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ORIGINAL ARTICLE

Effect of medium additives during liquid storage on developmental competence of *in vitro* matured bovine oocytes

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ABSTRACT

Our aim was to improve the developmental competence of bovine oocytes during their liquid storage by using additives. *In vitro* matured oocytes were stored for 20 h at 25°C in HEPES buffered TCM 199 medium (base medium). After storage, *in vitro* embryo development after *in vitro* fertilization was compared to those of non-stored (control) ones. Addition of 10% (v/v) newborn calf serum or 10.27 mmol/L pyruvate alone to the base medium did not improve blastocyst formation rates in stored oocytes; however, their simultaneous addition significantly improved the rate compared with those stored in base medium ($P < 0.05$). Supplementation of the holding medium with dithiothreitol (DTT) at any concentrations did not improve embryo development from stored oocytes. Although supplementation with cyclosporine A (CsA) significantly reduced apoptosis and membrane damage rates during storage, it did not improve the developmental competence of oocytes. 1,2-bis(2-aminophenoxy) ethane N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl ester and ruthenium red had no effect on oocyte apoptotic rates. Blastocyst formation rates in all stored groups remained significantly lower than that of the control. In conclusion, pyruvate and serum had a synergic effect to moderate the reduction of oocyte quality during storage, whereas mitochondrial membrane pore inhibitor CsA and the antioxidant DTT did not affect their developmental competence.

Key words: *additives, bovine, in vitro fertilization, oocyte, storage.*

INTRODUCTION

In vitro fertilization (IVF) of matured oocytes at the metaphase of the second meiotic division (metaphase-II: MII) has become an important technology for assisted reproduction in farm animals, allowing the efficient use of gametes cryopreserved in gene banks, gender-preselected semen with low fertilization ability or the genetic and health diagnosis of the resultant embryos. Nevertheless, IVF technology requires a laboratory which limits its availability.

Although MII oocytes remain arrested at this stage until fertilization, excessive delay of fertilization (insemination) causes the reduction of oocyte developmental competence due to a process called post-ovulatory oocyte aging (Fissore *et al.* 2002). Therefore, in each species, there is a time window for optimal fertilization. In cattle, signs of oocyte aging have been detected from approximately 5 h after reaching the MII stage (Agung *et al.* 2006; Koyama *et al.* 2014a). Prolonging the optimum time window for fertilization would greatly facilitate the use of oocytes for IVF such as allowing the transportation of oocytes from remote areas to laboratories and the flexible planning of experiments in time and space.

Post-ovulatory aging has been known to cause complex cellular and molecular changes in oocytes (Miao *et al.* 2009; Takahashi *et al.* 2013) which are detrimental for the developmental competence of oocytes (Wilcox *et al.* 1998) and also for the production of offspring (Tarin *et al.* 1999, 2002). These biochemical changes include: (i) the reduction of metaphase-promoting factor (MPF activity) in cytoplasm causing premature cortical granule exocytosis, zona hardening (Xu *et al.* 1997; Abbott *et al.* 1998) and parthenogenetic activation (Xu *et al.* 1997; Abbott *et al.* 1998; Petrova

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et al. 2005); (ii) apoptosis caused by the leakage of Ca^{2+} from mitochondria to the cytosol (Smaili *et al*. 2000); and (iii) various cellular damages such as DNA fragmentation and microtubule (spindle) deformation caused by an increase of the intracellular levels of reactive oxygen species (ROS) (Takahashi *et al*. 2003; Goud *et al*. 2008; Tang *et al*. 2013). The magnitude of these processes is affected by temperature and the composition of the medium (Wakayama *et al*. 2004; Li *et al*. 2012; Wang *et al*. 2014). Physiological temperature seems to be harmful during oocyte storage because it upregulates mitochondrial metabolism, causing the accumulation of its byproducts, ROS, thus the reduction of intracellular glutathione (GSH) (Li *et al*. 2012; Venditti *et al*. 2013), whereas low temperatures may cause the release of Ca^{2+} from mitochondria, triggering apoptosis (Giorgi *et al*. 2012). Recently, we have determined that the optimum temperature range for the temporary storage of IV maturation (IVM) bovine oocytes is 15-25°C (Suttirojpattana *et al*. 2015). Nevertheless even at this temperature, we observed significantly reduced levels of intracellular GSH and increased frequencies of apoptosis in stored oocytes associated with their reduced developmental competence. Moreover, the expression of the anti-apoptotic protein Bcl-2 was decreased and the rate of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling-positive oocytes was reportedly increased in aged oocytes (Ma *et al*. 2005; Tatone *et al*. 2006). Previous studies have demonstrated/suggested that the biochemical processes beyond oocyte aging can be moderated to some extent by specific modifications of medium composition. Increasing pyruvate concentration and neglecting glucose in medium combined with hypothermic conditions have been reported to prevent aging in mouse oocytes by the downregulation of mitochondrial metabolism (Li *et al*. 2012; Wang *et al*. 2014). Under hypothermic conditions serum also seems to act positively on the viability of bovine embryos (Ideta *et al*. 2013). Antioxidants such as α-tocopherol, cysteamine, cysteine (Wang *et al*. 2014), melatonin (Lord *et al*. 2013) or dithiotreitol (DTT) (Tarin *et al*. 1998; Rausell *et al*. 2007) have been reported to prevent/moderate the detrimental effects of ROS in aged mouse oocytes. Furthermore, in bovine oocytes, Zhao *et al*. (2015) reported that 1-octanol and 1,2-bis(2-aminophenoxy) ethane N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM) which reduce cytosolic free Ca^{2+} levels in oocytes could improve the proportion of blastocyst formation rates in stored oocytes after parthenogenetic activation.

