Resveratrol protects against age-associated infertility in mice

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STUDY QUESTION: Does resveratrol counteract age-associated infertility in a mouse model of reproductive aging?

SUMMARY ANSWER: Long-term-oral administration of resveratrol protects against the reduction of fertility with reproductive aging in mice.

WHAT IS KNOWN ALREADY: Loss of oocytes and follicles and reduced oocyte quality contribute to age-associated ovarian aging and infertility. Accumulation of free radicals with age leads to DNA mutations, protein damage, telomere shortening, apoptosis and accelerated ovarian aging. Increasing evidence shows that resveratrol, enriched in certain foods, for example red grapes and wine, has anti-tumor and anti-aging effects on somatic tissues by influencing various signaling pathways, including anti-oxidation, as well as activating Sirt1 and telomerase. We investigated the potential of resveratrol to stave off ovarian aging in the inbred C57/BL6 mouse model.

STUDY DESIGN, SIZE, DURATION: Young C57/BL6 females (aged 2–3 months) were fed with resveratrol added to drinking water at 30 mg/l (providing ≏7.0 mg/kg/day) for 6 or 12 months, and the fertility and ovarian functions were compared among mice treated with or without resveratrol, and young mice served as reproductive controls. Experiments were repeated three times, with an average of 25 females randomly allocated to each treatment group for each repeat.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Reproductive performance of female mice was determined by litter size, ovarian follicles and oocyte quantity and quality, and compared with age-matched controls. The impact of resveratrol on telomeres and telomerase activity, and expression of genes associated with cell senescence also was evaluated.

MAIN RESULTS AND THE ROLE OF CHANCE: Young mice fed with resveratrol for 12 months retained the capacity to reproduce, while age-matched controls produced no pups. Consistently, mice fed with resveratrol for 12 months exhibited a larger follicle pool than controls (P < 0.05). Furthermore, telomerase activity, telomere length and age-related gene expression in ovaries of mice fed with resveratrol resembled those of young mice, but differed (P < 0.05) from those of age-matched old mice. Resveratrol improved (P < 0.05) the number and quality of oocytes, as evidenced by spindle morphology and chromosome alignment. Also, resveratrol affected embryo development in vitro in a dose-dependent manner.

LIMITATIONS, REASONS FOR CAUTION: The doses of resveratrol and the experimental conditions used by different research groups have varied considerably, and the dosage influences both the effectiveness and toxicity of resveratrol. Fine-tuning the dosage of resveratrol likely will optimize its anti-aging effects on ovarian function.

WIDER IMPLICATIONS OF THE FINDINGS: Our data provide a proof of principle of the fertility-sparing effect of resveratrol in female mice. Although depletion of the ovarian reserve of high-quality oocytes also contributes to increased infertility with reproductive aging in women, the data obtained using a mouse model may not extrapolate directly to human reproduction, and more extensive research is needed if any clinic trials are to be attempted.

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Key words: ovarian aging / oocyte / oxidative stress / resveratrol
Introduction

Many women now postpone childbearing and a large part of this population becomes infertile by the time they attempt to conceive because of ovarian aging (Coccia and Rizzello, 2008; Mills et al., 2011). Ovarian aging includes a remarkable decline in the pool of follicles and oocytes as well as in oocyte quality (Gosden et al., 1983; Faddy et al., 1992; Tatone et al., 2008; Broekmans et al., 2009), which result from a lack of germ cell replenishment in post-natal ovaries (Bristol-Gould et al., 2006; Notarianni, 2011; Zhang et al., 2012). Cyclic folliculogenesis and ovulation, with massive follicular atresia and maintenance of oocyte quality could protect against ovarian aging and prolong the female reproductive lifespan.

Germ cells initiate meiosis and arrest at the dictyate stage of prophase I in the fetal ovary and the post-natal germ cells remain arrested for weeks to months in mice, and for 10–50 years in women (Nagaoka et al., 2012). During this prolonged interval reactive oxygen species (ROS) accumulate and negatively affect oocyte quality and quantity (Tarin, 1996; Behrman et al., 2001). ROS can damage mitochondria DNA (mtDNA) and contribute to mtDNA mutations (Keefe et al., 1995). Telomeres are especially sensitive to ROS, which accelerate telomere shortening even in non-dividing cells (Lansdorp, 2005; Huang et al., 2010). Telomere shortening and dysfunction impair meiosis and embryo development, resulting in reproductive failure and infertility (Keefe and Liu, 2009).

