

Age-associated events in bovine oocytes and possible countermeasures

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Abstract Maternal aging profoundly affects oocyte quality. This has become common knowledge in industrialized countries and extensive studies addressing the causes and possible countermeasures against age-associated deterioration of oocytes suggest that mitochondrial dysfunction is a causal factor in infertility. However, almost all studies addressing age-associated events in oocytes have used mice as an animal model, and the reproductive life of mice is very short, making it difficult to study the gradual decline in fertility observed in humans. In the present review, age-associated changes in the quality and quantity of bovine oocytes and possible countermeasures related to mitochondrial quality control are introduced.

Keywords Aging · Cows · Mitochondria · Oocyte · SIRT1

Introduction

Age-associated decline in fertility is common in mammals. In industrialized countries, the age of women's first birth has increased to approximately 30 years; in women, fertility declines after 35 years of age [1, 2]. The causal factors of age-associated infertility and possible countermeasures against infertility have become social concerns. Clinical reports from *in vitro* fertilization have shown that the transfer percentage of women's embryos that result in live birth declines after 35 years, while it

remains constant in the case of donor embryos [3], which indicates that the main cause of age-associated decline in fertility is low number or quality of oocytes and/or embryos. However, ethical and physical restrictions hamper studies of age-associated events in human oocytes and embryos, and there is, therefore, a need for appropriate animal models.

Cows have a longer reproductive life (approximately 13 years) compared with other model animals, including rodents [4], and they show similar follicular wave, follicle selection, ovulation patterns as well as age-associated endocrinal changes to humans. [5–9]. In addition, many oocytes can easily be collected from slaughterhouse-derived ovaries or *in vivo* by using ovum pick-up (OPU) technology. Thus, cows have been suggested to be a good animal model for reproductive aging in humans. Moreover, in Japan, cows are tracked at a high degree of detail, allowing the breeds, age (in months) and farms of origin to be identified at the slaughterhouse. Thus, we have studied age-associated events in bovine oocytes and embryos. This review introduces age-associated changes in quality and quantity of bovine oocytes and suggests possible countermeasures against age-associated events in bovine oocytes using mitochondrial quality control.

Follicle number in bovine ovaries

In general, age-associated infertility is believed to be related to a decline in the oocyte pool; the decline in oocyte reserves occurs on the premise that the follicle pool is established before birth and no *de novo* synthesis of oocytes occurs during a woman's life. Although the presence of germ cells in epithelial cells of ovaries or bone marrow is inferred [10, 11], the age-associated decline in follicle number has been supported by the facts that the

