworld Technology, Inc., (Shanghai, China) for the immunohistochemical analyses or from Cell Signaling Technology, Inc., for western blotting. Malondialdehyde (MDA), glutathione peroxidase (GSH-Px), catalase (CAT), hydrogen peroxide (H_2O_2) and superoxide dismutase (SOD) assay kits were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and treatment. Female Sprague Dawley rats were purchased from the Southern Medical University Animal Center (Guangzhou, China) at 3 (n=6) and 16-18 months of age (n=35). Animal care and use was performed in accordance with the Animal Research Institute Committee guidelines of Southern Medical University Animal Center. The study was approved by the Southern Medical University Animal Care and Use Committee. Estrous cycling in adult female rats was evaluated every morning for a minimum of 14 days using vaginal cytology (9). Following the evaluation of estrous cycling, the selected aging rats (n=30) with irregular cycles were divided into five experimental groups (n=6 per group): O-C group (Old control group); DT group (rats were treated with diethylstilbestrol, 0.05 mg/kg); YFN-H group (rats were treated with the high dose of YFN, 2.0 g/kg); YFN-L group (rats were treated with the low dose of YFN, 1.0 g/kg); Y-C group (the 3 months old rats were kept as the young control group). The animals were maintained under controlled light (12 h light/dark cycle) and temperature (20-24˚C) throughout the study, including during the treatment period, and received a standard diet and water *ad libitum*. The rats were treated with DT and YFN for 6 weeks. All non-treated animals received corresponding doses of the vehicle (vegetable oil).

All animals received humane care according to the Guidelines for the Ethical Care of Experimental Animals of the European Union (26). Following treatment, the rats were sacrificed under 10% chloral hydrate anesthesia, followed by abdominal aortic blood collection, and the ovarian tissues were collected and immediately frozen in either liquid nitrogen (for western blot determinations), RNAlater® (for PCR determinations), or 4% formaldehyde (for the immunohistochemical analyses).

Determination of biochemical parameters. Blood samples were collected and centrifuged at 3000 x g for 15 min at 4˚C, and serum estradiol (E_2), testosterone (Te) and progesterone levels were measured using electrochemiluminescence immunoassays (Roche Diagnostics GmbH, Mannheim, Germany). Oxidative stress (OS) indicators GSH-Px, MDA, SOD, H_2O_2, and CAT activity were measured according to the manufacturer's recommended instructions (Nanjing Jiancheng Bioengineering Institute).

Immunohistochemical staining. Immunohistochemistry was performed as previously described (27). Following dewaxing, antigens were retrieved in 0.01 M sodium citrate buffer for 5 min by pressure cooking. Sections were blocked for 30 min in normal 16.6% swine serum, which was diluted 1:5 in Tris-buffered saline (pH=7.4), supplemented with 5% bovine serum albumin, then further blocking with a streptavidin/biotin blocking kit (Vector Laboratories, Ltd., Peterborough, UK). The sections were incubated overnight with 8-OHDG and p53 primary antibodies at 4˚C (diluted 1:100 in blocking solution). At room temperature the primary antibodies were detected following incubation with biotinylated-swine anti-rabbit secondary antibody and streptavidin-horseradish peroxidase (cat. nos. MP-7451 and SA-5014; Vector Laboratories, Ltd.) mixtures for 30 min. Using a 3,3'-diaminobenzidine staining kit (Gene Tech, Ltd., Shanghai, China) the bound antibodies were visualized. The slides were counterstained with hematoxylin, dehydrated and coverslipped. They were photographed using a Nikon Eclipse light microscope (Nikon Corporation, Tokyo, Japan). Similar areas of staining between groups were compared to assess the effect.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the ovarian tissues using RNA TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 5 μg of total RNA for each sample was reverse-transcribed into cDNA using a cDNA synthetisis kit (Takara Bio, Inc., Otsu, Japan). The cDNA was diluted 1/100, then 5 μl was used as a template for each PCR reaction. The PCR reaction parameters were as follows: 95˚C for 10 min, 35 cycles of denaturing at 94˚C for 30 sec, annealing at 60˚C for 30 sec, extension at 72˚C for 60 sec and 7 min at 72˚C. qPCR was performed on an ABI PRISM 7300 PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBRGreen Taq qPCR Mastermix (Takara Bio, Inc.). The expression levels of the target genes (p19, p21, p53 and Rb) were used to generate standard curves, which were normalized against an endogenous reference

gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequences of the primers used in qPCR were as follows: GAPDH forward, 5'-ATTGTCAGCAATGCATCCTG-3' and reverse, 3'-ATGGACTGTGGTCATGAGCC-5'; p19 forward, 5'-GGTCACCGACAGGCATAACT-3' and reverse, 3'-CCAGAAGTGAAGCCAAGGAG-5'; p53 forward, 5'-GTTCCGAGAGCTGAATGAGG-3' and reverse, 3'-AGGATGCAGAGGCTGTCAGT-5'; p21 forward, 5'-TGCAATGAGGGACCAGTACA-3' and reverse, 3'-CCTGAGCCTGTTTCGTGTCT-5'; and Rb forward, 5'-TTGGCTAACGTGGGAGAAAG-3' and reverse, 3'-AATGGCATCTCATCCAGGTC-5'. The mRNA sequences of GAPDH, p19, p53, p21 and Rb were obtained from the UCSC Genome Bioinformatics website (https://genome.ucsc.edu/), and the primers were designed by Invitrogen (Thermo Fisher Scientific, Inc.). GAPDH was used as a housekeeping gene to compare the samples. PCR was performed three times for each sample and each gene. Expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (28).

Western blotting analyses. Ovarian tissues were powdered with liquid nitrogen and lysed in lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP40, 20 mM Tris base, pH 7.5) containing protease-inhibitor (1 mM of phenylmethane sulfonyl fluoride). Then, the homogenized tissues were centrifuged at 4˚C 14,000 x g for 30 min and supernatants were subjected to western blot analysis. Protein was quantified using a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology; Thermo Fisher Scientific, Inc.). Denatured samples (20 μg) were resolved on 5-12% SDS-PAGE gels and 5% stacking gel. The proteins were then transferred nitrocellulose membranes. Non-specific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20 (TBS-T) for 1 h at room temperature. Membranes were washed 3 times with TBS-T for 10 min and incubated with a specific primary antibodies (p19, p21, p53 and Rb; dilution 1:1,000) overnight at 4˚C. The membranes were then washed with TBS-T and incubated with horseradish-peroxidase conjugated goat anti-rabbit (dilution 1:10,000; cat. no. 7074) and goat anti mouse (dilution 1:10,000; cat. no. 7077) secondary antibody (Cell Signaling Technology, Inc.) for 1 h at room temperature. Using an enhanced chemiluminescence (ECL) detection system the immune complexes were visualized. Protein expression levels were determined using an image analyzer (LAS-3000; Fujifilm, Tokyo, Japan).

Statistical analyses. Statistical analyses were performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Differences in estrous cycles, sex hormones and OS indicators were evaluated using one-way analysis of variance. The transformed data and ovarian mRNA expression levels were analyzed using general linear regression procedures. The data are presented as the mean ± standard deviation.

Results

Estrous cycling and reproductive organ weights. As shown in Fig. 1A, the results indicated that the estrous cycles of rats in the O-C group were prolonged compared with rats in the Y-C group (P<0.001). The estrous cycles of rats in the DT- and YFN-treated groups were markedly shorter compared with the O-C group (P=0.047). The weights of the rats in the Y-C group significantly differed from the weights in the O-C group (P=0.025), however, the body weights of the rats in the DT, YFN-L and YFN-H-treated groups did not differ from the weights in the O-C group (P=0.063), as shown in Fig. 1B.

The ovarian index is equal to the weight of the ovaries divided by the body weight of the rat multiplied by 100, while the uterine index is equal to the uterine quality divided by the body weight of the rat multiplied by 100. As shown in Fig. 1C and D, following 6 weeks of lavage, the ovarian index of the O-C group did not differ from that of the Y-C group (P=0.053). The ovarian index of the YFN-H group was significantly improved compared with that of the O-C group (P=0.001). The uterine index of the O-C group was significantly reduced compared with that of the Y-C group (P<0.001). The DT and YFN-H-treated groups displayed marked improvements in uterine index compared with the O-C group (P=0.045 and P=0.001, respectively), and the uterine index of the YFN-H group was greater than that of the DT-treated group (similar to that of the Y-C group).

Effects of YFN treatment on serum E_2, Te and progesterone levels. As shown in Fig. 1E-G, it was observed that YFN treatment increased the levels of sex hormones in the natural aging model rats. The levels of E_2 and Te in the O-C group were significantly reduced compared with those in the Y-C group (P<0.001). The levels of progesterone were reduced in the O-C group compared with the Y-C group but did not differ significantly (P=0.055). DT and YFN treatment altered the levels of sex hormones in the rats, increasing the serum E_2 and Te levels. The serum levels of E_2 and Te in the DT-treated, YFN-H, and YFN-L groups were significantly increased compared with the O-C group (P=0.035, P=0.001 and P=0.005, respectively).