The purpose of this study was to test if supplementation of the holding medium with pyruvate, serum, the disulfide reducing agent DTT, the Ca^{2+} chelating agent BAPTA-AM or mitochondrial membrane pore inhibitors cyclosporine A (CsA) and ruthenium red (RR) can prevent the aging process in bovine oocytes during a 20 h storage and improve their ability to develop to the blastocyst stage after IVF.

MATERIALS AND METHODS

Oocyte collection and IVM

Collection and IVM of bovine oocytes were performed as described by Imai *et al*. (2006). In brief, bovine ovaries were collected at a slaughterhouse and transported to the laboratory and then washed in 0.9% (w/v) sodium chloride solution. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (2-8 mm in diameter) using a 10 mL syringe with an 18 gauge needle. The maturation medium was HEPES buffered TCM 199 medium (Medium 199, 12340-030; GIBCO BRL, Grand Island, NY, USA) supplemented with 5% newborn calf serum (NCS, S0750-500; Biowest SAS, Nuaillé, France) and 0.02 Armor Units/mL follicle stimulating hormone (FSH; Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan). Only COCs with compacted cumulus cells were selected, washed twice with maturation medium and then cultured in 100 μL droplets of maturation medium under paraffin oil (Paraffin liquid, Nacalai Tesque Inc., Kyoto, Japan) (20 COCs per droplet) for 20-21 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In vitro preservation of oocytes

The medium for oocyte preservation consisted of TCM 199 medium (Medium 199 powder, 31100-035; GIBCO Invitrogen) buffered with 11 mmol/L HEPES, 9 mmol/L Na-HEPES, and supplemented with 5 mmol/L sodium bicarbonate with a pH adjusted to 7.3 and the osmolarity was approximately 0.290 Osm. The storage medium was supplemented with chemicals which were described in experimental designs. After oocyte maturation, the COCs were washed twice in the storage medium and then transferred into 1.5 mL Eppendorf microfuge tubes in 1 mL of storage medium. The tubes were then closed, sealed air tight with parafilm and kept at 25°C for 20 h.

IVF

IVF was performed according to a previous report (Suttirojpattana *et al*. 2015). Briefly, frozen semen of Japanese Black bulls was thawed in 37°C water for 40 sec and centrifuged in 3 mL of 90% Percoll (Sigma-Aldrich Co., St. Louis, MO, USA) solution at 740 × *g* for 10 min. Then the pellet was re-suspended in 5.5 mL of IVF 100 medium (Research Institute for Functional Peptides Co., Ltd., Yamagata, Japan) and centrifuged at 540 × *g* for 5 min. The pellet was resuspended with IVF 100 medium and the final concentration was adjusted to 3×10^6 sperm/mL. The mature oocytes were washed twice in IVF 100 medium and co-cultured with sperm for 4 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air (20 oocytes/100μL droplet).

In vitro embryo culture (IVC)

After IVF, the putative zygotes were gently denuded from cumulus cells with a fine glass pipette. Fifteen to 20 zygotes were cultured in each 100 μL droplet of Charles Rosenkrans 1 (CR1) medium (Rosenkrans *et al*. 1993) supplemented with amino acids (CR1aa; Imai *et al*. 2002) and 5% NCS at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for up to 9 days.

Evaluation of total cell number in blastocysts

Blastocysts obtained on day 9 (day 0 = IVF) were fixed in 99.5% ethanol supplemented with 10 μg/mL Hoechst 33342 (Calbiochem, San Diego, CA, USA) overnight at 4°C. The embryos were then washed in ethanol, mounted on glass slides in glycerol droplets and flattened by cover slips. The total numbers of nuclei were counted under UV light with excitation at 330–385 nm and emission at 420 nm under an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan).

