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Lipid-rich bovine serum albumin is beneficial for the early development of bovine embryos, but induces lipid droplet formation in the blastocyst

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Abstract: Lipid-rich bovine serum albumin (LR-BSA) has been reported to increase the survival rate of blastocysts after freezing and thawing. However studies on the early development of *in vitro* fertilised (IVF) embryos are still insufficient in cattle. This study investigated the blastocyst productivity of *in vitro* culture (IVC) medium (synthetic oviductal fluid medium with amino acids, mSOFaa) supplemented with 5% fetal bovine serum (FBS, Control) or LR-BSA (1, 2.5, 5 mg/ml dosages, respectively) and the qualitative characteristics of produced blastocysts. Cleavage rates on day 2 were similar between all groups. Significant differences were obtained in the blastocyst rate in the 2.5LR-BSA group compared with the control (23.5% vs 35.7%, $P < 0.05$). Hatching rates were significantly higher in all treatment groups than in control ($P < 0.05$). The cell number in blastocysts did not differ among groups, but the apoptotic index in the 5.0LR-BSA group was higher than the control ($P < 0.05$). The expression of embryo quality-related markers was affected. Octamer-binding transcription factor 4 (Oct4), placenta associated 8 (Plac8), and sex-determining region Y-box 2 (Sox2) genes expression in the 2.5LR-BSA group were significantly up-regulated compared to the control ($P < 0.05$). Cytoplasmic lipid contents and the lipid droplet formation-related gene, sterol regulatory element binding transcription factor 1 (*Srebf1*), were significantly increased in the 5.0LR-BSA group ($P < 0.05$). Therefore, the LR-BSA supplementation (2.5 mg/ml) to mSOFaa medium could contribute to the early development of bovine IVF embryos and the qualitative improvement of the resulting blastocysts.

Keywords: cattle; apoptosis; embryo quality; *in vitro* fertilised embryo

The embryo transfer technology can obtain many fertilised eggs that preserve excellent genetic traits, which are then transplanted to recipients to effectively multiply individuals that have inherited excellent genetic traits. Fetal bovine serum (FBS) is generally used within *in vitro* production (IVP) culture medium to supply growth factors and energy sources. Many studies have reported that add-

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ing FBS enhances *in vitro* development of fertilised eggs. However, the composition of FBS is not well characterised, with a considerable batch variation that can increase the incidence of large offspring syndrome (Lazzari et al. 2002).

KnockOut Serum Replacement (KSR) is a defined serum replacement that utilises various organic molecules, trace elements, and proteins. It does not contain the unknown growth or differentiation-promoting factors found in FBS (Garcia-Gonzalo and Izpisua Belmonte 2008). KSR can be used as an FBS substitute in defined culture media in bovine IVP systems because it does not interfere with the development of fertilised eggs (Hoelker et al. 2009). Lipid-rich bovine serum albumin (LR-BSA), one of the primary proteins of KSR, contains essential fatty acids most mammals need and induces the production of active substances that affect various biological functions. For example, when LR-BSA was used late in culture (after culture day 5), indicators such as adenosine triphosphate (ATP) content, size, and expression levels of specific genes in pig blastocysts were improved (Suzuki et al. 2016). In bovine embryos, LR-BSA has been reported to improve survival after freeze-thawing of blastocysts (Lim et al. 2008), but research on *in vitro* culture of preimplantation embryos remains insufficient. In the embryo, the expression of genes involved in lipid metabolism is correlated with the lipid droplets (LDs) content in the cytoplasm. In the case of mouse embryos, the LDs content increased in the morula stage and decreased in the blastocyst stage, suggesting that the lipid metabolism of the embryo seems to be stage-specific (Sudano et al. 2016). However, the exceeded concentrations of lipids enhanced the expression of genes related to oxidative stress and the cellular inflammatory response, which could subsequently affect embryonic development and maternal recognition of pregnancy (Cagnone and Sirard 2016; de Andrade Melo-Sterza and Poehland 2021; Li et al. 2023).

The present study was performed to investigate the effects of LR-BSA added to the *in vitro* culture (IVC) media (synthetic oviductal fluid medium with amino acids, mSOFaa) on early embryo development and LDs distribution and determine whether it was possible to improve the *in vitro* culture medium that can enhance the quality of developed embryos by the addition of LR-BSA instead of FBS. LR-BSA was added to the IVC media to confirm the initial embryo development. Cleavage rate, cell death (apoptotic index), cell number and gene expression patterns were evaluated to analyse the blastocysts qualitatively.