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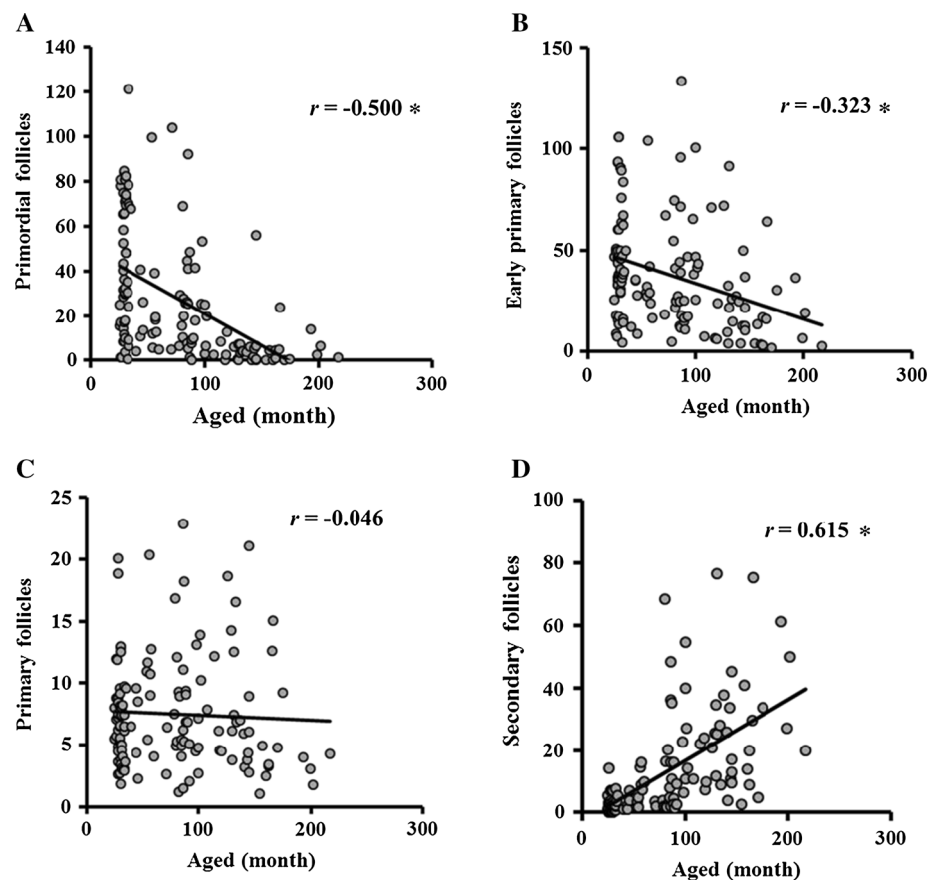
number of primordial follicles and antral follicles decreases as women age [12–14] and the follicle reserve is nearly exhausted by the age of 50 [15]. Singh et al. first reported that in cows, the number of small antral follicles with a 4–5-mm in diameter decreased with aged and the follicle-stimulating hormone (FSH) concentration in the plasma increases, as seen in aged women [16], and the negative relationship between the reduction in antral follicle number and the age of cows has also been reported in other studies [17, 18]. However, the chronological changes in the number of follicles at various developmental stages have not been clarified in cows. We then collected ovaries from a total of 131 Japanese black cows ranging in age from 25 to 217 months and histologically examined the number of follicles at various developmental stages. Figure 1 shows the relationship between donor age and the number of primordial, early primary, primary and secondary follicles. The number of primordial follicles and early primary follicles decreased as donor age increased and a significantly negative correlation between age of donor and the number of follicles was observed, whereas the negative correlation diminished for primary and secondary follicles. Interestingly, some aged donor cows possessed a large number of secondary follicles. Considering the small number of antral

follicles (3–6 mm in diameter) in the ovaries of aged cows [18], it is speculated that development of pre/early antral follicles to antral follicles is inactive in aged cows. In line with this, we examined the diameter of oocytes in antral follicles (AFs, 3–6 mm in diameter) and early antral follicles (EAFs, 0.5–0.7 mm) and compared them between young (25–50 months) and aged (>120 months) cows. In aged cows, the oocytes collected from antral follicles were smaller than those collected from younger cows, whereas oocytes of EAFs were larger than those of their younger counterparts [19]. An age-associated decline in the size of oocytes from antral follicles has been also reported in mice and humans [20, 21]. From these results, it is suggested that *in vivo* oocyte development is suspended after some development progression of the EAFs and the development of oocytes to the antral follicle stage is inactive in aged cows.

Age-associated decline in oocyte growth and factors underlying the impaired development

In vitro culture of small premature follicles is an easy method to investigate age-associated effects on oocyte growth. In some species, age-associated decline in *in vitro*

Fig. 1 Relationship between donor age and relative number of follicles. The *x* axis shows donor age in months, and *y* axis shows the relative number of primordial (a), early primary (b), primary (c) and secondary follicles (d). * $P < 0.01$. The number of follicles was counted in randomly selected areas under the same microscope, and the average value was multiplied by (the ovary weight)^{2/3}. These figures are modified from Itami et al. 2015 (Japanese Journal of Embryo Transfer 2016 Vol. 37)