Assay of live/dead status and apoptosis in oocytes

Staining was performed with an Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR, USA) according to Anguita *et al*. (2007). This assay is based on the annexin V binding which is applied to determine phosphatidylserine (PS) externalization on the plasma membrane. Annexin V, a member of the phospholipid-binding annexin family, binds most effectively to PS, which is externalized on the outer membrane of cells exposed to apoptotic stimuli. The assay also applies propidium iodide (PI), a membrane impermeable stain, to discriminate between live and dead (membrane damaged) cells. PI has the ability to enter the cell when the cytoplasmic membrane has lost its integrity. Oocytes were washed three times with phosphate-buffered saline and then transferred to 100 μL of binding buffer (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, Molecular Probes) with 5 μL of Alexa Fluor Annexin V, 1 μL of PI (100 μg/mL) and 0.2 μL of 5 mg/mL Hoechst 33342 for 20 min at 38.5°C in the dark. After incubation, the oocytes were washed three times in binding buffer and subsequently mounted on the glass slides. The samples were examined with an epifluorescence microscope (Eclipse E-600; Nikon) at an excitation wavelength of 480 nm and emission at 510 nm. Oocytes were classified in three groups (Anguita *et al*. 2007). Viable oocytes were characterized by PI negative nuclei (appearing in blue color by Hoechst 33342) and no annexin staining on the membrane (A^-/PI^-). Early apoptotic membrane intact (live) oocytes had PI negative nuclei (appearing in blue color by Hoechst 33342 at 350 nm excitation) and a homogeneous annexin positive signal on the membrane (A^+/PI^-). Dead oocytes which showed PI positive red nuclei, indicated membrane damage with or without annexin staining on the membrane (PI^+).

Experimental design

Experiment 1 investigated the effect of serum and/or pyruvate added to the holding medium (base medium) alone or in combination on the developmental competence of oocytes after storage. After IVM, COCs were stored for 20 h in base medium (HEPES-TCM 199) supplemented either with 10% (v/v) NCS or 10.27 mmol/L pyruvate or both or without any supplementation at 25°C. Then, the oocytes were subjected to IVF and IVC as described earlier. The cleavage rates, blastocyst formation rates, hatching/hatched rate of blastocysts and blastocyst cell numbers were checked on day 2, days 7-9, days 8-9 and day 9, respectively. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. Five replicates were performed.

Experiment 2 investigated the effect of the reducing agent DTT on developmental competence in stored oocytes. After IVM, COCs were stored for 20 h in base medium consisting of 10.27 mmol/L pyruvate with or without DTT (Sigma, St. Louis, MO, USA) supplementation at different concentrations (0.05 mmol/L, 0.5 mmol/L, 1.0 mmol/L and 5.0 mmol/L). Then, the oocytes were subjected to IVF and IVC as described earlier. The cleavage rates, blastocyst formation rates, hatching/hatched rate of blastocysts and blastocyst cell numbers were checked on day 2, days 7-9, days 8-9 and day 9, respectively. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. Three replicates were performed.

Experiment 3 investigated the effects of the Ca^{2+} chelating agent BAPTA-AM (Sigma), and mitochondrial membrane pore inhibitors RR (Sigma) and CsA (Sigma) on apoptosis in stored oocytes. After IVM, COCs were stored for 20 h at 25°C in base medium consisting of 10.27 mmol/L pyruvate supplemented with or without either 50 μmol/L BAPTA-AM (Tang *et al*. 2013; Zhao *et al*. 2015), 1 μmol/L RR (Nakagawa *et al*. 2008) or 33 μmol/L CsA (Thouas *et al*. 2004). The concentration for each reagent was selected according to previous studies given as references. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. The rates of apoptotic oocytes were compared among control and storage groups. Three replicates were performed.

Experiment 4 investigated the effect of CsA on developmental competence in stored oocytes. After IVM, cumulus-enclosed oocytes were stored for 20 h in base medium consisting of 10.27 mmol/L pyruvate supplemented with different concentrations of CsA

(1μmol/L and 33 μmol/L) according to previous studies (Nakagawa *et al*. 2008; Zhao *et al*. 2011). Then, the oocytes were IVF and IVC as described earlier. The cleavage rates, blastocyst formation rates, hatching/hatched rates of blastocysts and blastocyst cell numbers were checked on day 2, days 7-9, days 8-9 and day 9, respectively. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. Five replicates were performed.

Statistical analysis

All data are shown as mean ± SEM. Statistical analysis was performed with one-way analysis of variance by using software SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The percentage data were subjected to arcsine transformation. The significant differences between means were compared by a *post hoc* Fisher's protected least significant difference test. Different values were considered statistically significant when *P*-value was less than 0.05.

