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Original Article

Resveratrol intake by males increased the mitochondrial DNA copy number and telomere length of blastocysts derived from aged mice

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Abstract. The present study examined whether male resveratrol intake affected mitochondrial DNA copy number (mt-cn) and telomere length (TL) in blastocysts fathered by young and aged male mice. C57BL/6N male mice supplied with water or water containing 0.1 mM resveratrol were used for embryo production at 14–23 and 48–58 weeks of age. Two-cell-stage embryos were collected from the oviducts of superovulated female mice (8–15 weeks old) and cultured for 3 days until the blastocyst stage. Mt-cn and TL levels were measured by real-time polymerase chain reaction. Resveratrol intake did not affect body weight or water consumption. Resveratrol intake increased the expression levels of SIRT1 in the liver, the antioxidative ability of serum, and extended TL in the heart, whereas there was no significant difference in mt-cn in the heart or TL in sperm. The rate of blastocyst development was significantly lower in aged male mice than in younger mice, and resveratrol intake increased the total number of blastocysts derived from both young and aged males. Resveratrol intake did not affect mt-cn or TL in blastomeres of blastocyst-stage embryos derived from young mice, but significantly increased both mt-cn and TL in blastomeres of blastocysts derived from aged fathers. In conclusion, resveratrol intake increased mt-cn and TL levels in blastocysts derived from aged male mice.

Key words: Embryos, Mitochondria, Paternal aging, Resveratrol, Telomere

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The age of parents when they have their first child is increasing worldwide, and aging is a major cause of infertility. Maternal aging decreases oocyte quality and has been the focus of many studies. However, empirical evidence has shown that paternal aging affects semen concentration and quantity, sperm motility, DNA integrity, and chromosomal normality, resulting in poor pregnancy outcomes [1–5]. Clinical data have revealed that paternal aging affects live birth rates, pregnancy outcomes, and health risks in children [6]. In addition, paternal aging causes epigenetic changes in the sperm [7, 8], indicating long-term consequences. However, the extent of the changes in sperm and embryos induced by paternal aging remains unclear. Aging deteriorates organ function, including telomere length (TL), oxidative stress, inflammation, and mitochondrial axes [9]. Mitochondria are cellular energy houses that are important for oocyte maturation, fertilization, and embryonic development [10, 11]. Recently, we showed that paternal aging reduces mitochondrial DNA copy number (mt-cn) in mouse blastocyst stage embryos [12]. In addition, Aburada *et al*. confirmed a male age-associated decline in mt-cn expression in embryos. They conducted RNA sequencing of blastocysts derived from the same male mice at young and aged time points, and the differentially expressed genes identified were associated with mitochondria [13]. Furthermore, bull age affected genes related to mitochondrial function in embryos produced *in vitro* [14]. In general, all mitochondria are derived from oocytes; therefore, the paternally derived genome or certain molecules associated with sperm may affect the mitochondria in embryos. Telomeres are ribonucleic and protein complexes that maintain genome integrity and are lifespan markers as they shorten at every cellular division [15]. The TL of paternal aging. Sperm telomeres elongate as the donor ages in humans [16–18], whereas they shorten in mice [19]. Sperm TL is closely related to sperm quality in bulls and humans [20–22].

Recently, we reported that TL and mt-cn were lower in blastocysts fathered by aged C57BL/6N mice than in those fathered by the same mice at a younger age [12]. Furthermore, age-induced declines in mt-cn and TL levels have been confirmed in C57BL/6N mice [13].

Resveratrol is a natural phenol and phytoalexin that is found in grapes, peanuts, cocoa, and some berries [23]. Resveratrol has two phenolic rings and three hydroxyl groups and is an antioxidant [24]. Resveratrol has been studied in the field of anti-aging owing to its positive effects on cellular homeostasis and anti-inflammatory functions and is a well-known activator of SIRT1 [25–27]. In addition, resveratrol has been reported to increase mitochondrial biosynthesis and degradation in embryos and oocytes through SIRT1 activation [28, 29] and SIRT1 activation prevents the age-associated attrition of TL in the livers of mice [30]. Resveratrol protects against oxidative stress and promotes spermatogenesis in mice [31, 32]. Resveratrol is a possible countermeasure against aging-associated events, and the European Food Safety Authority has approved its use as a food supplement.

