Effects of pre-maturational culture duration on developmental competence of bovine small-sized oocytes

Mohammed. A. ABDEL-GHANI1, 2), Kenichiro SAKAGUCHI3), Chihiro KANNO3), Yojiro YANAGAWA1), Seiji KATAGIRI1) and Masashi NAGANO1)

1) Laboratory of Theriogenology, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
2) Department of Theriogenology, Faculty of Veterinary Medicine, Assuit University, Assuit, 71515, Egypt
3) Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Abstract. We investigated the effects of pre-maturational (pre-IVM) culture on the developmental competence of small-sized bovine oocytes (110 and < 115 μm). Oocytes were cultured with 3-isobutyl-1-methylxanthine (IBMX) for 0, 5, or 10 h and subjected to in vitro maturation, fertilization, and culture. The cleavage rate (73%) of small-sized oocytes with 5 h pre-IVM was higher than those with 0 and 10 h pre-IVM (61 and 62%, respectively). The blastocyst rate (16%) of embryos derived from small-sized oocytes with 5 h pre-IVM was higher than those with 0 and 10 h pre-IVM (9 and 8%, respectively). In addition, small-sized oocytes with 5 h pre-IVM had a higher mean cell number in blastocysts (134.1 ± 34.8) than those with 0 and 10 h pre-IVM (100.2 ± 17.2 and 107.8 ± 23.7, respectively). In conclusion, the pre-IVM of small-sized oocytes with IBMX for 5 h improved the developmental competence of bovine oocytes, as well as the quality of blastocysts.

Key words: IBMX, Oocytes, Pre-IVM duration
However, the proportion of oocytes at GV stage was higher (P < 0.05) when the oocytes were treated for 5 h than when they were treated for 10 h pre-IVM. Similarly, in large-sized oocytes, the percentage of MI oocytes and the overall meiotic resumption were higher (P < 0.05) in oocytes treated for 10 h than in those treated for 5 h pre-IVM, whereas lower percentages at the GV stage were observed in oocytes treated for 10 h than in those treated for 5 h pre-IVM (P < 0.05). After IVM culture, the overall meiotic resumption was similar between the small and the large-sized oocytes, regardless of the duration of pre-IVM treatment (Table 2). In addition, the percentage of metaphase II (MII) oocytes was similar among oocytes of the same size category that were treated for 0, 5, and 10 h pre-IVM. However, the percentage of MII oocytes among those treated for 0 and 10 h pre-IVM was higher among large-sized than among small-sized oocytes (P < 0.05), while the percentage of MII oocytes among those treated for 5 h pre-IVM tended to be higher among large-sized than among small-sized oocytes (P = 0.06). On the other hand, the rate of MI oocytes was higher among small-sized oocytes than among large-sized oocytes, regardless of the duration of pre-IVM treatment (P < 0.05).