RESULTS

Effects of serum and pyruvate supplementation during oocyte storage on developmental competence of stored bovine IVM oocytes and the quality of resultant embryos

As shown in Table 1, there was no difference in cleavage rates among the control and stored groups. However, the blastocyst formation rate in the control group was significantly higher than those of storage groups ($P < 0.05$) at all time points of IVC (Table 1). Compared within storage groups, supplementation of the base medium with NCS + pyruvate resulted in a significantly higher blastocyst formation rate ($P < 0.05$) compared with the group stored in base medium at all IVC time points. However, when oocytes were stored in base medium supplemented with either NCS or pyruvate, blastocyst formation rates did not differ significantly from those stored without supplementation (Table 1). The hatching ability of blastocysts after IVF and IVC of oocytes stored in base medium was significantly lower than that of the control group ($P < 0.05$, Table 2) whereas blastocysts of the other storage groups showed intermediate hatching values (Table 2). The total cell numbers of blastocysts on day 9 in stored groups (base medium, base medium + NCS, base medium + pyruvate and base medium + NCS + pyruvate) were significantly lower than those of the control group (120.7, 171.3, 170.4 and 167.2 vs. 262.9; $P < 0.05$).

Effect of DTT supplementation during oocyte storage on the developmental competence of bovine IVM oocytes and the quality of resultant embryos

As shown in Table 3, there was no significant difference in cleavage rates among the control and storage groups with DTT supplementation of 0.05 mmol/L to 1 mmol/L. However, the cleavage rate in the 5 mmol/L DTT treated group was 36.5% which was significantly lower than those in the other groups ($P < 0.05$). The incidences of embryos developing to the blastocyst stage in all stored groups were significantly lower than those of the control group at all time points ($P < 0.05$). Treatment of oocytes with 0.05 mmol/L, 0.5 mmol/L, 1.0 mmol/L and 5.0 mmol/L DTT could not improve the blastocyst formation rate compared with the group stored without DTT; moreover, 5 mmol/L DTT significantly reduced embryo development. The hatching ability of IVF-derived blastocysts was significantly reduced in 1.0 mmol/L and 5.0 mmol/L DTT treated groups compared with control ($P < 0.05$); however, significant differences were not detected among the control and the other treatment groups (Table 4). The total cell numbers of blastocysts on day 9 from stored oocytes were significantly decreased in all treatment groups (0 mmol/L, 0.05 mmol/L, 0.5 mmol/L, 1 mmol/L and 5 mmol/L) compared with the control group (184.9, 194.7, 193.8, 156.9 and 128.7 vs. 287.7; $P < 0.05$).

Table 1 Effect of additives in storage medium on development of stored[†] oocytes after *in vitro* fertilization (IVF)

Storage[†] of IVM oocytes	Total no. of oocytes used for IVF	No. of oocytes cleaved, % (mean ± SEM)	No. of oocytes developed to the blastocyst stage on		
			day 7, % (mean ± SEM)	day 8, % (mean ± SEM)	day 9, % (mean ± SEM)
Control (without storage)	210	155 (73.1 ± 4.6)	87 (40.8 ± 3.9^{a})	94 (44.0 ± 4.1^{a})	94 (44.0 ± 4.1^{a})
Stored in base medium	214	149 (68.9 ± 5.3)	14 (6.1 ± 2.2^{b})	16 (7.2 ± 2.3^{b})	16 (7.2 ± 2.3^{b})
Stored in base medium + NCS	208	159 (76.6 ± 4.7)	21 (10.3 ± 1.7^{bc})	23 (11.4 ± 2.0^{b})	23 (11.4 ± 2.0^{b})
Stored in base medium + Pyru	211	152 (71.4 ± 3.9)	28 (13.2 ± 1.2^{bc})	29 (13.8 ± 1.8^{bc})	30 (14.3 ± 1.7^{bc})
Stored in base medium + NCS + Pyru	219	180 (81.8 ± 4.0)	38 (17.1 ± 2.0^{c})	46 (21.0 ± 3.1^{c})	46 (21.0 ± 3.1^{c})

[†]Oocytes were stored for 20 h. Five replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). Day 0 = the day of IVF. NCS = 10% (v/v) newborn calf serum. Pyru = 10.27 mmol/L pyruvate.

Table 2 Effect of additives in storage medium for bovine oocytes on ability of blastocysts obtained from *in vitro* fertilization (IVF) of stored† oocytes to hatch during *in vitro* embryo culture (IVC)

Storage† of IVM oocytes	No. of blastocysts examined	No. of blastocysts hatching/hatched on day 8, % (mean ± SEM)	No. of blastocysts hatching/hatched on day 9, % (mean ± SEM)
Control (without storage)	94	57 (60.4 ± 5.8)	68 (73.4 ± 3.9^{a})
Stored in base medium	16	7 (28.0 ± 12.7)	8 (31.3 ± 15.4^{b})
Stored in base medium + NCS	23	11 (46.7 ± 11.0)	13 (56.7 ± 13.3^{ab})
Stored in base medium + Pyru	30	12 (37.8 ± 16.5)	16 (54.0 ± 15.2^{ab})
Stored in base medium + NCS + Pyru	46	16 (33.2 ± 7.6)	22 (46.2 ± 9.8^{ab})

†Oocytes were stored for 20 h. Five replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). Day 0 = the day of IVF. NCS = 10% (v/v) newborn calf serum. Pyru = 10.27 mmol/L pyruvate. IVM, IV maturation.

