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—Original Article—

Lipid-rich bovine serum albumin improves the viability and hatching ability of porcine blastocysts produced *in vitro*

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Abstract. The effects of lipid-rich bovine serum albumin (LR-BSA) on the development of porcine blastocysts produced *in vitro* were examined. Addition of 0.5 to 5 mg/ml LR-BSA to porcine blastocyst medium (PBM) from Day 5 (Day 0 = *in vitro* fertilization) significantly increased the hatching rates of blastocysts on Day 7 and the total cell numbers in Day-7 blastocysts. When Day-5 blastocysts were cultured with PBM alone, PBM containing LR-BSA, recombinant human serum albumin or fatty acid-free BSA, addition of LR-BSA significantly enhanced hatching rates and the cell number in blastocysts that survived compared with other treatments. The diameter, ATP content and numbers of both inner cell mass and total cells in Day-6 and Day-7 blastocysts cultured with PBM containing LR-BSA were significantly higher than in blastocysts cultured with PBM alone, whereas LR-BSA had no effect on mitochondrial membrane potential. The mRNA levels of enzymes involved in fatty acid metabolism and β-oxidation (*ACSL1*, *ACSL3*, *CPT1*, *CPT2* and *KAT*) in Day-7 blastocysts were significantly upregulated by the addition of LR-BSA. The results indicated that LR-BSA enhanced hatching ability and quality of porcine blastocysts produced *in vitro*, as determined by ATP content, blastocyst diameter and expression levels of the specific genes, suggesting that the stimulatory effects of LR-BSA arise from lipids bound to albumin.
Key words: ATP content, Fatty acids, Gene expression, Pig embryo

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In vitro production (IVP) of porcine embryos is an important tool for producing large numbers of embryos from abattoir-derived ovaries to reduce the time and cost of not only basic research such as reproductive physiology but also biotechnological research such as cloning and transgenesis [1]. Recent advances in porcine IVP systems, including *in vitro* oocyte maturation, fertilization and embryo culture, have enabled us to generate viable embryos that can develop to full term after transfer into recipients [2–4]. However, the current IVP systems still result in a low development rate and low quality of blastocysts compared with those produced *in vivo* [5].

We have developed an IVP system for porcine embryos, including a porcine oocyte medium (POM) for *in vitro* maturation (IVM), porcine fertilization medium (PFM) for *in vitro* fertilization (IVF) and porcine zygote medium (PZM)-5 for *in vitro* culture (IVC) of zygotes [6]. This system has enabled us to produce blastocysts that can produce live piglets born after embryo transfer. We have also developed a porcine blastocyst medium (PBM) for later stage development of porcine blastocysts [7]. This medium is PZM-5 supplemented with 5 mM glucose and 10 mM glycine, and it enhances development to the hatching and hatched blastocyst stages *in vitro*.

Hatching from the zona pellucida is a prerequisite physiologic event for attachment of the blastocyst to the endometrial epithelium and penetration towards the endometrium leading to pregnancy. Recently, we demonstrated that the addition of knockout serum replacement (KSR)—known as a substitute for serum or albumin—to PBM improved development to the hatching stage and blastocyst quality [8]. KSR has a beneficial effect on self-renewal in human embryonic stem (ES) cells [9] and has been utilized for diverse applications, such as the establishment of ES cell lines, freezing of ES cells and in the medium used for blastocoel microinjection [10]. Garcia-Gonzalo and Izpisúa Belmonte [9] suggested that the activity of KSR is responsible for albumin-associated lipids, which are available commercially as the form of lipid-rich bovine serum albumin (LR-BSA; AlbuMAX® I, Thermo Fisher Scientific, Waltham, MA, USA). Furthermore, fatty acids and the β-oxidation pathway have been demonstrated to be essential for oocyte and embryo development [11]. These findings indicated that LR-BSA might function as an energy source for development of porcine embryos. However, the mechanism of the stimulatory effect of LR-BSA on blastocyst development and the hatching remains unknown.

In the present study, we evaluated the effect of LR-BSA on viability and hatching of IVP porcine blastocysts. The quality of blastocysts was also evaluated based on ATP content, embryo diameter, total cell number, and mitochondrial membrane potential. A previous study reported that mRNA expression levels of genes encoding enzymes involved in fatty acid metabolism increased gradually with increasing concentrations of nonesterified fatty acids [12]. Therefore, we also examined the effect of LR-BSA on gene expression profiles of fatty acid metabolism- and β-oxidation-related enzymes.

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Materials and Methods

Reagents and culture media

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All culture media for IVP of porcine embryos were provided from the Research Institute for the Functional Peptides (Higashine, Japan).