As shown in Fig. 1, the major effects of the size of oocytes and the duration of pre-IVM treatment were evident through cleavage and blastocyst rates (P < 0.05), but not through the cell numbers of blastocysts (P > 0.05). Namely, the cleavage rate (73%) of embryos derived from the small-sized oocytes treated for 5 h pre-IVM was higher (P < 0.05) than that of those treated for 0 and 10 h pre-IVM (61 and 62%, respectively) (Fig. 1). However, the cleavage rates were lower in small-sized oocytes than in large-sized oocytes, regardless of the duration of pre-IVM treatment (P < 0.05). This difference may be caused by the lower maturation rate in small-sized oocytes than in large-sized oocytes. Based on inseminated oocytes, the blastocyst rate (16%) of embryos derived from small-sized oocytes subjected to 5 h pre-IVM treatment was higher (P < 0.05) than that of those subjected to 0 and 10 h pre-IVM treatment (9 and 8%, respectively), but was lower (P < 0.05) than that of large-sized oocytes (31%). In addition, blastocysts derived from small-sized oocytes treated for 5 h pre-IVM had a higher mean cell number (134.1 ± 34.8) than those derived from oocytes treated for 0 and 10 h pre-IVM (100.2 ± 17.2 and 107.8 ± 23.7, respectively). A previous mouse and bovine study using cumulus-oocyte complex (COC) [12] demonstrated that IBMX treatment for 1–2 h pre-IVM increased COC cAMP levels 100-fold and improved embryo cleavage, blastocyst rates, and embryo quality. Additionally, the pre-IVM treatment had a positive influence on the developmental competence of oocytes in pigs by improving cytoplasmic maturation [13]. In the present study, we also added low concentration of FSH during pre-IVM treatment, expecting to increase cAMP levels in oocytes. Sugimura et al. [14] reported that bovine oocytes treated with IBMX for 2 h followed by 22 h IVM with FSH significantly enhanced the ability of oocytes to develop to blastocysts. They suggested that pre-treatment with IBMX enhanced the effectiveness of FSH at improving oocyte developmental competence [14]. In the present study, we cultured bovine oocytes with simultaneous addition of IBMX and FSH for 5 h, and we did not observe a significant increase in blastocyst rate among large-sized oocytes. However, the synergistic effect of IBMX and FSH on developmental competence was observable in small-sized oocytes after 5 h of pre-IVM treatment. These results may suggest that the proper duration of pre-IVM treatment is different for large- and small-sized oocytes. We should thus determine the optimal pre-IVM treatment duration for oocytes of different sizes.
that the pre-IVM treatment of small-sized oocytes improved their developmental competence. The intracellular secondary messenger cAMP plays an important role in the regulation of mitochondrial activity in mammalian cells [16–18]. IBMX prevents the deprivation of cAMP in COCs, which significantly increases cAMP levels and further enhances meiosis progression in oocytes, similar to what occurs during in vivo oocyte maturation [19]. Furthermore, mitochondrial activity of bovine oocytes increases during follicular development, and stronger mitochondrial activity is accompanied by greater developmental competence of immature oocytes [15]. We showed that the mitochondrial activity of in vitro grown oocytes of 105.9–122.7 mm in diameter increased during pre-IVM treatment and was accompanied by the acquisition of developmental competence [20]. In the present study, cAMP concentrations in oocytes may have been increased by the addition of IBMX and a low concentration of FSH to the pre-IVM medium, and mitochondrial activity before the IVM culture may also have increased; it is possible that these phenomena improved the developmental competence of small-sized oocytes treated for 5 h pre-IVM. The extension of the pre-IVM treatment to 10 h may have resulted in the aging of oocytes, subsequently inducing oocyte or cumulus cell degradation and reducing not only mitochondrial activity before IVM culture, but also the developmental competence of oocytes.

In the case of oocytes with a diameter of ≥ 115 µm, 5- and 10-h pre-IVM treatment did not have any detrimental effects (aging) on the cleavage and blastocyst rates; however, the cell number in blastocysts significantly increased after the 5- and 10-h pre-IVM treatment (P < 0.05) compared to untreated oocytes. These results indicate that embryo quality may be improved by pre-IVM treatment due to improved cytoplasmic maturation.

In conclusion, the results of our study show that pre-IVM treatment with FSH and IBMX for 5 h improved the developmental competence of bovine oocytes of a diameter between 110 and < 115 µm as well as of those of a diameter of ≥ 115 µm. These results suggest that the approach to enhance the developmental competence of oocytes using the pre-IVM treatment for 5 h, regardless of oocyte diameter, will ultimately be useful in the management of IVEP in bovines. In the present study, we showed the benefits of pre-IVM treatment for only 5 and 10 h pre-IVM; therefore, the appropriate duration of pre-IVM treatment should be determined in a future study.

**Methods**

**Chemicals**

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

**Collection of cumulus oocyte complexes (COCs)**

Bovine ovaries were obtained from a local abattoir. They were transported to the laboratory within 6 h of collection in plastic bags at 20°C. After three washes in sterile physiological saline, follicles (2–8 mm in diameter) were aspirated using an 18-gauge needle attached to a 10-ml syringe containing TALP working medium, supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA). Aspirated follicular fluid was pooled in 50-ml conical tubes and allowed to settle. COCs were searched for under a

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![Image of bar charts showing cleavage rates, blastocyst rates, and blastocyst cell numbers for small-sized (110 to < 115 µm in diameter) and large-sized oocytes (≥ 115 µm in diameter) after 0, 5, and 10 h of pre-IVM treatment with IBMX. The number in the bar is the number of oocytes, and the number of replicates is in parentheses. * Asterisk indicates a significant difference between experimental groups (P < 0.05). a,b Different letters indicate a significant difference among small-sized oocytes (P < 0.05). x,y Different letters indicate a significant difference among large-sized oocytes (P < 0.05).](image-url)
stereomicroscope and washed three times in TALP working medium; those with more than three layers of cumulus cells and a uniform cytoplasm were selected for further processing. The diameters of oocytes (excluding the zona pellucida) were measured using an ocular scale attached to the stereomicroscope and then divided into COCs having oocytes with diameters between 110 and < 115 µm (small-sized) and those having oocytes with a diameter of ≥ 115 µm (large-sized).