Table 3 Effect of dithiothreitol (DTT) supplementation in storage medium for bovine oocytes on their subsequent development after *in vitro* fertilization (IVF)

Storage† of IVM oocytes	DTT concentration (mmol/L) during storage	No. of oocytes used for IVF	No. of oocytes cleaved, % (mean ± SEM)	No. of oocytes developed to the blastocyst stage on		
				day 7, % (mean ± SEM)	day 8, % (mean ± SEM)	day 9, % (mean ± SEM)
–	NA	149	105 (71.5 ± 5.2^{a})	55 (37.5 ± 5.9^{a})	55 (37.5 ± 5.9^{a})	55 (37.5 ± 5.9^{a})
+	0	146	112 (74.9 ±6.0^{a})	24 (16.2 ± 1.5^{bc})	26 (17.7 ± 2.2^{bc})	27 (18.2 ± 2.4^{c})
+	0.05	142	111 (78.8 ± 1.7^{a})	28 (20.2 ± 1.9^{c})	30 (21.3 ± 0.8^{c})	30 (21.3 ± 0.8^{c})
+	0.5	137	85 (63.8 ± 6.4^{a})	18 (13.3 ± 0.5^{bc})	19 (14.2 ± 1.3^{bc})	19 (14.2 ± 1.3^{bc})
+	1	131	86 (66.0 ± 1.6^{a})	23 (18.0 ± 2.2^{c})	23 (18.0 ± 2.2^{c})	23 (18.0 ± 2.2^{c})
+	5	104	39 (36.5 ± 8.8^{b})	9 (8.8 ± 1.8^{b})	9 (8.8 ± 1.8^{b})	9 (8.8 ± 1.8^{b})

†Oocytes were stored for 20 h. Three replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$).Day 0 = the day of IVF. The holding medium for oocyte storage was base medium supplemented with 10.27 mmol/L pyruvate. IVM, IV maturation.

Table 4 Effect of dithiothreitol (DTT) supplementation in storage medium for bovine oocytes on ability of blastocysts obtained from *in vitro* fertilization (IVF) of stored† oocytes to hatch after *in vitro* embryo culture (IVC)

Storage† of IVM oocytes	DTT concentration (mmol/L) during storage	No. of blastocysts examined	No. of blastocysts hatching/hatched on day 8, % (mean ± SEM)	No. of blastocysts hatching/hatched on day 9, % (mean ± SEM)
–	NA	55	40 (70.5 ± 6.6^{a})	47 (84.7 ± 4.0^{a})
+	0	27	16 (58.8 ± 5.6^{ab})	18 (65.1 ± 4.2^{ab})
+	0.05	30	18 (59.7 ± 2.0^{ab})	19 (62.5 ± 2.8^{ab})
+	0.5	19	11 (57.1 ± 12.0^{ab})	13 (68.2 ± 9.8^{ab})
+	1	23	8 (35.1 ± 11.5^{b})	12 (52.4 ± 7.6^{b})
+	5	9	3 (30.6 ± 19.4^{b})	5 (47.2 ± 23.7^{b})

†Oocytes were stored for 20 h. Three replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). Day 0 = the day of IVF. The holding medium for oocyte storage was base medium supplemented with 10.27 mmol/L pyruvate. IVM, IV maturation.

Effect of cytosolic Ca^{2+} modulating agents on the onset of apoptosis and membrane damage in oocytes during storage

In this experiment, the effects of 50 μmol/L BAPTA-AM, 1 μmol/L RR and 33 μmol/L CsA on apoptosis in stored oocytes were compared to those stored in base medium + pyruvate and control without storage (Table5). The percentage of viable non-apoptotic oocytes (A^-, PI^-) in the CsA treated group was not significantly different from that in the non-stored control group, whereas in all other treatment groups the rates were significantly lower than that in the control group ($P < 0.05$). A significantly increased rate of membrane intact apoptotic oocytes (A^+, PI^-) was detected in the RR treated group ($P < 0.05$), whereas the percentage of (A^+, PI^-) oocytes did not differ significantly among the other groups. A significantly increased proportion of membrane damaged oocytes (PI^+) were observed in the group stored in base

Table 5 Effects of cytosolic Ca^{2+} modulating agents during storage† of bovine oocytes on their live/dead status and apoptosis

