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Original article

The differential requirement of albumin and sodium citrate on the development of in vitro produced bovine embryos

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Abstract – In vitro culture for bovine embryos is largely not optimal. Our study was to determine the components necessary for early embryo development. In experiment 1, IVF embryos were cultured for two days in CR1aa medium containing sodium citrate and BSA from two sources (Sigma vs. ICPbio), subsequently for additional five days with cumulus monolayer in 10% FBS CR1aa. We found that supplementation with both Sigma-BSA and sodium citrate significantly increased total blastocyst (BL) development compared with the ICPbio-BSA groups (37% vs. 19–21%), and enhanced the total number of high quality (C1 BL, IETS standard) blastocysts (26% vs. 11–17%) ($P < 0.05$). In experiment 2 with serum free and/or somatic free culture, we found that CR1aa culture can support a comparable embryo development with a supplement of Sigma BSA. The addition of sodium citrate did not increase blastocyst development in either the Sigma-BSA or the ICPbio-BSA groups. An inferior blastocyst development occurring in ICPbio-BSA culture (1–3%) could be rescued by culture in CR1aa supplemented with 10% FBS (29%), more importantly, by culture in CR1aa with a replacement of Sigma BSA (24%) ($P < 0.05$). C1 blastocysts rescued by FBS and Sigma BSA in ICPbio-BSA culture possessed indistinguishable morphology to embryos developed in a Sigma-BSA, FBS and somatic co-culture system, showing similar cell number/blastocyst (129–180, $P > 0.05$). Our study found a beneficial effect of sodium citrate and BSA on the in vitro development of bovine IVF embryos during co-culture. We also determined that differential embryotrophic factor(s) contained in BSA and serum, probably not sodium citrate, is necessary for promoting competent morula and blastocyst development in cattle.

bovine IVF / BSA / sodium citrate / in vitro culture / embryo development

1. INTRODUCTION

The determination of components necessary to facilitate embryonic development in vitro is essential for the study of the mechanisms involved in early embryo development. However, there has not been a complete culture system optimized for supporting a competent pre-implantational development of IVF embryos in cattle. During early

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study of bovine embryo culture, embryonic development was hampered, and encountered an 8–16 stage block due to an inefficient culture condition. In the 1980's, the system of co-culturing bovine embryos with somatic cells was subsequently widely used to enhance development, and overcome this developmental block [1]. Somatic cells, such as oviduct epithelial or cumulus cells, provide unknown metabolic requirements and promoting factors that facilitate a high rate of blastocyst development. Nevertheless, IVF derived animals manifested the large offspring syndrome in sheep and bovine, which was possibly caused by the addition of serum and somatic cells in co-culture medium [2, 3]. Serum can provide proteins and other beneficial factors, such as growth factors, vitamins, amino acids, minerals, and energy substrates; however, it is known that serum inhibits the first cleavage division of bovine embryos although it promotes their blastocyst development [4]. Recently, the excess accumulation of abnormal cytoplasmic lipid droplets was found in bovine IVF embryos cultured in serum-supplemented medium [5]. Therefore, developing a capable embryo culture with somatic co-culture-free or even serum-free in the medium becomes an assurance for IVF embryos competent for cryopreservation and embryo transfer [5, 6]. Chemically defined and/or semi-defined media [7–10] such as the C. Rosenkrans medium (CR1) [11, 12], synthetic oviduct fluid (SOF), [13–15] and KSOM [16, 17], have been developed in an attempt to satisfy the metabolic requirements for embryonic development in vitro. Since then, research has focused on the selection of culture medium, amino acids, metabolic substrates, growth factors and protein supplementation for successful embryo production in vitro [15, 17–22].

Although the supplementation with a protein source is not absolutely necessary in a defined chemical medium [7, 16, 17], it has been shown that the complete lack of proteins in the medium causes inconsistent embryo development in vitro [23, 24]. The addition of the serum's biological derivative, bovine serum albumin (BSA), to protein free cultures reverses developmental inconsistency [20, 21, 25] and facilitates to a low extent of embryonic lipid inclusions in cultured embryos [5]. However, variations in commercially available BSA due to batch discrepancies and differences in methods of preparation have reinforced the difficulty of studying its function in embryo development [26–28]. The reason for batch discrepancies of albumins in promoting embryotrophic properties is due, at least in part, to the presence of contaminants. Gray et al. [27] found that commercial BSA was heavily contaminated by citrate that stimulated rabbit embryonic cell proliferation and the expansion of blastocysts. Citrate was thus found to be a prominent contaminant in commercial BSA that stimulates fatty acid synthesis, and is an important chelator of heavy metal ions [9, 29]. This may be necessary for sustaining junctional integrity and promoting compaction and blastocoel formation [15].