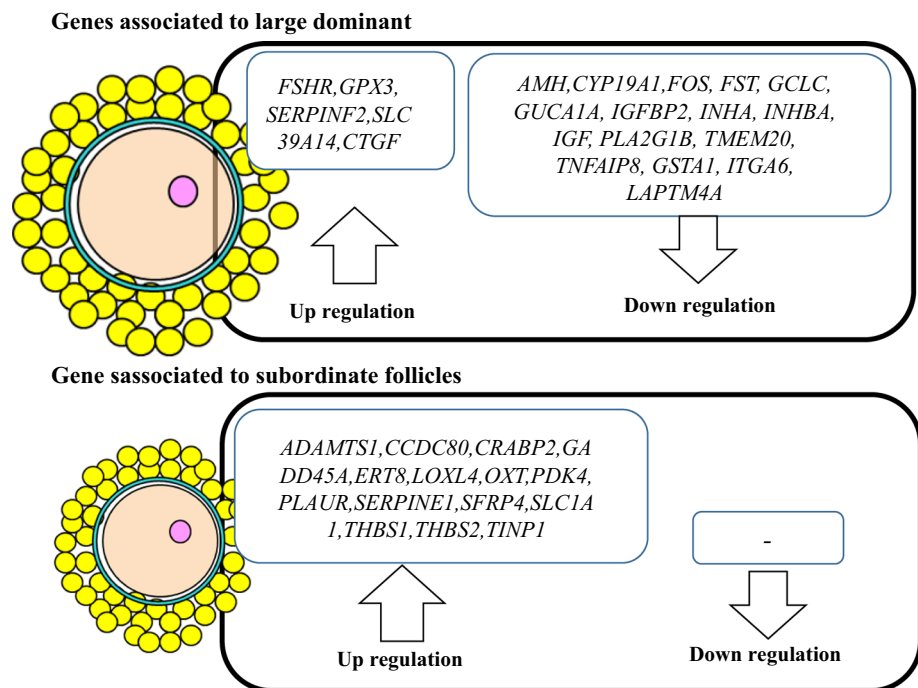
development of small follicles has been reported. Xue et al. reported that pre-antral follicles of aged rhesus monkeys develop slower than those of their younger counterparts [22]. Choi et al. reported that the number of follicles in the ovaries of mice decreased with age, and the ability for in vitro follicle formation is lower in aged mice than in young mice [23]. However, in large animals, oocyte growth requires a long period, and a culture system for in vitro growth of oocytes has not been established in cows or in almost all other large animals. EAFs are the final follicular stage, and give rise to a live calf following transfer of embryos derived from oocytes grown in vitro. To examine age-associated changes in the developmental ability of oocytes derived from EAFs, we collected oocytes and granulosa cell complexes (OGCs) from EAFs of 92 aged (>120 months) and 73 young (25–35 months) cows and cultured them for 16 days. The developmental ability of the OGCs, as determined by antrum formation, fertilization outcome and development to the blastocyst stage, was low for oocytes derived from aged cows, which showed low antrum formation, a high abnormal fertilization rate and a low total cell number of the blastocysts [19]. This result suggests that age-associated deterioration occurs even at the EAF stage in cows. To investigate the molecular mechanism underlying impaired oocyte growth, we examined the gene expression profiles of the granulosa cells of EAFs collected from 11 young (average 28.3 ± 0.7 months) and aged cows (164 ± 6.1 months) by using next generation sequencing (NGS) technology. To date, no study has reported the standard gene expression profiles in granulosa cells of healthy developing EAFs, and, therefore, we used a data set obtained from previous comparisons of gene expression in granulosa cells between large healthy follicles and subordinated follicles [24–30] (Fig. 2). The results of gene expression analysis showed that all genes reportedly associated with subordinate follicles were expressed at higher levels in aged cows, whereas many parts of genes associated with the large healthy follicles were expressed at lower levels in aged cows (Fig. 2). The results supports our hypothesis that development of EAFs to AFs is inactivated in aged cows; moreover, the results raises the possibility that the age-associated decline in oocyte development is partly caused by functional deterioration of surrounding cells. In addition, our comprehensive gene expression analysis showed that expression of genes related to glutathione appeared to be lower in granulosa cells of aged cows and that the glutathione (GSH) content of the EAFs was also lower in aged cows [19]. Similarly, it has been reported that the superoxide dismutase 1 and 2 (SOD1, SOD2) and catalase contents in granulosa cells are low in granulosa cells of aged women and that oxidative stress in granulosa cells, determined by phosphorylated p38 mitogen-activated protein kinase

(MAPK), is higher in aged women compared with their younger counterparts [31, 32]. Moreover, Goto et al. [33] reported another age-specific feature in granulosa cells, that proliferation activity and global DNA methylation is lower for aged cows than for young cows. Thus, age-associated decline in granulosa cell quality and function may be a causal factor of age-associated decline in oocyte growth.