In previous reports [12, 13] and the present study, we examined the effect of resveratrol intake in male mice on TL and mt-cn in embryos produced at younger and older time points.

Materials and Methods

Chemicals

Unless otherwise indicated, all the chemicals used in the present study were purchased from Nacalai Tesque (Kyoto, Japan).

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Animals

All animal experiments were approved by the Ethics Committee for Laboratory Animals of Tokyo University of Agriculture (No. 2023010). C57BL/6N male mice (Japan SLC Inc., Shizuoka, Japan) were housed individually, and female ICR mice (Japan SLC Inc.) were housed in groups (four mice per case). All mice had ad libitum access to food and water and were maintained at a controlled temperature of 23 ± 2°C with a 12 h light/12 h dark cycle. Body weight was measured weekly, and the total amount of water consumed per 7 days was measured. Resveratrol was diluted in ethanol (0.25 M) and added to water at a final concentration of 0.1 mM (1/2,500). For the control group, ethanol was added to water at 1/2500 (v/v).

Generation of blastocysts

Two-cell stage embryos were obtained from superovulated female mice according to previously described methods [13]. Superovulation was induced by intraperitoneal administration of 5 IU pregnant mare serum gonadotropin (ZENOAQ, Fukushima, Japan), followed by intraperitoneal administration of 5 IU human chorionic gonadotropin (hCG; Fuji Pharma, Tokyo, Japan) 46–48 h later. The superovulated females were subsequently mated with C57BL/6N males and examined for the presence of mating by visualization of copulation plugs (0.5 day post coitum [dpc]) 24 h post-hCG administration. Embryos were collected from the oviduct 43 h post-hCG administration. Two-cell stage embryos were used for further experiments, and non-cleaved oocytes and fragmented embryos were removed.

The embryos were cultured in 10 µl droplets of potassium simplex-optimized medium containing amino acids (KSOM-aa) for 72 h (4.5 dpc). *In vitro* embryo culture was maintained at 37.0°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Blastulation was conducted at 4.5 dpc, and blastocysts exhibiting clear, typical blastocyst morphology (not hatched) were selected for the experiments.

Total cell number in blastocysts and DNA extraction

Prior to DNA extraction, blastocysts were randomly selected and incubated with 1 µg/ml Hoechst 33342 in phosphate-buffered saline (PBS) for 5 min at 37.0°C, and images were captured under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) to evaluate the total number of cells in the blastocysts. Each blastocyst was then transferred to 20 µl of DNA extraction buffer (20 mM Tris, 0.4 mg/ml proteinase K, 0.9% Nonidet-40, and 0.9% Tween-20) and lysed at 55°C for 30 min, followed by 98°C for 10 min. The blastocyst lysate was then diluted in DNase-free water to a final volume of 50 µl and used for mt-cn and TL measurements. The mt-cn and TL values in the blastomeres were normalized to the total cell number of cells in the corresponding embryos.

Evaluation of the TL of embryos

TL was measured in duplicate using real-time polymerase chain reaction (PCR), according to a previously described protocol [12]. Before measurement, blastocysts with a narrow range of total cell numbers were selected to exclude more advanced-stage blastocysts and minimize measurement bias, given that the TL of the entire embryo was divided by the total cell number to obtain the blastocyst TL. The total cell number of the selected blastocysts was 72.1 ± 1.6 and 64.0 ± 2.0 for control young and aged groups, respectively, and 71.9 ± 1.2 and 73.8 ± 1.2 for the resveratrol-treated young and aged groups, respectively. Real-time PCR was conducted on a single plate to determine the effect of resveratrol on TL and to avoid interplate errors. The maximum sample size was 22. Real-time PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), 0.5 µM primer sets (Table 1), and a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR conditions were 95°C for 3 min, followed by 39 cycles of 98°C for 5 sec and 59°C for 60 sec. A standard curve was generated using ten-fold serial dilutions representing copies of the external standard, which was a synthesized oligonucleotide (84 bp in length; TTAGGG, repeated 14 times). The TL (bp) of each blastocyst was calculated using the following formula: detected copy number × 84/total cell number of the corresponding blastocyst/80 (total number of telomere regions in the diploid mouse genome). All sample measurements were within the standard range. The amplification efficiency of the standard was 0.85–0.95. Therefore, although the assay was an absolute measurement, the data are presented as relative TL, where the TL of embryos derived from young males was defined as 1.0.