Antioxidants reduce ROS-induced damage to oocytes and follicles. Indeed, the antioxidant N-acetyl-L-cysteine (NAC) delays ovarian aging in Kunming mice outbred from ICR mice (Liu et al., 2012). Mice fed with NAC produce larger litter sizes at 7–9 months of age compared with age-matched controls and litters similar to those of young mice at 2–3 months of age. Kunming mice still produce good litter size at 13–14 months of age with no further improvement from NAC. We suggest that the outbred Kunming mice may not be an appropriate model for ovarian aging. Quantitative cytological analyses of aging C57BL/6j mouse ovaries revealed that the populations of primordial and growing follicles are nearly exhausted by 13–14 months (Gosden et al., 1983). Moreover, increased ROS promotes apoptotic cell death in ovarian follicles and granulosa cells, and ovarian antioxidant defenses decrease and oxidative damage increases with age in C57BL/6 female mice (Lim and Luderer, 2011). We also found that C57BL/6 mice produce very small litter sizes (6–7) when young and no pups by the age of 1 year.

In addition to antioxidants, caloric restriction (CR) has been shown to prolong lifespan and delay aging and aging-associated diseases in various animal models (Mair and Dillin, 2008; Colman et al., 2009; Wang et al., 2009), and to prevent maternal aging-associated oocyte aneuploidy and meiotic spindle defects (Selesniemi et al., 2011). The mechanism underlying CR’s anti-aging effects could be explained by reduced oxidative stress and increased activation of SIRT1 (NAD+-dependent histone deacetylase) (Cohen et al., 2004; Roth et al., 2005; Guarente, 2007; Kume et al., 2010; Hu et al., 2011).

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenolic compound found in the skin of red grapes, red wine and other botanical extracts. Resveratrol initially was found to act as an activator of the mammalian ortholog of SIRT1 and yeast Sir2 in vitro (Howitz et al., 2003) and to partially mimic the physiological effects of CR (Hu et al., 2011). Further, resveratrol activates SIRT1 and protects mitochondrial function (Price et al., 2012). In addition, resveratrol has other biological activities, including anti-carcinogenic, anti-inflammatory and telomerase enhancing activity, to inhibit cell senescence, protect the cardiovascular system and influence apoptosis (Aluyen et al.; Baur and Sinclair, 2006; Xia et al., 2008; Yu et al., 2012).

We hypothesized that resveratrol could counteract age-associated infertility in a mouse model of reproductive aging. We performed a long-term administration of resveratrol in mice to test the effects of resveratrol on maintaining reproductive performance using C57BL/6 mice as a model.

Materials and Methods

Mice feeding and mating

Female C57 mice at 6 weeks old were purchased from Vital River Laboratory Animal Technology Co. Ltd. and housed under 12:12 h light:dark cycle in the specific pathogen-free animal facility at the experimental animal center of Nankai University, China. After the protocol was approved by the Institutional Animal Committee. Mice were randomly divided into two groups: half were fed with water as a control and the other half water supplemented with 30 mg/l resveratrol (DND Pharm-Technology Co. Inc., Shanghai, China). The stock resveratrol was stored at room temperature in the dark. Sterile drinking water was freshly prepared using the resveratrol powder and changed every 5 days. The bottles containing resveratrol were covered with aluminum foil to protect from light. Following treatment with resveratrol for 6–12 months (the ‘Res’ group, and ‘Old’ is the water control), some mice were randomly chosen to breed for evaluation of litter size to determine their fertility. Others were used for assessment of follicle reserve, oocyte number and quality, telomere length and telomerase activity, and expression of genes related to aging and DNA damage. Young mice at the age of 2–3 months served as controls (the Young group).