Age-associated changes in quality of oocytes derived from antral follicles of aged cows

The decline in oocyte quality has been demonstrated to be a major causal factor of infertility in aged women [34, 35]. However, due to physical and ethical restrictions, it is difficult to compare oocyte quality between young and aged healthy women. Singh et al. compared the oocytes of cows aged 13–16 years (years of age) and their daughters aged 3–6 years (years of age) and reported that follicle number and the responsiveness of ovaries to the hormonal stimulation was lower in aged cows, and that fertilization ability of the oocytes decreased based on the results that the number of oocytes and/or embryos collected was the same between the two age groups, whereas the rate of cleavage was decreased [36]. Su et al. [37] collected oocytes by ovum pick up (OPU) from aged (>15 years) and young (12 months) cows and found that the developmental ability of oocytes to reach the blastocyst stage, and the plasma concentrations of estradiol, were low in the aged groups. We collected oocytes from slaughterhouse-derived ovaries of Japanese black cows and compared in vitro maturation and fertilization ability between 338 aged cows (average age 156.7 months) and 323 young cows (average age 27.3 months). We observed that oocytes derived from aged cows showed premature progression of nuclear maturation and lower fertilization ability [18]. In addition, we examined the relationship between fertilization ratio and donor age in months using 65 cows ranging from 22 to 174 months and found a significant positive correlation between donor age and rate of abnormal fertilization [38]. After fertilization, embryos derived from aged cows cleaved at a low frequency and the percentage of embryos that reached the 4–8 cell stage at 48 h after fertilization was negatively correlated with donor age [39]. Furthermore, blastocysts derived from oocytes of aged cows had fewer number of blastomeres than those of their younger counterparts [40]. In this study, we also compared gene expression in oocytes and embryos between young and aged cows by using NGS, and revealed that differential expression of genes in oocytes between young and aged cows is related to oxidative phosphorylation and mitochondrial dysfunction. We also showed that at the 8–16 cell stage, when zygotic genome activation occurs in cows, comprehensive gene expression levels were lower in aged

Fig. 2 Upregulation or downregulation of genes of granulosa cells derived from aged cows compared with younger cows. Gene expression in granulosa cells of early antral follicles of young and aged cows was examined by using next generation sequencing and compared between the two age groups. In the granulosa cells of aged cows, expression of genes associated with subordinate follicles was higher in granulosa cells, and expression of many genes associated with large dominant follicles was lower in granulosa cells compared with the younger counterparts. Summarized from Itami et al. [19]



cows than in young cows. Furthermore, levels of reactive oxygen species (ROS) were higher in the oocytes of aged cows. These results suggest that mitochondrial function and quality are potential foci for study of age-associated events in bovine oocytes.

Age-associated changes in mitochondrial quality and quality in oocytes

Mitochondria are double-membraned cellular organelles that play crucial roles in cellular energy production, apoptosis, and calcium homeostasis [41]. In addition, mitochondria contain their own circular DNA, consisting of 13 genes that encode respiratory chain enzymes, 22 genes encoding tRNA genes, and 2 genes encoding rRNA and other proteins; while nuclear genes encode other mitochondrial proteins to be transported to mitochondria [42]. Interestingly, mitochondrial DNA (mtDNA) in cells turns over independent of cellular cycles [43], and mitochondria in somatic cells generally contain 2 to 10 copies of their DNA. Unlike in somatic cells, the mitochondrial number can be predicted by determining the DNA copy number on the premise that the mtDNA copy number in mitochondria of oocytes is only one or two [44]. Thus, the mitochondrial number in oocytes has been determined in humans [45], cows [39, 46], and pigs [47], horses [48], and sheep [49]. In the ovaries, almost all oocytes stay in the primordial follicle stage, and the mitochondrial number in oocytes of primordial follicles is expected to be in the thousands [50]. Once oocytes begin to develop, the

mitochondrial number, as determined by DNA copy number, increases with oocyte growth, finally reaching approximately 200,000–1,000,000 in oocytes in antral follicles of cows and pigs [38, 51]. The significance of mitochondria for development of oocytes has been evaluated based on the reports that low mitochondrial numbers are associated with oocytes and embryos with low fertilization and developmental ability and ovarian insufficiency [52–56]. In addition, Wai et al. [57] created germline-specific heterozygous *Tfam* knockout mice to reduce the mitochondrial number in oocytes and concluded that a mtDNA copy number below 50,000 copies impairs developmental abilities.