Pre-IVM treatment and IVM of COCs

COCs were submitted to IVM with or without pre-IVM treatment, as described previously [21]. Briefly, COCs were incubated in droplets of pre-IVM medium (approximately 10 COCs/50 µl), which was modified from IVM medium containing 0.5 mM IBMX, and a lower FSH concentration (2 × 10⁻⁶ units/ml, from the porcine pituitary), and covered with paraffin for 0, 5, or 10 h. The IBMX stock solution was diluted to 500 mM in dimethyl sulfoxide, and 10 µl of the stock solution was mixed with 10 ml pre-IVM medium, yielding a final concentration of IBMX of 0.5 mM. The maturation medium consisted of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1 µg/ml estradiol-17β, 10% FBS, and 50 µg/ml gentamicin sulfate, covered with paraffin at 39°C for 22 h in a 5% CO₂ atmosphere. The total culture periods were 22, 27, or 50 µg/ml gentamicin sulfate, covered with paraffin at 39°C for 22 h.

In vitro maturation (IVM) and subsequent culture (IVC)

IVF using frozen semen was performed according to a previously described procedure [22] with slight modifications. Briefly, motile sperm (5 × 10⁶ sperm/ml) separated in a Percoll gradient (45% and 90%) were incubated with COCs in a 100-µl droplet (approximately 10 COCs per droplet) of modified Brackett and Oliphant isotonic medium [23] containing 3 mg/ml fatty acid-free BSA and 2.5 mM theophylline [24] at 39°C for 18 h in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. IVC of inseminated oocytes (presumptive zygotes) was performed as previously described [25]. Briefly, after an incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured at 39°C for 6 days in 30-µl droplets of culture medium in a 5% CO₂, 5% O₂, and 90% N₂ atmosphere. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, seven non-essential amino acids for minimum essential medium, 10 µg/ml insulin, 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml fatty acid-free BSA. Cleavage and blastocyst rates were assessed after 2 days (approximately 30 h) and 6 days (approximately 150 h) of IVC, respectively. The total cell number in Blastocysts obtained after 6 days of IVC was counted using an air-drying method [24].

Evaluation of the oocyte nuclear status after pre-IVM treatment

After pre-IVM and/or IVM, oocytes were denuded from cumulus cells by vortexing and stained with 1% aceto-orcein. The nuclear status was classified as germinal vesicles (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) by observation under a phase-contrast microscope [3].

In vitro fertilization (IVF) and subsequent culture (IVC)

IVF using frozen semen was performed according to a previously described procedure [22] with slight modifications. Briefly, motile sperm (5 × 10⁶ sperm/ml) separated in a Percoll gradient (45% and 90%) were incubated with COCs in a 100-µl droplet (approximately 10 COCs per droplet) of modified Brackett and Oliphant isotonic medium [23] containing 3 mg/ml fatty acid-free BSA and 2.5 mM theophylline [24] at 39°C for 18 h in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. IVC of inseminated oocytes (presumptive zygotes) was performed as previously described [25]. Briefly, after an incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured at 39°C for 6 days in 30-µl droplets of culture medium in a 5% CO₂, 5% O₂, and 90% N₂ atmosphere. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, seven non-essential amino acids for minimum essential medium, 10 µg/ml insulin, 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml fatty acid-free BSA. Cleavage and blastocyst rates were assessed after 2 days (approximately 30 h) and 6 days (approximately 150 h) of IVC, respectively. The total cell number in Blastocysts obtained after 6 days of IVC was counted using an air-drying method [24].

Statistical analysis

All statistical analyses were performed using JMP software version 11.0.0 (SAS Institute, Cary, NC, USA). The oocyte nuclear status was analyzed by the chi-square test. Interactions among the size of collected COCs, duration of the pre-IVM culture, and developmental competence were compared by two-way ANOVA followed by Turkey-Kramer’s HSD as a post-hoc test. Differences of P < 0.05 were regarded as significant.

Conflict of Interests

Authors declare that they have no competing interests.

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