Evaluation of mt-cn in embryos

Mt-cn was measured using real-time PCR according to a previously described protocol [13]. Real-time PCR was conducted using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 0.5 µM primer set targeting mitochondrial ND2 (*mt-Nd2*; Table 1), and a CFX Connect™ Real-Time PCR Detection System. The PCR conditions were 95°C for 3 min, followed by 39 cycles of 98°C for 8 sec and 60°C for 10 sec. The primer set was designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated by sequencing the PCR products. In addition, the primer sequences designed for mt-cn were examined to determine whether similar sequences were detected in the genome using the GGGenome tool (https://gggenome.dbcls.jp/ja/) because the nuclear genome contained a mitochondrial sequence fragment. A standard curve was generated for each assay using ten-fold serial dilutions representing copies of the external standard. The PCR product was cloned into a vector using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). The identities of the standards were confirmed by sequencing prior to use. The mt-cn value of the standard was calculated using the Avogadro number, molecular weight, and plasmid concentration. The amplification efficiency for

Table 1. Primer sequences used for real-time polymerase chain reaction

Targets	Primer sequence (5'—3')	Amplicon size (bp)	Accession number
mt-Nd2	F: ACCCACGATCAACTGAAGCA	82	NC_005089.1
	R: AGTACGATGGCCAGGAGGAT		
Telomere	F: CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT		N/A
	R: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT		
ACTB	F: GATCGATGCCGGTGCTAAGA	67	NC_000071.7
	R: CCTTCTGACCCATTCCCACC		

all measurements was > 0.99. mt-cn per blastomere was calculated using the total number of cells in the corresponding blastocysts.

DNA extraction from tissues and semen

Hearts were frozen at –80°C, smashed into a powder, and transferred to 100 µl of DNA extraction buffer, followed by incubation at 55°C for 60 min. To extract DNA from semen, epididymides were incubated in human tubal fluid media (HTF) [33] for 30 min, and sperms were centrifuged in PBS containing 0.1% polyvinyl alcohol (PVA). To remove DNA from the contaminated cells, the resulting pellets were incubated in a DNA extraction buffer for 3 h, followed by centrifugation at 13,000 × *g* for 10 min. The resulting sperm heads were further incubated in a DNA extraction buffer containing 0.1 M dithiothreitol for 3 h. DNA in the lysates was purified using MagExtractor-PCR & Gel Clean Up (CosmoBio Co., Tokyo, Japan) according to the manufacturer's instructions.

Samples were diluted ten-fold in DNase-free water. To measure the number of cells in the samples, a single-copy gene (*ACTB*) was analyzed using real-time PCR. Real-time PCR was performed using KAPA SYBR® FAST qPCR Master Mix, 0.5 µM primer set targeting *ACTB* (Table 1), and a CFX Connect™ Real-Time PCR Detection System. The PCR conditions were 95°C for 3 min, followed by 39 cycles of 98°C for 8 sec and 60°C for 10 sec. The primer set was designed using the Primer3Plus software. A standard curve was generated for each assay using ten-fold serial dilutions of the standard, which was the PCR product of the corresponding gene cloned into a vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The products were confirmed by sequencing before use. The DNA copy number in the standard was calculated using the concentration of DNA, Avogadro's number, and molecular weight of the plasmid. All data were normalized to the number of cells.

Evaluation of mt-cn and TL in tissue samples

Mt-cn and TL levels in the tissues were measured as described above. To determine the mt-cn per cell, mt-cn obtained by real-time PCR was divided by the number of cells in the sample determined using PCR amplification of a single-copy (*ACTB*). Similarly, the TL of the sample was divided by the number of cells determined by single-copy single-copy-gene (*ACTB*) PCR. The TL of the samples was represented as the relative TL and the TL of the sample derived from a young male control was defined as 1.0.

Measurement of anti-oxidative ability in blood

Mouse serum samples were collected at euthanasia and antioxidative activity was measured using an antioxidant capacity assay kit (PAO: KPA050, Fujifilm, Tokyo, Japan) according to the manufacturer's instructions.