In vitro maturation, in vitro fertilization, and in vitro embryo culture

IVP of porcine blastocysts was conducted as described previously [13]. Briefly, intact cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) of ovaries of slaughtered prepubertal gilts. COCs were matured in POM supplemented with 0.5 IU/ml recombinant human follicle stimulating hormone (rhFSH; Gonal-F, Merck Serono, Geneva, Switzerland), 10 ng/ml recombinant human transforming growth factor-α (R&D Systems, Minneapolis, MN, USA) and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) for 20–22 h and subsequently matured in the same medium without rhFSH and dbcAMP for 24 h. IVF was performed using the same batch of frozen-thawed ejaculated semen. Matured COCs were incubated for fertilization in PFM with 1×10^6 Percoll (50/80%; GE Healthcare, Buckinghamshire, UK)-separated spermatozoa/ml for 10 h. After IVF, the presumptive zygotes were stripped of cumulus cells by vortexing for 4 min 30 sec in porcine oocyte/embryo collection medium (POE-CM). Presumptive zygotes were washed twice with POE-CM and with PZM-5 and cultured in 40 µl droplets of PZM-5 until Day 5. Each droplet contained approximately 25 presumptive zygotes. All cultures were maintained at 38.6 C in a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2. The rates of cleavage and blastocyst formation were evaluated under a stereomicroscope on Days 2 and 5, respectively. The Day-5 blastocysts were washed with PBM and then randomly assigned to the various treatment groups in each experiment.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

TUNEL assays were performed to determine the proportion of cells undergoing apoptosis as described previously [7]. Briefly, the blastocysts were washed three times in phosphate-buffered saline (PBS) containing 3 mg/ml polyvinyl alcohol (PVA), fixed at 4 C in 4% (w/v) paraformaldehyde overnight, permeabilized in 0.1% (v/v) Triton X-100 in PBS for 60 min and incubated in blocking solution (PBS containing 1% (v/v) BSA) at 4 C overnight. The embryos were incubated in fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT) (In Situ Cell Death Detection Kit, Fluorescein; Roche Applied Science, Indianapolis, IN, USA). Negative controls were incubated in fluorescein-conjugated dUTP in the absence of TdT. Positive controls were incubated in 50 U/ml deoxyribonuclease I (Promega, Madison, WI, USA) before TUNEL assay. After labeling, all embryos were counterstained with 10 µg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) for 20 min to label nuclei, washed with PBS-PVA, mounted with slight coverslip compression and examined under a fluorescence microscope (BZ-8000, Keyence, Osaka, Japan). The total numbers of nuclei and the numbers of TUNEL-labeled nuclei were recorded for each blastocyst. The ratio of the number of TUNEL-labeled nuclei to the total number of cells was defined as the apoptotic index.

Measurement of ATP content of blastocysts

The ATP content of each embryo was measured using a commercial assay kit based on the luciferin–luciferase reaction (ATPlite, PerkinElmer, Groningen, The Netherlands) as described by Mito *et al.* [7]. Briefly, blastocysts were washed three times in POE-CM and transferred individually to 50 µl aliquots of PBS in 1.5 ml tubes and stored at −80 C until assay. To determine ATP contents, 25 µl mammalian cell lysis solution was added to each tube, and the contents were vortexed for 5 min. Subsequently, 30 µl sample and 10 µl substrate solution (luciferase/luciferin) were added to each well of a 384-well microplate (PerkinElmer). After shaking for 5 min, the plates were held at room temperature in the dark for 10 min. Luminescence was measured using a luminometer (Wallac 1420 ARVO MX, PerkinElmer). The ATP content was calculated based on a standard curve (0–1,000 nM). Assays for all standards and samples were performed in duplicate.

Differential staining of inner cell mass and trophectoderm cells

The cell allocation of blastocysts was assessed by differential staining of inner cell mass (ICM) and trophectoderm (TE) cells as described previously [14]. The embryos were placed for 30 sec in PBS containing 0.2% (v/v) Triton X-100 and 100 µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA). They were then placed in ethanol containing 25 µg/ml Hoechst 33342 and were incubated at 4 C overnight in the dark for fixation and staining. After staining, the embryos were washed several times with glycerol (Merck, Darmstadt, Germany), placed on a glass microscope slide with a small amount of glycerol and covered with a coverslip. Stained embryos were observed using a fluorescence microscope (BZ-8000, Keyence). The numbers of blue nuclei in the ICM and pink nuclei in the TE were counted.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined by staining with the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR, USA) [15]. Briefly, blastocysts were incubated in PBM containing 10 µM JC-1 at 38.6 C in 5% CO_2, 5% O_2 and 90% N_2 for 30 min, washed in PBM, transferred into a 1 µl droplet on a slide and covered with a coverslip. The fluorescence of JC-1 J-aggregates (red) and monomers (green), which correspond to mitochondria with higher and lower membrane potentials, respectively, was acquired and analyzed using fluorescence microscope observations (BZ-8000, Keyence). The mitochondrial membrane potential of blastocysts was evaluated using the red/green fluorescence ratio.

