Application of intracytoplasmic sperm injection to the embryo production in aged cows

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ABSTRACT. Reduction in oocyte quality is a major factor responsible for declining fertility associated with maternal aging in cows. The objective of the present study was to determine whether intracytoplasmic sperm injection (ICSI) could increase the efficiency of embryo production in older cows. We used cows aged 30 to 50 months or >120 months, which were defined as young or aged, respectively. The distribution of cortical granules in oocytes was affected by age as older cows had lower proportion of oocytes with mature cytoplasm containing evenly dispersed cortical granules compared to young cows. Although fertilization rates did not differ significantly between the two groups after *in vitro* fertilization (IVF), the rate of abnormal fertilization was higher, and the numbers of total and diploid blastocysts were lower for aged cows compared to young cows. However, in the embryos produced by ICSI, there was no significant difference in these parameters between young and aged cows. Although ICSI did not improve the blastocyst development rate, ICSI increased the proportion of diploid blastocysts in aged cows compared to IVF. In conclusion, maternal aging may negatively affect cytoplasmic maturation of bovine oocytes, which could be associated with abnormal fertilization or low developmental competence of oocytes. Our data also suggests beneficial effects of ICSI on the production of chromosomally normal embryos in aged cows.

KEY WORDS: aneuploidy, bovine oocyte, intracytoplasmic sperm injection, *in vitro* fertilization, maternal age

In most mammals, female fertility declines with age. The main reasons for it include reduced follicle number, compromised oocyte quality, altered reproductive endocrine status, and increase in reproductive tract defects [17, 20]. Among them, the decline in oocyte quality is considered the major factor responsible for age-associated infertility [17], as the oocyte quality significantly affects fertilization rates, early embryonic survival, continuation of pregnancy, and fetal development [20].

In cattle, the use of oocytes from aged donors for assisted reproduction can have considerable commercial benefits as it would extend the reproductive life of cows with valuable genetic traits and promote breeding of animals with high market value. Especially in female, genetically superior aged cows are quite valuable because of the limitation of animal numbers and ability to produce embryos with good quality. However, more than half of the cows become infertile by the age of 15 years [7]. Superovulated aged cows (13 to 16 years old) had a higher proportion of uncleaved embryos compared with their young daughters (3 to 6 years old) [16]. Moreover, cleavage and blastocyst formation rates among oocytes recovered by ovum pick up (OPU) were lower in aged donors (more than 15 years old) than in young donors (one year old) [24]. These data suggest that reproductive aging in cattle results in the reduced developmental competence of oocytes.

Successful fertilization, which is a crucial step in embryonic development, is known to be negatively affected by age in cattle. The rate of abnormal oocyte fertilization in aged cows was reportedly higher than that in young cows [12], suggesting that abnormal fertilization might reflect low oocyte competence in older females. Polyspermy is one of the most commonly observed types of abnormal fertilization in mammalian oocytes, and cortical granule distribution under the oolemma and proper exocytosis are key factors responsible for successful blocking of polyspermic fertilization [26]. However, in cattle, the effect of reproductive aging on the cortical reaction of oocytes is still unclear.

Among assisted reproduction technologies used for humans, the application of intracytoplasmic sperm injection (ICSI) has increased dramatically in recent years. ICSI has been proposed to decrease the incidence of fertilization failure as it offers the advantage of bypassing the barriers responsible for any block in the process of fertilization, either of the oocyte or spermatozoan

origin [8]. However, despite the increased use of ICSI, studies that examined ICSI benefits in aging cattle are lacking. Here, we tested the hypothesis that in cows, age-related abnormal fertilization might be associated with the impaired cortical reaction of oocytes and that assisted fertilization by ICSI might improve the developmental ability of these oocytes. Therefore, the objectives of this study were to determine: 1) the effect of maternal aging on the developmental competence of bovine oocytes and 2) whether ICSI could improve embryo productivity in aged cows.

MATERIALS AND METHODS

Materials

Ovaries of multiparous Holstein cows were obtained from a local slaughterhouse and transported to the laboratory in sterile saline solution (0.85% NaCl) at 20°C. Information of ages of cows was obtained from the slaughterhouse, and cows aged 30 to 50 months or >120 months were defined as young or aged, respectively. Animals with organ disorders diagnosed by experienced veterinarians as well as ovaries with overt abnormalities such as follicular cysts and atrophy were not included in the study. All materials were purchased from Sigma-Aldrich Japan Inc. (Tokyo, Japan), unless stated otherwise.