For breeding, male Kunming mice at 8 weeks old were purchased from Vital River Laboratory Animal Technology Co. Ltd. Males with proven fertility were used for breeding experiments. Late in the afternoon, one female Kunming mouse at 6 months of age was mated with a proven fertile male Kunming mouse. The inseminated female mouse was checked for a plug the following morning and treated as pregnant if there was a plug. Due to the specificity of the newborn Kunming mice, pregnant females were closely monitored for the presence of newborn Kunming mice. Young adult Kunming mice were bred in pairs, and pregnant females were checked for plug every 2 days. Dead newborn Kunming mice were recorded as postmortem stillborns. Total number of newborn Kunming mice delivered each day was recorded. However, the number of newborn Kunming mice that survived to day 3 was the number of newborn Kunming mice to be raised in the subsequent 3 days. The numbers of newborn Kunming mice that survived to day 3 were used to calculate the number of newborn Kunming mice per group. The number of newborn Kunming mice per group was recorded and calculated from the total number of newborn Kunming mice delivered each day for 3 days after birth.

Ovary serial section and follicle counting

Ovaries were randomly collected from the different groups (treated, untreated and young control mice). After immersion in 4% paraformaldehyde for 8–24 h, tissues were embedded in paraffin wax. Based on previous methods (Bolon et al., 1997), the serial sections (5 μm) from each ovary were aligned in order on glass microscope slides, stained with hematoxylin and eosin Y and analyzed for the number of follicles at four different developmental stages in every fifth section with random start in the first five sections. The total number of follicles per ovary was calculated.
by combining the counts of every fifth section throughout the whole ovaries.

The follicles were categorized as primordial and primary, secondary and mature or antral and atretic according to a previous study (Myers et al., 2004) (Supplementary data Fig. S1). Follicles were classified as primordial and primary if they contained an oocyte surrounded by a single layer of squamous or cuboidal granulosa cells. Follicles at an intermediate-stage also were scored in this group. Secondary follicles were identified as having more than one layer of granulosa cells with no visible antrum. Mature or antral follicles possessed small areas of follicular fluid (antrum) or a single large antral space. Only those follicles containing an oocyte with a visible nucleus were scored. Follicles at early (immature) stages of development were scored as atretic if the oocyte was degenerating (convoluted, condensed) or fragmented but still staining brightly with eosin Y. Grossly atretic follicles lacking oocyte remnants were not included in the analysis.

Oocyte collection
Female mice from the different groups were superovulated by injection of 5 IU pregnant mare's serum gonadotrophin (PMSG), followed 46–48 h later by 5 IU hCG. Females were humanely sacrificed and oocytes encased in cumulus masses collected into potassium simplex optimized medium (KSOM) by releasing from oviduct ampullae 14 h after hCG injection. Cumulus cells were removed by pipetting after brief incubation in 0.03% hyaluronidase prepared in KSOM containing 14 mM HEPES and 4 mM sodium bicarbonate (HKSOM) (Liu and Keefe, 2002).

Immunofluorescence microscopy
Oocytes were processed for indirect immunofluorescence with anti-a-tubulin (Sigma) for microtubules as previously described (Liu and Keefe, 2002). Chromosomes were stained with Hoechst 33342 and images captured using a Zeiss Axio Imager Z1. For the detection of Oct4-positive cells, embryos were fixed in freshly prepared 3.7% paraformaldehyde, permeabilized, incubated with anti-Oct4 mouse monoclonal antibody (sc5279, Santa Cruz), then labeled with a fluorescence secondary antibody. To assay apoptosis, fixed embryos were reacted with TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Roche). Embryos were stained with 1 µg/ml Hoechst 33342, and mounted in Vectashield mounting medium (Vector Laboratories, CA, USA). Oct4-positive, TUNEL-positive and total cell nuclei were imaged using a Zeiss Axio Imager and counted.

Telomere length measurement by quantitative real-time PCR
Average telomere length was measured from total genomic DNA using a real-time PCR assay. PCR reactions were performed on the iCycler iQ real-time PCR detection system (Bio-Rad), using telomeric primers, primers for a reference control gene (the mouse 36B4 single-copy gene) and PCR settings as previously described (Liu et al., 2007). For each PCR reaction, a standard curve was made by serial dilutions of known amounts of DNA from the same tissues. The telomere signal was normalized to the signal from the single-copy gene to generate a T/S ratio indicative of relative telomere length.