RNA isolation and quantitative reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from pools of 10 blastocysts using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Briefly, each group of 10 blastocysts was washed in PBS three times, transferred into 400 µl ISOGEN and then stored at −20 C until RNA extraction. The quantity and quality

Table 1. Sequences of the PCR primers used for quantitative RT-PCR

Gene	Primer sequences (5′–3′)	Product size (bp)	GenBank accession number
FATP4	F: AGGGTTGGAATTAGGGGTCA	108	EU703768
	R: CGATGAGGAGGGTGACAGAT		
ACSL1	F: AGGCCTGAGTGGGTGATCATT	100	AY690660
	R: TGATGTAGGTGATGGCCTCAGT		
ACSL3	F: GGACCCACAGGACTTCCAAA	179	NM_001143698
	R: CGGCATCCATGAGAAAGAC		
CPT1	F: GGACCGCCACCTGTTCTG	172	AF288789
	R: CCCTCCGCTCGACACATAC		
CPT2	F: CAAGGCCTACCCTCTGGATA	175	NM_001246243
	R: GCTCACAATCTTCCCGTCTT		
KAT	F: CCAAGAAGGCACAGGATGAA	144	AF028007
	R: AGGGCTTGATGAATGCAGGT		
UBB	F: TGTTGGCGGTTTCGCTGTTG	248	NM_001105309
	R: AGTGCGGCCATCCTCCAGCT		

of extracted total RNA were determined using a NanoDrop ND-1000 (Thermo Fisher Scientific). Samples were reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) with random hexamer primers according to the manufacturer's instructions. Real-time quantitative PCR was carried out with LightCycler® 2.0 systems (Roche Applied Science) using a LightCycler FastStart DNA Master SYBR Green I Kit (Roche Applied Science) in duplicate, according to the manufacturer's instructions. Primer sequences, sizes of amplified products and GenBank accession numbers are shown in Table 1. To amplify the cDNA for long-chain acyl-CoA synthetase-1 (*ACSL1*), *ACSL3*, carnitine palmitoyltransferase 1 (*CPT1*), *CPT2*, fatty acid transport protein 4 (*FATP4*), 3-ketoacyl-CoA thiolase (*KAT*) and ubiquitin B *(UBB)*, aliquots (2.5 µl) of cDNA from reverse transcription were amplified by PCR in 10 µl volumes containing a final concentration of 3 mM $MgCl_2$, 0.5 µM of each primer and 10% LightCycler DNA Master SYBR Green I Master mix (Roche Applied Science). The conditions for the real-time PCR reactions were as follows: initial denaturation at 95 C for 10 min followed by 40 amplification cycles of denaturation at 95 C for 10 sec; annealing at 53 C (*CPT2*), 55 C (*ACSL3*), 58 C (*ACSL1* and *KAT*) or 62 C (*CPT1*) for 10 sec or at 60 C (*FATP4*) or 67 C (*UBB*) for 5 sec; and final extension at 72 C for 4 sec (*ACSL1*), 6 sec (*FATP4* and *CPT1*), 7 sec (*CPT2* and *KAT*) or 8 sec (*ACSL3* and *UBB*) with a single fluorescence detection point at the end of the relevant extension segment. Quantification was done using the second derivative maximum method of the LightCycler ver. 3.5 Software (Roche Applied Science), which determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration. The mRNA levels of target genes were normalized to those of *UBB* [8]. The specificity of each primer set was confirmed by both electrophoresis of the PCR products on a 2.0% agarose gel and analysis of the melting (dissociation) curve using the LightCycler Software after each real-time PCR run.

Experimental design

In Experiment 1, the effects of various concentrations of LR-BSA added to PBM from Day 5 on the subsequent viability and hatching of embryos were examined. Day-5 blastocysts were cultured with PBM containing 0, 0.5, 1, 2 or 5 mg/ml LR-BSA for an additional 48 h in 50-µl droplets (12 to 14 blastocysts per droplet). The numbers of surviving, partially hatched and completely hatched blastocysts were determined under a stereomicroscope at 24-h intervals up to Day 7. Blastocysts with a clear blastocoel were defined as surviving, whereas those in the process of emerging and those that had emerged from the zona pellucida were classified as hatching or hatched blastocysts, respectively [7]. The total cell numbers of blastocysts on Day 7 were counted by an air-drying method, as described previously [3].

The objective of Experiment 2 was to investigate whether the effects of LR-BSA on development to the hatching and hatched stage depended on albumin itself or its lipid constituents. Day-5 blastocysts were cultured with PBM alone or PBM containing 1 mg/ml recombinant human serum albumin (rHSA; StemCell Research Laboratories, Carlsbad, CA, USA), 1 mg/ml fatty acid-free BSA (FAF-BSA) or 1 mg/ml LR-BSA for an additional 48 h. The numbers of surviving, hatching and hatched blastocysts were assessed under a stereomicroscope at 24-h intervals. The total numbers of cells and the apoptotic index in Day-7 blastocysts were also evaluated after staining using the TUNEL assay.

Experiment 3 was conducted to determine the effect of LR-BSA on blastocyst diameter and ATP content in blastocysts. Day-5 blastocysts were cultured with PBM alone or PBM containing 1 mg/ml LR-BSA for an additional 24 or 48 h. The diameter of each surviving blastocyst on Day 6 or 7 was measured using the ImageJ software (version. 1.47, NIH, Bethesda, MD, USA). The diameter of each blastocyst was taken as the mean of two measurements made perpendicularly to each other. After measurement, blastocysts were used immediately for determining the ATP content.

The objective of Experiment 4 was to determine the effects of LR-BSA on the numbers of ICM and TE in blastocysts. Day-5 blastocysts were cultured as in Experiment 3. The ICM and TE of each surviving blastocyst on Day-6 or Day-7 was stained using a

differential staining procedure as described above, and the numbers of ICM and TE cells were counted.