Effects of YFN treatment on GSH-Px, SOD and CAT activity and MDA and H_2O_2 levels. As shown in Fig. 1H-J, SOD, GSH-Px, and CAT activity in the serum of O-C group were significantly reduced compared with those of the Y-C group (P<0.001, P=0.003 and P<0.001, respectively). In addition, the MDA and H_2O_2 concentrations in the serum of the O-C group were significantly increased compared with those of the Y-C group (P=0.001 and 0.014, respectively). Treatment with different doses of YFN markedly increased SOD activity compared with the O-C group (P<0.001). Similarly, SOD activity levels were significantly increased compared with the O-C group (P<0.01). However, GSH-Px activity was not significantly upregulated in the DT, YFN-H and YFN-L-treated groups compared with the O-C group (P=0.055). CAT activity in the YFN-H group was markedly increased compared with the O-C group (P=0.007) and was similar to the activity in the Y-C group. In addition, compared with the O-C group, MDA and H_2O_2 expression levels in the YFN-H (P=0.025 and P=0.001, respectively) and YFN-L groups (P=0.015 and P<0.001, respectively) were significantly reduced. The DT-treated group also displayed significantly lower levels of H_2O_2 than the O-C group (P<0.001), as shown in Fig. 1K and L.

Effects of YFN on oxidative DNA damage and p53 protein expression. Compared with the Y-C group, it was observed that oxidative damage in the O-C group was significantly increased