The objectives of this study were, first, to evaluate the effects of albumin (Sigma vs. ICPbio) and sodium citrate, a known albumin contaminant, on the development of co-cultured bovine embryos. Secondly, under complete somatic and serum-free culture conditions, we examined the direct effect of BSA and sodium citrate on the subsequent development of in vitro produced bovine embryos. We report here, an effect of sodium citrate and BSA on the in vitro development of bovine IVF embryos during cumulus co-culture. We also determined that another embryotrophic factor, probably not sodium citrate, retained in the extraction of BSA and present in bovine fetal serum is necessary for promotion of competent morula compaction and blastocyst development in cattle.

2. MATERIALS AND METHODS

2.1. Collection, maturation and fertilization of oocytes in vitro

Chemicals for in vitro maturation/fertilization/culture in cattle were purchased from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise indicated.

The maturation medium consisted of Medium 199 (M199) with Earle's salts, L-glutamine, 2.2 $g \cdot L^{-1}$ sodium bicarbonate, and 25 mM HEPES (Gibco, 12340-014, Grand Island, NY) containing 7.5% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT, SH0070.03) supplemented with 0.5 $\mu g \cdot mL^{-1}$ ovine FSH (NIDDK), 5.0 $\mu g \cdot mL^{-1}$ ovine LH (NIDDK) and 1.0 $\mu g \cdot mL^{-1}$ estradiol (E-8875). The cumulus oocyte complexes (COCs) used in this study were aspirated from antral follicles of slaughterhouse ovaries as described previously [30]. Oocytes with at least four intact layers of cumulus cells were selected, washed three times in Dulbecco phosphate buffered saline (D-PBS; Gibco, 15240-013) supplemented with 0.1% polyvinyl alcohol (PVA; P-8136). Once washed in the maturation medium, the oocytes were transferred as groups of 20 to 25 per drop into Falcon 35 × 10 mm Petri dishes (Becton Dickinson, Franklin Lakes, NJ, 1008) containing 75 µL maturation medium covered with mineral oil, and subsequently cultured for 20–22 h in 5% CO_2 humidity saturated air at 39 °C.

Commercially available frozen semen (0.25 mL per straw) from a single ejaculate of a Holstein bull was used for the fertilization procedure. After 10 s gentle shaking in air (20 °C), the semen straw was thawed for 10 s in a 37 °C water-bath. Spermatozoa were washed in 8 mL Brackett and Oliphant solution (BO) [31], and medium was added containing 3 $mg \cdot mL^{-1}$ BSA (A-6003, see detail below) and 10 mM caffeine (P-0750) (BO sperm wash solution), before centrifugation for 8 min at 1000× *g*. The washing and centrifugation steps were repeated at least twice. The washed spermatozoa pellet was resuspended in BO sperm wash solution at a concentration of $1.0 \times 10^6 \cdot mL^{-1}$, ready for subsequent fertilization. After maturation, COCs with expanded cumulus cells were transferred, and washed twice in BO medium containing 6 $mg \cdot mL^{-1}$ BSA (A-6003) and 10 $\mu g \cdot mL^{-1}$ heparin (H-3125) (BO fertilization solution). The oocytes were allocated into groups of 20–25 and placed into a 50 µL drop of BO fertilization solution pre-equilibrated for two hours in 5% CO_2 at 39 °C. One 50 µL volume of sperm suspension with final sperm concentration of $0.5 \times 10^6 \cdot mL^{-1}$ was added to each oocyte-containing fertilization droplet before finally incubation for 6 h at 39 °C in 5% CO_2 in humidified air.

2.2. In vitro culture

The base culture medium for all embryos was defined as CR1 [6], consisting of 114.7 mM NaCl, 3.1 mM KCl, 26.2 mM $NaHCO_3$, 1 mM L-Glutamine, 0.4 mM sodium pyruvate (P-2256) and 5.5 mM hemicalcium lactate (L-4388). The CR1 medium was supplemented with 1X MEM (M-7145) and 1X BME amino acid (B-6766), and referred to as CR1aa. Following IVF, presumptive zygotes were stripped of cumulus cells by vortexing for 1 min in 100 µL 7.5% FBS M199 medium, and washed three times in M199 and twice in CR1aa medium. The embryos were then randomly allocated to different treatment groups according to the experimental design described below. Two sources of BSA, one from Sigma, A-6003, (essentially fatty acid free, initially fractionated from fraction V by cold alcohol precipitation), and another from ICPbio ABRZ-010 (ICPbio Living science, Auckland, New Zealand; freeze-dried powder irradiated to 2.5 MRads), were tested. The concentration of BSA was prepared as 6 $mg \cdot mL^{-1}$ for all treatments in the study. For all CR1aa culture media containing fetal bovine serum (FBS), 10% (v/v) FBS (Hyclone, SH0070.03) was used. For somatic cell co-culture of embryos, culture dishes used for maturation, which contained a layer of

Table I. Effect of BSA and sodium citrate on subsequent development of bovine IVP embryos under somatic co-culture.