In Experiment 5, the effect of LR-BSA on the mitochondrial membrane potential of blastocysts was examined. Day-5 blastocysts were cultured as in Experiment 3. The mitochondrial membrane potential of Day-6 or Day-7 blastocysts was determined using JC-1 dye. Only surviving blastocysts were used for this measurement.

The objective of Experiment 6 was to determine the effects of LR-BSA on mRNA expression of genes encoding enzymes involved in lipid metabolism and β-oxidation in the blastocysts by real-time RT-PCR. Day-5 blastocysts were cultured as in Experiment 3. Total RNA was extracted from surviving blastocysts on Day 6 or 7, and the mRNA levels of *FATP4, ACSL1*, *ACSL3*, *CPT1*, *CPT2* and *KAT* were analyzed by quantitative RT-PCR.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Data were analyzed using the general linear model procedure in the Statistical Analysis System software (version 9.4; SAS Institute, Cary, NC, USA). Treatment differences were determined using the Tukey–Kramer honestly significant difference post hoc test. Percentage data and the total number of cells per blastocyst were subjected to arcsine and logarithmic transformation, respectively, before statistical analysis. $P < 0.05$ was considered statistically significant.

Results

In our IVF system, the mean percentage of penetrated oocytes (with one or more swollen sperm head(s) and/or male pronuclei with a corresponding sperm tail) and the normal fertilization rate (with a second polar body, a pair of pronuclei and corresponding sperm tail) were 80.7% and 68.7% of examined oocytes, respectively. Polyspermy was identified in 12.0% of the oocytes. A total of 7509 presumptive zygotes were cultured in PZM-5 following IVF: 5145 embryos (76.4%) were cleaved at Day 2 post IVF and 1594 (23.2%) developed to the blastocyst stage on Day 5.

Experiment 1

The hatching rates of Day-6 blastocysts cultured with 1 or 5 mg/ml LR-BSA and of Day-7 blastocysts cultured with 0.5 to 5 mg/ml LR-BSA were significantly higher ($P < 0.05$) than those of blastocysts cultured with PBM alone (Fig. 1). The addition of 5 mg/ml LR-BSA significantly enhanced the survival rate of blastocysts on Day 7 compared with PBM alone ($P < 0.05$). The percentages of completely hatched blastocysts cultured with 5 mg/ml LR-BSA on Day 6 or with 1 or 5 mg/ml LR-BSA on Day 7 were also increased ($P < 0.05$) compared with those of blastocysts cultured with PBM alone. The total cell numbers of Day-7 blastocysts cultured with 0.5 to 5 mg/ml LR-BSA were significantly greater than that of blastocysts cultured with PBM alone. Figure 2 shows representative images of embryos cultured with PBM alone or PBM containing 1 mg/ml LR-BSA on Days 6 and 7. Briefly, the highest total cell numbers in Day-7 surviving blastocysts were obtained by addition of LR-BSA to PBM at a concentration of 1 mg/ml LR-BSA, and the rates of surviving, at least partially hatched, and completely hatched blastocysts cultured with LR-BSA at this concentration were not significantly different compared with those at higher concentrations (2 and 5 mg/ml). Thus, 1 mg/ml LR-BSA was used throughout the following experiments.

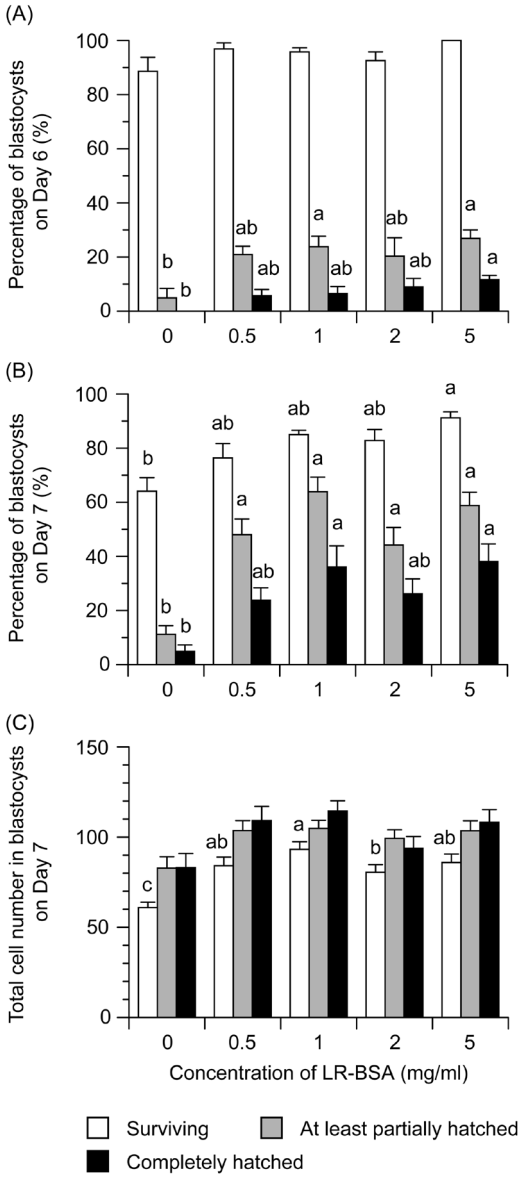

Fig. 1. *In vitro* development of Day 6 (A) and Day 7 (B) porcine blastocysts cultured in PBM containing 0 to 5 mg/ml LR-BSA from Day 5. (C) Total cell numbers of porcine blastocysts on Day 7. Blastocysts were classified as surviving, at least partially hatched, and completely hatched. Values are shown as the mean ± SEM. Values with different superscript letters among media within blastocyst classifications differ significantly. The experiment was performed six or seven times with 77 to 93 blastocysts per treatment group.