MATERIAL AND METHODS

Production of bovine blastocysts

The ovaries of Hanwoo cows were obtained at a slaughterhouse, and immature oocytes were collected from follicles with diameters of 2–7 mm. Only cumulus-oocyte complexes (COCs) with homogeneous cytoplasm and cumulus cells were used for the experiment. For *in vitro* maturation (IVM), 30–50 COCs were cultured in 500 µl of BO-IVM^{TM} medium (IVF Bioscience, Falmouth, UK) overlaid with mineral oil at 38.5 °C, with 5% CO_2 for 20–22 h.

Matured oocytes were subjected to *in vitro* fertilisation (IVF) using BO-IVF^{TM} medium (IVF Bioscience, UK). Groups of 10–20 COCs were transferred into 90 µl of IVF medium overlaid with mineral oil. Motile sperm were enriched for by the Percoll gradient (45/90%), injected into IVF droplets containing COCs (final concentration: 5×10^4 spermatozoa/COC), and co-cultured for 18 h at 38.5 °C with 5% CO_2.

After insemination, 25 to 40 presumptive zygotes were cultured in 200 µl of mSOFaa (Gutierrez-Adan et al. 2001), supplemented with either 5% FBS (ThermoFisher Gibco^{TM}, Waltham, MA, USA), or 1, 2.5 or 5 mg/ml LR-BSA (AlbuMAX^{TM} I Lipid-Rich BSA; ThermoFisher Gibco^{TM}, Waltham, MA, USA), respectively, at 38.5 °C with 5% CO_2 and 5% O_2 for nine days. The IVC experiments were performed at least four times. The developmental status of the embryos was evaluated using an inverted epifluorescence microscope (ICX41, Sunny Optical Technology, China). Cleaved embryos were recorded on day two post-insemination (pi). Developmental rates were based on the number of embryos that developed at day 5 (morula) and day 9 (blastocyst) pi and were calculated based on the total number of cultured embryos. The hatching rate was calculated as the hatched blastocysts (H-BL) ratio to total blastocysts (T-BL) at day nine pi.

Fluorescence microscopy for TUNEL assay and lipid droplet staining

TUNEL assay was performed to examine the number of TUNEL-positive cells blastocysts

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from each group. Randomly selected blastocysts, five to eight, from each treatment group at day nine pi were fixed in 4% (w/v) paraformaldehyde for one hour and permeabilised and in 0.5% Triton X-100 solution for 30 min at room temperature (RT). Fixed blastocysts were then labelled using in situ cell death detection kit (TMR red kit; Roche, Basel, Switzerland) for 1 h at 38.5 °C in darkness. For DNA counterstaining, blastocysts were incubated in 4',6-diamidino-2-phenylindole, (DAPI; NucBlue™ Fixed Cell ReadyProbes™ Reagent, Invitrogen, Waltham, MA, USA) for 30 min at 38.5 °C and then mounted on glass slides in VECTASHIELD antifade medium (Vector Laboratories, Newark, CA, USA) under cover slides. Images of each blastocyst were captured under an inverted epifluorescence microscope. The number of TUNEL-positive cells and the total number of cells per blastocyst were recorded. The apoptotic index was calculated as the ratio of TUNEL-positive cells to the total number of cells per blastocyst.

For LD staining, blastocysts (n = 10 per group) at day seven pi were washed in PBS and fixed in 4% formaldehyde for 20 min at RT. For lipid droplet staining, 2 µg/ml of BODIPY 493/503 dye (Thermo Fisher Scientific, United States) was used at RT for 20 min in the dark. Subsequently, the nuclei of blastocysts were stained with DAPI for 20 min at RT and then mounted on glass slides in VECTASHIELD antifade medium (Vector Laboratories, Newark, CA, USA) undercover slides. Images of each blastocyst were captured under an inverted epifluorescence microscope at a magnification of 200 X. The semi-quantitative lipid droplet content was estimated using ImageJ software (v1.54d; National Institutes of Health, Bethesda, MD, USA). The intensity mean was recorded, and the embryo's area was calculated using the freehand selection tool.

