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Detrimental Effect of Bovine Serum Albumin in a Maturation Medium on Embryonic Development after Somatic Cell Nuclear Transfer in Pigs

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ABSTRACT

This study was designed to evaluate the effect of bovine serum albumin (BSA) in a maturation medium on oocyte maturation and embryonic development in pigs. Immature pig oocytes were matured for 44 h in a medium supplemented with 0.4% (w/v) BSA, 0.1% (w/v) polyvinyl alcohol (PVA), or 10% (v/v) pig follicular fluid (PFF). After IVM, oocytes reached metaphase II stage were activated for parthenogenesis (PA) or used as cytoplasts for somatic cell nuclear transfer (SCNT). Nuclear maturation (89.5%, 90.7% and 91.3% for BSA, PVA and PFF, respectively) and intraoocyte glutathione contents (1.20, 1.16 and 1.00 pixels/oocyte for BSA, PVA and PFF, respectively) were not altered by the macromolecules added to maturation medium. IVM of oocytes in a medium containing BSA (21.4%) and PVA (20.7%) showed significantly lower blastocyst formation after PA than culture in medium with PFF (39.2%). After SCNT, oocytes matured in medium with BSA showed decreased embryonic development to the blastocyst stage (9.2%) compared to those matured in medium with PFF (28.9%), while 23.6% of SCNT oocytes matured in medium with PVA developed to the blastocyst stage. When the effect of BSA in a maturation medium during the first 22 h and the second 22 h of IVM in combination with PFF or PVA was examined, PVA-BSA showed a higher nuclear maturation (94.1%) than BSA-PFF (84.5%). However, there was no significant difference in the blastocyst formation among tested combinations (47.3, 52.2, 50.0, 44.4 and 49.0% for PFF-PFF, PFF-BSA, PVA-BSA, BSA-PVA and BSA-PFF, respectively). Our results demonstrate that BSA and PVA added to maturation medium can support oocyte maturation comparable to PFF-supplemented medium. However, maturation of oocytes in a BSA-containing medium decreases embryonic development after PA and SCNT when compared with the medium supplemented with PFF.

(Key words : bovine serum albumin, oocyte maturation, embryonic development, somatic cell nuclear transfer, pig)

INTRODUCTION

In vitro maturation (IVM) is a technique allowing immature oocytes to mature *in vitro*. Generally, IVM medium for pig oocytes is supplemented with gonadotrophic hormones, carbohydrates, growth factors, macromolecules, and other substrates. Especially, protein sources such as serum, serum albumin, or follicular fluid are commonly used as macromolecules. The protein supplement reduces embryotoxicity of other medium supplements or metabolic products generated from embryos and provides sources for nutritive requirements for early embryonic development. In addition, proteins play as a source of growth factors promoting embryonic development and increase cumulus-cell proliferation (Eckert and Niemann, 1994). Porcine follicular fluid (PFF) is commonly included as a source of macromolecules in an IVM medium for porcine oocytes. PFF contains steroid hormones such as estrogen and progesterone, growth factors, amino acids and other undefined nutrients. Physiologically, oocytes are maturing in the PFF-filled follicles which provide oocytes with desirable environment for nuclear and cytoplasmic maturation. Even *in vitro*, PFF that is supplemented to IVM medium enhances oocyte maturation and subsequent embryonic development after *in vitro* fertilization (IVF), parthenogenetic activation (PA), and somatic cell nu-