Age-associated changes in mitochondrial quality and quantity have been used to explain the age-related deterioration of cellular functions [58, 59]. In older women, oocytes have been reported to show low number of mitochondria, as determined by the fraction of the ooplasm occupied by mitochondria [60]. In line with this, Chan et al. [61] reported an age-associated decline in mtDNA copy number in human oocytes. Contrary to these reports, Reynier et al. [52] reported that due to the high variation in mitochondrial number among oocytes it is difficult to precisely evaluate the relationship between donor age and mitochondrial number. Furthermore, Barritt et al. [62] examined 87 oocytes collected from 29 women and showed no relationship between donor age and mitochondrial number. A major drawback of this method based on mtDNA is that mitochondrial number varies widely among the cohort of oocytes, even those collected from a single

donor. Thus, a large number of oocytes collected from the same donor is needed to precisely predict the mitochondrial copy number for an individual donor. Based on this notion, we collected 20 oocytes from each donor cow and pig and divided them into two groups, each consisting of 10 oocytes. When the average mitochondrial number of oocytes was predicted using the two oocyte groups individually, the two predicted DNA copies were highly correlated, which indicates that using 10 oocytes from an individual donor is sufficient to predict the average mitochondrial number for each donor cow or pig [38, 51]. We then collected 10 oocytes from 180 donor cows ranging from 20 to 197 months of age and determined the average mitochondrial number of in vitro-matured oocytes for each donor cow. We found that once the age of a donor exceeded 70 months, there was a significantly negative relationship between mtDNA copy number and donor age [38]. A reduction in mtDNA has been reported in mice; oocytes collected from mice older than 300 days contained a lower number of mitochondria; In addition, oocytes from aged females contained differentiated and elongated mitochondria, whereas oocytes from young mice contained immature undifferentiated mitochondria. [63] Similar aged-associated mitochondrial features were also reported in equine oocytes [48]. Another marker of age-associated decline in mitochondrial quality is adenosine triphosphate (ATP) content, and a low ATP content in oocytes of aged females has been reported in mice and hamsters [64]. This decrease in mitochondrial function is speculated to be a factor in abnormal meiosis, as mitochondrial dysfunction disturbs spindle integrity [65, 66]. These data indicate that low mitochondrial quantity as well as quality are major causes of age-associated decline in oocyte quality.

Mitochondrial quality control in cells

Mitochondrial quality is important for cellular homeostasis and is maintained through a quality control system that includes antioxidants, chaperones, proteases, and ubiquitin–proteasomes. [67]. In addition, mitochondrial morphology changes via fission and fusion in response to mitochondrial and cellular conditions [68, 69]. When mitochondria can no longer function, and their function can't be restored through the quality control systems, they are removed from cells via mitophagy. One major mitochondrial removal system is the PARKIN–PINK1 pathway. In normal mitochondria PINK1, a serine/threonine kinase is imported into the inner mitochondrial membrane (IMM) and cleaved by the IMM protease, PARL, and subsequently degraded [70, 71]. However, in dysfunctional mitochondria with a dispersed membrane potential, PINK1 binds to the outer mitochondrial membrane and the activated PINK1 which recruits PARKIN, an E3 ubiquitin ligase, triggering

autophagic degeneration of the mitochondria [72, 73]. In line with this, carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) treatment disperses the mitochondrial membrane potential and induces recruitment of PARKIN [74, 75] and autophagy-related proteins, including p62/SQSTM1, NRB1, LC3, and LC3 family members [76], to the mitochondrial outer membrane. Overexpression of PARKIN removes abnormal mitochondria from the cells and improves mitochondrial functions [77–79]. Although many studies have been performed on mitochondrial quality control in somatic cells, how mitochondrial quality is maintained in oocytes remains unclear, and most studies have focused on the fate of sperm mitochondria following fertilization [80–83].