RNA extraction and quantitative polymerase chain reaction

RNA extraction and transcription of individual blastocysts (five blastocysts from each treatment group at day 9 pi) were performed using FastLane Cell cDNA Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Quantitative PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the QuantStudio Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: 95 °C, 5 min, followed by 35 cycles of 95 °C for 5 s and 60 °C for 10 s. The primer sets shown in Table 1 were used. The data were analysed by QuantStudio™ Design & Analysis Software v1.5.2 (Carlsbad, CA, USA).

Statistical analysis

Data were analysed using IBM SPSS Statistics package v22.0 (Chicago, IL, USA). Embryo development, apoptosis, and gene expression data were analysed by one way analysis of variance and Duncan's multiple range test. Differences in fluorescence intensity were compared using the Student's t-test. A statistically significant difference was determined as $P < 0.05$.

Table 1. Primer sets for real-time PCR

Gene		Primer sequences (5' to 3')	Product size (bps)	Accession number
Oct4	F-	CCACCCTGCAGCAAATTAGC	68	NM_174580
	R-	CCACACTCGGACCACGTCTT		
Sox2	F-	GGTTGACATCGTTGGTAATTTATAATAGC	88	NM_001105463
	R-	CACAGTAATTTCATGTTGGTTTTTCA		
Plac8	F-	CGGTGTTCCAGAGGTTTTTCC	163	NM_016619
	R-	AAGATGCCAGTCTGCCAGTCA		
Srebf1	F-	GTGCATTTCACCGAACCGAG	117	NM_001113302.1
	R-	AGTAACGCCTGACCCTTGAG		
18S rRNA	F-	GACTCATTGGCCCTGTAATTGGAATGAGTC	87	AF176811.1
	R-	GCTGCTGGCACCAGACTTG		

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Table 2. Effect of LR-BSA on *in vitro* development of embryos

Treatment (mg/ml)	No. of embryos	No. (%) of embryos developed to*			
		2-cell	morula		blastocyst
			day 5	day 7	
Control	143	95 (66.4)	18 (12.6)[a]	35 (25.7)[b]	32 (23.5)[b]
1.0	128	84 (65.6)	22 (17.2)[ab]	28 (25.7)[b]	25 (22.9)[b]
2.5	154	106 (68.8)	32 (20.8)[b]	56 (39.2)[a]	56 (35.7)[a]
5.0	116	85 (73.8)	25 (21.6)[b]	31 (28.4)[ab]	29 (26.6)[b]

*Developmental rates were based on the number of embryos that developed by day two (2-cell; cleaved embryos), 5 (morula), 7 (total morula) and 9 (blastocysts) pi of culture and were calculated based on the total number of cultured embryos. The hatching rate was calculated as the ratio of hatched blastocysts (H-BL) to total blastocysts (T-BL) at day 9 pi

[a,b]Values with different superscripts within a column differ significantly ($P < 0.05$)

RESULTS

There was no significant difference in the cleavage rate among the groups on day two pi (Table 2). The developmental rate to the morula stage on days 5 and 7 pi was 20.8 (32/154) and 39.2% (56/154), respectively, in the 2.5LR-BSA, which was significantly higher than in the control ($P < 0.05$). 82.3% (32/56) of the total number of morulas formed a compact mass at day 5 in the 2.5LR-BSA. However, significantly ($P < 0.05$) fewer embryos formed a compact mass in the control (67.5%, 18/35). The blastocyst development rate on day 9 pi in the 2.5LR-BSA was 35.66% (51/154), which was significantly higher than those in the other groups ($P < 0.05$). The hatching rate in all LR-BSA treatment groups was significantly higher than in the control group ($P < 0.05$, Figure 1A).