Experiment 2

Addition of LR-BSA to PBM enhanced the hatching rate of blastocysts compared with PBM alone on Day 6 ($P < 0.05$; Table 2),

Table 2. Effect of different types of serum albumin reagents on development of porcine blastocysts

Addition to PBM	No. of blastocysts cultured	Day 6			Day 7		
		Surviving (%)	At least partially hatched (%)	Completely hatched (%)	Surviving (%)	At least partially hatched (%)	Completely hatched (%)
None	123	109 (88.1 ± 5.4)	3 (2.4 ± 1.2) [b]	2 (1.6 ± 1.0)	55 (44.6 ± 4.9) [b]	11 (8.8 ± 2.4) [c]	5 (3.9 ± 1.7) [b]
1 mg/ml rHSA	127	121 (95.4 ± 2.0)	19 (15.4 ± 4.4) [ab]	4 (3.2 ± 1.7)	87 (68.2 ± 5.6) [ab]	32 (25.6 ± 5.0) [b]	13 (10.3 ± 2.7) [b]
1 mg/ml FAF-BSA	126	123 (97.5 ± 1.8)	13 (13.8 ± 3.6) [ab]	5 (4.0 ± 1.7)	91 (71.9 ± 5.0) [a]	27 (21.8 ± 3.2) [bc]	20 (16.1 ± 3.0) [ab]
1 mg/ml LR-BSA	124	121 (97.6 ± 1.2)	19 (18.4 ± 5.3) [a]	4 (3.2 ± 1.7)	90 (71.9 ± 5.0) [a]	58 (46.7 ± 2.3) [a]	35 (28.1 ± 5.0) [a]

Data are from nine replicates. Percentages are expressed as the mean ± SEM. [a–c] Within each column, values with different superscript letters are significantly different ($P < 0.05$).

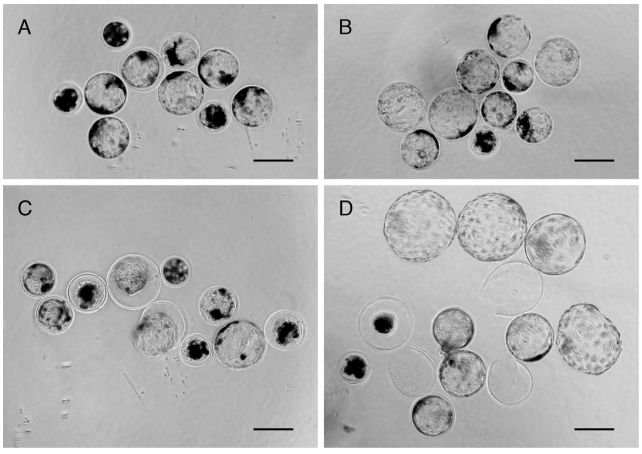

Fig. 2. Porcine blastocysts cultured with PBM alone (A, C) and PBM containing 1 mg/ml LR-BSA (B, D) on Day 6 (A, B) and Day 7 (C, D). Scale bars = 200 μm.

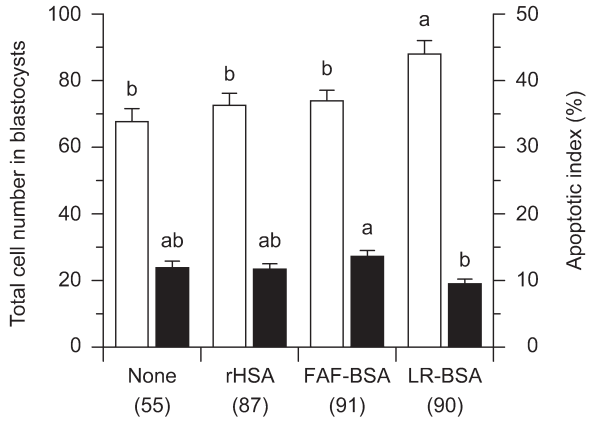

Fig. 3. Total cell numbers (white bars) and apoptotic indexes (black bars) of Day-7 blastocysts cultured with PBM alone, PBM supplemented with 1 mg/ml rHSA, 1 mg/ml FAF-BSA or 1 mg/ml LR-BSA from Day 5. Values are expressed as the mean ± SEM. Values with different superscript letters within a subject differ significantly ($P < 0.05$). The experiment was replicated nine times. Numbers in parentheses indicate the number of embryos tested per group.

