

Age-associated changes in granulosa cells and follicular fluid in cows

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Abstract. Age-associated decline in oocyte quality is common in mammals. Oocytes take a long time to reach their full-grown size in large animals, and maternal physical conditions profoundly affect follicle development. Aging affects the oocyte itself as well as the surrounding environment, such as granulosa cells and follicular fluid. This review discusses age-associated changes that occur in granulosa cells and follicular fluid in cows and suggests that age-associated decline in granulosa cells and follicular fluid hampers proper oocyte development.

Key words: Aging, Granulosa cells, Follicular fluids

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In industrialized countries, changes in working patterns and late marriages decrease the opportunities that women have to become pregnant. In humans, fertility declines once women exceed 35 years of age [1, 2], and accumulating evidence has shown that the quality of oocytes declines as the donor age increases [3]. To manage the rapidly aging population and falling birth rate, it is of great importance to determine the factors involved with this age-associated decline in oocyte quality. Assisted reproductive technologies (ART), such as artificial insemination, embryo transfer, and *in vitro* production of embryos, have advanced in domestic industries, especially in cattle production, and almost all cows are now derived from artificial insemination and embryo transfer in Japan. Cows are known to have follicular wave, follicle selection, and ovulation patterns similar to those in humans and have a long reproductive life compared to rodents [4–8]. In the 1970s, a field-based study demonstrated age-associated decline in bovine reproductive performance [9]. In addition to developments in reproductive technologies, age-associated decline in the reproductive performance of cows is supported by evidences; for example, the number of embryos collected from aged cows (13–16 years old) following super-ovulation was lower than that collected from their younger daughters [10]. We have previously reported specific features of bovine oocytes that are associated with aging: premature progression of nuclear maturation [11], high abnormal fertilization rate following *in vitro* fertilization [12], low developmental ability [13], shorter telomere length [14], low lipid content in oocytes (unpublished data), and a low level of histone acetylation in germinal vesicle-stage oocytes [15]. In line with this, we conducted a comprehensive gene expression analysis of oocytes using next-generation sequencing technology (NGS) and compared between young (25–40 months) and aged (> 120 months) cows. The results showed that differentially expressed genes between *in vitro*-

matured oocytes from aged and young cows were associated with mitochondrial dysfunction [16]. That study also showed that oocytes from aged cows had higher levels of reactive oxygen species (ROS) compared with those from their younger counterparts. In studies in mammals, age-related decline in oocyte quality is associated with mitochondrial DNA damage, decreased mitochondrial membrane potential, and downregulation of both mitochondria- and ATP-related genes [17–21]. Consistent with this, we observed that *in vitro*-matured oocytes collected from aged cows had a lower mitochondrial DNA copy number compared to that in their younger counterparts, and there was a negative correlation between mitochondrial DNA copy number in oocytes and donor age [12]. Similarly, eight-cell-stage embryos derived from cows of advanced age (> 180 months) had a low mitochondrial DNA copy number [13]. However, despite accumulating findings on age-associated abnormalities in oocytes, the primary question remains unsolved: whether these abnormalities in oocytes are caused by lifelong accumulation of molecular and cellular damages in the oocytes, or if they are induced by other factors such as the surrounding environment that support oocytes growth. This review focuses on age-associated changes in granulosa cells and follicular fluid, as well as the relationship between the environment and oocyte qualities.

Granulosa Cells Collected from Aged Cows

In ovaries, most oocytes are quiescent in the form of primordial follicles, which contain several granulosa cells. Once follicles are activated, the morphology of granulosa cells changes from flattened to cuboidal, and they proliferate to become multi-layered, surrounding the oocytes. At the preantral follicle stage, oocytes and granulosa cell complexes form an antral like cavity, which is filled with follicular fluid. The interactions among oocytes and granulosa cells, as well as follicular fluid, are crucial for oocytes growth [22]. Oocyte growth and granulosa cell number are affected by physical conditions. When the diameters of oocytes collected from antral follicles (AF; 3–5 mm in diameter) were compared between young and aged cows, those from aged cows were smaller than those from younger cows [23], which is consistent with previous reports in humans [24]. Interestingly, when comparing the diameter of bovine oocytes collected from early antral

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follicles (EAFs; 0.5–0.7 mm in diameter), oocytes from aged cows had larger diameters than those from their younger counterparts. In line with this, the number of granulosa cells surrounding the oocyte in AFs was lower in aged cows than in their younger counterparts, whereas those in EAFs were similar between the two age groups [23]. Furthermore, the developmental ability of oocyte-granulosa cell complexes collected from the EAFs of aged cows was lower than that in the younger cows. These results prompted us to address the relationship between granulosa cells and oocyte growth.