Gene expression by real-time PCR
Total RNA was isolated from tissues using RNeasy minikit (Qiagen), and subjected to cDNA synthesis using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). PCR reactions were set up in duplicate using the FastStart Universal SYBR Green Master (Roche) and run on the iCycler iQ5 real-time PCR detection system (Bio-Rad). At least three parallel samples were run for analysis of each gene. Primers were designed using the IDT DNA website and are listed in the Supplementary data Table S1. The final PCR reaction volume of 20 µl contained 10 µl SYBR Green PCR Master Mix, 1 µl cDNA template, 3 µl primer mixture and 6 µl water. Thermal cycling was carried out with a 10 min denaturation step at 94°C, followed by two-step cycles: 15 s at 94°C and 59 s at 58°C. Amplification data were collected and analyzed using the iCycler IQ5 2.0 Standard Edition Optical System and the Software V2.

For embryo analysis, total RNA was isolated using RNeasy microkit (Qiagen), with additional carrier RNA to reduce RNA degradation during the extraction process. The RNA was then subjected to cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen).

Telomerase activity assay
The telomerase activities of ovaries of each group were determined by the telomerase (TE) ELISA kit (CSB-E08022, CUSABIO, Wuhan, China). The microtiter plate provided in this kit was pre-coated with an antibody specific to telomerase. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for telomerase and Avidin conjugated to horse-radish peroxidase added to each microplate well. Then a TMB (3,3′,5,5′ tetramethyl-benzidine) substrate solution was added to each well. Only those wells that contained telomerase, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution and the color change measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentration of TE in the samples was then determined by comparing the optical density of the samples to a standard curve.

Collection of zygotes and in vitro development
Female ICR mice at 7 weeks old were purchased from Vital River Laboratory. When 8 weeks old, they were injected with 5 IU PMSG (Calbiochem) and then 5 IU hCG (Sigma) 46–48 h later, and mated with fertile ICR males. Successfully mated females were humanely sacrificed the following morning by cervical dislocation and zygotes collected into KSOM by release from the oviduct ampullae. Embryos were cultured in 50 µl droplets of KSOM with additional amino acids, covered with mineral oil at 37°C in a humidified atmosphere of 6.0% CO2. At 36 h, 2-cell embryos were separated into different groups with supplemented resveratrol at various concentrations. Resveratrol was dissolved in dimethyl sulfoxide (DMSO) and stored as stock solution at 4°C. Resveratrol was used at the concentrations of 0.1, 0.5, 1 µM or higher in the embryo culture medium and percentage of embryos developing to blastocysts at 108 h, cell number, apoptosis and Oct4 expression in embryos were examined and counted.

Statistical analysis
Percentages were transformed using arcsin transformation. Percentage transformed data and other data were analyzed by analysis of variance, and the means were compared by Fisher’s protected least-significant difference using the StatView software from SAS Institute Inc. (Cary, NC, USA).
Results

Resveratrol counteracts age-related fertility decline

Litter size was counted in successfully mated females and in every pregnant female. After oral intake of resveratrol for 6 months, treated mice showed litter size similar to that of age-matched controls but reduced compared with young 2-month-old mice. Old mice (7–9 months) exhibited significantly reduced litter size compared with that of young mice (P < 0.001), about two to three pups per litter (Fig. 1A and B). Resveratrol did not significantly improve litter size at this time period. Notably, the pregnancy failure was increased in aging females, as well as in the aging females treated with resveratrol (Res), following successful mating as indicated by the presence of mating plugs. Most plugged young females delivered pups, whereas a majority of aged plugged females did not.

By 14–16 months of age, control (Old) mice produced no pups despite successful mating. Remarkably, females that received resveratrol for 12–14 months delivered pups albeit with a reduced litter size, about two per pregnancy (Fig. 1C and D). Litter size was less than that of females in the Young group, but significantly more than old females. These data show that resveratrol prevented infertility associated with ageing.