Storage† of IVM oocytes	Additive during storage	No. of oocytes examined	No. of oocytes categorized as A^-/PI^-, % (mean ± SEM)	No. of oocytes categorized as A^+/PI^-, % (mean ± SEM)	No. of oocytes categorized as PI^+, % (mean ± SEM)
–	**NA**	140	131 (94.0 ± 3.5^a)	6 (4.0 ± 3.0^a)	3 (2.0 ± 1.2^a)
+	**-**	144	108 (74.6 ± 6.0^{bc})	13 (9.2 ± 3.6^a)	23 (16.1 ± 2.8^c)
+	**BAPTA**	146	118 (80.9 ± 2.0^{bc})	18 (12.3 ± 1.9^a)	10 (6.8 ± 0.3^{ab})
+	**RR**	141	87 (61.7 ± 3.2^b)	37 (26.2 ± 3.6^b)	17 (12.0 ± 0.6^{bc})
+	**CsA**	143	129 (90.0 ± 5.9^{ac})	9 (6.5 ± 3.4^a)	6 (4.3 ± 2.2^a)

†Oocytes were stored for 20 h. Three replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). The holding medium for oocyte storage was base medium supplemented with 10.27 mmol/L pyruvate.IVM, IV maturationBAPTA = 1,2-bis(2-aminophenoxy) ethane N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl esterRR = ruthenium redCsA = cyclosporin AA^-/PI^- = viable non-apoptotic oocyte; A^+/PI^- = early apoptotic oocyte; PI^+ = dead oocyte

medium + pyruvate and the RR treated group compared with those of the other groups, whereas the BAPTA-AM treated group showed intermediate values.

Effect of CsA on developmental competence of bovine oocytes and the quality of resultant embryos

Since in the previous experiment, status of stored oocytes treated with CsA most resembled non-stored control oocytes, we tested the effect of CsA treatment of oocytes at different concentrations during storage on their developmental competence after IVF and IVC. As shown in Table 6, at all time points of culture the blastocyst formation rate of the control group was significantly higher than those of all the stored groups irrespective of the medium additive ($P < 0.05$). Supplementation of the holding medium with 1 μmol/L or 33 μmol/L CsA did not affect cleavage and blastocyst formation rates. No significant difference ($P > 0.05$) was observed between control and preserved oocytes in either hatching ability or total cell number, irrespective of the use of CsA (Tables 7 and 8).

DISCUSSION

The results of Experiment 1 indicated that the supplementation of the base medium with either serum or pyruvate alone did not significantly improve blastocyst formation rates compared with oocytes stored in base medium. Previous studies reported the benefit of serum supplementation during oocyte storage at 4°C in mice, but not at 37°C (Tsuchiya *et al.* 2001; Wakayama *et al.* 2004). Similarly, serum supplementation during the hypothermic storage of bovine embryos was reported to maintain their viability (Ideta *et al.* 2013). Furthermore, supplementing a basic holding medium with serum was reported to maintain the viability of immature porcine oocytes preserved under ambient temperatures (Yang *et al.* 2010). In contrast with these reports, serum supplementation alone did not enhance the developmental competence of bovine oocytes during 20 h storage at 25°C in the present study. Since serum has been reported to exert its preventive effect on bovine embryos concentration-wise, in an additional experiment we have increased the concentration of serum in holding medium during oocyte storage up to 50%; however, there were no significant effects on blastocyst formation rates (data not shown). It is possible that serum exerts its positive effect to support viability of oocytes/embryos only under low temperatures such as 4°C.

In previous studies, an increased concentration (10.27 mmol/L) of pyruvate could maintain the developmental potential of matured mouse oocytes stored under 25°C for 24 h by regulating intracellular redox status and energy supply (Li *et al.* 2012; Wang *et al.* 2014). Furthermore, Liu *et al.* (2009) reported that pyruvate supplementation could delay the aging process by increasing the MPF activity and blocking the premature exocytosis of cortical granules. In addition,

Table 6 Effect of cyclosporin A (CsA) supplementation in storage medium for bovine oocytes on subsequent development after *in vitro* fertilization (IVF)

Storage† of IVM oocytes	CsA concentration (μmol/L) during storage	No. of oocytes used for IVF	No. of oocytes cleaved, % (mean ± SEM)	No. of oocytes developed to the blastocyst stage on		
				day 7, % (mean ± SEM)	day 8, % (mean ± SEM)	day 9, % (mean ± SEM)
–	NA	284	196 68.5 ± 2.6^a	102 (35.6 ± 2.2^a)	108 (37.6 ± 1.9^a)	113 (39.3 ± 1.7^a)
+	0	273	197 72.9 ± 4.0^a	46 (18.1 ± 3.8^b)	50 (19.8 ± 4.2^b)	50 (19.8 ± 4.2^b)
+	1	267	189 71.1 ± 2.0^a	29 (10.9 ± 2.0^b)	32 (11.9 ± 1.6^b)	34 (12.6 ± 1.2^b)
+	33	278	230 83.9 ± 3.1^b	52 (18.7 ± 2.5^b)	55 (19.9 ± 2.1^b)	55 (19.9 ± 2.1^b)

†Oocytes were stored for 20 h. Five Replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). Day 0 = the day of IVF. The holding medium for oocyte storage was base medium supplemented with 10.27 mmol/L pyruvate. IVM, IV maturation.

