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The effect of temperature during liquid storage of *in vitro*–matured bovine oocytes on subsequent embryo development

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ABSTRACT

The aim of the present study was to optimize the temperature for the temporal storage of matured bovine oocytes. *In vitro*–matured bovine oocytes were preserved in HEPES-buffered TCM199 medium supplemented with 10% newborn calf serum at different temperatures (4 °C, 15 °C, 25 °C, and 38.5 °C) for 20 hours. Embryo development and blastocyst quality after *in vitro* fertilization, cytoplasmic ATP and glutathione levels in oocytes, and the frequency of apoptotic oocytes were compared among storage groups and a control group without storage. Among the storage groups, those at 25 °C and 38.5 °C showed the highest rates of blastocyst development (19.3% and 24.5%, respectively) compared with those stored at 4 °C and 15 °C (8.5% and 14.9%, respectively); however, blastocyst formation rates in all storage groups were lower than that in the control group (39.8%; $P < 0.05$). Storage at 38.5 °C and 15 °C was associated with reduced cell numbers in resultant blastocysts compared with the control and the 25 °C storage groups. Storage at 4 °C reduced metabolic activity of oocytes characterized by their lower ATP levels compared with the other groups. Storage for 20 hours significantly reduced the glutathione content in oocytes in all groups in a similar manner, irrespective of the temperature. Storage at 4 °C or 15 °C but not at 25 °C and 38.5 °C significantly increased the percentage of apoptotic oocytes compared with the control group. In conclusion, 25 °C was found to be the most suitable temperature for the temporal storage of matured bovine oocytes regarding both the developmental competence of oocytes and the quality of resultant blastocysts.

1. Introduction

In vitro production (IVP) of embryos employing IVM of oocytes and subsequent IVF and IVC is a technology that allows the efficient production of bovine embryos available for embryo transfer in large quantities [1,2]. Using either IVM oocytes or *in vivo*–matured ones (obtained by Ovum Pick-Up [OPU] from preovulatory follicles), IVP technology has been proven to be a cost-effective way to produce embryos using sex-sorted sperm [3–5]. The matured mammalian oocyte is arrested at the metaphase stage of the second meiotic division (also known as the metaphase II or MII stage) by a high level of maturation-promoting factor until it is penetrated by a spermatozoon. The sperm entry into the oocyte induces Ca^{2+} oscillations lasting for several hours [6–8] which trigger oocyte activation, pronucleus formation, and hence embryo

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development [9]. However, if matured oocytes are not fertilized for an excessive duration, they undergo a time-dependent aging process which greatly reduces their developmental competence [10,11]. The aging process is characterized by profound, complex, and harmful cytoplasmic changes such as the reduction of maturation-promoting factor activity leading to spontaneous activation causing cortical granule exocytosis and zona hardening, the alterations of mitochondrial activity and ATP levels, altered redox status caused by the accumulation of reactive oxygen species (ROS), the disruption and displacement of microtubules and microfilaments, and the activation of the apoptotic cascade (reviewed by Miao et al. [12]). Accordingly, the developmental competence of bovine oocytes has been reported to decrease significantly from approximately 5 hours after they reached the MII stage [13–16]. Because of the aging of matured oocytes mentioned previously, the optimum timing for their use for IVF is predominantly determined by the timing of their retrieval. This fact appears to be a hitch for the planning of IVP programs when MII oocytes are obtained at awkward time points or spatially far from the site of IVF. Therefore, preservation of matured oocytes without reducing their competence even for an extended period would be greatly advantageous by making the planning of IVF programs more flexible in time and space. Efficient storage of matured oocytes could also reduce the production costs of embryos. For instance, when bovine oocytes are collected from superstimulated cows by OPU from follicles larger than 5 mm, approximately 25% of the oocytes are still immature and can be used by IVM [5]. In our IVM system, nuclear maturation of bovine oocytes is finished after 20 hours of culture (Supplementary Fig. 1). Storage of already matured oocytes during this period would make it possible to use all oocytes for IVF at once, using a single straw of frozen sperm which would reduce production costs of each transferable embryos especially when expensive (such as sex-sorted) sperm is to be used.