Western blotting

Frozen liver samples were crushed into a powder, and proteins were extracted using lysis buffer (Complete Lysis-M; Roche, Basel, Switzerland) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) and ultrasonication (Ultrasonic disruptor, UD-100, Tomy, Tokyo, Japan), and protein concentrations were determined (Protein Assay Kit; Thermo Fisher, Rockford, IL, USA). The protein samples (10 µg) were co-incubated with Laemmli sample buffer containing 2-mercaptoethanol at 95°C for 5 min. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Trans-Blot Turbo Mini Transfer Packs; Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad). The primary antibodies used were anti-SIRT1 (sc-15404; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-actin (#4970S; Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated donkey anti-rabbit antibodies (ab6802; Abcam, Cambridge, UK) were used as secondary antibodies. The membranes were digitized using an ImageQuant LAS 4000 biomolecular imager and ImageQuant software (GE Healthcare, Buckinghamshire, UK). Expression of each protein was normalized to that of β-actin.

Experimental design

Figure 1 illustrates the experimental design. Male C57BL/6N mice (n = 3) were housed from 8 to 58 weeks of age and embryos were generated when the males were 14–23 weeks old (young) or 48–58 weeks old (aged). Resveratrol was administered to the experimental group from 8 weeks of age until the end of the experiment. At the end of the experiment, SIRT1 expression levels in the liver, mt-cn and TL in the heart, TL in sperm, and the antioxidant ability of the serum were measured. Mt-cn and TL in the blastomeres of blastocysts produced by young and aged male mice were compared between the resveratrol-treated and untreated groups.

Statistical analysis

The measured data were analyzed using the Kolmogorov-Smirnov test. Parametric data were analyzed using Student's *t*-test, nonparametric data were analyzed using the Mann-Whitney U test, and the developmental rate was analyzed using the chi-square test. The total cell number of the blastocysts was analyzed using the Kruskal-Wallis test, followed by the Scheffe test. All data are presented as the mean ± standard error of the mean. Statistical analyses were performed using the Bell Curve for Excel software. Statistical significance was set at $P < 0.05$.

Results

Effect of resveratrol intake on male mice

Supplementation of water with resveratrol did not affect the amount of water consumed or weight of the mice (Figs. 1B–C). As resveratrol is a well-known antioxidant and SIRT1 activator, we confirmed the significantly higher antioxidative ability of the serum and expression levels of SIRT1 in the liver of resveratrol-treated mice compared to their non-treated counterparts (Figs. 2A–C). Furthermore, resveratrol intake extended TL in the heart but did not affect mt-cn (Figs. 3A–B). In addition, TL in the sperm of resveratrol-treated mice tended to be longer ($P = 0.07$) than that in non-treated mice (Fig. 3C).

Effects of resveratrol on blastocyst cell number, TL, and mt-cn

Resveratrol intake did not affect the rate of blastulation but increased the total cell number of blastocysts fathered by both young and aged mice (Table 2). In addition, the chi-square test showed that the blastulation rate was significantly lower in the aged control group than in the young control group (63% *vs*. 93%, $P < 0.05$, Table 2). In addition, resveratrol intake did not affect either TL or mt-cn in embryos fathered by young mice but significantly increased those in embryos fathered by aged mice (Fig. 4). Although we did not directly compare mt-cn and TL levels between the two age groups on the same PCR plate, mt-cn and TL levels were significantly lower in the aged group than in their younger counterparts (mt-cn: aged male, 4,976.8 ± 150.0 *vs*. young male, 6,729.8 ± 607.1; TL: aged male, 0.71 ± 0.03 *vs*. young male, 1.0 ± 0.05; $P < 0.05$).