Resveratrol increases primary and growing follicles

As expected, many growing and mature follicles were located at the ovarian cortex, whereas atretic follicles and blood vessels localized to the medulla. Young C57 females exhibited a large number of primary and primordial follicles, as well as growing and mature follicles (Fig. 2A). Aging females (Old) showed a markedly reduced number of primordial and primary follicles, and few growing and mature follicles (Fig. 2A). The number of primordial and primary follicles was remarkably reduced in aging females at 6 months of age, whereas age-matched females treated with resveratrol had significantly more primordial and primary follicles compared with aged controls (Fig. 2B). Resveratrol did not improve the number of growing and mature follicles following treatment for 6 months (Fig. 2C). Also, the number of atretic follicles did not differ between the old mice treated with resveratrol or not treated with resveratrol (Old)—both groups had fewer atretic follicles than young mice (Fig. 2D). The number of primordial and primary follicles, and growing and mature follicles continued to decline with age but increased in mice fed with resveratrol for 12–14 months (Fig. 2E and F). The number of atretic follicles also was reduced in aging females compared with young mice (Fig. 2G).

Resveratrol prevents telomere shortening and increases telomerase activity

Telomere length estimated by T/S ratio was shorter (P < 0.01) in ovaries from old mice at 12 months old compared with young controls (Fig. 3A). However, the telomere length was longer (P < 0.05) in ovaries from mice treated with resveratrol for 12 months compared with ovaries from age-matched females (Old group), and comparable to those from young females (Young group). Ovaries from mice at 12 months showed markedly reduced telomerase activity compared with those from young controls. Age-matched mice treated with resveratrol exhibited increased telomerase activity, comparable to that of young mice (Fig. 3B).

Consistent with the idea of cellular senescence, expression of p21 was increased in ovaries from 12 month old females, whereas levels of p21 mRNA were significantly reduced in ovaries from age-matched mice treated with resveratrol, comparable to ovaries from young mice (Fig. 3C). In contrast, expression of Sirt1 was reduced in ovaries from old mice compared with young mice, and resveratrol increased expression levels of Sirt1 (Fig. 3D). In addition, the expression of genes related to apoptosis (p53, Bcl2, and Bax, mTOR, Pen and TOR), aging (Sirt2, Sirt6), germ cell development (Lin28 and cohesion (Rec8 and SMCB1)) was analyzed but showed no appreciable differences in mRNA levels among young, resveratrol-treated and age-matched control mouse ovaries (Supplementary data Fig. S2).
Resveratrol treatment enhances oocyte quantity and quality in aged mice

The number of oocytes collected from aging mice with or without resveratrol administration for 6 months was slightly reduced, though the difference was not significant, compared with that of young mice (Fig. 4A). In experiments for 12 months, old mice exhibited remarkably fewer oocytes compared with young mice (1.3 versus 12, \( P < 0.0001 \)). Following treatment with resveratrol for 12 months, old mice showed more oocytes than age-matched old controls not administered resveratrol but still fewer than young mice (Fig. 4B).

Oocyte quality was determined by spindle morphology and chromosome alignment. Properly aligned chromosomes often were accompanied by a compact spindle with normal morphology (Fig. 4C, Young), whereas chromosome misalignment was accompanied by one or more chromosomes detached from the equatorial spindle and various spindle dysmorphologies (Fig. 4C, Old). The number of oocytes with abnormal spindles and improper chromosome alignment increased in aging mice, such that the frequency of normal oocytes was markedly reduced compared with young mice (Fig. 4D). In contrast, mice treated with resveratrol for 6 months exhibited a higher frequency of normal oocytes than age-matched untreated controls, demonstrating that chronic administration of resveratrol prevented age-associated decline in oocyte quality.

By 12 months the frequency of normal oocytes from old mice declined markedly (\( P < 0.05 \)), compared with young mice (Fig. 4E and F), though these old mice had fewer oocytes (1.3 on average) (Fig. 4B). In contrast, the frequency of normal oocytes did not differ (\( P = 0.38 \)) between old mice treated with resveratrol and young mice. These data show that resveratrol prevents aging effects on both number and quality of oocytes in mice.

Doses of resveratrol affect embryo development in vitro

In initial experiments, embryos failed to reach blastocyst stage when cultured with resveratrol at a concentration of 1 \( \mu \text{M} \) or higher. Most embryos arrested at early cleavage stages (Supplementary data).
improves healthy follicle number, telomere length and telomerase activity, as well as oocyte quantity and quality, suggesting that it staves off ovarian aging. Resveratrol has beneficial effects on the follicle pool and oocyte quality, and these are likely associated with reduction in age-associated somatic and germ cell dysfunction, as shown by increased telomerase activity and telomere length, and activation of Sirt1.