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clear transfer (SCNT) (Seli *et al.*, 1998; Liu *et al.*, 2002; Hong and Lee, 2007). On the other hand, PFF contains many undefined substances and its composition is varied depending on the follicular size, estrous stages, and seasons. The variation in the PFF composition may be attributed to the inconsistent results depending on the various batches of PFF (Hong and Lee, 2007; You *et al.*, 2009). Thus, it is desirable to use a simple defined medium to evaluate effect of a specific substance on oocyte maturation and embryonic development (Abeydeera *et al.*, 1998). To prepare a simple medium for oocyte maturation, PFF or serum can be replaced with polyvinyl alcohol (PVA) or serum albumin (Abeydeera *et al.*, 1998; Wang and Day, 2002). PVA is a non-biological alternative to protein supplements and has been commonly used as a macromolecule in a chemically medium to aid handling of oocytes and embryos by preventing oocytes or embryos from sticking to culture dishes in the protein-free medium (Duque *et al.*, 2003). Although it is not a completely defined substance, bovine serum albumin (BSA) has been widely used in the culture medium for bovine and porcine embryos because albumin is a physiological component in the animal serum and shows beneficial effect on embryonic development by protecting embryos from toxic substances present in the medium and also by providing nutrients (Gray *et al.*, 1992; Sung *et al.*, 2004; Wydooghe *et al.*, 2014). To date, there have been many studies examined the effect of PFF, serum and PVA on the porcine oocyte maturation *in vitro* (Abeydeera *et al.*, 1998; Wang and Day, 2002). However, a few reports are available on the effects of BSA in a maturation medium on the oocyte maturation and embryonic development after PA and SCNT in pigs. The objective of this study was to examine the effect of BSA in IVM medium in comparison with PFF and PVA on nuclear maturation, intraoocyte glutathione (GSH) contents in IVM oocytes, and preimplantation development of PA and SCNT embryos in pigs.

MATERIALS AND METHODS

1. Culture Media and Reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. The base medium for IVM was Medium-199 (M-199) (Invitrogen, Grand Island, NY, USA) that was supplemented with 0.91 mM pyruvate, 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 1 μg/ml insulin and 75 μg/ml kanamycin. This medium was further supplemented with 10% (v/v) PFF, 0.1% (w/v) polyvinyl alcohol (PVA) or 0.4% (w/v) BSA (A-6003) according to the experimental design. The medium for *in vitro* culture (IVC) of PA and SCNT embryos was porcine zygote medium (PZM)-3 containing 0.3% (w/v) BSA. The IVC medium was modified in this study by adding 0.34 mM tri-sodium citrate, 2.77 mM myo-inositol, and 10 μM β-mercaptoethanol (You *et al.*, 2012).

2. Oocyte Collection and IVM

Pig ovaries from prepubertal gilts were obtained at a local abattoir. Follicular contents containing cumulus-oocyte complexes (COCs) were aspirated from superficial follicles of 3～8 mm in diameter. The COCs that had more than 3 unexpanded cumulus cell layers were selected and washed in HEPES-buffered Tyrode's medium containing 0.05% (w/v) PVA (TLH-PVA). The COCs were placed into each well of a four-well culture dish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium with 80 μg/ml follicle stimulating hormone (Antrin R-10, Kyoritsu Seiyaku, Tokyo, Japan) and 10 IU/ml human chorionic gonadotrophin (Intervet International BV, Boxmeer, Holland). The COCs were cultured at 39℃ under humidified atmosphere of 5% CO_2 and 95% air. After 22 h in the maturation culture, the COCs were washed properly and then cultured in hormone-free IVM medium for an additional 22 h and 20 h for PA and SCNT, respectively.

3. Experimental Design

In experiment 1, effects of PFF, PVA and BSA in IVM medium on nuclear maturation and intraoocyte GSH contents were examined. Effects of macromolecules in IVM medium on embryonic development after PA and SCNT were examined in experiments 2 and 3, respectively. Finally, in experiment 4, effects of PFF, PVA and BSA that were supplemented in combinations to maturation medium during the first (0～22 h) and the second (22～44 h) halves of IVM culture on oocyte maturation and embryonic development after PA were examined with 5 experimental groups. Each treatment group was designated as PFF-PFF, PFF-BSA, PVA-BSA, BSA-PVA and BSA-PFF, respectively.