Molecular Background of Granulosa Cell Proliferation

To better understand the molecular background of follicular development, we conducted RNA-seq of porcine granulosa cells of EAFs (0.5–0.7 mm in diameter) and AFs (1–3 mm in diameter). The analysis predicted that inhibition of TP53 and SP1 is a key top upstream regulator, indicating that downregulating apoptosis is necessary during rapid granulosa proliferation along with follicular development [25]. In addition, those authors showed that HIF1 activation is predicted to be an upstream regulator. HIF1 is a basic helix-loop-helix transcriptional factor comprised of a heterodimeric complex of alpha (HIF1A) and beta (ARNT) subunits, which regulates versatile cellular metabolisms, including glycolysis and angiogenesis. In addition, most genes associated with glycolysis were upregulated in granulosa cells during the transition of EAFs to AFs [25]. In the follicle, oxygen concentration is maintained at low levels [26], which is the normal condition for granulosa cell proliferation *in vivo*. To examine the effect of hypoxic conditions on the metabolism and proliferation of granulosa cells, granulosa cells were cultured under high or low oxygen concentrations, and gene expression was compared between the two oxygen levels using NGS [27]. In that study, hypoxia induced cellular proliferation and increased the expression levels of HIF1 and HIF1-regulated genes concomitant with the activation of glycolytic genes and the suppression of oxidative phosphorylation (OXPHOS)-related genes. In line with this, hypoxia increased the protein levels of HIF1 and the activation of PFK. It was also reported that low oxygen stimuli in mice enhanced the expression of glycolytic genes in GCs, as well as the survival rate and development of follicles [28]. Furthermore, oocytes themselves activate glycolytic genes through the secretion of cytokines in mice and pigs [29–31]. Contrary to the activation of glycolysis, hypoxic conditions decreased mitochondrial functions and quantity, as determined by mitochondrial mass and DNA copy number [27]. These data indicate that high glycolysis and low OXPHOS represent a primary metabolic state of granulosa cells for follicle growth (Fig. 1). During follicular development, the number of granulosa cells expands such that EAFs contains 8000 granulosa cells, which increases to 1390000 in AFs in pigs [32]. Gene expression analysis shows the apparent upregulation of VEGF during the EAF–AF transition [25]. Furthermore, Shiratsuki *et al.* [27] showed that low oxygen-induced granulosa cell proliferation is dependent on the HIF1-VEGF-AKT-mTOR pathways (Fig. 1). The importance of VEGF on the level of AKT phosphorylation required for granulosa cells proliferation was examined in rats [33]. Furthermore, Rico *et al.* [34] used Vegfa δ/δ mice to show that VEGFA deficiency

decreases HIF-1 α -dependent signaling and consequently decreases antrum formation and luteal formation. From these findings, we can speculate that hypoxia-induced activation of HIF1, activation of the VEGF-AKT-mTOR pathway, and the metabolic shift from OXPHOS to glycolysis are important events for granulosa cell proliferation in developing follicles. However, how aging affects this pathway in granulosa cells remains unclear.

Gene Expression in Granulosa Cells

Several studies have investigated granulosa cell marker genes associated with the high developmental competence of oocytes by comparing large healthy follicles and subordinate follicles [35–39]. We conducted RNA-seq using granulosa cells collected from the EAFs of young and aged cows and found that all marker genes related to subordinate follicles were expressed at higher levels in the granulosa cells of aged cows than in those from young cows. Furthermore, many genes related to healthy large follicles were expressed at lower levels in the granulosa cells of aged cows [26]. These results suggest that granulosa cells from the EAFs of aged cows have similar gene expression profiles to those from subordinate follicles. Furthermore, Ingenuity Pathway Analysis (IPA; Qiagen) showed that the genes that were significantly differentially expressed between the granulosa cells of young and aged cows were associated with the oxidative stress response. In line with this prediction, the expression levels of genes related to anti-oxidative ability were lower in granulosa cells from aged cows, and the GSH content in the granulosa cells of aged cows was also significantly lower than that in their younger counterparts [26]. Decreased levels of SOD1, SOD2, and catalase mRNA and proteins in granulosa cells derived from the AFs of older women (≥ 38 years) have also been reported [40]. These findings raise the possibility that age-associated events in oocytes result from the deterioration of granulosa cells surrounding the oocytes. To examine this hypothesis, when oocytes and granulosa cell complexes (OGCs) derived from the EAFs of aged cows were co-incubated with granulosa cells collected from the EAFs of aged or young cows (Fig. 2), only co-incubation with GCs derived from young cows improved the development of OGCs *in vitro*, the ATP content, and the development of *in vitro*-grown oocytes (Table 1). These results show that the ability of granulosa cells to support oocyte development deteriorates in aged cows. In the following section, we address how low granulosa cell number and quality affect oocytes, and what is responsible for the low quality of granulosa cells.