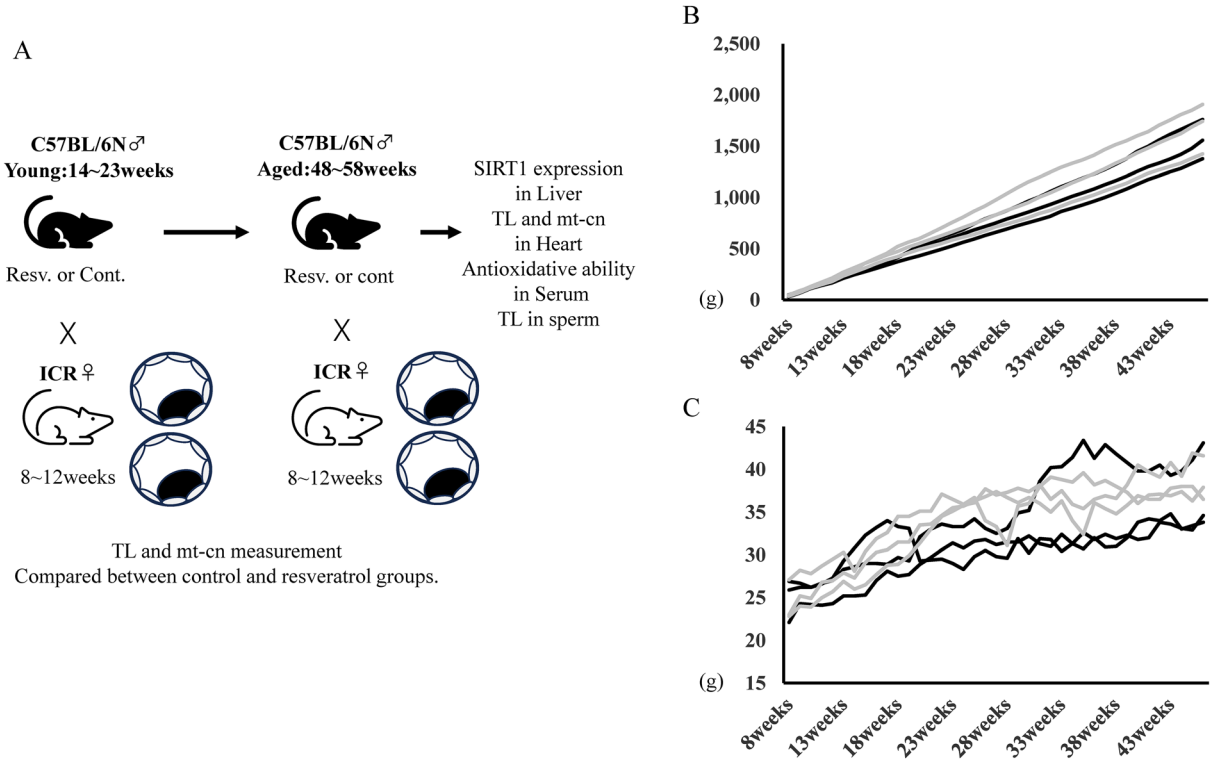

Fig. 1. Experimental design and effect of resveratrol supplementation on male mice. A: C57BL/6N mice were given a water-containing vehicle or resveratrol (three mice per group). Embryos were produced from young (14–23 weeks) or aged (48–58 weeks) male mice. Embryos were subjected to telomere length (TL) and mitochondrial DNA copy number (mt-cn) measurements. At the end of the experiment, the liver, heart, and sperm were collected and used for the experiments. B: Amount of water consumed (g), and C: Weight (g) of the mice. Cont., control group; Resv., resveratrol groups. The black line indicates the control group and the gray line indicates the resveratrol group.