Oocyte maturation

Cumulus-oocyte complexes (COCs) were aspirated from 2 to 7-mm ovarian follicles of young (40 ± 7 months, n=89) and aged (136 ± 12 months, n=55) cows. Only COCs of grade 1 or 2 [4] were selected for *in vitro* maturation. COCs were cultured for 23 hr in a droplet of 100 μl of tissue culture medium 199 (TCM199) containing 25 mM HEPES (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 5% fetal bovine serum (FBS; MP Biomedicals, Illkirch, France) in 4-well tissue culture plates (Nunc, Nalge Nunc International, Roskilde, Denmark) under sterile paraffin oil (Nacalai Tesque, Kyoto, Japan) at 38.5°C in an atmosphere of saturated humidity containing 5% CO_2 and 95% air (10 COCs per a droplet).

Assessment of oocyte nuclear maturation

Meiotic progression was evaluated based on the presence of the first polar body (PB) and nuclear morphology. The oocytes were denuded of cumulus cells, mounted on a slide, fixed for 24 hr in ethanol:acetic acid (3:1 v/v), and stained with 1% acetic orcein for 20 min. Nuclear stages were distinguished by chromatin morphology as described previously [11]: germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII). In total, 101 and 98 oocytes from the young and aged cows, respectively, were evaluated for nuclear maturation in the course of 4 independent experiments.

Assessment of cortical granule distribution

To examine cytoplasmic maturation of oocytes, cortical granules were stained as previously described [1, 5] with minor modifications. Briefly, at the end of maturation, the denuded oocytes were treated with 2.5 mg/ml protease type XIV from *Streptomyces griseus* to remove the zona pellucida. After washing three times in Dulbecco's phosphate buffered saline (PBS; Thermo Fisher Scientific, Yokohama, Japan) supplemented with 5% FBS (PBS-FBS), the oocytes were fixed in 4% (w/v) paraformaldehyde for 30 min at 25°C, washed again three times in PBS-FBS, and placed in blocking solution containing 0.1% (v/v) Triton X-100, 2% (v/v) normal goat serum, 0.1 M glycine, 1% (w/v) powdered skim milk, and 0.5% (w/v) BSA in PBS for 1 hr at 39°C. Cortical granules were stained with 100 mg/ml FITC-peanut agglutinin (PNA) in PBS-0.1% (w/v) polyvinylpyrrolidone (PVP) for 30 min at 39°C, and oocytes were washed three times in PBS-PVP, mounted on slides in fluoromount, covered with coverslips, and examined under a Leica DM LB fluorescence microscope (Leica, Wetzlar, Germany) at the excitation/emission wavelengths of 488/530 nm. The oocytes were classified into three types according to the observed distribution pattern of cortical granules [13]: class 1, large aggregates of cortical granules distributed over the entire cytoplasm; class 2, cortical granules localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates; and class 3, cortical granules almost evenly dispersed in the cortical cytoplasm aligning with the oolemma (Fig. 1A). In total, 172 and 191 oocytes were examined in the young and aged groups, respectively, in 5 independent experiments.

IVF

IVF was performed in IVF100 medium (Research Institute for the Functional Peptides, Yamagata, Japan), according to the manufacturer's instructions. Briefly, spermatozoa obtained from a bull were washed twice by centrifugation ($600 \times$ g for 10 min) in IVF100 medium, and the resultant sperm pellet was diluted in the same medium to a final concentration of 2.5×10^6 sperm/ml. After maturation, COCs were washed three times with IVF100 medium, added into sperm suspension droplets (10 oocytes per 100-μl droplet under sterile paraffin oil), and incubated for 14 hr at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air. After 14 hr of fertilization, any remaining cumulus cells were removed from presumptive zygotes by vortexing for 4 min in PBS-FBS and washing three times in the same buffer, prior to embryo culture.