Number of Granulosa Cells and Oocytes

Granulosa cells proliferate along with follicle development and support oocyte development via gap junctional communication and cytokines secreted from each cell in an autocrine- and paracrine-dependent manner [41]. Although myriad studies have addressed the mutual communication that occurs between oocytes and granulosa cells [42, 43], the significance of granulosa cell number for oocyte quality has not been determined. It is well known that metabolites derived from surrounding cells are used to generate ATP in the mitochondria of oocytes [44]. During the growth phase, oocytes accumulate lipids as small droplets in their cytoplasm [45], and oocytes use these lipids

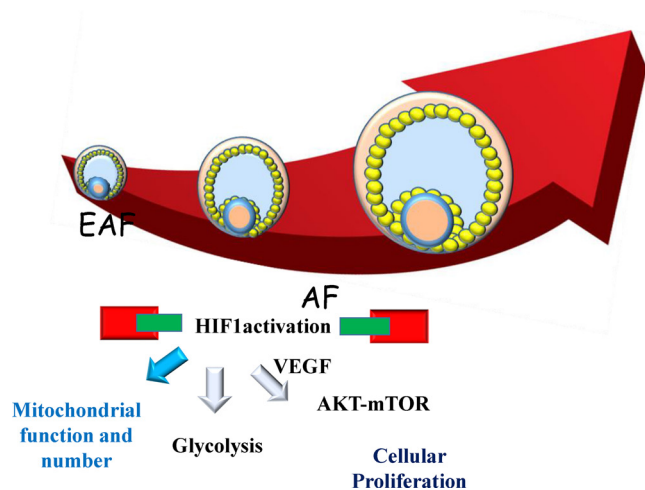


Fig. 1. Comprehensive gene expression analysis and protein analysis of granulosa cells show that granulosa cells proliferate during follicle development from an early antral follicle (EAFs; 0.5–0.7 mm in diameter) to an antral follicle (AFs; 1–3 mm in diameter), depending on the activation of the HIF1-VEGF-AKT-mTOR pathway and glycolysis as well as the suppression of mitochondrial functions. [25, 27].

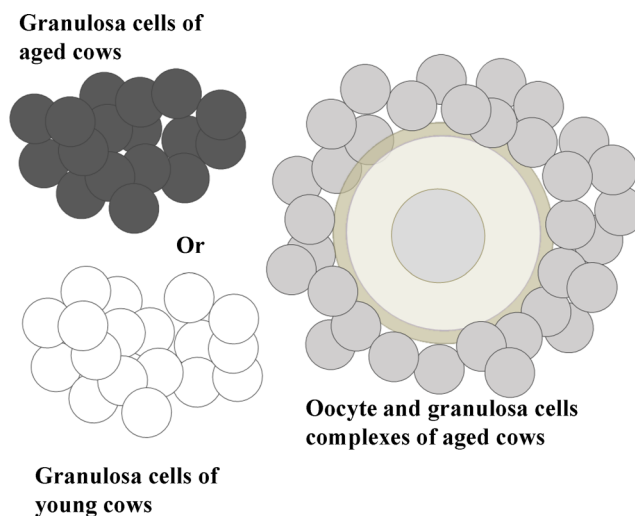


Fig. 2. Oocyte and granulosa cell complexes (OGCs) were collected from the early antral follicles of aged cows and cultured for 16 days. OGCs were cultured with or without a granulosa cell mass, which was collected from randomly selected OGCs of either young or aged cows. The granulosa cell mass was incorporated into OGCs and assisted the development of OGCs.