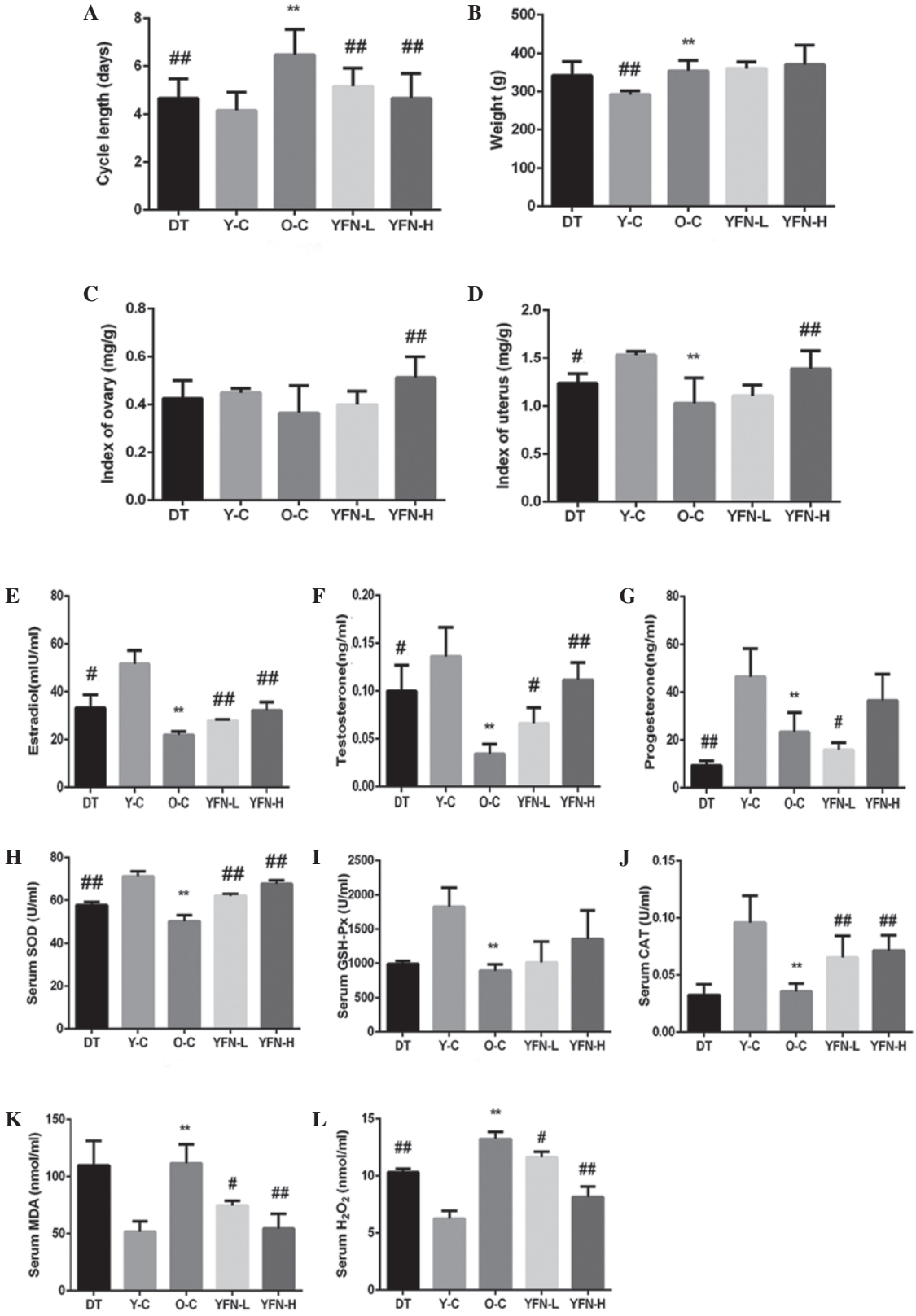

Figure 1. Body weights, organ indices, and estrous cycle data. (A) Following 6 weeks of administration, the DT, YFN-L and YFN-H-treated groups had 3-5 day regular estrous cycles, which significantly differs from the cycles in the O-C group. (B) The body weights were not significantly different between the DT-treated, O-C, YFN-L and YFN-H groups, and only the body weights in the Y-C group differed significantly from those of the O-C group (P=0.025). (C) Ovarian weights, adjusted for body weight, in the YFN-H group increased significantly from those in the O-C group (P=0.001). (D) The uterine indices in the DT-treated and YFN-H groups differed significantly compared with those of the O-C group (P=0.045 and 0.001, respectively). The YFN-H group displayed larger improvements in the uterine index, with values that were similar to those of the Y-C group. Comparison of serum (E) estradiol, (F) testosterone and (G) progesterone levels in the rats. (H) SOD, (I) GSH-Px and (J) CAT activities and (K) MDA and (L) H_2O_2 levels in the sera of the rats. The SOD, GSH-Px, and CAT activities and the MDA and H_2O_2 levels differed significantly among the groups ($F(4,25)=73.300$, $P<0.001$; $F(4,25)=9.133$, $P<0.001$; $F(4,25)=29.787$, $P<0.001$; $F(4,25)=22.593$, $P<0.001$; and $F(4,25)=71.087$, $P<0.001$, respectively). Values are presented as the mean ± standard deviation, n=6 rats per group. $^{**}P<0.01$ vs. the Y-C group, and $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the O-C group. DT, diethylstilbestrol; YFN-L, yifuning-low dose; YFN-H, YFN-high dose; O-C, old control; Y-C, young control; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde.