Group	No. of replications	Treatment				No. of embryos	Cleavage (%)	6–8 Cell (%)	Morula (%)	D7 Blastocyst (%)	
		Days 1–2		Days 3–7							
		BSA	Sodium citrate (mM)	FBS (%)	Sodium citrate (mM)					Total BL	C1 BL
A	5	Sigma	0.34	10	0.34	818	565 (69)	473 (58)	424 (52)	299 (37)[a]	212 (26)[a]
B	5	Sigma	0	10	0	790	586 (74)	480 (61)	271 (34)	223 (28)[ab]	132 (17)[b]
C	5	ICPbio	0.34	10	0.34	406	278 (68)	237 (58)	144 (35)	84 (21)[b]	61 (15)[b]
D	5	ICPbio	0	10	0	619	452 (73)	362 (58)	158 (26)	116 (19)[b]	65 (11)[b]

[a,b] Values with different superscripts within columns are significantly different ($P < 0.05$). BL, blastocysts; BSA, bovine serum albumin; FBS, fetal bovine serum; C1, grade C1 blastocyst (IETS standard); D7, day 7 of culture. The BSA concentration was 6 $mg \cdot mL^{-1}$ in the treatments where supplementation of albumin was applied. Embryos in all treatments were cultured in CR1aa supplemented with corresponding BSA during Days 1–2, subsequently in additive 10% FBS CR1aa with a cumulus monolayer during Days 3–7 (5 days).

80–90% confluent cumulus cells, were washed and supplemented with 10% FBS CR1aa culture medium for embryo culture. All other cell-free or serum-free embryo culture was conducted in 4-well dishes (Nunc, Nalge Nunc International, NY, 176740) with 450 μL medium covered with 200 μL mineral oil. Whenever specified, 0.34 mM trisodium citrate (C-3434) was added. The embryos were cultured under either 5% CO_2, 5% O_2 and 90% N_2, or 5% O_2 in air with high humidity.

The percentage of cleavage to the two and six-eight cell stages, and subsequent development to the morula and blastocysts (BLs), were recorded on Day 2, Day 5 and Day 7, respectively. Expanded blastocysts with tight compaction and integrity of the inner cell mass were regarded as grade C1, large blastocysts as grade C2, according to the standard of the International Embryo Transfer Society (IETS, 2003). The total number of blastocysts is accumulated as a total of grade C1 and C2 embryos. The cell number of the blastocysts was evaluated by fluorescent microscopy following staining with 10 $\mu g \cdot mL^{-1}$ Hoechst 33342.

2.3. Experiment 1: Effect of sodium citrate and BSA source on IVF embryos co-cultured with somatic cells

The experiment, as described in Table I, was designed to determine the effect of supplemented BSA in serum-free CR1aa medium for the first two days culture on further developmental potential of IVF embryos co-cultured with cumulus cells. Briefly, the embryos were cultured for 2 days (Day 1–2) in CR1aa medium added with two different sources of BSA (Sigma and ICPbio) and two levels of sodium citrate (0, and 0.34 mM) under the humidified condition of 5% CO_2, 5% O_2 and 90% N_2 at 39 °C. Embryos cleaved to the 4–8 cell stage were subsequently washed three times in 10% FBS CR1aa, then cultured for an additional 5 days (Day 3–7) with a cumulus monolayer in 10% FBS CR1aa medium under 5% O_2 in the air. During additional somatic co-culture, BSA was removed while the sodium citrate treatment was continued according to the experimental design.