4. SCNT and PA

Porcine fetal fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) with the nutrient mixture F-12

(Invitrogen, Grand Island, NY, USA), which was supplemented with 15% (v/v) fetal bovine serum, until a complete monolayer of cells formed. Cells were synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 72～96 h. A suspension of single cells was prepared by trypsinizing the cultured cells and resuspending the cells in TLH containing 0.4% (w/v) BSA (TLH-BSA) prior to nuclear transfer.

SCNT was performed as described previously (You *et al.*, 2012; Lee *et al.*, 2014). Briefly, IVM oocytes were incubated for 15 min in manipulation medium containing Hoechst 33342, washed twice with fresh medium, and transferred in a droplet of medium containing 5 μg/ml cytochalasin B covered with warm mineral oil. Oocytes were enucleated by aspirating the polar body (PB) and metaphase II (MII) chromosomes using a 17-μm beveled glass pipette (Humagen, Charlottesville, VA, USA). A single cell was inserted into the perivitelline space of each oocyte. Oocyte-cell couplets were placed on a 1-mm fusion chamber covered with 1 ml of 280 mM mannitol solution containing 0.001 mM $CaCl_2$ and 0.05 mM $MgCl_2$, as previously described (Song *et al.*, 2009). Membrane fusion was induced by applying an alternating current field of 2 V cycling at 1 MHz for 2 sec, followed by two pulses of 175 V/mm direct current (DC) for 30 μsec using a cell fusion generator (LF101; NepaGene, Chiba, Japan). The oocytes were then incubated for 30 min in TLH-BSA and were examined for fusion under a stereomicroscope. Immediately after incubation, the reconstructed oocytes were activated with two pulses of 120 V/mm DC for 60 μsec in a 280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. For PA, oocytes at the MII stage were activated using a pulse sequence identical to that used to activate SCNT oocytes.

5. Post-activation Treatment and Embryo Culture

After electrical activation, the PA and SCNT embryos were treated, respectively, with 7.5 μg/ml CB and 0.4 μg/ml demecolcine combined with 1.9 mM 6-dimethylaminopurine in IVC medium for 4 h. The SCNT and PA embryos were washed in fresh IVC medium, transferred into 30-μl IVC droplets under mineral oil, and then cultured at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 days. Embryo cleavage and blastocyst formation were observed on Days 2 and 7, respectively, with the day of SCNT or PA designated as Day 0. The mean cell number in blastocysts was counted after Hoechst 33342 staining under an epifluorescence microscope.

6. Measurement of Intraoocyte GSH Contents

GSH contents in IVM oocytes were measured as described previously (Sakatani *et al.*, 2007; You *et al.*, 2010). Briefly, CellTracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen) was used to detect intraoocyte GSH as blue fluorescence. After IVM, 6～10 denuded oocytes at the MII stage were incubated for 30 min in the dark in TLH-PVA supplemented with 10 mM CellTracker. After incubation, oocytes were washed with D-PBS (Invitrogen) containing 0.1% (w/v) PVA and observed for fluorescence using an epifluorescence microscope (TE-300; Nikon) with a UV filter (370 nm). Fluorescent images were recorded and saved in TIFF format. The fluorescence intensities of oocytes were analyzed by ImageJ software (version 1.45r; National Institutes of Health, Bethesda, MD, USA) (Fig. 1).

7. Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (version 9.3; SAS Institute, Cary, NC, USA). The data were analyzed by the general linear model procedure followed by the least square method when the treatments di-