Table 1. Effect of origins of granulosa cells origin on oocyte growth

GC addition	Day 16 Antrum formation	Oocyte ATP (pM)	24 h after IVF > 8 cell (Mean ± SE%)
–	56.2 ± 4.2 ^a	1.94 ± 0.12 ^a	12.0 ± 3.0 ^{ab}
Aged	69.4 ± 4.1 ^{ab}	2.21 ± 0.14 ^{ab}	5.0 ± 3.1 ^a
Young	73.3 ± 5.3 ^b	2.41 ± 0.10 ^b	25.0 ± 5.7 ^b

Oocyte and granulosa cell complexes (OGCs) collected from aged cows were cultured with or without additional granulosa cells (GC) for 16 days (Fig. 2), at which antrum formation was determined. GC were collected from OGCs of young or aged cows. *In vitro* grown oocytes were examined for their ATP content and developmental ability following *in vitro* maturation and fertilization.

as an important energy source for nuclear maturation [46] and early development [47]. We addressed the relationship between the lipid content of oocytes and the number of surrounding cells in porcine ovaries and found a close significant relationship among the average number of granulosa cells contained within a follicle, the average number of cumulus cells surrounding an oocyte, and the average lipid content in an oocyte [48]. Furthermore, this relationship was confirmed in *in vitro*-developed OGCs. In that study, we cultured OGCs derived from the EAFs of gilts for 16 days; after which, the granulosa cell number, the glucose consumption of the OGCs, and the lipid and ATP content in the enclosed oocytes were compared. The ATP and lipid content in oocytes grown *in vitro* were significantly correlated with the number of granulosa cells surrounding the oocytes and glucose consumption of the OGCs. Interestingly, granulosa cell number in OGCs was related to the level of histone H4K12 acetylation

in enclosed oocytes grown *in vitro* [48]. During oocyte growth, histone acetylation increases with chromatin condensation [49, 50], and a high level of H4K12 acetylation was observed in human, bovine, and porcine germinal vesicle-stage oocytes [51–53]. Energy sufficiency results in high ATP generation, as well as protein acetylation, in cells [54, 55]. Furthermore, acetyl-CoA is derived from citrate, which originates from the mitochondria, and is used for protein acetylation [56]. Next, we hypothesized that the number of granulosa cells that surround the oocyte is a determinant factor for the energy status of the oocytes and that the number of granulosa cells affects the lipid and ATP content in oocytes, as well as their acetylation levels. To examine this notion, we examined the relationship between the energy status of oocytes and the granulosa cell number by *in vitro* culture of OGCs from the following three possibilities (Fig. 3): 1) Does artificially increasing the number of granulosa cells result in the high-energy status of oocytes grown *in vitro*? 2) Does chemically upregulating granulosa cell proliferation increase the energy status of oocytes grown *in vitro*? 3) Does modifying the culture conditions to enhance cellular proliferation result in the high-energy status of oocytes grown *in vitro*? In all trials, we found that granulosa cell number positively correlated with increased lipid, ATP, and histone acetylation levels in oocytes grown *in vitro* [15, 57, 58]. Thus, we conclude that the number of granulosa cells surrounding oocytes profoundly regulates the energy status of oocytes, which reflects the ATP, lipid, and acetylation levels found in oocytes (Fig. 4). Together with these results, we speculated that the low acetylation levels and low lipid content of oocytes derived from aged cows were attributable to the low number of granulosa cells in the follicle and that increasing the number of granulosa cells surrounding the oocyte may improve the quality of oocytes in older females.