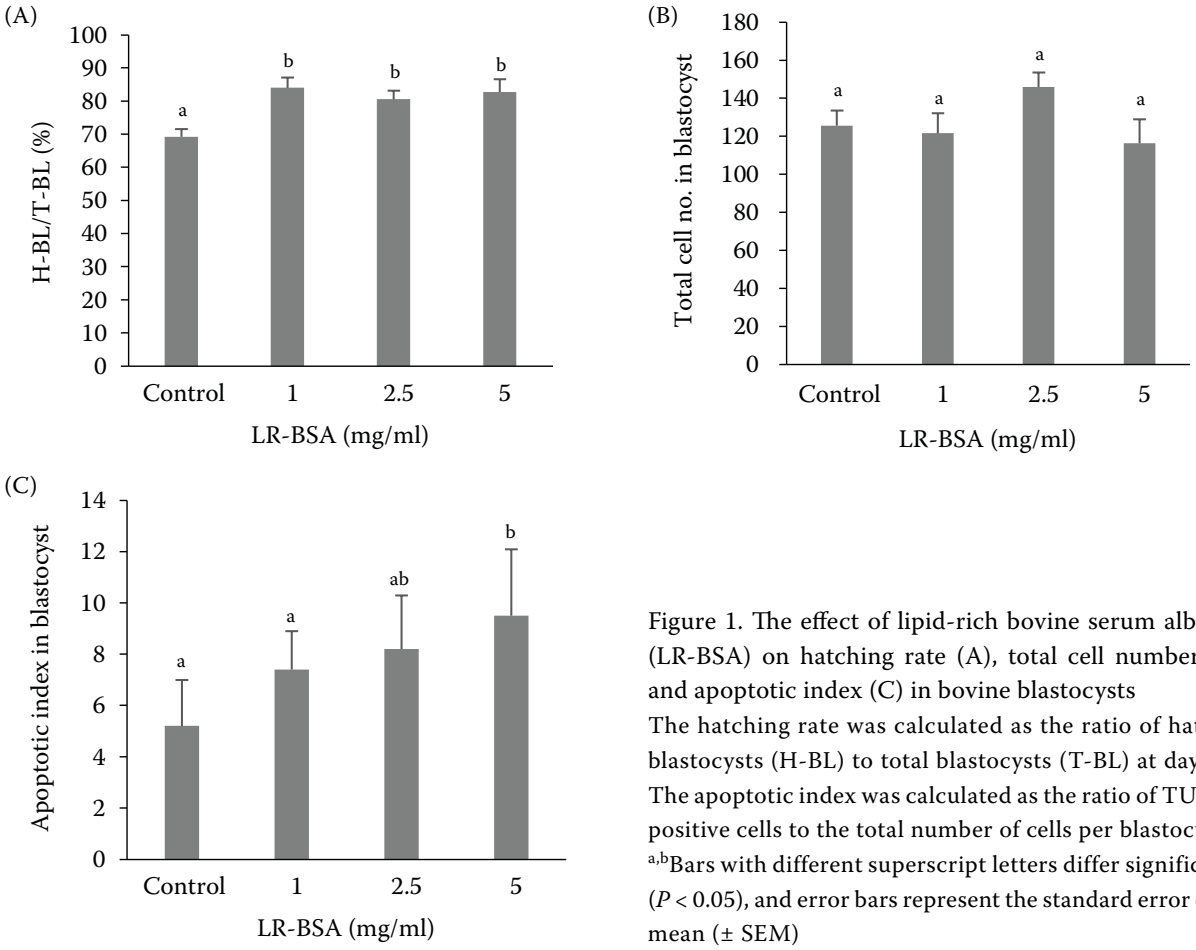

Figure 1. The effect of lipid-rich bovine serum albumin (LR-BSA) on hatching rate (A), total cell numbers (B) and apoptotic index (C) in bovine blastocysts

The hatching rate was calculated as the ratio of hatched blastocysts (H-BL) to total blastocysts (T-BL) at day 9 pi. The apoptotic index was calculated as the ratio of TUNEL-positive cells to the total number of cells per blastocyst

[a,b]Bars with different superscript letters differ significantly ($P < 0.05$), and error bars represent the standard error of the mean (± SEM)

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TUNEL analysis was performed using blastocysts on day 9 pi of culture to examine the apoptotic index in blastocysts. The total number of cells in the blastocysts was analysed simultaneously (Figures 1B and 1C). There were no significant differences in the total cell numbers in the blastocysts on day 9 pi (122.2 ± 27.4~142.1 ± 33.2). The apoptotic index in the 5.0LR-BSA (9.0 ± 2.2) differed significantly from the control ($P < 0.05$, Figure 1C).

Oct4 gene expression in the 2.5LR-BSA group was significantly higher than in all other groups, including the control ($P < 0.05$, Figure 2). *Sox2* expression was significantly higher in all LR-BSA treatment groups ($P < 0.05$), with the highest expression in the 2.5LR-BSA group ($P < 0.05$). The *plac8* gene is an important marker related to the survival of embryos and is primarily used to predict embryo quality in IVP. 2.5 and 5.0LR-BSA group showed significantly higher *plac8* expression than the control and 1.0LR-BSA ($P < 0.05$). Expression of *Serbf1* was significantly higher than the control in all treatment groups ($P < 0.05$).