Assessment of the fertilization rate

Fertilization rates were evaluated at 15 hr post-insemination by counting the number of pronuclei (PNs). For this, presumptive zygotes denuded of cumulus cells were fixed for 24 hr in ethanol:acetic acid (3:1, v/v), stained with 1% acetic orcein, and PNs were observed under an inverted microscope (Leica DM LB). The oocytes were divided into three groups based on the number of PNs: those with two PNs were considered to have undergone normal fertilization, whereas those with ≥ three PNs were considered
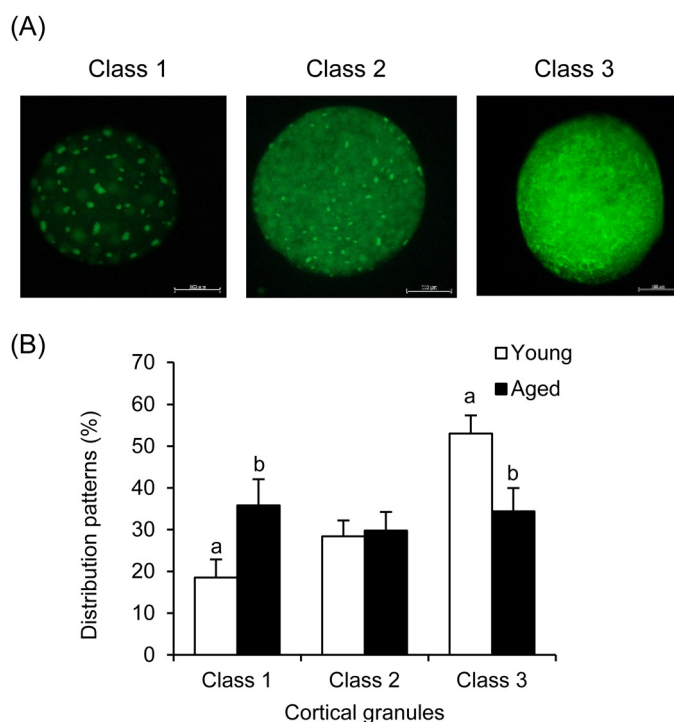
(A)

(B)

Fig. 1. (A) The oocytes were classified into three types according to the observed distribution pattern of cortical granules. Class 1, large aggregates of cortical granules distributed over the entire cytoplasm; class 2, cortical granules localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates; and class 3, cortical granules almost evenly dispersed in the cortical cytoplasm aligning with the oolemma. (B) Effect of maternal aging on cortical granules of bovine oocytes collected from young or aged cows. All values are shown as means ± SEM. Values with different letters (a, b) are different between groups (*P*<0.05).

to have undergone abnormal fertilization, and those with none or one PN were defined as non-fertilized. In total, 123 and 90 oocytes from the young and aged cows, respectively, were evaluated for fertilization in the course of 4 independent experiments. Oocytes that were penetrated by sperm but did not form pronuclei were divided into unfertilized.

Sperm preparation for ICSI

Spermatozoa from a bull used for IVF were separated following a swim-up procedure described previously [27]. Briefly, five 0.2-m*l* sperm aliquots were each layered under 1 m*l* of Sp-TALP medium supplemented with 5 mM dithiothreitol, incubated for 1 hr at 38.5°C, and the top 0.5 m*l* from each tube was aspirated, pooled, and washed in the same medium by centrifugation at 500 × g for 5 min. The pellet was washed by centrifugation in fresh Sp-TALP medium, and the final pellet was reconstituted in 0.2 m*l* of the same medium and the suspension was mixed with PVP90 (final concentration, 5% [w/v]).

ICSI

Oocytes were denuded of cumulus cells and selected for ICSI only if they had a visible first PB. Oocytes were transferred to an injection PBS-FBS drop under sterile paraffin oil, and ICSI was performed with a piezo-micromanipulator (model PMM-MB-A, Prime Tech, Tsuchiura, Japan). The injection microcapillary was moved into the sperm drop, and a single sperm was aspirated in the tail-first position. An oocyte was placed so that the first PB was located at the 6 or 12 o'clock position, and the zona pellucida was drilled by applying several piezo pulses. Then, the injection microcapillary was advanced mechanically into the cytoplasm of the oocyte, the oolemma was punctured by applying a single piezo pulse, and the sperm was deposited into the cytoplasm. The injected oocytes were transferred into TCM199 supplemented with 5% FBS under paraffin oil and maintained at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air until all the injection was performed (about 1 hr in maximum) and then transferred into the culture medium. Injected oocytes were not activated, since the oocyte activation treatment might induce parthenogenetic development [15].

Embryo culture

Presumptive zygotes were cultured for 4 days (IVF) or 5 days (ICSI) in 100-μl droplets of CR1aa medium [21] with 2% FBS and then in 100-μl droplets of USU6 medium [28] with 5% FBS for 3 days in 4-well tissue culture plates under sterile paraffin oil at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (10 zygotes per a droplet). Cleavage rate was calculated as the proportion of oocytes that cleaved at day 2 post-fertilization. Blastocyst rate was calculated as the proportion of oocytes that developed to the blastocyst stage at day 8 post-fertilization. Following IVF, 172 and 191 oocytes for the young and aged groups were cultured in 5 independent experiments, respectively. Following ICSI, 195 and 127 oocytes for the young and aged groups were cultured in 10 and 9 independent experiments, respectively.