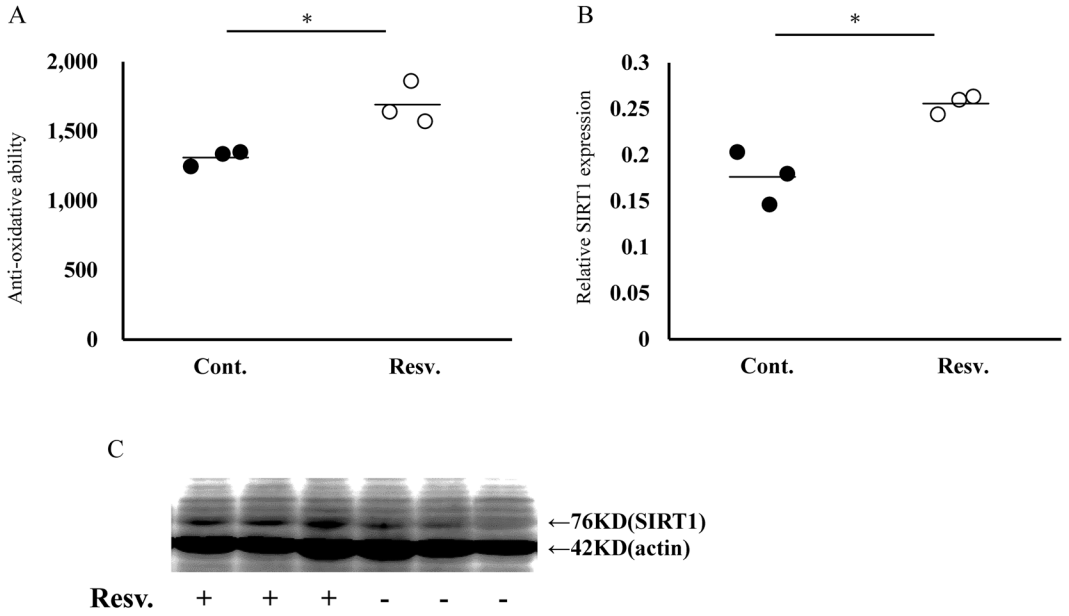

Fig. 2. Effect of resveratrol intake on anti-oxidative ability of serum and expression levels of SIRT1 in the liver. A: Anti-oxidative ability (Y axis: Cu antioxidant power reflecting Cu+ levels derived by the reduction of Cu^{++} using the action of antioxidants in serum [μmol/l]). B: SIRT1 expression in the liver was normalized to actin expression. C: Representative images of the bands. Data analysis was conducted using Student's *t*-test. * $P < 0.05$. Cont, control; Resv, resveratrol.

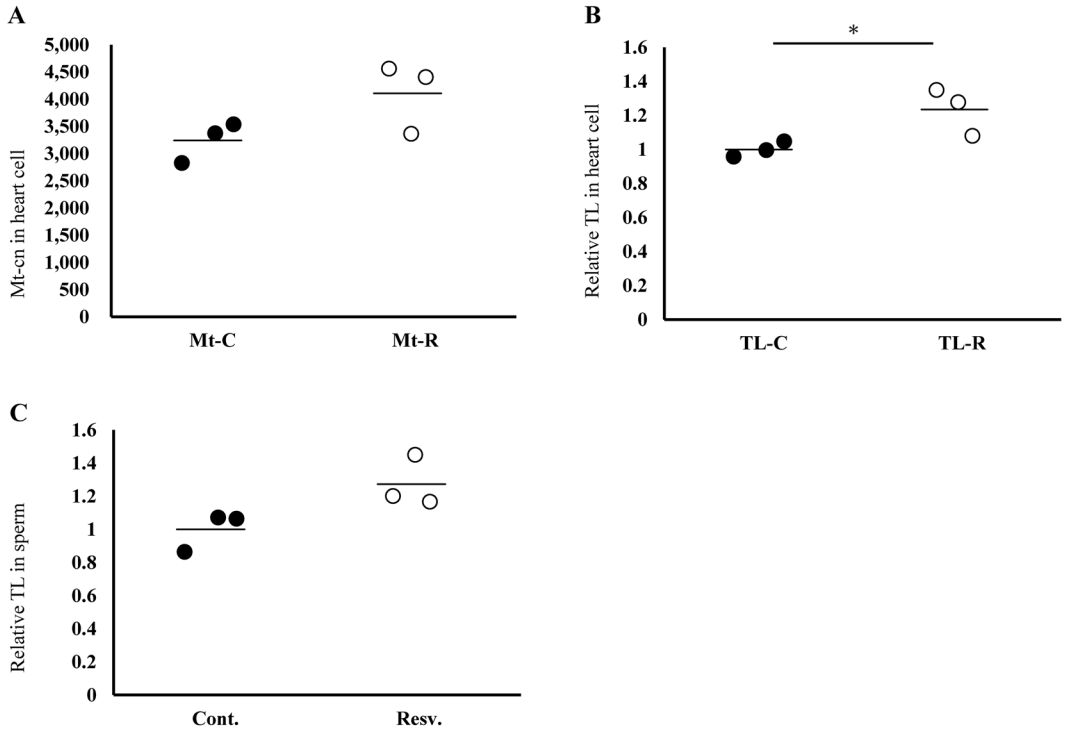

Fig. 3. Effect of resveratrol intake on telomere length (TL) and mitochondrial DNA copy number (mt-cn) in the heart and sperm of male mice. A: mt-cn in heart cells, and B: Relative TL in heart cells. C: Relative TL in sperm. B, C: The value for the control group was defined as 1.0. Data were analyzed using Student's *t*-test. * $P < 0.05$. Mt-C, mt-cn in the control group; Mt-R, mt-cn in the resveratrol group; Cont., control group; Resv., resveratrol group.