Table 7 Effect of cyclosporin A (CsA) supplementation during oocyte storage for 20 h on the hatching ability of resultant blastocysts

Treatment	CsA concentration (μmol/L) during storage	Total	Hatching day 8, % (mean ± SEM)	Hatching day 9, % (mean ± SEM)
Control	NA	113	80 (75.6 ± 3.9)	90 (79.8 ± 4.2)
Stored	0	50	30 (61.4 ± 8.6)	31 (62.9 ± 7.6)
Stored	1	34	21 (70.9 ± 9.6)	24 (72.5 ± 9.0)
Stored	33	55	33 (60.2 ± 4.6)	34 (61.3 ± 4.7)

Five replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). Day 0 = the day of IVF. The holding medium for oocyte storage was base medium supplemented with 10.27 mmol/L pyruvate.

pyruvate also increased the levels of antiapoptotic Bcl-2 proteins (Liu *et al*. 2009) and the ratio of reduced and oxidized glutathione (GSH/GSSH) in aged mouse oocytes (Kim & Schuetz 1991; Liu *et al*. 2009). According to Li *et al*. (2012), addition of 10.27 mmol/L pyruvate could improve developmental competence in stored mouse oocytes by maintaining GSH levels which have an important role in coping with oxidative stress (Deneke & Fanburg 1989). These reports in mice contradict our results in cattle oocytes. Recently, we demonstrated significant decrease of GSH contents in matured bovine oocytes after storage for 20 h, irrespective of the storage temperature compared with oocytes without storage (Suttirojpattana *et al*. 2015). However, in the present study the blastocyst formation rates and embryo quality of stored bovine oocytes were not significantly improved by supplementing the base medium with 10.27 mmol/L pyruvate. This may suggest either a difference in the biology of the aging process in oocytes between cattle and mice or that the optimum (effective) concentrations of pyruvate may differ for mouse or bovine oocytes. Interestingly, the combination of 10% (v/v) serum and 10.27 mmol/L pyruvate supplementation significantly improved the development of stored bovine oocytes to the blastocyst stage, suggesting a synergic effect of these additives on developmental competence of the oocytes. Furthermore, the hatching ability which is an important criteria of embryo quality (Balaban *et al*. 2000; Yuan *et al*. 2003) was significantly reduced when oocytes were stored in base medium compared with the control without storage. However, the supplementation of the base medium with either serum or pyruvate during oocyte storage prevented the significant reduction of hatching ability of resultant blastocysts. Based on these results, in further experiments of the study we used a HEPES-buffered TCM 199 medium supplemented with 10.27 mmol/L pyruvate as a defined storage medium to avoid possible interactions of the tested reagents with undefined factors of serum.

Table 8 Effect of cyclosporin A (CsA) supplementation during oocyte storage for 20 h on total cell number of resultant blastocysts derived from *in vitro* fertilization (IVF)

Treatment	CsA concentration (μmol/L) during storage	Total	Total cell number, mean ± SEM
Control	NA	110	254.7 ± 20.4
Stored	0	45	228.3 ± 27.6
Stored	1	33	214.8 ± 21.9
Stored	33	55	223.7 ± 12.5

Five replicates were performed. Values in the same column with no common superscripts differ significantly ($P < 0.05$). The holding medium for oocyte storage was base medium supplemented with 10.27 mmol/L pyruvate.

In animal cells, the mitochondria are the primary intracellular site of oxygen consumption and the major source of ROS (Mari *et al*. 2009). Previously, Tarin (1996) reported that a mechanism based on ROS related to mitochondrial dysfunction during aging. Acceleration of ROS damages mitochondria, resulting in decreased redox potential of GSH/GSSH, which leads to the loss of potential to prevent the detrimental effects of excessive ROS (Mari *et al*. 2009). In accordance, a number of studies have demonstrated increased levels of ROS associated with reduced levels of GSH in aged oocytes (Hao *et al*. 2009; Wang *et al*. 2014). Also, in recent experiments we have observed significantly reduced GSH levels in IVM bovine oocytes after 20 h storage in base medium supplemented with 10% serum, irrespective of the temperature (Suttirojpattana *et al*. 2015). DTT, a disulfide reducing agent, is known to be effective in diminishing thiol oxidative damage in mouse zygotes and blastocysts (Tarin *et al*. 1998; Liu *et al*. 1999). DTT has been reported to delay the aging process in mouse (Tarin *et al*. 1998; Liu *et al*. 1999; Rausell *et al*. 2007) and ovine oocytes (Ye *et al*. 2010). To date, there have been no reports on the effect of DTT on developmental competence of aging bovine oocytes. Therefore, we have tested if this compound was an effective additive to maintain blastocyst developmental competence of bovine IVM oocytes after storage. Our results indicated that DTT added at various concentrations did not improve the developmental competence of bovine oocytes, which was inconsistent with the results of previous reports in other species (Tarin *et al*. 1998; Liu *et al*. 1999; Rausell *et al*. 2007; Ye *et al*. 2010). Moreover, at a high concentration (5 mmol/L) DTT exerted a negative effect on blastocyst development for stored oocytes.