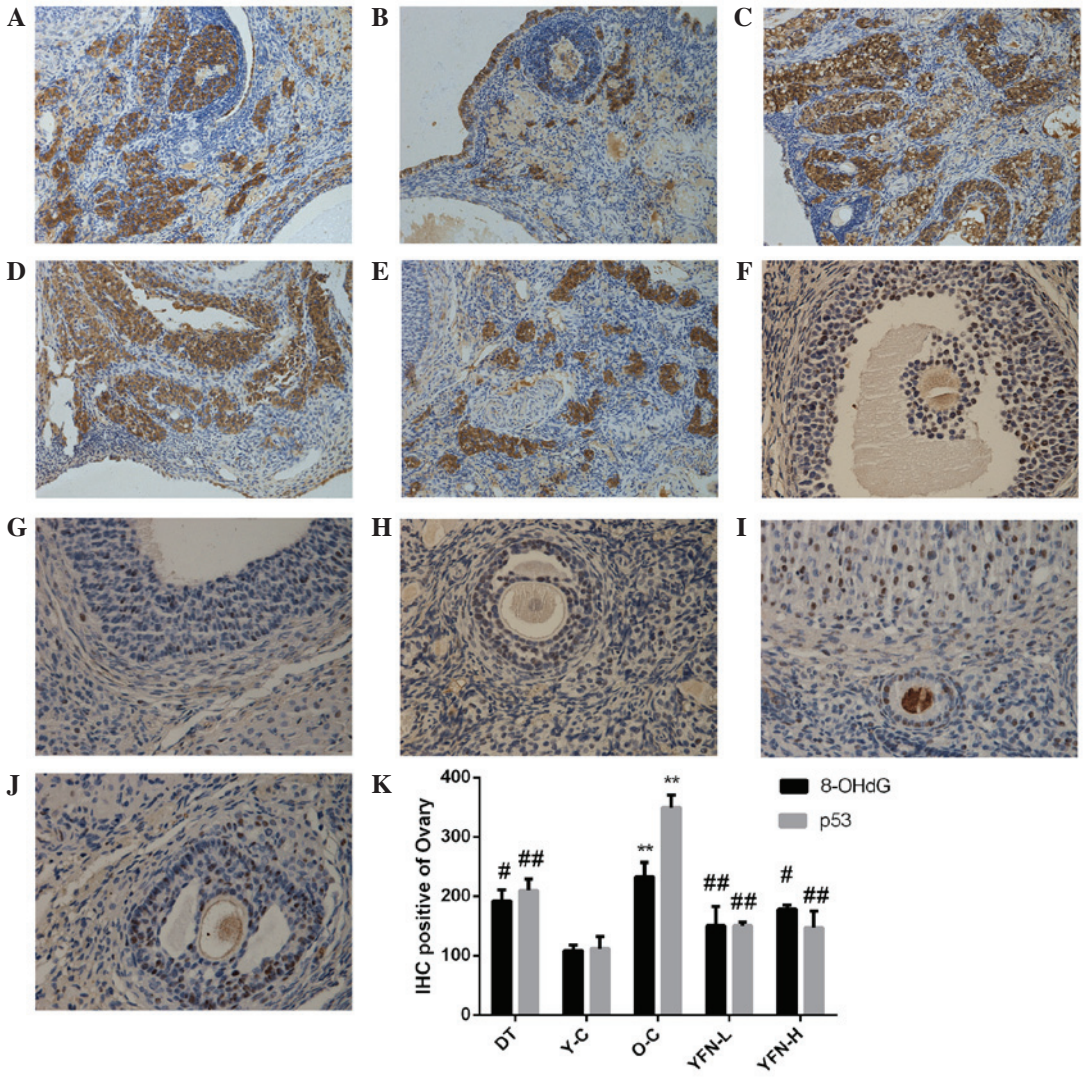

Figure 2. Effects of YFN on 8-OHDG and p53 protein expression in the ovaries. Immunostaining was used to detect the protein expression of the oxidative damage marker 8-OHDG. Representative immunostaining images show DNA damage (8-OHDG) in the (A) DT-treated, (B) Y-C, (C) O-C, (D) YFN-L and (E) YFN-H groups and p53 expression in the (F) DT-treated, (G) Y-C, (H) O-C, (I) YFN-L and (J) YFN-H groups. (K) Graph showing the number of 8-OHDG and p53 positive cells in the ovarian tissues from each group. The data are presented as the mean ± standard deviation (n=3) of the percentage of ovarian cells that stained positive. Positive 8-OHDG staining differed significantly among the groups ($F(4,10)=14.478$, $P<0.001$). Positive p53 staining differed significantly among the groups ($F(4,10)=45.033$, $P<0.001$). $^{**}P<0.01$ vs. the Y-C group. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the O-C group. Magnification, x200. YFN, yifuning; 8-OHDG, ; DT, diethylstilbestrol; Y-C, young control; O-C, old control; YFN-L, YFN-low dose; YFN-H, YFN-high dose; IHC, immunohistochemistry.

as shown in Fig. 2A-E. The number of 8-OHDG-positive cells in the ovaries of rats in the O-C group was significantly higher than in the Y-C group ($P<0.001$). In addition, a marked reduction in 8-OHDG expression was observed following treatment with the different doses of YFN and in the DT-treated group compared with the O-C group ($P=0.045$). Fewer 8-OHDG-positive cells were observed in the ovaries from the YFN-H group compared with the O-C group, resembling the results that were observed in the Y-C group.

To observe ovarian oxidative stress in the O-C group and to further elucidate the mechanisms of action underlying the anti-oxidant activity of YFN, p53 protein expression was evaluated using immunohistochemistry, as presented in Fig. 2F-J. This indicated that p53 was predominantly localized to the nuclei of cells in the follicles, oocytes, corpus luteum and mesenchyme. p53 was expressed in a higher percentage of cells in luteal cells than in follicular cells. The number of cells that stained positive for p53 protein expression in the ovarian tissue in the O-C group was significantly higher than the number in the Y-C group ($P<0.001$). Positive p53 staining in the ovaries of the YFN-H and YFN-L-treated groups was reduced compared with the O-C group, and these differences were significant ($P<0.001$). Similarly, positive p53 staining in the DT-treated group was significantly reduced compared with the O-C group ($P<0.001$). p53 expression in both YFN-treated groups was significantly lower than in the DT-treated group and was similar to the Y-C group.

p19, p53, p21, and Rb mRNA expression. p19, p53, p21, and Rb are involved in age-associated signal transduction pathway arrest. As shown in Fig. 3A-D, p19, p53, p21 and Rb mRNA levels were significantly reduced in the rats in the O-C group ($P=0.011$, $P=0.007$, $P<0.001$ and $P=0.008$) compared with the Y-C group. However, significant elevations were observed in