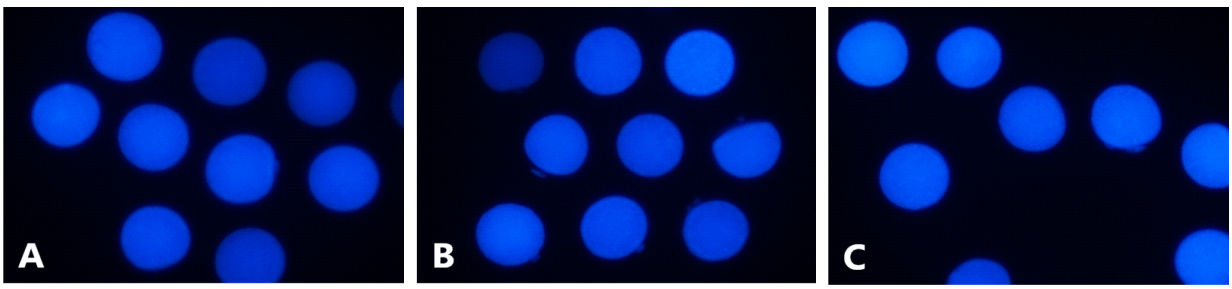

Fig. 1. Epifluorescent photomicrographic image of *in vitro*-matured porcine oocyte. Oocytes were matured in medium supplemented with 10% (v/v) pig follicular fluid (A), 0.1% (w/v) polyvinyl alcohol (B) or 0.4% (w/v) bovine serum albumin (C). After maturation culture, oocytes were stained with CellTracker Blue to detect intracellular glutathione contents of oocytes.

ffered at $P<0.05$. The percentage data were subjected to arcsine transformation before analysis to maintain homogeneity of variance. The results are expressed as the mean ± standard error of the mean (SEM).

RESULTS

1. Effect of Macromolecules in IVM Medium on Nuclear Maturation and Intraoocyte GSH Contents

Morphology of COCs after IVM was influenced by the source of macromolecules in IVM medium. The cumulus cell layer of oocytes expanded more when COCs were matured in medium containing PFF compared to maturation culture in medium supplemented with BSA and PVA (Fig. 2). However, nuclear maturation and intraoocyte GSH contents were not altered by the source of macromolecules added to IVM medium (Table 1).

2. Effect of Macromolecules in IVM Medium on Embryonic Development after PA

PFF (94.4%) in IVM medium significantly increased ($P<0.05$) the proportion of cleaved embryos after PA compared to PVA (75.2%) but there was no significantly differences in embryo cleavage between PFF and BSA treatment (85.4%) during IVM. The blastocyst formation in oocytes matured in medium containing PFF (39.2%) was significantly higher ($P<0.05$) than that in oocytes matured in medium supplemented with PVA (20.7%) and BSA (21.4%). However, the mean cell numbers in blastocysts (34.9～37.1 cells/blastocyst) were not altered by the macromolecules supplemented to IVM medium (Table 2).

3. Effect of Macromolecules in IVM Medium on Embryonic Development after SCNT

The results on the effect of macromolecules in IVM medium on oocyte-cell fusion and embryonic development after SCNT are shown in Table 3. Oocyte-cell fusion (74.5%, 57.4% and 61.3% in oocytes matured with PFF, PVA and BSA, respectively) was not influenced by the supplemented macromolecules. PFF supplementation showed significantly higher ($P<0.05$) cleavage (93.0%) than PVA (84.0%) and blastocyst formation (28.9%) than BSA supplementation (9.2%). The number of cells in SCNT blastocyst (32.1～36.1 cells/blastocyst) was not altered by the macromolecules examined (Table 3).