Blastocyst karyotyping

The effect of maternal aging on ploidy was evaluated by counting chromosome numbers in blastocysts. Preparation of

Table 1. Effect of age on nuclear maturation of bovine oocytes and fertilization folowing IVF

Group	No. of oocytes (Replicates)	Meiotic stage		No. of oocytes (Replicates)	Fertilization type		
		GV/MI (%)	MII (%)		Normal (%)	Abnormal (%)	Unfertile (%)
Young	101 (4)	19 (18.9 ± 1.9)	82 (81.1 ± 1.9)	123 (4)	78 (62.9 ± 2.5)[x)]	18 (14.8 ± 1.1)[a)]	27 (22.3 ± 1.6)
Aged	98 (4)	22 (22.7 ± 2.2)	76 (77.3 ± 2.2)	90 (4)	36 (38.6 ± 2.6)[y)]	29 (32.8 ± 6.2)[b)]	25 (28.6 ± 5.5)

Values with different superscripts within the same column are significantly different (x, y: $P<0.01$ and a, b: $P<0.05$). Percentage data are shown as mean ± SEM.

Table 2. Effect of age on embryo development following *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI)

	Group	No. of oocytes (Replicates)	Cleaved (%)	Blastocyst (%)
IVF	Young	172 (5)	129 (73.7 ± 2.3)	67 (37.0 ± 3.2)[a)]
	Aged	191 (5)	131 (67.1 ± 4.7)	50 (25.7 ± 1.7)[b)]
ICSI	Young	195 (10)	102 (56.5 ± 6.6)	37 (17.5 ± 3.3)[b)]
	Aged	127 (9)	60 (45.2 ± 6.8)	26 (16.3 ± 6.9)[b)]

Values with different superscripts within the same column are significantly different (a, b: $P<0.05$). Percentage data are shown as mean ± SEM.

chromosome spreads in embryos was performed as described previously with minor modifications [29]. Briefly, after *in vitro* culture for 6 days, blastocysts were incubated for 14 hr in CR1aa medium containing 5% FBS and 60 n*g*/m*l* vinblastine sulfate (Wako Pure Chemical Industries, Osaka, Japan). The blastocysts were washed, incubated in 0.4 m*l* of 1% (w/v) sodium citrate solution for 15 min, and fixed with 0.02 m*l* acetic methanol (acetic acid:methanol=1:1). Then, each blastocyst was placed on a glass slide, immediately covered with a very small droplet of acetic acid to separate cells, and then re-fixed with 3 to 4 drops of acetic methanol (acetic acid:methanol=1:3). Slides were dried, and chromosome spreads were observed under a light microscope (Leica DM LB) after staining with 2% (v/v) Giemsa solution (Wako Pure Chemical Industries, Osaka, Japan) for 20 min. Blastocysts with one (n=30), two (2n=60), and more than two (3n, 4n, 5n etc.) sets of chromosomes were considered haploid, diploid, and polyploid, respectively (Fig. 1A). In total, we analyzed 46 and 56 IVF-derived blastocysts for the young and aged groups in 7 and 5 independent experiments, respectively, and 26 and 22 ICSI-derived blastocysts for the young and aged groups in 10 and 9 independent experiments, respectively.

Statistical analyses

Statistical analyses were performed using Stat View 5.0 (SAS Institute Inc., Cary, NC, U.S.A.). Homogeneity of variance was examined by *F*-test. Cortical granule distribution was evaluated by Mann-Whitney *U*-test. Meiotic progression, fertilization rate, embryo development, and chromosome numbers were analyzed by Chi-squared test. The proportion data were subjected to arcsine transform prior to analysis. Differences were considered statistically significant at $P<0.05$.