Table 2. Effect of resveratrol intake on the rate of blastocysts and total cell number

Male age	Groups	Rate of blastocysts (%)	Total cell number	
			No.	Cell number
Young	Cont.	81/87 (93) [a]	81	70.0 ± 2.3 [ab]
	Resv.	60/64 (94) [a]	60	74.3 ± 2.2 [c]
Aged	Cont.	58/77 (75) [b]	58	62.1 ± 2.5 [ad]
	Resv.	40/46 (87) [ab]	40	73.1 ± 2.0 [bc]

Blastocysts were produced from young or aged male mice fed with water (Cont.) or resveratrol-containing water (Resv.). The total cell number is presented as mean ± standard error of the mean. Different scripts indicate significant differences $P < 0.05$.

Discussion

The present study showed that paternal aging reduces mt-cn and TL in embryos. Oral intake of resveratrol did not affect mt-cn or TL in blastocysts derived from young males but increased them in those derived from aged males.

In our previous studies, mt-cn and TL were lower in blastocysts fathered by aged C57BL/6N mice than in those fathered by the same mice at a younger age [12]. Consistent with this finding, our recent study confirmed a male age-associated decline in mt-cn and TL in blastocysts [13]. This study revealed that paternal aging affects the expression of mitochondria-associated genes in blastocysts. Gene expression analysis of embryos fathered by differentially aged bulls showed that male aging affected metabolism-related pathways, including oxidative phosphorylation and mitochondrial dysfunction [14]. Studies in humans have shown that increased paternal age at conception affects mitochondrial-function-related genes in the peripheral blood mononuclear cells of the offspring [34]. These reports suggest that mitochondria in embryos are a major target of paternal aging and that this effect persists for a long time.

Mt-cn expression increases from the oocyte to the blastocyst stage in cows [35]. Similarly, the TL is longer in the blastocyst stage than in the early cleavage stage of human embryos [36], and the TL elongates from the oocyte to the two-cell stage in mice [37, 38]. Furthermore, our previous report showed a close correlation between mt-cn and TL in mouse and cow blastocysts [12]. In addition, we recently showed that TL regulates mt-cn in blastocysts because either the inhibition of telomerase or treatment with TERT-siRNA reduced mt-cn in blastocyst-stage embryos [39]. Considering that all mitochondria in embryos are derived from oocytes, TL plays a regulatory role in mt-cn in embryos.

In line with this notion, de Frutos et al. showed that the TL is shorter in embryos and offspring fathered by aged male mice than in their younger counterparts, and that the TL of sperm regulates mt-cn in mouse embryos [19]. Although the sperm TL increases with age in humans, studies in mice have shown that the sperm TL decreases with donor age [19, 40]. In the present study, we could not measure sperm TL at a younger age, but age-associated changes in sperm TL may be a possible causal factor for the shorter TL in embryos; thit requires further investigation. Conversely, paternal aging has been reported to induce epigenetic changes in embryos, affecting placental characteristics in mice [8] and induce hypomethylation of mouse sperm DNA, which might affect offspring health [41]. Another possible factor underlying the short TL in embryos is paternal aging-associated epigenetic changes in the sperm genome, which may