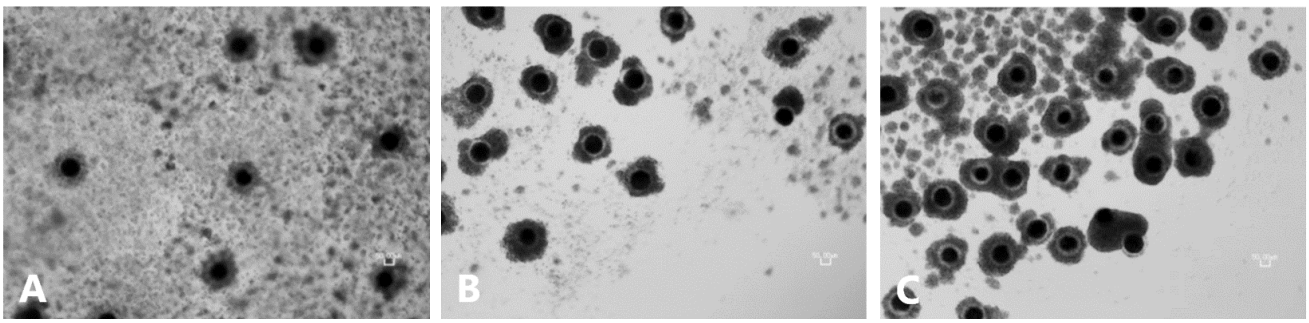

Fig. 2. Morphology of porcine cumulus oocyte complexes (COCs) after *in vitro* maturation. COCs were cultured for 44 h in a maturation medium that were supplemented with 10% (v/v) pig follicular fluid (A), 0.1% (w/v) polyvinyl alcohol (B) or 0.4% (w/v) bovine serum albumin (C).

Table 1. Intracellular glutathione (GSH) contents of oocytes matured in a medium supplemented with PFF, PVA or BSA

Macromolecule in medium*	No. of oocytes cultured for IVM	% of oocytes reached metaphase II (MII)	No. of MII oocytes examined for GSH	Relative level (pixels/oocyte) of GSH
PFF	213	90.7 ± 2.1	31	1.00 ± 0.08
PVA	222	91.3 ± 2.1	29	1.16 ± 0.08
BSA	209	89.5 ± 3.6	31	1.20 ± 0.08

Five replicates.
* PFF: 10% (v/v) porcine follicular fluid, PVA: 0.1% (w/v) polyvinyl alcohol, BSA: 0.4% (w/v) bovine serum albumin.

Table 2. Effect of PFF, PVA or BSA in a maturation medium on embryonic development of pig oocytes after parthenogenetic activation

Macromolecule in medium*	No. of embryos cultured	% of embryos developed to		No. cells in blastocyst
		≥ 2-cell	Blastocyst	
PFF	176	94.4 ± 1.5[a]	39.2 ± 5.4[a]	37.1 ± 1.9
PVA	163	75.2 ± 7.5[b]	20.7 ± 5.1[b]	34.9 ± 2.0
BSA	147	85.4 ± 4.0[ab]	21.4 ± 3.8[b]	35.7 ± 2.8

Five replicates.
* PFF: 10% (v/v) porcine follicular fluid, PVA: 0.1% (w/v) polyvinyl alcohol, BSA: 0.4% (w/v) bovine serum albumin.
[a,b] Values in the same column with different superscript letters are different (P<0.05).

Table 3. Effect of PFF, PVA or BSA in a maturation medium on embryonic development of pig oocytes after somatic cell nuclear transfer (SCNT)

Macromolecule in medium	No. of cell-oocyte couplets produced	% of oocytes fused	No. of SCNT embryos cultured	% of embryos developed to		No. cells in blastocyst
				≥ 2-cell	Blastocyst	
PFF	170	74.5 ± 4.5	124	93.0 ± 1.5[a]	28.9 ± 2.9[a]	36.1 ± 2.7
PVA	145	57.4 ± 6.3	96	84.0 ± 6.7[b]	23.6 ± 6.7[ab]	32.1 ± 2.0
BSA	155	61.3 ± 7.3	86	85.7 ± 3.2[ab]	9.2 ± 4.4[b]	35.8 ± 5.9

Four replicates.
* PFF: 10% (v/v) porcine follicular fluid, PVA: 0.1% (w/v) polyvinyl alcohol, BSA: 0.4% (w/v) bovine serum albumin.
[a,b] Values in the same column with different superscript letters are different (P<0.05).