The fluorescence intensity of LDs and cytoplasmic lipid contents in bovine blastocysts derived from different culture conditions were analysed (Figure 3). The blastocysts from the control ($n = 10$) and 5.0 LR-BSA group ($n = 10$) were stained using BODIPY 493/503 dye to evaluate lipid distribution and cytoplasmic lipid contents in the blastocysts. As shown in Figure 3B, the fluorescence intensity of BODIPY 493/503 dye in the 5.0LR-BSA group was significantly higher than in the control ($P < 0.05$).

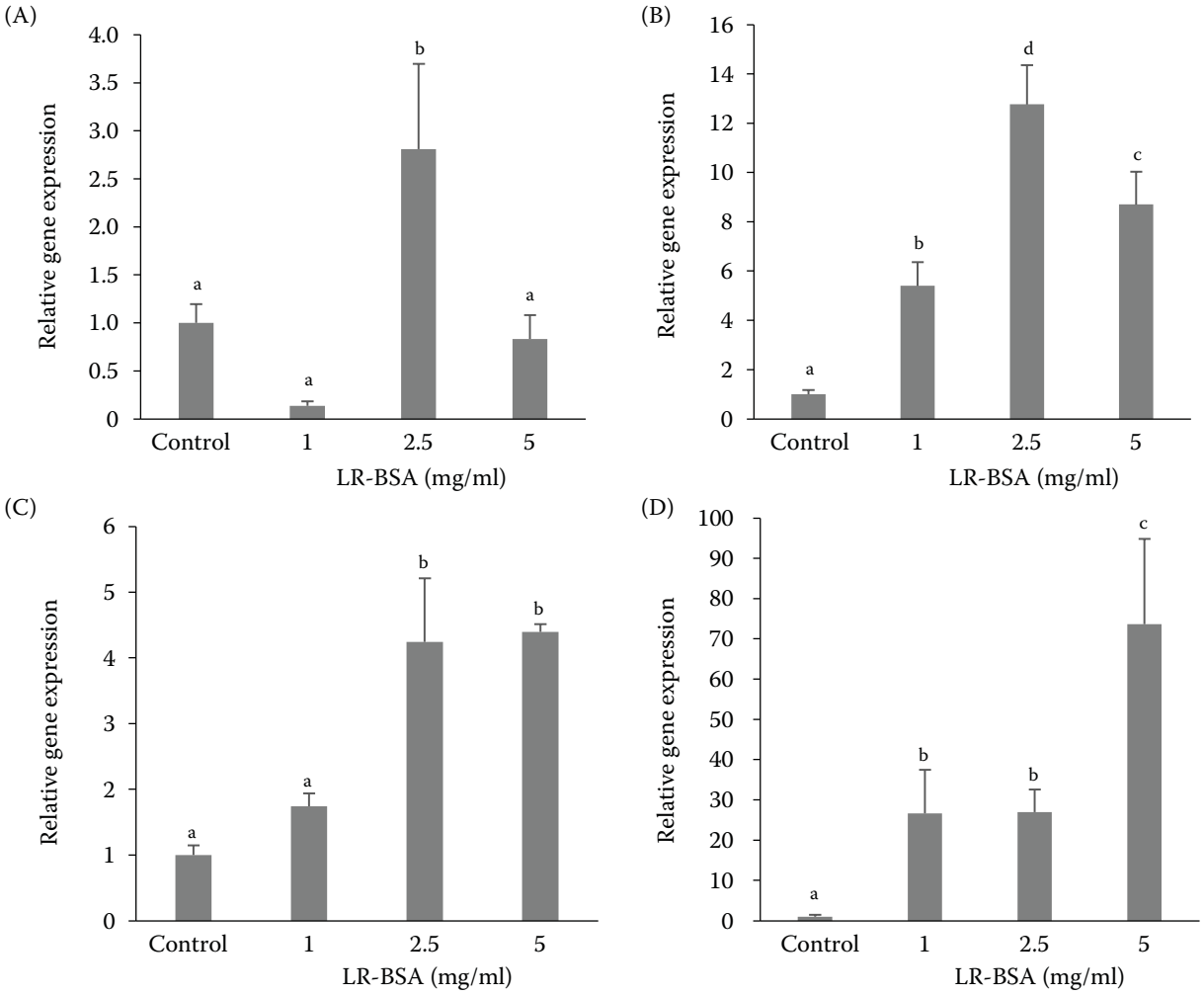

Figure 2. Relative gene expression in bovine blastocysts cultured in a medium supplemented with FBS (control) and lipid-rich bovine serum albumin (LR-BSA)