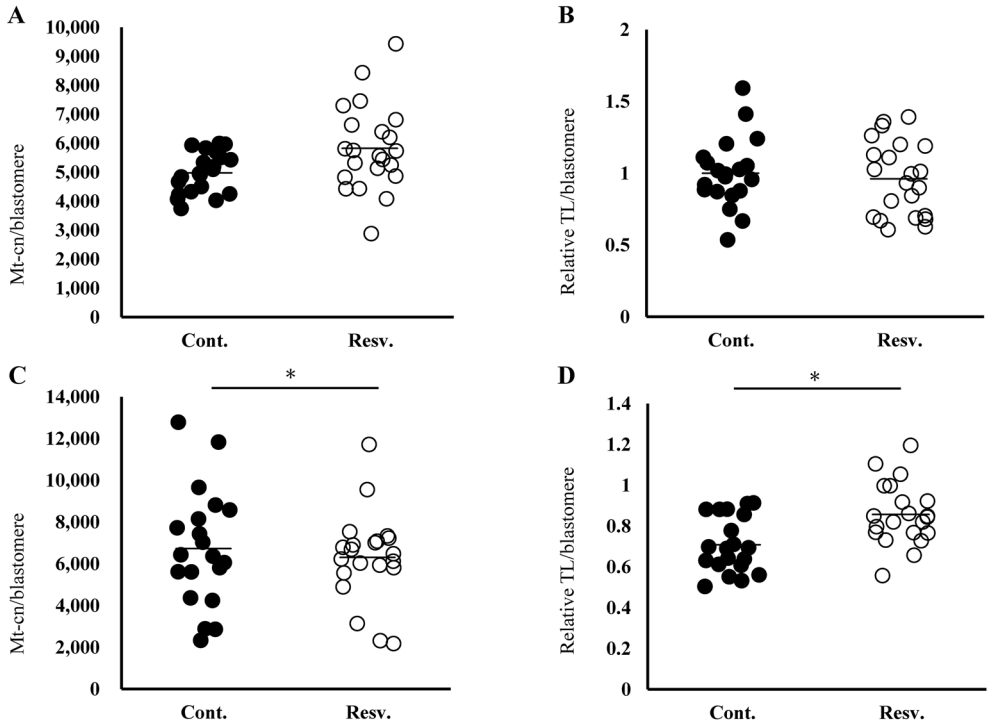

Fig. 4. Effect of resveratrol intake by male mice on mitochondrial DNA copy number (mt-cn) and telomere length (TL) in blastocyst-stage embryos. A, B: mt-cn and TL, respectively, in young mouse blastocysts. C, D: mt-cn and TL in the blastocysts of aged mice. B, D: TL in the control group of young mice was defined as 1.0. Data were analyzed using Student's *t*-test, * $P < 0.05$. Cont., control; Resv., resveratrol.

affect mt-cn and TL kinetics during early embryonic development and should be examined in further experiments.

Resveratrol is a well-known antioxidant and activator of SIRT1 [42]. In the present study, resveratrol intake increased the protein levels of SIRT1 in the liver and enhanced the antioxidative properties of serum. Decreases in mt-cn and TL levels in the heart are specific features of aging [9]. Oral resveratrol activates mitochondrial biosynthesis via SIRT1 activation in mice [43]. In addition, oxidative stress affects TL in primary fibroblasts (MRC-5 cells) [44], and resveratrol protects human and rat sperm from oxidative stress [32]. We showed that resveratrol significantly increased the mt-cn and TL levels in the hearts of aged male rats. This beneficial effect may be due to the antioxidant properties of resveratrol and/or activation of SIRT1. Interestingly, the oral intake of resveratrol improved embryo quality with a greater cell number, irrespective of the father's age, indicating that resveratrol changes the embryonic developmental ability of the sperm. It has been reported that oral intake of resveratrol increases sperm synthesis and gonadotropin and testosterone concentrations in the serum ofrats [45]. Moreover, resveratrol administration in aged male mice activates autophagy and increases hormone synthesis in Leydig cells [46]. Future studies should focus on the effects of resveratrol on sperm quality.

Oral resveratrol intake did not affect mt-cn or TL in embryos of young fathers, but increased them in embryos of aged fathers. These results suggest that the TL of young paternal embryos increases during early embryonic development, which masks the effect of resveratrol, or that resveratrol prevents age-associated attrition of sperm TL, resulting in greater TL in embryos derived from resveratrol-treated aged mice. The present study showed that oral resveratrol intake tended to increase the TL levels in sperm ($P = 0.08$). Based on these results, we hypothesized that resveratrol intake affects the TL of embryos through changes in sperm TL; however, further studies are required.

Currently, male aging is a risk factor for childhood diseases, and the factors that prevent male age-associated events in embryos need to be examined. The results of the present study suggest that resveratrol is a potential molecule for countering the age-associated disadvantages of mouse embryos with short TL and low mt-cn.

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Data availability: The corresponding author will disclose all data on the proper requests.

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