To date, oocyte storage is possible either by cryopreservation or by keeping them in a medium (liquid storage). Cryopreservation by vitrification is a relatively simple and rapidly developing technique [17]; however, the current vitrification methods greatly reduce the developmental competence of bovine oocytes [18–20]. Another alternative way to store oocytes for a short term is to keep them in a medium without freezing under conditions that prevent the aging process and thus prolong the developmental competence (usability for IVF) of matured oocytes. Nevertheless, to date, few efforts have been reported for the storage of matured bovine oocytes. Therefore, the optimum environment for the liquid storage of matured bovine oocytes and the cytoplasmic alterations that may compromise competence during the process have remained unknown. In a recent report, matured mouse oocytes were successfully stored in medium without reducing their developmental competence for up to 36 hours under low temperatures which seemed to prevent aging of oocytes [21]. The aim of the present study was to define the optimum storage temperature for liquid preservation of MII stage bovine oocytes for the first time and to reveal cytoplasmic alterations during the process. We stored cumulus-enclosed IVM oocytes for 20 hours in a simple HEPES-buffered tissue culture medium at different temperatures and compared their developmental competence in terms of (1) fertilization and embryo development after IVF, (2) the quality of resultant blastocysts characterized by their hatching ability and cell numbers, (3) overall metabolic activity (ATP levels), (4) cytoplasmic redox status (levels of glutathione; a natural antioxidant), and (5) the onset of apoptosis in oocytes.

2. Materials and methods

2.1. Oocyte collection and IVM

The oocyte collection and IVM were performed as described by Imai et al. [22]. Briefly, bovine ovaries were obtained from slaughterhouse and kept in plastic bag at 25 °C. Then, the ovaries were transported to laboratory. After bovine spongiform encephalopathy (BSE) test according to Abattoir Law of Japan was confirmed to be negative, the ovaries were washed several times in 0.9% (w:v) sodium chloride solution. Cumulus–oocyte complexes (COCs) were aspirated from small follicles (2–6 mm in diameter) with an 18-gauge hypodermic needle connected to a 10-mL syringe. Groups of 20 COCs which had homogeneous cytoplasm and compacted cumulus cells were selected and cultured in 100-μL droplets of HEPES-buffered TCM-199 medium (Medium 199, 12340-030, GIBCO Invitrogen; Life Technologies, Auckland, New Zealand) supplemented with 5% newborn calf serum (NCS, S0750-500; Biowest SAS, Nuaillé, France) and 0.02 Armor U/mL FSH (Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan) under paraffin oil (Paraffin liquid; Nacalai Tesque Inc., Kyoto, Japan) overlay for 20 to 21 hours at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. In vitro preservation of oocytes

The medium for oocyte preservation was a TCM-199 medium (Medium 199 powder, 31100-035, GIBCO Invitrogen) buffered with 11 mmol/L of HEPES (Sigma–Aldrich Co., St. Louis, MO, USA), 9 mmol/L of Na-HEPES (Sigma–Aldrich Co.), and supplemented with 5 mmol/L of sodium bicarbonate and 10% (v:v) NCS with a pH adjusted to 7.3 and the osmolarity to approximately 0.290 osm. After maturation, the cumulus-enclosed oocytes were washed twice in the storage medium, and then they were transferred into 1.5-mL Eppendorf microfuge tubes in 1.3 mL of storage medium. The tubes were then closed, sealed air tight with parafilm, and kept at either 4 °C, 15 °C, 25 °C, or 38.5 °C for 20 hours.

2.3. In vitro fertilization

Frozen semen of a single proven Japanese Black bull was thawed in 37 °C for 40 seconds and centrifuged in 3 mL of 90% Percoll (Sigma–Aldrich Co.) solution at 740 × *g* for 10 minutes. Then, the pellet was resuspended in 5.5 mL of IVF 100 medium (Research Institute for Functional Peptides Co., Ltd., Yamagata, Japan) and centrifuged at 540 × *g* for 5 minutes. The pellet was resuspended with IVF 100