2.4. Experiment 2: Effect of sodium citrate, BSA and FBS on the development of IVF embryos using a somatic co-culture or serum free system

Since the effects of sodium citrate and BSA may be masked by the metabolic and physiological complexity from somatic monolayers, the second experiment was designed to evaluate the direct effect of sodium citrate and BSA on the developmental potential of IVF embryos in a somatic cell free system (Tab. II). Randomly selected presumptive zygotes were subjected to the following culture treatments supplemented with different sources of BSA, sodium citrate and/or FBS as follows: (A) complete serum-free culture in 6 $mg \cdot mL^{-1}$ Sigma-BSA CR1aa for 7 days, (B) complete serum-free culture in 6 $mg \cdot mL^{-1}$ Sigma-BSA, 0.34 mM sodium citrate CR1aa for 7 days, (C) culture in 6 $mg \cdot mL^{-1}$ Sigma-BSA for 2 days, then in 10% FBS CR1aa for an additional 5 days, (D) complete serum-free culture in 6 $mg \cdot mL^{-1}$ ICPbio-BSA CR1aa for 7 days, (E) complete serum-free culture in 6 $mg \cdot mL^{-1}$ ICPbio-BSA, 0.34 mM Sodium citrate CR1aa for 7 days, (F) culture in 6 $mg \cdot mL^{-1}$ ICPbio-BSA for two days, subsequent 5 days in 10% FBS CR1aa, (G) complete serum-free culture in 6 $mg \cdot mL^{-1}$ ICPbio-BSA CR1aa for 2 days, then 6 $mg \cdot mL^{-1}$ Sigma-BSA CR1aa for an additional 5 days. Except for Groups C, F, and G, the culture medium containing of designed supplements was not changed during the 7 days of in vitro culture. In groups (C) and (F), the CR1aa media was changed to 10% FBS after 48 h culture in CR1aa BSA in order to determine the effect of serum on embryo development. Group (G) was designed to determine whether any embryonic promoting factor

Table II. Effect of BSA, sodium citrate and FBS on the development of bovine IVP embryos under somatic free culture.

Group	No. of replications	Treatment					No. of embryos	Cleavage (%)	6–8 Cell (%)	Morula (%)	D7 Blastocyst (%)	
		Days 1–2		Days 3–7								
		BSA	Sodium citrate (mM)	BSA	FBS (%)	Sodium citrate (mM)					Total BL	C1 BL
A	4	Sigma	0	Sigma	0	0	158	125 (79)	99 (62)	53 (34)[a]	32 (20)[a]	23 (15)[a]
B	4	Sigma	0.34	Sigma	0	0.34	156	124 (80)	104 (67)	35 (22)[b]	28 (18)[a]	22 (14)[a]
C	4	Sigma	0	0	10	0	157	127 (81)	101 (64)	64 (41)[a]	48 (31)[a]	42 (27)[a]
D	4	ICPbio	0	ICPbio	0	0	161	124 (77)	98 (61)	16 (10)[c]	1 (1)[b]	0 (0)[b]
E	4	ICPbio	0.34	ICPbio	0	0.34	158	126 (80)	101 (64)	18 (11)[c]	5 (3)[b]	0 (0)[b]
F	4	ICPbio	0	0	10	0	158	124 (79)	101 (64)	50 (32)[ab]	45 (29)[a]	32 (20)[a]
G	4	ICPbio	0	Sigma	0	0	165	134 (81)	99 (60)	53 (32)[ab]	40 (24)[a]	36 (22)[a]

[a,b,c] Values with different superscripts within columns are significantly different ($P < 0.05$). BL, blastocysts; BSA, bovine serum albumin; C1, grade C1 blastocyst (IETS standard); D7, day 7 of culture; FBS, fetal bovine serum. The BSA concentration was 6 $mg \cdot mL^{-1}$ in the treatments. In Groups A, B, D, and E the embryos were continuously cultured in corresponding serum-free CR1aa medium for 7 days. The embryos in Groups C and F were first cultured in serum-free CR1aa-BSA during Days 1–2, subsequently in 10% FBS additive CR1aa for 5 days. The embryos in Group G cultured in ICPbio BSA CR1aa during Days 1–2, subsequently in CR1aa medium supplemented with Sigma BSA for an additional 5 days.

remained in the Sigma prepared BSA, but was absent in ICPbio-BSA.

2.5. Statistical analysis

Each experiment was repeated five times. The treatment effects on cleavage, and development to morulae and blastocysts were statistically analyzed using a General Linear Model (GLM, Univariate, SPSS 9.0, SPSS Inc., Chicago, IL60606) or student *t*-test; the mean number of nuclei per blastocyst for each treatment was compared by one-way ANOVA. *P* values less than 0.05 are considered significant.