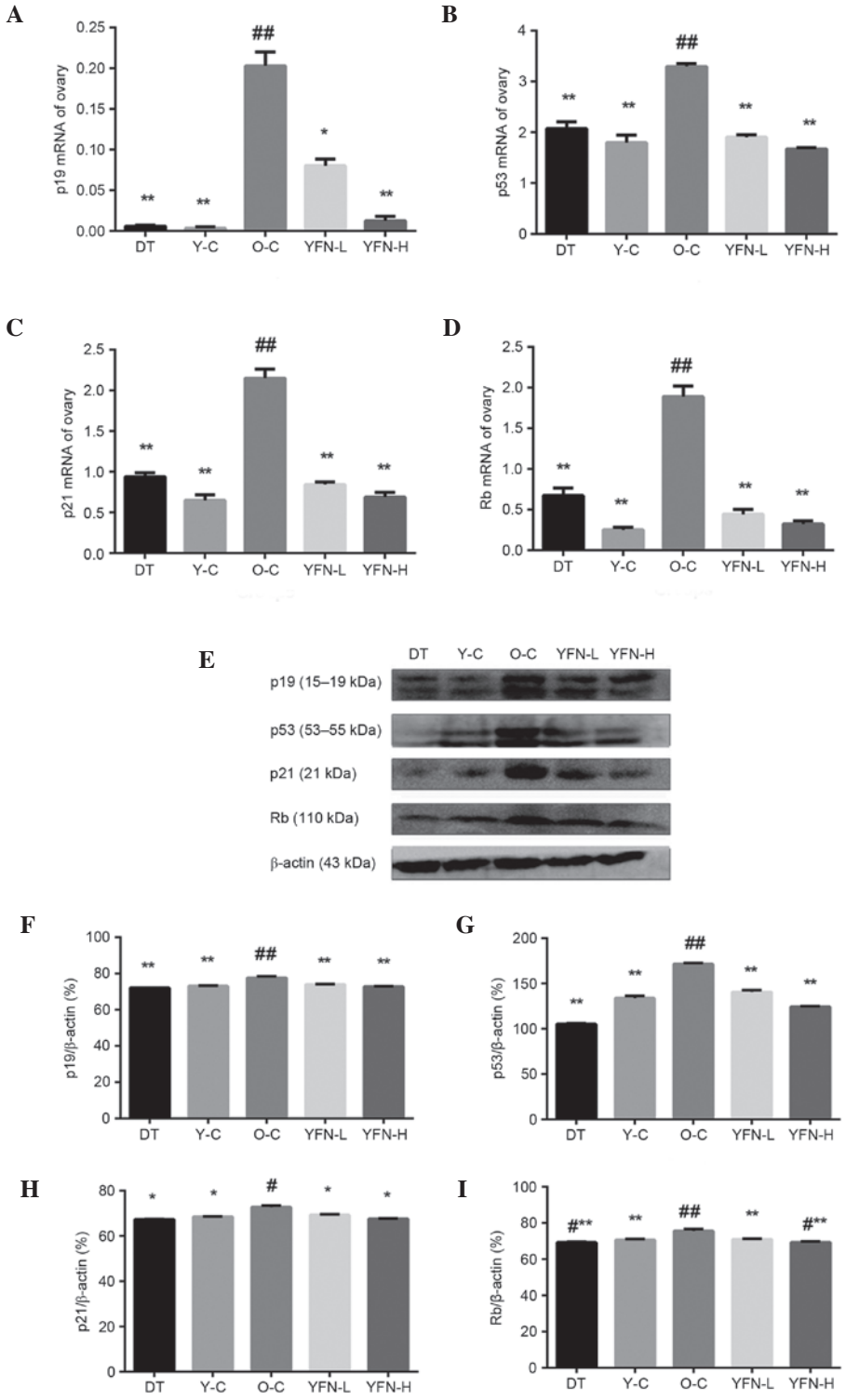

Figure 3. mRNA expression levels of (A) p19, (B) p53, (C) p21 and (D) Rb mRNA expression in the ovaries. The values were normalized against GAPDH as a reference gene. p19, p53, p21 and Rb mRNA expression differed significantly among the groups ($F(4,10)=234.014$, $P<0.001$; $F(4,10)=158.515$, $P<0.001$; $F(4,10)=283.181$, $P<0.001$; and $F(4,10)=224.121$, $P<0.001$). (E) Image of western blotting for p19, p53, p21, and Rb protein expression in the ovaries. (F) p19, (G) p53, (H) p21 and (I) Rb protein expression significantly differed among the groups ($F=98.099$, $P<0.001$, $F=956.654$, $P<0.001$; $F=80.415$, $P<0.001$; and $F=20.563$, $P<0.001$, respectively). Values are presented as the mean ± standard deviation, n=3. $^{*}P<0.05$ and $^{**}P<0.01$ vs. the Y-C group. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the O-C group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DT, diethylstilbestrol; Y-C, young control; O-C, old control; YFN-L, yifuning-low dose; YFN-H, YFN-high dose.