RNA was extracted from individual blastocysts (five from each treatment group at day nine pi)

[a–d]Bars with different superscript letters differ significantly ($P < 0.05$), and error bars represent the standard error of the mean (± SEM)

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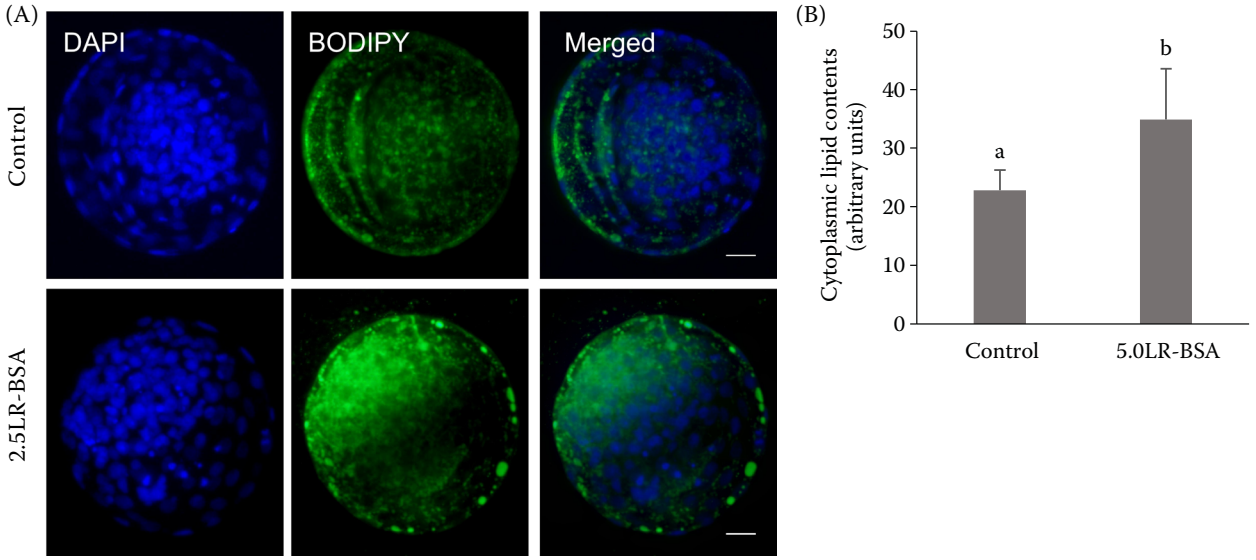

Figure 3. Fluorescence images of lipid staining (A) and cytoplasmic lipid contents (B) in bovine blastocysts derived from different culture conditions

Blastocysts from control (n = 10) and the 5.0 lipid-rich bovine serum albumin (LR-BSA) group (n = 10) were stained using BODIPY 493/503 dye (green) and DAPI (blue) to evaluate lipid distribution and cytoplasmic lipid contents in embryos

[a,b]Bars with different superscript letters differ significantly ($P < 0.05$), and error bars represent the standard error of the mean (± SEM)

Scale bar: 50 μm

DISCUSSION

The present study was performed to investigate the effects of LR-BSA added to the IVC media (1, 2.5, 5 mg/ml dosages, respectively) on early embryo development and LDs distribution. The cleavage rate on day two pi did not differ between the treatment groups. In contrast, the observed developmental rate of blastocysts formed on day 9 post-insemination of culture was higher in the 2.5LR-BSA group than in the control group. The hatching rate of blastocysts developed on day 9 pi was higher in all of the LR-BSA treatments than the control, consistent with results from IVF pig embryos (Suzuki et al. 2016). The effect of LR-BSA on the self-renewal of stem cells has been reported to be caused by the lipids in LR-BSA, not by albumin (Garcia-Gonzalo and Izpisua Belmonte 2008). Exogenous supplied fatty acids enhance the growth and development of embryos in various animals, which use exogenous fatty acids between the 8-cell and the late blastocyst stages to prepare for blastocyst hatching (Sudano et al. 2016). The total number of cells in the resulting blastocysts is reduced if embryos are cultured in an IVC culture medium with added fatty acid inhibitors (Hewitson et al. 1996).