3. RESULTS

3.1. Experiment 1

A beneficial effect of sodium citrate and the BSA source on the development of IVF embryos was shown when embryos were co-cultured with a somatic cell monolayer during the last 5 days of culture (Tab. I). In this experiment, sodium citrate was contained throughout seven-day culture while BSA was initially supplemented in CR1aa during the first two-day culture, subsequently replaced by the addition of FBS. There were no significant differences ($P > 0.05$) in the four treatments regarding the rates of cleavage (68–74%), 6–8 cell (58–61%), or morula (26–52%), and total blastocyst rate between the Sigma-BSA groups (28% vs. 37%) and the ICPbio-BSA groups (19% vs. 21%). However, CR1aa medium supplemented with both Sigma-BSA and sodium citrate significantly increased total blastocyst development when compared with ICPbio-BSA (37% vs. 19–28%, $P < 0.05$). Furthermore, a significant difference was found in that the CR1aa medium with both Sigma-BSA and sodium citrate added resulted in the highest percentage of C1 quality blastocysts (26% vs. 11–17%, Tab. I).

3.2. Experiment 2

We then determined the effect of the BSA source and levels of sodium citrate on embryo development in a serum free or somatic-cell free system. Embryos cultured in both ICPbio-BSA (Fig. 1A) and Sigma-BSA for 2 days had cleaved normally to the 6–8 cell stage (Tab. II). In the serum free system, when Sigma-BSA (Group A and B) was used (Tab. II), we found significant improvements over ICPbio-BSA (Group D and E) ($P < 0.05$) on the development to morulae (22–34% vs. 10–11%), total blastocysts (18–20% vs. 1–3%), and C1 blastocysts (14–15% vs. 0%) (Fig 1B), respectively. Furthermore, the addition of sodium citrate did not significantly increase blastocyst development in either the Sigma-BSA or the ICPbio-BSA groups. Continuous culture of embryos in ICPbio-BSA for 7 days resulted in significantly lower development of blastocysts (Tab. II) with inferior morphology (Fig. 1D). Most of the embryos in ICPbio-BSA culture terminated at the 8–16 cell stage indicating a typical developmental block (Fig. 1D). Although FBS did not improve blastocyst development in the Sigma-BSA group (Group C), its inclusion along with ICPbio-BSA in culture (Group F) significantly increased the total blastocyst rate (29% vs. 1–3%, $P < 0.05$) and the number of C1 grade blastocyst (20% vs. 0%, $P < 0.05$). More interestingly, in a serum free system (Group G), when ICPbio-BSA was replaced by Sigma-BSA with the same concentration of 6 $mg \cdot mL^{-1}$ after embryos were cultured in CR1aa ICPbio-BSA for two days, a developmental rescue was observed since blastocyst development of the ICPbio-BSA treated embryos (Fig. 1E) was at levels comparable to those supported by FBS (Group F) and Sigma-BSA without the addition of FBS (Group A) (Fig. 1B).

3.3. Nuclear analysis of blastocysts derived from different cultures

As shown in Table III, following embryo staining and epi-fluorescent microscopy, the total number of nuclei in expanded C1 grade blastocysts between sigma-BSA groups was not significantly different, regardless whether the embryos were derived from serum free