the YFN-L (P=0.012) and YFN-H (P=0.008) groups compared with the O-C group, and a partial, but significant, elevation was observed in the DT-treated group (P=0.003) compared with the O-C group.

Effects of YFN on p19, p53, p21, and Rb protein expression. The protein expression levels of p19, p53, p21, and Rb were also investigated. As shown in Fig. 3E-I, the levels of p19, p53, p21 and Rb increased as a result of aging (the O-C group vs. the

Y-C group; P<0.001, P<0.001, P=0.046 and P<0.001). However, treatment with YFN significantly reduced the age-induced p19, p53, p21 and Rb activity (all vs. the O-C group; P<0.001, P<0.001, P=0.031 and P<0.001). In addition, p19, p53, p21 and Rb expression levels were significantly increased compared with the O-C and DT group.

Discussion

Ovarian aging is one of the characteristics of perimenopausal symptoms. Regular estrous cycles represent ovarian function. Alterations in estrous cycles are a convenient and effective index which can be used to monitor reproductive conditions. Estrus in rats typically occurs in regular 4 to 5 day cycles, and continues to occur until the rats reach the age of 10-12 months. During aging, the cycle grows longer or becomes irregular, following which estrus eventually stops. In the present study, the estrous cycles of rats in the YFN-treated groups had markedly shortened and YFN groups displayed marked improvements in ovarian index and uterine index compared with the O-C group, which suggests that YFN may promote cell recovery and delay ovarian aging to a certain extent.

At present, estrogen replacement therapy is widely used to treat perimenopausal symptoms in which a number of deficiencies and side effects exist. In a previous study, it was reported that the influence of YFN on E_2 was similar to that of DT, therefore, DT was selected as a control in the present study, in which it exhibited the same effect. Reproductive endocrine hormone levels and the ovarian and uterine indices in the aging rats were observed to be improved following treatment with YFN. In addition, YFN-L treatment was superior to YFN-H in restoring reproductive endocrine hormone activity. The effects of YFN treatment on progesterone differed from the effects of DT treatment. It has been suggested that the common symptoms of the menopause are caused by dramatic declines and fluctuations in the levels of estrogen and, to some extent, P (29). Approaches that are aimed at alleviating symptoms generally focus on restoring these hormones in the body. It has been suggested that Asian women experience fewer menopausal symptoms because their diets are rich in soy proteins and phytoestrogens, and because they experience less stress and lead healthier lifestyles (30-32). The influence of YFN on hormones was similar to that of DT, however, YFN contains lower natural estrogens, with estrogen levels ten times lower than in DT. This indicates that the formation of reproductive endocrine hormones was not only affected by estrogens in YFN. YFN may affect the feedback that controls pituitary hormone synthesis and release. YFN may affect the level of hormones such as DT, but YFN contains lower levels of estrogen, suggesting that YFN treatment may reduce the need for hormone replacement therapy and reduce its associated side effects. We will further study these indications.

YFN is rich in nutritional antioxidant ingredients. In the present study, YFN significantly increased the levels of enzymatic antioxidants and reduced the accumulation of lipid peroxidation. The O-C group exhibited higher MDA and H_2O_2 levels and lower GSH-Px, SOD and CAT activity compared with Y-C group. Certain previous studies have indicated that oxidative stress is higher in women who are experiencing perimenopausal symptoms than those who are not experiencing these symptoms (3,33). The levels of lipoperoxides in perimenopausal women have been reported to be increased and the activities of antioxidant enzymes which can protect the body against oxidation damage reduced (2,34).

The present study also examined an indicator of oxidative DNA damage, 8-OHDG. Sai *et al* (35) found that during aging the process of the accumulation of oxidative DNA damage varies among organs. Kaneko *et al* (36) reported that 8-OHDG begins accumulating in the DNA of rat organs at 24 months of age or older. However, certain other studies have reported no significant alterations in oxidative DNA damage with age (37-39). A previous study reported that 8-OHDG is expressed in ovarian interstitial cells and follicles with increasing age (10). The data from the current study indicates an increase in 8-OHDG expression levels in the ovaries of the O-C group. YFN treatment protected against increased oxidative stress in the aging rats, reducing 8-OHDG expression during age-related ovarian oxidative DNA damage. We speculate that elevated repair activity may contribute to the protective effects of YFN. Therefore, one of the mechanisms underlying the ability of YFN to delay ovarian aging may be defense against oxidative stress.