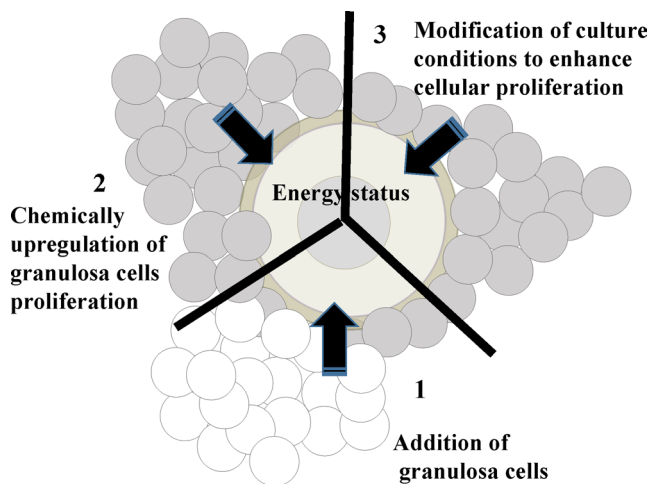


Fig. 3. 1: Effect of adding granulosa cells to the oocytes and granulosa cell complexes (OGCs) on the energy status of oocytes grown *in vitro*. OGCs derived from the early antral follicles (EAFs) of young cows were cultured with an additional granulosa cell mass collected from other young cows for 16 days; after which, ATP and lipid content and histone acetylation levels were examined [15]. 2: Effect of chemical stimulation of granulosa cell proliferation on the energy status of oocytes grown *in vitro*. OGCs of porcine EAFs were cultured in medium supplemented with or without high glucose and insulin, after which the ATP and lipid contents of oocytes grown *in vitro* were examined [58]. 3: Effects of modifying culture conditions for the proliferation of granulosa cells on the energy status of oocytes grown *in vitro*. OGCs of EAFs were cultured on acrylamide gels, which enhanced granulosa cell proliferation; after which, ATP and lipid contents as well as the acetylation levels of proteins and histones were compared to those cultured without acrylamide gels [57].

Age-associated Changes in the Characteristics of Granulosa Cells

The characteristics of granulosa cells change as the donor ages. In the AFs of aged cows, the number of granulosa cells in follicles was lower than that contained by their younger counterparts [15]. Consistent with this, the levels of proliferation, as measured by the BrdU assay, was low in the granulosa cells of AFs from aged cows [59]. The telomere length of cells is closely linked to their proliferation activity, and an age-associated reduction in telomere length was observed in the granulosa cells of both the EAFs and AFs of cows [14]. The telomere length of granulosa cells or cumulus cells may represent a potential marker for oocyte ability in older women [60]. A comparison of telomere length between oocytes and granulosa cells revealed significant positive correlations in the AFs of cows [14]. Telomeres shorten owing to the end replicable problem, and telomeres are vulnerable to DNA damage due to their GC-rich sequence [61]. The extended exposure of oocytes and granulosa cells to oxidative stress during their lifespan may damage telomeres in both cell types. In line with this, the frequency of double-stranded breaks in DNA from granulosa cells was higher in aged monkeys than in their younger counterparts [62]. Moreover, the amount of reactive oxygen and oxidative damage was higher in the ovaries of

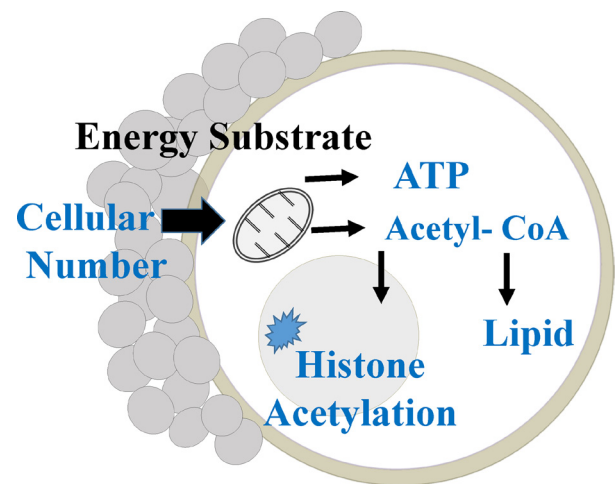


Fig. 4. Schematic design of factors affecting ATP levels, lipid content, and acetylation of oocytes. Granulosa cells provide energy substrates to oocytes; mitochondria use these substrates to generate ATP, and acetyl CoA derived from mitochondrial citrate is used to generate lipids and acetylation of proteins in oocytes.

aged mice than in those of younger counterparts [63], and low ovary anti-oxidative activities were observed in the granulosa cells of aged humans [40]. Furthermore, high levels of p38 MAPK expression induced by high levels of ROS were observed in human granulosa cells [64]. Consistent with this report, we observed higher p38 MAPK expression in granulosa cells from aged cows compared to that in young cows [65]. Together with these results, it is speculated that age-associated high ROS generation in ovaries plays a role in the high level of DNA damage and telomere shortening that occurs in granulosa cells. Thus, the accumulation of molecular and cellular damage, including shorter telomere lengths, prevents granulosa cells from actively proliferating; however, the molecular targets of high ROS and the causal factor for high ROS generation remain elusive.