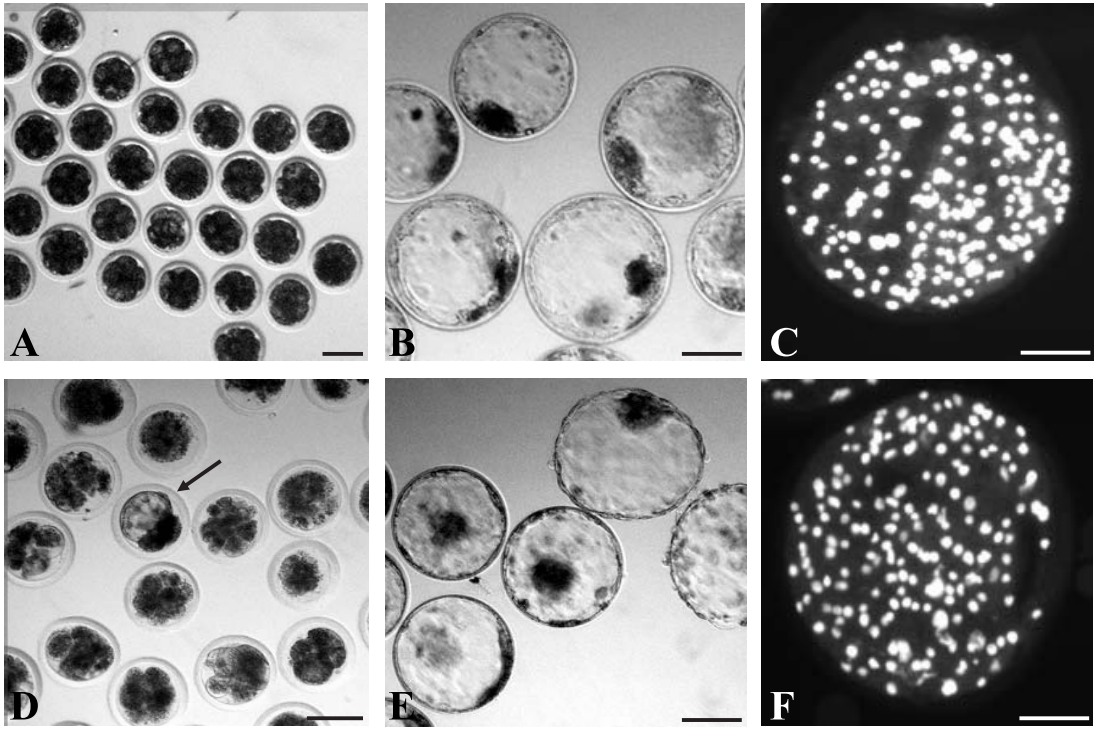

Figure 1. In vitro development of bovine embryos cultured in ICPbio and Sigma BSA. Fertilized bovine embryos show morphologically normal 6–8 cell stage (**A**) after culture for 2 days in CR1aa supplemented with 6 mg·mL^{-1} ICPbio-BSA. After continuous culture in ICPbio-BSA medium for another 5 days, most embryo development has been terminated and shows 8–16-cell developmental block and fragmented cell debris (**D**), an arrow indicating a low-grade early blastocyst with some fragmented cells attached. However, when ICPbio-BSA is replaced with Sigma BSA in CR1aa medium post a two-day culture in CR1aa supplemented with ICPbio-BSA, embryos can resume to develop into expanded blastocysts (**E**) on Day 7 with a morphology indistinguishable from those cultured for a continuous seven days in Sigma-BSA CR1aa medium (**B**). Those blastocysts (**E**) rescued via Sigma BSA had possessed a similar cell number, shown as (**F**) to those derived from a complete Sigma BSA culture, shown as (**C**), by fluorescent microscopy. Bar = 100 μm.

Table III. Nuclear counting of IVP blastocysts derived from different culture systems.

Blastocyst (D7)	No. embryos	Cells/BL	No. (%) pycnotic cells
Sigma BSA[1]	15	129 ± 8[a]	4 ± 0.5 (3)[a]
Sigma BSA/FBS[1]	14	157 ± 10[a]	6 ± 1 (4)[a]
Sigma BSA/FBS/co-culture[2]	16	134 ± 13[a]	5 ± 1 (4)[a]
ICPbio BSA/FBS[1]	15	173 ± 7[a]	8 ± 1 (5)[a]
ICPbio BSA/FBS/co-culture[2]	22	180 ± 15[a]	6 ± 1 (3)[a]
ICPbio BSA/Sigma BSA[1]	14	134 ± 9[a]	5 ± 2 (4)[a]

[a] Values within columns with the same superscript do not differ, $P > 0.05$.
[1] Embryos were continuously cultured at 39 °C in 5% CO_2, 5% O_2 and 90% N_2 for 7 days.
[2] Embryos were cultured at 39 °C in 5% CO_2, 5% O_2 and 90% N_2 for 2 days, subsequently 5 days at 5% CO_2 in air. Sigma BSA, embryos were continuously cultured in 6 mg·mL^{-1} Sigma BSA CR1aa for 7 days under serum/somatic free culture. Sigma BSA/FBS, embryos were cultured in 6 mg·mL^{-1} Sigma BSA CR1aa for 2 days, subsequently 5 days in 10% FBS CR1aa but under somatic-free culture. Sigma BSA/FBS/co-culture, embryos were cultured for 2 days in 6 mg·mL^{-1} Sigma BSA CR1aa, subsequently 5 days in 10% FBS CR1aa under somatic co-culture. ICPbio BSA/FBS, culture same as that used in Sigma BSA/FBS treatment except for ICPbio BSA was used. ICPbio BSA/FBS/co-culture, culture same as that used in Sigma BSA/FBS/co-culture treatment except for ICPbio BSA was used. ICPbio BSA/Sigma BSA, embryos were cultured in CR1aa supplemented with ICPbio BSA for two days, subsequently in Sigma BSA CR1aa for an additional 5 days under serum/somatic free culture.

(Fig. 1 C), serum supplemented in somatic free culture, or FBS somatic co-culture after initial two-day culture in BSA CR1aa. None of the expanded C1 blastocysts were developed and available for staining from the culture group in CR1aa with ICPbio-BSA supplement alone. No differences was found regarding the total nuclear number between ICPbio-BSA treated blastocysts that were developed from a rescued culture system supplemented with either Sigma-BSA (Fig. 1 F), FBS or FBS surplus somatic co-culture (Tab. III).