A comparison of developmental status demonstrated that LR-BSA treatment groups progressed faster after cleavage than did the control. Additionally, blastocyst cell numbers did not differ among groups, but the apoptotic index in the 5.0LR-BSA was higher than the control. Notably, an increased cell number in blastocysts facilitates the expansion and hatching of blastocysts (Montag et al. 2000). It has also been reported that the content of ATP in blastocysts produced *in vitro* increased rapidly during the hatching period. ATP is the energy source of all cells and is known to be a major factor in embryo development (Stojkovic et al. 2001). In order to determine whether the cause of the high apoptotic index in the 5.0LR-BSA group was related to the cytoplasmic LDs, the LD distribution in blastocysts of the control and 5.0LR-BSA group were compared. A higher intensity of LDs was observed in the 5.0LR-BSA group than in the control. Under conditions of high levels of intracellular lipids, free fatty acids undergo lipid peroxidation within the cytoplasm, producing excess reactive oxygen species (ROS) and toxic lipid peroxides and depleting protective glutathione levels in a process known as lipotoxicity (Igosheva et al. 2010).

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Furthermore, excess ROS is highly cytotoxic and is harmful to mitochondrial and nuclear DNA, leading to oocyte mitochondrial and spindle abnormalities (Luzzo et al. 2012; Bradley and Swann 2019). It is postulated that a high concentration of LR-BSA can induce DNA fragmentations during embryo development *in vitro*, but fatty acids added by LR-BSA may be utilised as an energy source to increase ATP. As a result, the proliferation of cells in the blastocysts increases, which then increases hatching rates.

Oct4 mRNA expression was higher in the 2.5LR-BSA group than in all other treatment groups. The expression of pluripotency-related factors is known to affect the development of early embryos (Eckardt and McLaughlin 2004), so it is utilised as a qualitative indicator for embryos (Jeong et al. 2013). In bovine embryos, the expression of *Oct4* begins after zygotic genome activation and increases rapidly in morula (Kurosaka et al. 2004). Oct4 inhibits the expression of extraembryonic lineage-specific genes in trophectoderm cells during early embryogenesis, which induces active cell proliferation before implantation. Plac8 is a protein with a molecular weight of 12.5 kDa, and its intracellular distribution is dynamically and highly regulated in a developmental stage-dependent manner (Li et al. 2014). A high expression of *Plac8* has been reported in bovine blastocysts that succeeded in pregnancy, compared with those that were reabsorbed, and in the endometrium of pregnant cows compared with lower expression in nonpregnant ones (Galaviz-Hernandez et al. 2003; Silva et al. 2013). In this study, the expression levels of *Plac8* in the 2.5LR-BSA and 5.0LR-BSA groups were higher than those seen in the control and 1.0LR-BSA groups. Cytoplasmic LDs are known to affect the development of early embryos and the cryotolerance of blastocysts (Sudano et al. 2011). The SREBF1 proteins are crucial in regulating lipid metabolism (Shimano and Sato 2017). Berberine promotes lipid metabolism in porcine oocytes by down-regulating SREBF1 and peroxisome proliferator-activated receptor gamma (PPARG), thus improving the IVM of porcine oocytes (Dai et al. 2021). In the present study, the expression level of *Srebf1* mRNA increased as LR-BSA increased. Given the results and the information presented above, it can be assumed that adding LR-BSA to the culture medium could improve blastocysts production and promote cytoplasmic LD formation.

CONCLUSION

In conclusion, this study confirmed that adding LR-BSA (2.5 mg/ml) to IVC medium has beneficial effects on the quality of blastocysts and the production efficiency of bovine blastocysts. However, the treatment with a high concentration of LR-BSA affects the quality of blastocysts, inducing high levels of cytoplasmic lipids and DNA fragmentation. LR-BSA might function as an energy source for developing and hatching bovine embryos. However, high lipid content may have a negative effect on mitochondrial function, which likely affects lipid metabolism. Further research is needed to understand the relationships between LR-BSA components, energy metabolism and mitochondrial function.

Conflict of interest

The authors declare no conflict of interest.

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