4. Effect of Combined Addition of PFF, BSA and PVA to IVM Medium on Oocyte Maturation and Embryonic Development after PA

When the effect of BSA supplementation to IVM medium during the first 22 h and the second 22 h of IVM in combination with PFF and PVA was examined, PVA-BSA showed a higher nuclear maturation (94.1%) than BSA-PFF (84.5%). However, there were no significant differences in the blastocyst formation (47.3, 52.2, 50.0, 44.4 and 49.0% for PFF-PFF, PFF-BSA, PVA-BSA, BSA-PVA and BSA-PFF, respectively) and the number of cells in blastocyst (36.1～41.5 cells/blastocyst) among macromolecule combinations (Table 4).

DISCUSSION

Table 4. Effect of combined supplementations of a maturation medium with BSA and PVA or PFF on embryonic development of pig oocytes after parthenogenetic activation

Macromolecule during IVM		No. of oocytes matured	% of oocytes reached MII	No. of oocytes cultured	% of embryos developed to		No. cells in blastocyst
0～22 h	22～44 h				≥ 2-cell	Blastocyst	
PFF	PFF	205	88.5 ± 3.2[ab]	163	93.9 ± 2.2	47.3 ± 5.4	41.5 ± 1.6
PFF	BSA	204	91.8 ± 1.9[ab]	173	94.3 ± 2.0	52.2 ± 4.5	39.1 ± 1.4
PVA	BSA	206	94.0 ± 1.7[a]	161	90.1 ± 1.5	50.0 ± 3.9	36.1 ± 1.4
BSA	PVA	206	91.1 ± 3.0[ab]	164	92.0 ± 2.1	44.4 ± 3.6	38.2 ± 1.5
BSA	PFF	207	84.5 ± 2.0[b]	139	91.3 ± 1.4	49.0 ± 6.6	40.0 ± 1.7

Four replicates.
* PFF: 10% (v/v) porcine follicular fluid, PVA: 0.1% (w/v) polyvinyl alcohol, BSA: 0.4% (w/v) bovine serum albumin.
[a,b] Values in the same column with different superscript letters are different (P<0.05).

BSA has been frequently used as a protein supplement in IVC medium for the development of *in vitro*-produced bovine and porcine embryos (Suzuki and Yoshioka, 2006; George *et al.*, 2008; Wydooghe *et al.*, 2014). However, there have been limited reports available on the effect of BSA in IVM medium on oocyte maturation and later developmental competence in pigs. In the present study, we compared the effect of PFF, BSA and PVA in a maturation medium on oocyte maturation and embryonic development after PA and SCNT. Our results demonstrated that nuclear maturation of oocytes and intraoocyte GSH contents were not influenced by the macromolecules supplemented to IVM medium but embryonic development of IVM oocytes after PA and SCNT was altered by the macromolecules added to the IVM medium. Even though BSA could support nuclear maturation and embryonic development to the cleavage stage comparable to PFF but showed decreased blastocyst formation of PA and SCNT embryos compared to PFF.

Cumulus cells play important roles in protecting oocytes from harmful environments and transferring nutrients and signals to oocytes, and also involve in fertilization process (Zhang *et al.*, 1995; Yamauchi and Nagai, 1999; Yong and Lee, 2007). The cumulus expansion of COCs is one of the important morphological criteria of IVM oocytes and has been used as an indicator of oocyte maturation (Eppig, 1982; Marei *et al.*, 2012). When the morphology of COCs was examined after IVM in the present study, cumulus expansion was more prominent in COCs matured in medium containing PFF than in oocytes cultured in medium with BSA and PVA. Hyaluronic acid is one of the main substrates contributing to the expansion of cumulus cell layer. This substrate is produced from cumulus cells and stabilizes cumulus cells by combining intracellular matrix among cells (Chen *et al.*, 1990; Camaioni *et al.*, 1993). Thus, it is likely that hyaluronic acid present in the PFF might have contributed to the increased expansion of cumulus cells in the present study. On the other hand, the cumulus cells have trans-zonal cytoplasmic processes (TZP) which penetrate through the zona pellucida and reach the oolemma. The signal and substrates are transferred to oocytes through the gap junctions at the ends of these TZP and this process is essential for successful maturation of oocytes and subsequent development of embryos (Eppig, 1991; Sutton *et al.*, 2003). Interestingly, expansion of cumulus cell layer was closely related with embryonic development; oocytes matured in PFF-supplemented medium showed increased blastocyst formation compared to oocytes with less expanded cumulus cells that were matured in the presence of BSA and PVA. This result indicates again that the degree of cumulus cell layer expansion can be used to predict the ability of oocytes to support later embryonic developmental competence.