RESULTS

Effect of maternal aging on nuclear and cytoplasmic maturation of bovine oocytes

The average number of follicles per cow were 36.7 ± 25.4 in the young group (39.6 ± 6.8 months, Mean ± SD, n=89) and 37.3 ± 18.5 in the aged group (135.6 ± 11.9 months, Mean ± SD, n=55). Oocyte distribution into nuclear meiotic stages (GVBD/MI and MII) did not differ significantly between the young and aged groups ($P=0.26$, Table 1). At the same time, the distribution of matured oocytes into different cortical granule classes was affected by age as evidenced by a lower proportion of class 3 (mature cytoplasm) oocytes in aged cows (34.4 ± 5.6%, Mean ± SEM) compared to young cows (53.0 ± 4.3%, Mean ± SEM) ($P<0.05$; Fig. 1B). In the young and aged group, the proportion of class 1 were 18.5 ± 4.3% and 35.8 ± 6.3% (Mean ± SEM), and that of class 2 were 28.4 ± 3.8 and 29.8 ± 4.4% (Mean ± SEM), respectively.

Effect of maternal aging on the fertilization rate following IVF

Although the proportion of unfertilized oocytes did not differ significantly between the two groups ($P=0.29$, Table 1), the rate of normal fertilization was lower ($P<0.01$) and that of abnormal fertilization (appearance of more than two PNs in the zygote) was higher in the aged group ($P<0.05$).

Effect of maternal aging on embryo development

After IVF, the number of cleaved embryos was similar in both groups ($P=0.17$, Table 2), whereas the rate of oocyte development into blastocysts was lower in aged cows compared to young cows ($P<0.05$). However, after ICSI, no significant difference was observed in the number of cleaved embryos or blastocysts between the two groups ($P=0.24$ and $P=0.74$, respectively).

Table 3. Effect of age on the chromosome complementation in IVF- and ICSI-derived embryos

Fertilization	Group	No. of embryos (Replicates)	Chromosome complementation			
			Haploid (%)	Diploid (%)	Polyploid (%)	Mixploid (%)
IVF	Young	46 (7)	0 (0.0)	35 (73.8 ± 8.7)[a]	1 (2.0 ± 0.8)	11 (25.2 ± 8.8)[a, b]
	Aged	56 (5)	4 (9.0 ± 4.8)	25 (45.0 ± 4.2)[b]	7 (6.3 ± 3.0)	20 (39.7 ± 4.6)[a]
ICSI	Young	26 (10)	0 (0.0)	21 (85.2 ± 6.2)[a]	0 (0.0)	5 (14.8 ± 6.2)[a, b]
	Aged	22 (9)	0 (0.0)	19 (90.2 ± 5.3)[a]	0 (0.0)	3 (6.3 ± 5.4)[b]

Values with different superscripts within the same column are significantly different (a, b: $P<0.05$). Percentage data are shown as mean ± SEM.

Chromosome numbers in blastocysts

Following IVF, the proportion of diploid blastocysts in the aged group was lower than that in the young group ($P<0.05$, Table 3). The rate of mixoploidy did not differ between aged and young cows ($P=0.12$), and mixoploidy was mostly commonly represented by the diploid/triploid combination. Following ICSI, no significant difference between aged and young cows was observed in the number of diploid blastocysts ($P=0.87$) or the rate of mixoploidy ($P=0.56$). In the aged group, the proportion of diploid blastocysts in the IVF group was lower than that in the ICSI group ($P<0.05$).

DISCUSSION

The present study examined the effects of maternal aging on the developmental competence of bovine oocytes. Our data confirmed the well-documented age-induced decline in embryo competence, which corresponded to an increase in chromosomal abnormalities. The present study demonstrated for the first time that distribution of cortical granules was impaired in the oocytes of aged cows, which could be associated with abnormal fertilization or low developmental competence of the oocytes following IVF. Another new finding of the present study is that ICSI increased the proportion of diploid blastocysts compared to IVF, although the use of ICSI did not affect the blastocyst development rate in aged cows.

It is well known that aneuploidy in the *in vitro*-produced embryos increases with female age [9, 14]. Previous findings indicate that the aneuploidy rate was elevated from 47% among women aged 27 to 37 years to 78% among those aged 38 to 47 years [9]. However, there is very little information on the relationship between reproductive aging and chromosomal aneuploidy in cattle. In the present study, the proportion of diploid blastocysts was significantly lower among the IVF-derived embryos from the aged group compared to those from the young group, suggesting maternal age-induced aneuploidy in bovine embryos. Chromosomal aneuploidy is considered to be a major underlying cause of abortion in humans [23]. Similarly, in cattle, it has been suggested that chromosomal abnormalities are associated with spontaneous abortions and neonatal losses [22]. Thus, the chromosomal aneuploidy of embryos in aged cows could be one of the key factors responsible for age-related infertility in cattle. The nuclear maturation rates in the oocytes from young and aged cows were similar, and the use of ICSI decreased the proportion of aneuploid embryos in the aged group. These findings indicate that the increase in chromosomal abnormalities among the IVF-derived embryos from aged cows is probably indicative of fertilization failure rather than meiotic errors.