The well-established paradigm of reproduction in mammals holds that females are born with a fixed number of oocytes which continuously decline until few or none remain (Faddy et al., 1992). Our study confirms, as so many other papers have shown, that aging ovaries exhibit reduced numbers, as well as reduced quality of oocytes. We show that resveratrol may provide long-term benefit by maintaining female reproductive capacity in mice. At the age of 7–9 months, mice treated with resveratrol produce comparable numbers of pups compared with old controls. The beneficial effects of resveratrol gradually emerge after prolonged treatment: mice receiving resveratrol in drinking water for 12–14 months still produce pups, in contrast to age-matched mice not treated with resveratrol, which produced no pups. Reduction in litter size with age is associated with a reduced number of oocytes. Abrogation of ovarian aging by resveratrol presumably slowed the rate of depletion of the non-renewable female germ cells.

We show that aging mice, by the age of 6 months, ovulate slightly fewer oocytes compared with young mice, but the quality of the oocytes from the aging mice is noticeably reduced. The number of oocytes with abnormal spindles and chromosome misalignment increases markedly with age. Resveratrol improves the quality of oocytes of mice after treatment for 6 months. While age-matched control mice show remarkably reduced number as well as quality of oocytes, resveratrol treatment for 12 months improves both number and quality of oocytes. Resveratrol therefore appears to protect against ovarian aging.

In agreement with the mouse data shown here, rats treated with resveratrol for 4 months have more healthy follicles and fewer atretic follicles than controls, indicating that resveratrol increases ovarian follicular reserve and prolongs ovarian lifespan (Chen et al., 2010).

Telomere shortening is considered a biomarker of cellular senescence and has been associated with a wide range of age-related diseases (von Zglinicki and Martin-Ruiz, 2005). Telomere dysfunction may contribute to reproductive aging-associated meiotic defects, miscarriage and infertility (Keefe and Liu, 2009). Telomeres are particularly susceptible to oxidative damage and oxidative stress accelerates telomere shortening in preimplantation embryos (Liu et al., 2003; Huang et al., 2010), possibly explaining, at least in part, the association between telomere shortening and physiological aging (Starr et al., 2008). Telomerase activity and maintenance of telomere length are critical for survival and proliferation of germ cells and granulosa cells, and reduced telomerase activity plays an integral role in granulosa cell apoptosis and follicular atresia (Lavranos et al., 1999; Yamagata et al., 2002; Liu and Li, 2010). Resveratrol was shown to stimulate proliferation of rat granulosa cells (Ortega et al., 2012). Telomerase activity declines in the human ovary with age (Kinugawa et al., 2000). Telomerase deficiency and shorter telomeres could contribute to the age-related decline in ovarian follicle number and infertility (Broekmans et al., 2009). Consistent with a recent report in HeLa

Discussion

We show that resveratrol protects against the reduction of fertility with aging in mice as indicated by litter size. Furthermore, resveratrol

Figure 3 Telomere length, telomerase activity and expression of p21 and Sirt1 in the ovaries of mice following administration of resveratrol for 12 months. (A) Relative telomere length shown as the T/S ratio by quantitative PCR (qPCR) analysis. (B) Telomerase activity of ovaries by enzyme-linked immunosorbent assay analysis. (C) Relative expression levels of p21 in ovaries by qPCR. (D) Relative expression levels of Sirt1 by qPCR. n = number of mice. The bars show the mean ± SEM. *P < 0.05; **P < 0.01. The P value is labeled if P > 0.05.
cells (Uchiumi et al., 2011), our data show that resveratrol also promotes telomerase activity and reduces telomere shortening of ovaries from aging mice.