while there were no significant differences between the addition of no supplement, rHSA or FAF-BSA. On Day 7, the survival rate of blastocysts cultured in the presence of LR-BSA or FAF-BSA was higher than that of blastocysts cultured with PBM alone ($P < 0.05$). The hatching rate of blastocysts cultured with LR-BSA on Day 7 was increased compared with other treatments ($P < 0.05$), while the rate was higher in blastocysts cultured with PBM containing rHSA than in those cultured with PBM alone ($P < 0.05$). Addition of LR-BSA also enhanced the percentage of blastocysts that completely hatched on Day 7 compared with PBM alone and addition of rHSA to PBM ($P < 0.05$). The total cell number of Day 7 blastocysts that survived in culture with LR-BSA was significantly greater than in other treatments (Fig. 3). The apoptotic index of Day-7 blastocysts cultured with FAF-BSA was higher than that of Day-7 blastocysts cultured with LR-BSA ($P < 0.05$).

Experiment 3

The mean diameter of blastocysts cultured with LR-BSA was significantly greater ($P < 0.05$) than that of blastocysts cultured with PBM alone on both Days 6 and 7 (Fig. 4). Addition of LR-BSA to PBM also increased the ATP content of blastocysts on Days 6 and 7 significantly compared with no addition ($P < 0.05$).

Experiment 4

Addition of LR-BSA to PBM increased the total cell numbers, cell numbers of the ICM and TE, and ratios of ICM cells/total cells of blastocysts, which were significantly higher ($P < 0.05$) than those of blastocysts cultured with PBM alone, except for the number of TE cells in Day-6 blastocysts (Fig. 5).

Experiment 5

The mitochondrial activities assessed by membrane potential using JC-1 dye in Day 6 or 7 blastocysts cultured with or without LR-BSA are shown in Fig. 6. There was no significant difference between treatments.

Experiment 6

When the mRNA expression levels of *FATP4, ACSL1*, *ACSL3*, *CPT1*, *CPT2* and *KAT* in Day 6 or 7 blastocysts were analyzed by real-time quantitative RT-PCR, there were no significant differences in the expression levels of *FATP4* mRNA among treatments and days (Fig. 7). The mRNA expression level of *ACSL3* in Day-6 blastocysts cultured with LR-BSA was significantly lower ($P < 0.05$) than in

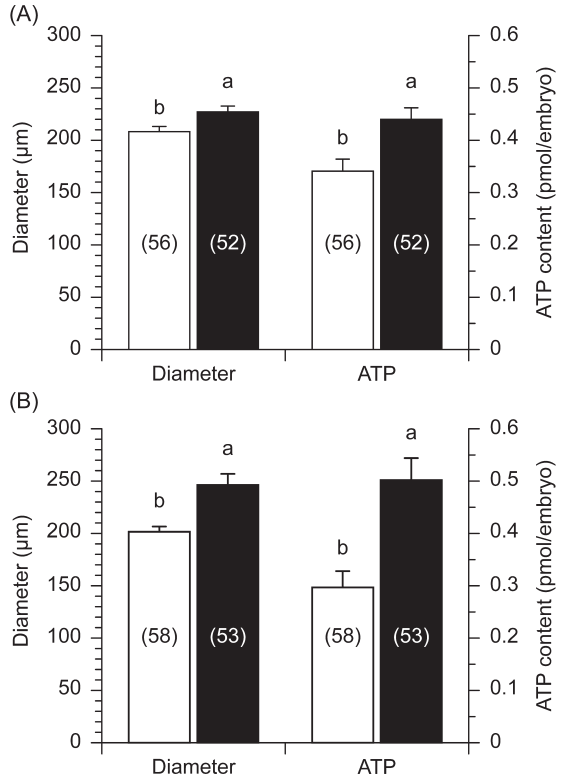

Fig. 4. Diameters and ATP contents of Day-6 (A) and Day-7 (B) blastocysts, cultured with PBM alone (white bars) or PBM with 1 mg/ml LR-BSA (black bars) from Day 5. Values are expressed as the mean ± SEM. Values with different superscript letters within a subject differ significantly ($P < 0.05$). Numbers in parentheses indicate the number of embryos tested per group.