Mitochondrial quality control in oocytes

In somatic cells, accumulating evidence shows that mitochondrial quality and quantity are regulated through mitochondrial fusion and fission, along with de novo synthesis and degradation by proteasomes and mitophagy according to the cellular conditions [84]. In addition, it is believed that the mitochondrial quality control system deteriorates with aging [68]. However, how mitochondrial quality is maintained in oocytes or how maternal aging affects the quality control system is unclear. To examine mitochondrial de novo synthesis and degradation, monitoring the total mitochondrial mass is insufficient. As such, a sophisticated technique that assesses an embryo's mitochondria-targeted green fluorescent protein (mito-GFP) has been developed [85]. However, this technique can't be used in oocytes of large mammals. Proteasomes play a role in mitochondrial degeneration in cells, and proteasome inhibitors suppress mitochondrial degeneration, increasing the mitochondrial mass in cells [76, 86, 87]. When oocytes are cultured in medium containing MG132 (a proteasome inhibitor), mtDNA copy number as well as ubiquitinated proteins are increased in porcine oocytes [51], indicating that mitochondrial generation can be examined in a medium that inhibits mitochondrial removal. We collected oocytes from porcine ovaries and treated them for 2 h with CCCP which disperses the mitochondrial membrane potential; the ATP content of oocytes was reduced, whereas the amount of ROS increased in CCCP-treated oocytes. When these oocytes were further incubated in a general maturation medium, mtDNA copy number did not differ between CCCP-treated and un-treated oocytes. Whereas, when these oocytes were cultured in medium containing MG132, mtDNA copy number significantly increased in CCCP-treated oocytes compared with un-treated oocytes, indicating induced mitochondrial dysfunction stimulates mitochondrial de novo synthesis and degeneration [47]. This notion is supported by the

upregulation of the expression level of *TFAM* and increased puncta of LC3, a marker of autophagosomes, observed in CCCP-treated oocytes [88]. These data suggest that oocytes possess a mechanism for monitoring mitochondrial functions and/or cellular conditions, and if mitochondrial dysfunction is detected, de novo synthesis and degradation will be up regulated to replenish the mitochondrial pool. In addition, treatment of oocytes with CCCP for 2 h enhanced the expression levels of phosphorylated 5' adenosine monophosphate-activated protein kinase (AMPK) and silent mating type information regulation 1 (SIRT1) in oocytes. SIRT1 and AMPK are key cellular energy sensors, and SIRT1 has been reported to show protective effect through enhanced mitochondrial generation in ischemic injury [89]. Given these results, we hypothesized that controlling SIRT1 activity may impact the mitochondrial quality control system in oocytes.

Enhanced SIRT1 expression improves the quality of oocytes derived from aged cows

SIRT1 is a sirtuin family member that dependently acetylates proteins (NAD⁺) and affects various cellular metabolic functions, including regulating mitochondrial biogenesis and degeneration [90, 91]. Resveratrol (trans-3,4',5'-trihydroxystilbene) is a polyphenol present in a wide variety of plants, including grapes (skin), mulberries, and peanuts, and it has attracted much attention for its therapeutic potential. Resveratrol activates SIRT1 and AMPK and triggers mitochondrial biogenesis and autophagy, and regulates glucose and lipid homeostasis as well as immune response [92].

Considering the stimulative effect of resveratrol on mitochondrial biogenesis and degradation, one can speculate that upregulation of mitochondrial replenishment aids in maintenance of cellular viability [93]. To date, several studies have demonstrated that resveratrol mediates the maintenance of cellular functions; for example, resveratrol enhanced SIRT1 and AMPK activity and ameliorated mitochondrial dysfunction through autophagy in SH-SY5Y cells [94], and resveratrol activated SIRT1 in H9C2 cells and ameliorated H₂O₂-induced cytotoxicity by upregulation of mitochondrial biogenesis through SIRT signaling [95]. Furthermore, resveratrol enhanced survivability of H9c2 cardiac cells by inhibition of mTOR and activation of autophagy [96]. In this context, we examined the effect of resveratrol on oocytes of pigs and cows.