Sirt1 can increase telomerase promoter activity and gene expression (Uchiumi et al., 2011). SIRT1 is also a positive regulator of telomere length in vivo and attenuates telomere shortening associated with aging, an effect dependent on telomerase activity (Pallocios et al., 2010). Exposure to resveratrol increases mRNA levels of SIRT1 in rat granulosa cells (Morita et al., 2012). Telomerase activity is reduced in mice exposed to oxidative stress, and antioxidant

**Figure 4** Resveratrol improves oocyte number and quality. (A) Average number of oocytes collected from each mouse group by ovulation induction after administration of resveratrol for 6 months. (B) Average number of oocytes collected from each mouse by ovulation induction after administration of resveratrol for 12 months. (C) Morphology of spindle and chromosome alignment of oocytes collected from mice after administration of resveratrol for 6 months. The spindle was stained by α-tubulin antibody (green), and chromosomes stained by Hoechst33342 (blue). (D) Statistical analysis of frequency of normal oocytes from each mouse group after administration of resveratrol for 6 months. (E) Morphology of spindle and chromosome alignment of oocytes collected from mice after administration of resveratrol for 12 months. (F) Frequency of normal oocytes from mice after administration of resveratrol for 12 months. n = number of mice. The bars show the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. The P value is labeled if P > 0.05.
therapy attenuates myocardial telomerase activity reduction (Makino et al., 2011). Consistent with the findings that resveratrol is an activator of Sirt1 in many cell types (Lagouge et al., 2006; Sun et al., 2007; Baur et al., 2012), our study also shows that resveratrol increases the expression of Sirt1 in mouse ovaries, coinciding with increased telomerase activity and telomere length. Moreover, expression levels of p21 are significantly reduced in ovaries from resveratrol-treated mice. Oxidative stress resulting from enhanced cell turnover during the proliferation of granulosa cells during folliculogenesis might also directly shorten telomeres and accelerate ovarian aging when telomerase is reduced. Telomere length can be used as a biomarker of chronic oxidative stress (Houben et al., 2008). It is likely that several factors together, including activation of Sirt1 and telomerase, as well as reduction of oxidative damage, contribute to telomere maintenance and reduced cell senescence in the ovaries of mice treated with resveratrol.

Previously, the doses of resveratrol used by different research groups and the experimental conditions varied considerably (Baur and Sinclair, 2006; Baur et al., 2012). For instance, resveratrol at 2.5 mg/kg/day attenuated insulin resistance in vivo induced by a high-fat diet (Sun et al., 2007). Also, oral administration of resveratrol at 22.4 mg/kg/day improved insulin sensitivity and reduced body weight of 1-year-old mice fed on a high-fat diet (Baur et al., 2006). Resveratrol at higher doses (400 mg/kg/day) prevented diet-induced obesity and alleviated obesity-related insulin resistance (Lagouge et al., 2006). Notably, a low dose of resveratrol (4.9 mg/kg/day) mimics CR at the transcriptional level, and delays some aspects of aging under normal diet conditions (Barger et al., 2008). We reasoned that a lower dose of resveratrol would be appropriate for our study on physiological ovarian aging and so mice were fed with normal diets and provided with water supplemented with 30 mg/l resveratrol (~7.0 mg/kg/day).

**Figure 5** Dosage effects of resveratrol on embryo cleavage and development in vitro. (A) Morphology of embryos cultured for 108 h in potassium simplex optimized medium (KSOM) supplemented with 0.1 or 0.5 μM resveratrol, compared with vehicle, which served as a control. (B) Rates of blastocyst formation under culture with or without resveratrol. ‘KSOM’ is the control group with normal embryo culture condition without DMSO or resveratrol. (C) Representative immunofluorescence images for Oct4 (red) and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) (green) in embryos cultured for 108 h. Nuclei stained with Hoechst (blue). (D) Total cell number of each embryo estimated by counting nuclei. (E) Number of Oct4-positive cells indicative of inner cell mass cell number of an embryo. (F) Number of TUNEL-positive cells of an embryo. D, E, F, n = number of embryos counted. The bars show the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. The P value is labeled if P > 0.05.
The above in vivo experiments suggest that chronic treatment with low dose resveratrol can exert anti-aging effects on fertility. It is too costly and time consuming to test dosage effectiveness of chronic treatment with resveratrol using animals, particularly considering that a minimum of 12 months is needed for aging experiments. Although in vitro data cannot be simply extrapolated to the in vivo situation, we assessed effects of acute treatment of resveratrol at various doses on embryo cleavage development in vitro. When porcine embryos were incubated with 0.5 μM resveratrol, higher frequencies of blastocyst formation and elevated cell numbers were achieved by the end of the 7-day culture period (Lee et al., 2010). Pretreatment with 10 μM resveratrol for 1 h prevented ethanol-induced disruption of mouse embryonic development in vitro (Huang et al., 2007). Resveratrol at 0.01 μM activates telomerase activity within 24 h in subpopulations of human Li–Fraumeni syndrome-derived breast epithelial cells (Pearce et al., 2008). It appears that resveratrol has different effects based on cell type, culture conditions and concentration (Signorelli and Ghidoni, 2005; Lanzilli et al., 2006; Ortega et al., 2012). Higher concentrations usually are more effective on highly proliferative cells, such as cancer cells (Le Corre et al., 2005; Signorelli and Ghidoni, 2005). Low concentrations (10^{-8} M) of resveratrol activate telomerase in slow growing mammary epithelial progenitor HMESO cell lines but resveratrol at 10^{-7} M or 10^{-9} M fails to promote telomerase activity (Pearce et al., 2008). Thus, the effective resveratrol concentration depends on the duration of treatment and the cell type.