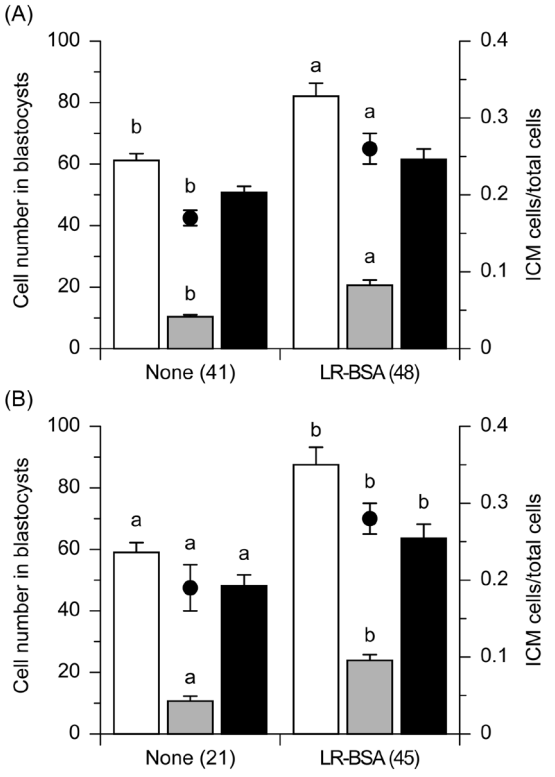

Fig. 5. Total cell numbers (white bars), numbers of ICM cells (gray bars), numbers of TE cells (black bars) and ratios of ICM cells/total cells (black circles) in Day-6 (A) and Day-7 (B) blastocysts, cultured with PBM alone or PBM with 1 mg/ml LR-BSA from Day 5. Values are expressed as the mean ± SEM. Values with different superscript letters between media within a subject differ significantly ($P < 0.05$). The experiment was performed four times. Numbers in parentheses indicated the number of embryos tested per group.

blastocysts cultured with PBM alone, while the mRNA levels of *ACSL1*, *CPT1*, *CPT2* and *KAT* did not differ between treatments. In Day-7 blastocysts, the expression levels of *ACSL1*, *ACSL3*, *CPT1*, *CPT2* and *KAT* mRNA were significantly higher ($P < 0.05$) when LR-BSA was added to PBM compared with no addition.

Discussion

Our study demonstrated that the addition of LR-BSA to PBM from Day 5 enhanced the *in vitro* viability and hatching of porcine IVP blastocysts. In addition, the presence of LR-BSA improved blastocyst quality, as determined by total blastomere numbers, embryo diameter, ATP content and mRNA expression level of enzymes involved in fatty acid metabolism and β-oxidation. These stimulatory effects on blastocyst hatching were probably caused by lipids bound to BSA.

The addition of LR-BSA to PBM enhanced porcine embryo development after blastocyst formation, especially in terms of the hatching rate. The AlbuMAX® I employed in this study is a commercially available LR-BSA containing naturally occurring fatty acids such as α-linolenic acid, linoleic acid, oleic acid, stearic acid and palmitic acid. It has been suggested that the stimulatory effect of AlbuMAX® I on human ES cell self-renewal is caused by albumin-associated lipids but not by albumin itself [9]. Endogenous and exogenous fatty acids play

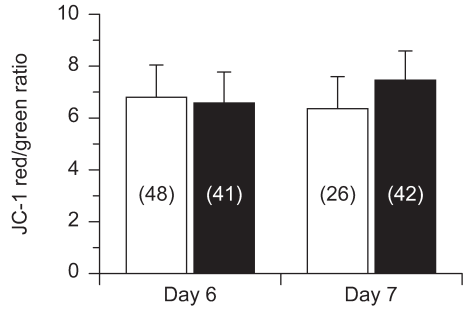

Fig. 6. Ratio of JC-1 staining of Day-6 and Day-7 blastocysts cultured in PBM alone (white bars) or PBM with 1 mg/ml LR-BSA (black bars) from Day 5. Data are presented as the mean ± SEM. The experiment was replicated four times. Numbers in parentheses represent the numbers of blastocysts tested per group. There were no statistically significant differences among the treatments.

various roles in metabolism, oxidative stress, membrane composition, cell signaling events and gene expression in the development of mammalian oocytes and embryos [16]. Mouse embryos incorporate

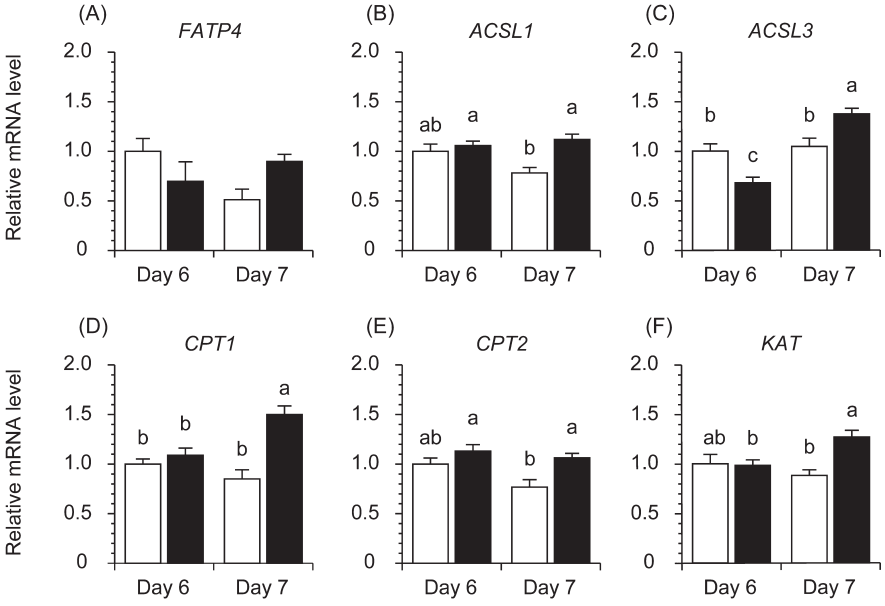

Fig. 7. Relative mRNA abundance of genes encoding enzymes involved in fatty acid metabolism or β-oxidation among blastocysts cultured in PBM alone (white bars) or PBM with 1 mg/ml LR-BSA (black bars). The respective values were normalized to those of *UBB* to obtain the relative abundance of the targets (arbitrary units). Data are presented as the mean ± SEM of five replicates. Values with different superscript letters among treatments (media × culture days) differ significantly ($P < 0.05$).