As a tumor suppressor protein, p53 is also a redox-active transcription factor that organizes and directs cellular responses, in the face of a variety of stresses that lead to genomic instability. Oxidative factors are activated in response to stress signals. p53 regulates its target genes and initiates stress responses, which include cell cycle arrest, apoptosis and/or senescence (40-43). In unstressed mammalian cells, p53 has a short half-life and is normally maintained at low levels (44), while it is activated upon oxidative stress or DNA damage. Studies have indicated that cellular reactive oxygen species (ROS) can be modulated by p53 in a number of ways. Multiple pathways exist that integrate redox and p53 signaling, in which various redox signals converge on p53 target genes to determine cell fate, such as apoptosis, cell cycle arrest and DNA repair. For example, excess ROS production in the mitochondria resulting from treatments with chemotherapeutic agents has been demonstrated to lead to apoptosis (45,46), whereas oxidative stress that occurs in the nucleus stimulates p53-dependent DNA repair (47). As a redox-sensitive protein, p53 is also under redox regulation that determines cell fate via the selection of target genes. Other parameters, including cell type, source of stress and the intensity of stimuli, also determine the outcomes of the interaction between ROS and p53, which may limit any generalized mechanistic explanation of interaction between p53 and ROS. High levels of ROS activate p53 under severe oxidative stress conditions or irreparable damage, then in turn leads to p53-mediated apoptosis and senescence (14,15). Furthermore, activated p53 protein induces the expression of a set of pro-oxidant genes, which include p53-inducible gene 3 (PIG3), PIG6, ferredoxin reductase, Bax and p53 upregulated modulator of apoptosis (15,48). The induction of these genes can increase intracellular ROS levels and further sensitize cells to oxidative stress to eliminate damaged cells through apoptosis and p53-mediated senescence (49). In the present study, the number of p53-positive cells in the ovaries of rats in the O-C group increased, while YFN treatment resulted in a significant reduction. We speculate that p53 was activated by oxidative stress and irreparable DNA damage

in aged rats and that YFN improves the ability to repair DNA and alter redox regulation.

In addition, p53 is involved in the G_1/S checkpoint of the $p19^{ARF}$-p53-p21-pRb cell cycle pathway, which ensures that eukaryotic cells proliferate and divide in an orderly and programmed manner. Alterations in $p19^{ARF}$ expression which leads to the activation of p53 is a critical step in the senescence response (50). In response to DNA damage, the tumor suppressor gene p21 is activated by p53 and inhibits the activation of cyclin/Cdk complexes, then blocking cell cycle progression from G_1 to S phase (51). Rb regulates the transition between the G_1 and S phases of the cell cycle (52). Dephosphorylation of Rb during G_1 progression results in cell cycle arrest. This dephosphorylation may be caused by the overexpression of p21, which inhibits Cdk (53). The $p19^{ARF}$-p53-p21-pRb pathway is activated in a variety of cell types in response to a number of cellular stresses, including senescence. In the present study, it was demonstrated that the $p19^{ARF}$-p53-p21-pRb pathway was activated in the model rats, and that YFN was able to reduce the expression of p19, p53, p21 and Rb. We speculate that another regulatory effect that is mediated by YFN to defer aging in the ovaries may be the blockade of the $p19^{ARF}$-p53-p21-pRb pathway to promote cell differentiation and proliferation, thereby slowing the aging process in the ovaries.

The results of the present study suggest that YFN defers ovarian aging by upregulating antioxidants and downregulating negative regulators of proliferation, including p19, p53, p21 and Rb, in ovarian tissues. In addition, YFN reduces ovarian damage, promotes cell proliferation and restores the ability of the ovaries to produce sex hormones, thereby relieving the symptoms of the menopausal transition. To conclude, the anti-aging effects of YFN in the ovaries may be mediated by promoting antioxidant activity and proliferation. However, the possibility cannot be excluded that natural ovarian aging mediates endogenous oxidative stress that blocks cell cycle progression, thus leading to senescence. Further investigation is required to clarify the association between oxidative stress and the cell cycle. At present, the ability of p53 to function as an inducer of antioxidant genes, which may suppress p53-activated reductions in oxidative stress and enhance ovarian function, remains to be elucidated.

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