We demonstrate that resveratrol at 0.1 μM exhibits beneficial effects on early mouse embryo development in vitro, has no effects at 0.5 μM, and adverse effects, as shown by cleavage arrest and death, at concentrations >1 μM. Also, relatively low concentrations of resveratrol (0.5–3 μM) inhibited neuronal differentiation of neural stem cells whereas higher concentrations (> or = 10 μM) induced cell death (Wallenborg et al., 2009). Moreover, while 25 μM resveratrol increases ATP production and mitochondrial membrane potential in C2C12 cells, 50 μM dose reduces mitochondrial membrane potential and cellular ATP levels, indicative of mitochondrial dysfunction (Price et al., 2012). Consistently, 50 μM resveratrol was found to promote proliferation and migration of endothelial progenitor cells, and reduce cell senescence, whereas 100 μM dose of resveratrol showed no beneficial effects but instead increased apoptosis (Xia et al., 2008). Additionally, these studies indicate that the anticancer effects of resveratrol are dose and duration dependent (Aluyen et al., 2012). Resveratrol has a direct, dose dependent, anti proliferative and pro-apoptotic effect in breast cancer and colon cancer cells, and relatively high concentrations of this compound are able to substantially down-regulate telomerase activity (Fuggetta et al., 2006; Lanzilli et al., 2006). Our in vitro embryo development experiments also may explain the previous paradoxical data showing that appropriate (lower) doses of resveratrol increase cell proliferation and prevent cell senescence in association with activation of Sirt1 and telomerase and telomere elongation but high doses inhibit cell proliferation and increase apoptotic cell death, thereby inhibiting tumorigenesis.

Notably, treatment of young, outbred CD-1 mice for 130 days with resveratrol provided at 3 mg/l in drinking water did not show significant effects on litter size (Kyselova et al., 2003). Presumably, the 3 mg/l dose in drinking water was not sufficient for resveratrol to affect reproduction in vivo. Compared with CD-1 mice, C57BL/6 females have a smaller litter size and much smaller litter size with age. Short-term treatment may be insufficient for resveratrol to achieve the anti-aging effects. Indeed, we also did not find beneficial effects of resveratrol on litter size of C57BL/6 females by 6 months. Fine-tuning the dosage of resveratrol likely will optimize its anti-aging effects on ovarian function but our data provide a proof of principle of the fertility-sparing effect of resveratrol in mammalian females. Although depletion of the ovarian reserve of high-quality oocytes also contributes to increased infertility with reproductive age in women, the data obtained using a mouse model may not extrapolate directly to human reproduction, and more extensive research is needed if any clinic trials are to be attempted.

### Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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### Authors’ roles

M.L. performed the experiments and analyzed the data. Y.Y assisted the experiments and helped analyze the data. X.Y, M.Z, Q.Z. assisted the experiments. D.L.K. provided critical input on the interpretation of data and revision of the manuscript and participated in critical discussions. L.L. designed the study, analyzed the experiments and wrote the manuscript.

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### Conflict of interest

None declared.

### References