Normal fertilization depends on appropriate cortical granule distribution in matured oocytes [26]. Fertilization triggers the process termed 'cortical reaction', i.e., exocytosis of cortical granules, which contain enzymes that modify and thereby harden the zona pellucida, making it impermeable to additional sperm; as a result, sperm binding affinity is decreased [3]. In human oocytes, reproductive aging is shown to be associated with the decrease in the number of cortical granules [2]. Moreover, internalization of cortical granules was observed in oocytes collected from aged mice, indicating the inability of oocytes to develop normal cortical reaction in aged females [6]. Similarly, in the present study, the proportion of class 3 oocytes, in which cortical granules are evenly dispersed and align with the oolemma, was reduced in the aged group, indicating the deleterious effect of maternal aging on cytoplasmic maturation of oocytes in cattle. Thus, increased abnormal fertilization in the aged group might reflect age-associated increase of polyspermy due to impaired cortical reaction. The mechanism by which maternal aging affects the distribution of cortical granules is still unclear. A recent study revealed two separate mechanisms driving translocation of cortical granules: myosin Va-dependent movement along actin filaments and an unexpected vesicle hitchhiking phenomenon, when cortical granules bind to Rab11a vesicles powered by myosin Vb [3]. It is possible that the age-related changes in the oocyte cytoskeleton might be associated with impaired distribution of cortical granules and subsequent fertilization failure.

ICSI has been suggested as a routine approach to decrease the incidence of fertilization failure [8]. In cattle, ICSI advantages include effective utilization of spermatozoa for livestock improvement and production of animals with superior qualities. It has been shown that OPU-ICSI improved the blastocyst development rate in donors with low embryo production compared with the standard OPU-IVF [18]. In the present study, the number of total and diploid blastocysts produced by IVF were lower for aged cows compared to young cows. However, there was no significant difference in these parameters between young and aged cows in the embryos produced by ICSI. Following ICSI, the blastulation rates of young and aged cows were 19.0 and 20.5%, respectively. These are comparable to the previous report in which the proportion of embryos developed to blastocysts was 22.7% [27]. However, further efforts are still needed to investigate more appropriate conditions to achieve better results of ICSI in cattle, since the blastocyst development rates of ICSI were not higher than that of IVF. In aged cows, the proportion of diploid

blastocysts was considerably higher in ICSI compared to IVF (86.4 vs. 47.2%, ICSI vs. IVF), indicating the high implantation potential of ICSI derived embryos. This finding might suggest that assisted fertilization by ICSI could have beneficial effects on embryo production in aged cows by overcoming fertilization failure associated with compromised polyspermy block. Although ICSI equipment are expensive, application of ICSI on aged cows could have economic benefit since ICSI increased the production of chromosomally normal embryos and thus might reduce early embryonic losses or abortion. However, embryos derived from aged cows still have some risk for early embryonic loss because of the impaired cytoplasmic maturation such as changes in the cortical granule distribution or mitochondrial dysfunction [25]. Thus, further study for embryo transfer of ICSI-derived embryos is necessary to evaluate the implantation potential. In contrast to the present study, the prevalence of zygotes with ≥3 PNs did not differ significantly between IVF and ICSI treatments in women at the advanced reproductive age [10]. The inconsistency between findings in humans and our results may be attributed to different conditions of oocyte maturation in women and cows. In the present study, oocytes were matured *in vitro* without any hormonal treatment, whereas assisted reproduction in women is usually conducted with oocytes matured *in vivo* following controlled ovarian stimulation. A previous study suggested that negative processes occurring in the oocytes from older women could be aggravated *in vivo* through age-related alterations in follicular fluid [19]. Our results suggest that ICSI could be a helpful approach to improving the efficiency of production of chromosomally normal embryos for women of advanced reproductive age, if compromised polyspermy block is the key factor for the abnormal fertilization of oocytes.

In conclusion, the results of the present study indicate that maternal aging in cows impairs the distribution of cortical granules during oocyte maturation, which could result in abnormal fertilization, low developmental competence of oocytes, or/and increased aneuploidy. Although ICSI did not improve the blastocyst development rate, ICSI increased the proportion of diploid blastocysts in aged cows. Our data suggests the beneficial effects of ICSI on the production of chromosomally normal embryos in aged cows.

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