In animal cells cytoplasmic Ca^{2+} plays important roles in signal transduction. When mammalian oocytes are fertilized, the penetrating sperm triggers repetitive Ca^{2+} oscillations in the oocyte cytoplasm which have a crucial

role for inducing oocyte activation and thus embryonic development (Bos-Mikich *et al.* 1997; Swann & Lai 1997; Miyazaki *et al.* 1998; Gordo *et al.* 2000). In normal cases, each of the fertilization-related Ca^{2+} oscillations are characterized by a step rise to the peak followed by a quick return to the base line since extended increase in cytosolic Ca^{2+} triggers apoptotic events in animal cells (Gordo *et al.* 2002). However, abnormal Ca^{2+} oscillations were found in aged oocytes (Jones & Whittingham 1996; Igarashi *et al.* 1997; Gordo *et al.* 2000; Hao *et al.* 2009; Takahashi *et al.* 2009; Tang *et al.* 2013) which was associated with abnormal activation and apoptosis (Gordo *et al.* 2002; Tatone *et al.* 2006; Koyama *et al.* 2014b), resulting in poor embryo development (Takahashi *et al.* 2009). In oocytes, the level of cytosolic Ca^{2+} is regulated by the smooth endoplasmic reticulum (SEM) and mitochondria (Bootman *et al.* 2001). It has been speculated that during aging, Ca^{2+} might leak from the intracellular stores (i.e. the SEM and mitochondria) into the cytosol which may trigger apoptosis (Takahashi *et al.* 2013). In accordance, in a recent report, Zhao *et al.* (2015) treated bovine oocytes with 1-octanol to diminish the extracellular Ca^{2+} and BAPTA-AM to control the intracellular Ca^{2+} and both of these substances could enhance the embryo development in aged oocytes. Regarding the above-mentioned, we attempted to test the effects of different substances which affect cytosolic Ca^{2+} levels and regulation on the onset of apoptosis during oocyte storage. The membrane permeable Ca^{2+} chelator BAPTA-AM was used to block cytosolic free Ca^{2+}. Furthermore, inhibitors of mitochondrial permeability transition (mPT) were tested. CsA is a chemical inhibitor of mPT pore formation (Montero *et al.* 2004), thus it restrains the release of pro-apoptotic factors (e.g. cytochrome-c) and Ca^{2+} which are potent apoptotic stimulation factors (Zamzami *et al.* 1996; Green & Reed 1998). RR is a non-competitive inhibitor of mitochondrial Ca^{2+} uniporter which diminishes Ca^{2+} influx (Kruman & Mattson 1999; Belous *et al.* 2003) and specifically prevents the release of cytochrome c and activation of caspase 3 (Bae *et al.* 2003). Our results have revealed significantly lower rates of viable non-apoptotic (A^-, PI^-) oocytes in stored groups compared with the non-stored control group, except for the CsA treated and stored group which showed similar rates of A^-, PI^- oocytes to the control. Moreover, the CsA treated group presents the lowest percentages of live apoptotic oocytes (A^+, PI^-) and dead (membrane damaged, PI^+) oocytes. Although a significant increase in the percentage of live apoptotic oocytes was only detected when oocytes were stored in the presence of RR, the percentage of oocytes with damaged membrane were significantly increased when oocytes were stored without additive or with RR. It is possible that oocytes undergoing apoptosis during the early stages of storage might lose the ability to maintain membrane integrity by the end of the storage period.

Based on the results of Experiment 4, we tested the effects of CsA addition during oocyte storage on subsequent embryo development after IVF. We hypothesized that CsA would enhance the embryo developmental competence of stored bovine oocytes; however, irrespective of the concentration used it had no effect on the blastocyst formation rate and blastocyst quality. It is possible that under the present oocyte storage conditions apoptotic events in oocytes might not be the primarily cause of reduced embryo development. Further research will be needed to clarify and address other mechanisms involved in the aging process to improve the developmental ability of stored oocytes.

In conclusion, our results demonstrate that supplementation of the oocyte storage medium with serum and pyruvate synergistically improved the developmental competence of stored bovine oocytes, whereas treatment with only serum or pyruvate did not enhance the blastocyst rate. Supplementation of the storage medium with the antioxidant agent DTT did not improve the blastocyst formation rate. Among the cytosolic Ca^{2+} modulating agents, only CsA could maintain the percentage of non-apoptotic live oocytes in storage groups at a level similar to that of non-stored oocytes. However, when applied during the storage of matured oocytes, CsA had no effect on subsequent embryo development.

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