GSH is a low molecular thiol compound and protects cells or oocytes by alleviating harmful action of reactive oxygen species. Intraoocyte GSH content is frequently used as a marker of cytoplasmic maturation of bovine and porcine oocytes. It has been reported that the intraoocyte GSH content affects oocyte maturation and embryonic development (Abeydeera *et al.*, 1998; De Matos *et al.*, 2000). In the present study, despite of greater cumulus cell expansion and higher blastocyst formation after PA in oocytes that were matured in PFF-supplemented medium, GSH contents of those oocytes were not different from oocytes matured in BSA- and PVA-supplemented medium. It is considered that undefined substrates only present in PFF, not in BSA or PVA either, might improve cytoplasmic maturation without influencing GSH contents and then stimulated PA embryonic development. Embryonic development to the blastocyst stage after PA and SCNT was decreased in oocytes matured in medium containing BSA compared to oocytes matured in PFF-supplemented medium. The detrimental effect of BSA was not attributed to suboptimal concentration of BSA used in this study because PA embryonic development was not altered by various BSA concentrations in IVM medium (0.08, 0.4 and 2% (w/v) BSA) in our preliminary study (data not shown). BSA consists of albumin, unknown low molecular substrates, growth factors, and estradiol (Eckert and Niemannt, 1994; Mingoti *et al.*, 2002). It has been shown that estradiol is present at high levels in BSA which can induce sufficient nuclear and cytoplasmic maturation (Mingoti *et al.*, 2002). There were controversial results on the effect of BSA. BSA in a culture medium stimulated embryonic development in cattle and sheep (Wan *et al.*, 2009; Wydooghe *et al.*, 2014) while toxic effect of BSA on rabbit embryonic development was also reported (Kane, 1985). Although it was unclear in this study, presence of undesirable substances such as estradiol or lack of maturation-promoting substances in BSA might influence cytoplasmic maturation and decrease subsequent embryonic development after PA and SCNT.

Non-protein substrate such as PVA is commonly used for the preparation of a defined IVM medium instead of serum or follicular fluid. However, oocytes matured in medium supplemented with PVA show lower competence of blastocyst for-

mation compared to oocytes matured in the presence of proteins (Abeydeera *et al.*, 2000; Kishida *et al.*, 2004). This problem can be resolved by supplementing PVA-based IVM media with hormones (follicular stimulating hormone and/or luteinizing hormone), growth factors, and other beneficial factors (Abeydeera *et al.*, 2000; Geshi *et al.*, 2000; Mizushima and Fukui, 2001). Combined treatment of oocytes with PVA, BSA and PFF by supplementing IVM medium differently during the first (22 h) and the second (22 h) halves of IVM did not influence PA embryonic development. Interestingly, the combined treatment of oocytes with BSA and PVA or PFF showed a comparable level of blastocyst formation to PFF treatment; supplementation of IVM medium with BSA just during the first or the second 22 h of IVM did not decrease blastocyst formation compared to PFF. This result indicates that the influence of macromolecules in IVM medium may vary depending on the early or later stages of IVM. In summary, our results demonstrate that BSA added to the maturation medium can support oocyte maturation comparable to PFF-supplemented medium but maturation of oocytes in a BSA-containing medium decreases embryonic development after PA and SCNT compared to medium supplemented with PFF. Further studies are needed to improve the IVM system using BSA.

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