exogenous fatty acids between the 8-cell and late blastocyst stage, in preparation for hatching [17], and exogenously supplied fatty acids enhance the growth and development of embryos in rabbits [18], mice [19] and rats [20]. Moreover, addition of a β-oxidation inhibitor during IVM of bovine oocytes reduced the rate of development to the blastocyst stage [21], and IVC medium containing an inhibitor of fatty acids reduced the total cell numbers in mouse blastocysts [22]. These previous findings indicate that fatty acids are important energy sources for embryo development. In our present study, when LR-BSA was replaced with rHSA or FAF-BSA, the percentage of blastocysts that developed to at least partially hatched blastocysts on Day 6 or to completely hatched blastocysts on Day 6 and Day 7 did not increase compared with PBM alone. Thus, the stimulatory effect of LR-BSA on blastocyst hatching might be caused by lipids bound to this additive.

In the present study, not only the hatching rates but also the total cell numbers, diameter and ATP contents of blastocysts were significantly enhanced by the addition of LR-BSA. During the hatching process *in vitro*, blastocysts significantly increase their cell numbers, enabling blastocyst expansion and zona shedding [23]. Thus, LR-BSA would increase the blastocyst diameter by increasing the cell numbers. It has been shown that the ATP contents were markedly increased at the hatching blastocyst stage in *in vitro* cultured bovine [24] and feline [25] embryos. Increased cell numbers in blastocysts cultured with LR-BSA could be associated with enhanced ATP contents in the blastocysts, because ATP is the primary energy source of all living cells and is a key indicator of the developmental potential of oocytes and embryos [26, 27]. A single molecule of glucose produces the equivalent of about only 30 ATP molecules, whereas β-oxidation of a fatty acid such as palmitate generates 106 molecules [28]. Although the PBM used in the present study already contains 5 mM glucose, the addition of LR-BSA to the medium might enhance hatching via utilization of the supplementary fatty acids as energy sources.

In the present study, there were no differences in the mitochondrial membrane potential assessed using JC-1 dye among the different culture conditions and sampling days. JC-1 is the potentiometric probe of choice to distinguish cytoplasmic regions containing high- or low-polarized mitochondria. While mitochondria in normal cells usually maintain a relatively high polarity in order to establish a transmembrane potential sufficient to derive ATP synthesis by oxidative phosphorylation, a sudden loss of polarization has been considered one of the first detectable events in initiation of the apoptotic cascade [27]. This result from JC-1 staining confirms the result indicating that the apoptotic index did not differ in blastocysts cultured with or without LR-BSA. Moreover, total cell numbers, cell numbers of the ICM and TE (except for Day-6 TE) in blastocysts and ratios of ICM cells/total cells cultured with LR-BSA were greater than those of blastocysts cultured in PBM alone. Therefore, our data suggest that the addition of LR-BSA to PBM causes an increase in ICM and TE cell numbers in blastocysts, which may result in an increase in the ATP contents of blastocysts.

The female reproductive tract contains plenty of albumin [29]. The addition of FAF-BSA has been shown to increase the hatching rate of porcine embryos [30]. Culture with rHSA has been shown to increase the blastocyst development and hatching rates of mouse embryos [31]. In the present study, the Day-7 hatching rate of blastocysts cultured with rHSA was greater than that of blastocysts cultured with PBM alone. Albumin has several functions, such as a chelator that

binds growth factors and as a source of nitrogen in a simple medium lacking amino acids [32]. The increase in the frequency of hatching with rHSA on Day 7 may be also partly responsible for increasing the developmental competence of embryos. The results of Experiment 2 indicated that the effect of LR-BSA on the development of porcine IVP blastocysts could be caused by albumin-associated lipids. The metabolism of fatty acids via β-oxidation occurs in the mitochondrial matrix via a multistep pathway [33]. Here, analysis of *ACSL1*, *ACSL3*, *CPT1*, *CPT2* and *KAT* gene expression levels of blastocysts cultured in LR-BSA on Day 7 showed overall increased levels of genes encoding enzymes involved in lipid metabolism and the β-oxidation of fatty acids. These are key enzymes that regulate the activation, translocation and β-oxidation of fatty acids in the mitochondria, and in particular, KAT plays a role in the last key step in β-oxidation [34]. Although enzyme activities were not measured directly here, the significant upregulation of mRNA levels for these enzymes in blastocysts cultured with LR-BSA might have been partly responsible for increasing their ATP levels and developmental competence.

In summary, the addition of LR-BSA to culture medium following the blastocyst stage enhanced the hatching ability and quality of porcine IVP blastocysts, as determined by ATP content, blastocyst diameter and mRNA expression levels of genes encoding enzymes involved in fatty acid metabolism and β-oxidation. Recombinant HSA and FAF-BSA had no beneficial effect on blastocyst hatching. Therefore, we conclude that the stimulatory effects of LR-BSA might arise from lipids bound to albumin.

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