4. DISCUSSION

Our study has demonstrated a beneficial effect of sodium citrate and BSA on the development of bovine IVF embryos when embryos were cultured with somatic cells, with BSA from the Sigma Chemical Company. This positive correlation appeared at the post-compaction stage, leading to a higher quality of derived blastocysts. Citrate was one of the cell growth stimulating agents discovered as a significant contaminant in preparations of BSA [24, 27]. Indeed, sodium citrate added to SOF defined medium has been reported to enhance blastocyst development [15, 24]. The mechanism(s) involved has yet to be revealed, although the fact that citrate stimulates fatty acid synthesis during embryonic development is noteworthy [29, 32, 33]. In addition, citrate is a chelator of some heavy metal ions [9], thereby acting to eliminate the risk of toxicity to the embryos posed by these ions. Heavy metal chelation can also promote the maintenance of cellular junctional integrity that is important for compaction and blastocoel formation [15, 27]. Sodium citrate has played a role as an energy substrate. Lane et al. [34] have found that sodium citrate promotes blastocyst expansion and hatching, and the beneficial effect of citrate depends on the type of albumin used [34]. Interestingly, on the contrary to the report of Holm et al. [15], the beneficial effect of sodium citrate was not apparent in our CR1aa-BSA/cell-free-culture system. Holm et al. [15] demonstrated that the addition of citrate and myo-inositol under defined SOF culture condition improved blastocyst development to a rate comparable to that obtained with serum

and co-culture. Therefore, we propose that the beneficial effect of sodium citrate on embryonic development becomes significant during co-culture where it acts by absorbing potential metabolites and toxicants secreted by the somatic cells, assuming these toxic substances are present in concentrations high enough to be detrimental. Moreover, citrate becomes ineffective in somatic-cell-free culture systems where the extent of toxicants is under the threshold of a harmful level.

Bovine serum albumin (BSA) has been found in high concentrations within cattle blood and reproductive tracts [21, 25]. BSA functions as a surfactant and nutrient in embryo culture in vitro and improves embryo post-compaction development in mice and cattle [25, 34–37]. We found that blastocyst development from serum-free, but Sigma-BSA containing CR1aa medium showed comparable levels of blastocyst development to those with serum or somatic co-culture. This result was in accordance to that of Krisher et al. [10] where a complete removal of serum from the culture medium, during the final stages of culture, while continuing to supply BSA resulted in a development equivalent to that seen with serum.

However, the use of BSA produced by different isolating methods and from different lots, compounds the studies of the basic requirements for competent embryonic development in vitro [28], and some batches of BSA have even been found to be toxic [8]. We tested BSA from two commercial sources, Sigma and ICPbio, and found that Sigma-BSA supports a higher blastocyst formation than does the ICPbio-BSA. The possibility that BSA from ICPbio contains embryonic inhibiting factors is low, because ICPbio BSA is purified and certified as being low in endotoxin; in addition, it is widely used as a surfactant in preparing media for embryo transfer as well as for culture of bovine embryos in vitro [15]. We therefore hypothesize that the compound necessary for the development of morulae to blastocysts is present in Sigma-BSA but is lacking in ICPbio-BSA. Both ICPbio-BSA and Sigma-BSA are fatty-acid free. ICPbio-BSA is chromatographically fractionated, resulting in a minimal denaturation of the albumin structure, followed by charcoal treatment to remove endotoxin. On the contrary, Sigma-BSA is isolated by alcohol precipitation and charcoal treatment [38]. It is believed that the removal of impurities may not be complete by organic solvent precipitation and charcoal [38, 39], and that varying amounts of impurities remain in BSA prepared by this method. Although chromatographical fractionation can give rise to much purer albumin, some associated molecules can also be removed during this process. The observation that BSA from ICPbio supported lower blastocyst formation may result from the likelihood that certain compounds, required for embryo development, were removed during its preparation. However, the effect of the missing compound can be compensated for by the addition of FBS into the media during Days 3–7. More importantly, in experiment 2, replacement of ICPbio-BSA with Sigma-BSA in serum-free CR1aa medium (Group G) during Days 3–7 of culture resulted in a rescued blastocyst development similar to that observed with FBS, clearly indicating that the embryonic promoting factor is retained in Sigma prepared albumin as well as serum. Interestingly, the missing compound did not seem to affect cleavage development, and we did not observe any difference in embryo development up to the 8-cell stage. The exact nature of the missing compound is very unlikely to be sodium citrate because the addition of sodium citrate in ICPbio-BSA did not improve blastocyst development in either somatic co-culture or cell-free systems. Holm et al. [15] demonstrated an acceptable level of blastocyst development by using ICPbio-BSA in modified SOF medium that is in contrast to the results in our study with CR1aa basal medium. This discrepancy is probably due to the differences between specific in vitro maturation/fertilization/culture conditions, including those of medium composition, ionic and metabolic