Follicular Fluid Affects Granulosa Cell Proliferation and Oocyte Maturation

Oocytes grow in follicles, which are filled with follicular fluid derived from serum and granulosa cell secretions. The characteristics of follicular fluid reflect those of the serum and are affected by physical conditions and aging in cows [65, 66]. Follicular fluid contents include hormones, proteins, peptides, amino acids, and molecules with antioxidant and anti-apoptotic abilities [67]. An imbalance in the pro-oxidant and antioxidative ability of follicular fluid is suggested as a causal factor for low oocyte quality [22], because low glutathione peroxidase and superoxide dismutase were reported in the follicular fluid and cumulus cells of aged women [68, 69]. Furthermore, when culturing oocytes in medium containing follicular fluid derived from aged cows, the ROS content in oocytes was greater compared with that in oocytes cultured in follicular fluid derived from younger cows [65]. Moreover, low anti-oxidative abilities in the follicular fluid of aged cows were observed (Fig. 5-A). Takeo *et al.* [65] reported that supplementation of *in vitro*-maturation medium with follicular fluid

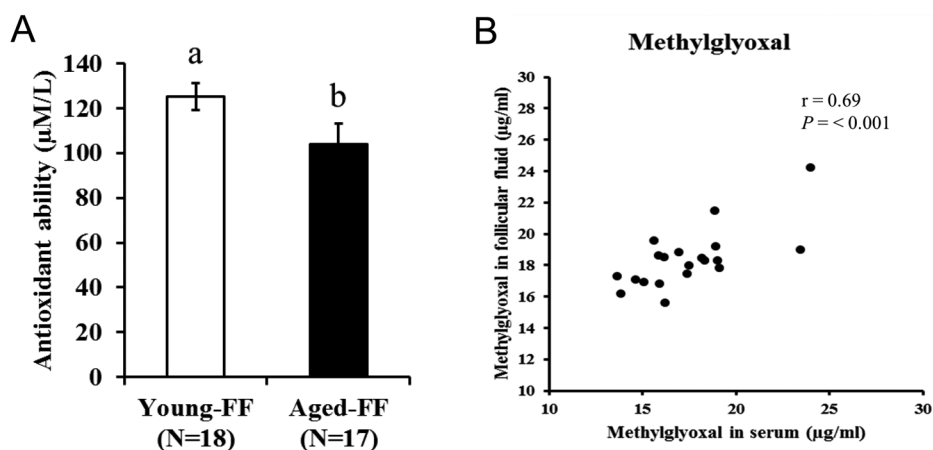


Fig. 5. A: Antioxidant ability of follicular fluid. Follicular fluid was collected from the antral follicles of ovaries, which were collected from young (27–50 months of age) and aged (> 120 months age) Japanese Black cows from a slaughterhouse. Antioxidative abilities were measured using a test kit for potential antioxidant (PAO; Japan Institute for the Control of Aging, NIKKEN SEIL, Shizuoka, Japan). B: Correlation of methylglyoxal between follicular fluid and serum. Follicular fluid was collected from Japanese Black cows following collection of serum and slaughter. Methylglyoxal was measured using the Methylglyoxal (MG) competitive ELISA kit (Cell Biolabs, San Diego, CA, USA).

from aged cows induced fast closure of gap-junctions, high abnormal fertilization, and low developmental competence in oocytes, similar to age-induced abnormalities in bovine oocytes. In the context of a possible causal candidate for the age-associated deleterious effects of follicular fluid, it was reported that supplementation of maturation medium with advanced glycation end products (AGEs) induced oocyte abnormalities in similar ways to induction by the follicular fluid of oocytes from aged cows [65]. Consistent with this, a positive correlation between pentosidine (Pent), a type of AGE, and aging in women was reported [70]. In addition, Pent accumulation in oocytes from primordial and primary follicles was observed in aged women [71]. Considering that AGEs in the follicular fluid of cows were closely associated to that in the serum (Fig. 5-B), age-associated physical changes indirectly hamper proper oocyte development.

Perspective

In large domestic animals, oocytes take a long time to reach their full growth size, and the surrounding environment is crucial for their proper development. Studies have revealed that aging affects granulosa cell quality and quantity as well as the characteristics of follicular fluid, which in turn deteriorates oocyte development. This raises the possibility that, under proper conditions, some of the age-associated decline in oocytes associated with older females could be improved.

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