A culture of porcine oocytes in in vitro maturation medium containing 10- μ M resveratrol enhances SIRT1 expression in oocytes and increases generation and degeneration of mitochondria during the maturation period;

this effect was diminished by supplementation of the medium with the SIRT1 inhibitor, EX527. Furthermore, treatment of oocytes with resveratrol improved mitochondrial functions, including ATP generation and developmental ability of oocytes to the blastocyst stage [51]. These results indicate that upregulation of SIRT1 results in replenishment of mitochondria in oocytes and, hence, improves oocyte quality. In line with this, supplementation of maturation medium with resveratrol improves fertilization ability of oocytes collected from ovaries of both young and aged cows [40]. Furthermore, SIRT1 closely interacts with AMPK [91], and upregulation of the AMPK activity of oocytes by aminoimidazole carboxamide ribonucleotide (AICAR) treatment improves fertilization outcome and mitochondrial functions of bovine oocytes [97]. Mitochondria are actively generated and increase in number during oocyte growth. As mentioned above, in vitro growth of oocytes derived from EAFs and the developmental ability of oocytes grown in vitro was low in aged cows; in addition, the characteristics of the granulosa cells are similar to those collected from subordinated follicles [19]. Supplementation with resveratrol of an in vitro culture medium of OGCs collected from EAFs of aged cows (>120 months) enhanced the expression level of SIRT1 in both oocytes and granulosa cells, and increased the mtDNA copy number in fully developed oocytes compared with those grown without resveratrol, whereas markers of autophagosomes including LC3 band patterns, as detected by western blot analysis, and the number of LC3-positive dots following immunostaining were increased by resveratrol treatment. Furthermore, oocytes grown with resveratrol showed greater developmental ability to reach the blastocyst stage than oocytes grown without resveratrol. Gene expression analysis using NGS revealed that the gene expression profiles of granulosa cells were improved from the subordinate follicle pattern to the large healthy follicle pattern [98]. These results indicate that upregulation of SIRT1 by resveratrol improves the condition of both granulosa cells and oocytes derived from EAFs of aged cows, partly through replenishment of mitochondria.

Perspective

Mitochondrial dysfunction, including mutation of mtDNA, is a causal factor not only for age-associated infertility but also for several serious diseases. Pronuclei, spindle, and polar body transfer have been proposed as clinical countermeasures for oocytes with mtDNA mutations [99]. In addition, cryopreservation of oocytes collected at a younger age has been proposed to bypass age-associated cytosolic deterioration [100]. Here, we propose

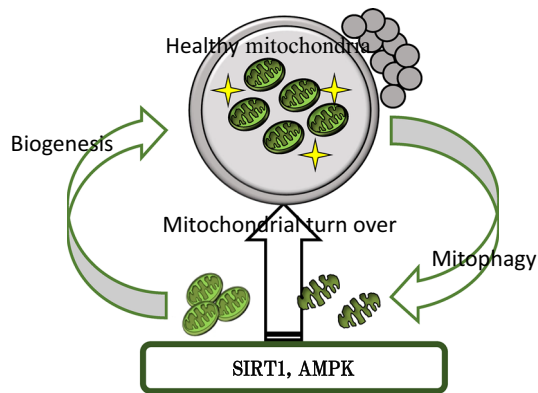


Fig. 3 Schematic diagram of regeneration of oocytes through mitochondrial biogenesis and degradation via SIRT1 and/or AMPK activation

recuperation of oocytes from aged cows by replenishment of mitochondria through activation of the quality control system (Fig. 3). However, fundamental questions remain, such as; the molecular mechanism underlying the age-associated deterioration of mitochondria, whether these mitochondrial dysfunctions can be restored through the quality control system, and whether aging affects the mitochondrial quality control system in oocytes. It is clear that further studies are needed to better understand age-associated events in oocytes.

Compliance with ethical standards

Conflict of interest Hisataka Iwata declares that he has no conflicts of interest.

Human studies This article does not contain any studies with human subjects performed by any of the authors.

Animal studies All institutional and national guidelines for the care and use of laboratory animals were followed.

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