balance between modified SOF and CR1aa. C. Wrenzycki et al. [40] proposed that basic medium composition has a profound effect on the amounts of specific transcripts in bovine embryos. Compared to modified SOF medium [13, 15], CR1aa is a much simpler medium formula specific for the study of physiological requirements of bovine embryos in vitro such as the metabolism of amino acids and vitamins [12] and energy substrates [11]. Potassium phosphate, magnesium sulfate and myo-inositol, a categorized vitamin that prepared in modified SOF medium were not included in the CR1aa medium during our study. Myo-inositol was likely to be one contaminant present in commercially prepared BSA that is essential for cellular signaling [41] and embryonic development [15]. Imbalance of the intracellular ionic environment [42] and altered calcium homeostasis [43] are also associated with disrupted and impaired developmental competence and cellular organization in hamster embryos. The other possible components for compounds that support embryo development include the following: growth factors such as epidermal growth factor (EGF) [44, 45], FDF [46], platelet-derived growth factor (PDGF) [35, 47], cysteamine [48], acetoacetate/D-beta-hydroxybutyrate [22]. It appears that the alcohol precipitated BSA, although less pure, retained more of this unidentified compound necessary for embryo growth.

A significant finding of this study is that the missing compound in ICPbio-BSA does not appear to be necessary for cleavage divisions. Due to the fact that embryos incubated in continuous ICPbio-BSA CR1aa have shown developmental termination and degenerative processes at the 8–16 cell stage (Fig. 1 D), we propose that the extent of this embryonic promoting factor seems to be necessary for embryos overcoming the 8–16 cell block, and to facilitate specifically morula compaction and blastocyst formation. Additionally, blastocyst development of embryos cultured for two days in BSA from ICPbio was similar to that of those cultured in Sigma-BSA when FBS, rather than ICPbio-BSA, was used during a later stage of embryo development. This suggests that ICPbio-BSA does not affect embryo cleavage division during the first two days of culture, and that there is no lingering effect on the subsequent development from early culture in this BSA. The identification of these compounds, necessary for further embryonic development, is important for the improvement of the bovine embryo culture system.

Although co-culture shows a beneficial effect on bovine embryo development [1, 48, 49], this culture condition remains largely undefined. The variability and complexity of co-culture systems makes it problematical to define the specific nutritional requirements for embryos. In addition, employing serum or co-culture may introduce unknown toxic components or pathogens [10, 19]. Abe et al. [5] reported a significant accumulation of intracellular granules and lipid vesicles in blastocysts when serum was included in the culture medium. These data are indicative of an adverse effect of serum on the morphology of embryos and may be related to subsequent abnormal fetal development, such as the large offerspring syndrome [2, 3, 50, 51], and fetal loss following conception [21, 52]. Replacing fetal calf serum with BSA as a protein source in a variety of culture media, along with using somatic-cell free culture, has provided an effective alternative for the successful development of IVF and nuclear transfer embryos [10, 52, 53]. The nuclear and cell counting revealed that embryos derived from serum-free CR1aa culture either with supplement of continuous Sigma-BSA or ICPbio-BSA/Sigma-BSA regime possessed a similar cell number to those embryos produced from serum additive culture or somatic co-culture. This suggests that our CR1aa with serum free culture is suitable for producing competent or at least embryos comparable to those derived from the culture with serum or somatic cells. Our embryo transfer trial at TransOva Genetics, Iowa, USA showed (by ultrasound examination) the pregnancy rate of vitrified/thawed IVF embryos, produced by either BSA or BSA/FBS culture

systems, to be as high as 76% (16/21) on Day 60 after embryo transfer [54]. Further studies with this culture system on pregnancy and calving rates are under investigation.

In summary, we demonstrated a beneficial effect of sodium citrate and Sigma-BSA on the development of bovine IVF embryos in CR1aa medium co-cultured with somatic cells. Bovine embryos produced in serum free CR1aa medium showed a competent blastocyst development with morphological and cytological features indistinguishable from those derived from serum-containing medium. Further experiments, using embryos cultured in a somatic-cell free system, revealed an unknown factor necessary for embryonic post-compaction development. This factor is also present in FBS and BSA from Sigma possibly as an unremoved contaminant while it is likely removed in ICPbio-BSA by chromatographical fractionation and related purifications. Future studies to identify the factor important for embryo post-compaction development of bovine IVF embryos are underway.

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