Theriogenology 140 (2019) 109–116

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Magnesium is a critical element for competent development of bovine embryos

Liyou An [a], Sadie L. Marjani [b], Zhisong Wang [a], Zhihui Liu [a], Ruihai Liu [c], Fei Xue [d], Jie Xu [d], Tshimangadzo L. Nedambale [e], Lan Yang [f], X. Cindy Tian [g], Lei Su [h, **], Fuliang Du [a, d, *]

[a] Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing, 210023, PR China
[b] Department of Biology, Central Connecticut State University, New Britain, CT, 06050, USA
[c] Department of Food Science, Cornell University, Ithaca, NY, 14853, USA
[d] Renova Life, Inc., College Park, MD, 20742, USA
[e] Tshwane University of Technology, Faculty of Science, Department of Animal Science, Private Bag X680, Pretoria, 0001, South Africa
[f] Lannuo Biotechnologies Wuxi Inc., Wuxi, 214000, PR China
[g] Department of Animal Science and Center for Regenerative Biology, University of Connecticut, Storrs, CT, 06269, USA
[h] Yunnan Zhongke Embryo Biotechnology Co., LTD, Kunming, Yunnan, 650505, PR China

ARTICLE INFO

Article history:
Received 15 May 2019
Received in revised form
19 July 2019
Accepted 12 August 2019
Available online 13 August 2019

Keywords:
In vitro fertilization (IVF)
Bovine serum albumin
Magnesium
Embryo development

ABSTRACT

The study was designed to determine the impact of magnesium (Mg^{2+}) on bovine embryo development. We found that two commercially available sources of bovine serum albumin (BSA) and fetal bovine serum (FBS) contained different amounts of Mg^{2+} residue: 4 ppm in ICPbio BSA, 114 ppm in Sigma BSA, and 44 ppm in FBS. When CR1 was used as basal medium, PVA and ICPbio BSA produced the lowest blastocyst yield (2.2–2.3%), whereas Sigma BSA increased blastocyst yield to 18.9% ($P < 0.05$). Supplementation of 1.4 mM $MgCl_2$ into the medium increased the blastocyst rate in the ICPbio BSA group (29.4%) but not in the PVA group (5.4%; $P < 0.05$) to a level comparable to that of the FBS group (33.7%; $P > 0.05$). We next found that increasing concentrations of $MgCl_2$ in the culture medium (ICPbio BSA) elevated blastocyst rate from 2.6% (0 mM), 38.4% (0.35 mM) to 50.2% (1.4 mM; $P < 0.05$), further maintained at 44.9% (2.1 mM) and 43.4% (2.8 mM) ($P > 0.05$). However, blastocyst rate was reduced to 31.4% (4.2 mM) and 29.4% (5.6 mM) when $MgCl_2$ supplement was increased ($P < 0.05$). Comparable blastocyst development was achieved in both ICPbio BSA (30.0–33.1%) and Sigma BSA (37.4–38.7%) groups when 1.4 mM Mg^{2+} was supplemented regardless of its source ($MgCl_2$ vs. $MgSO_4$; $P > 0.05$). In embryo transfer experiments, higher rates of pregnancy (54.3 vs. 41.5%) and calving (44.3 vs. 32.5%) were achieved in the CR1-Mg^{2+}-supplemented BSA group compared with the FBS group with co-culture, respectively ($P < 0.05$). These results demonstrate that Mg^{2+} is a key ion that promotes competent blastocyst and term development. Therefore, a simple and efficient defined medium (CR1-Mg^{2+}-BSA) can successfully replace complex serum and somatic cell co-culture.

1. Introduction

Successfully culturing fertilized embryos is important for the study of biological mechanisms controlling reproduction and embryo development. Several chemically defined or semi-defined media, such as C. Rosenkrans medium (CR1) [1–3], synthetic oviduct fluid [4–7], KSOM [8,9], and G1/G2 [10], have been developed to satisfy the metabolic requirements of embryonic development post fertilization in vitro (IVF) and to enable the discovery of basic biological mechanisms regulating pre-implantation embryonic development.

Magnesium (Mg^{2+}) is an important ion that maintains normal cellular physiology and function [11]. Mg^{2+} regulates calcium dynamics and hemostasis in cells and is a cofactor of many enzymes related to ATP binding, protein kinases, ion transport ATPases, and proteins for nucleic acid metabolism and RNA stability [11]. In the nucleus, Mg^{2+} is used for DNA polymerase, DNA repair, and

* Corresponding author. Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing, 210023, PR China.
** Corresponding author.
E-mail addresses: suleiynzk@aliyun.com (L. Su), fuliangd@njnu.edu.cn (F. Du).

https://doi.org/10.1016/j.theriogenology.2019.08.015
0093-691X/

structure maintenance [12,13]. However, the inherent requirement of Mg^{2+} for early embryo development and its impact on term development after embryo transfer (ET) are unknown.

We previously reported that bovine serum albumin (BSA) can support normal embryo development [14]. Also, variations in commercial BSA due to batch-to-batch differences can produce inconsistencies in embryo development [15]. Although a protein source in culture medium is not absolutely necessary, a complete lack of proteins in a defined chemical medium causes inconsistent embryo development in vitro [16,17]. Furthermore, it is uncertain whether commercially available BSA contains the key chemical components critical for successful embryo development without serum or cell co-culture.

In this study, we examined the embryonic-promoting chemical factors contained in two commercial sources of albumin, Sigma and ICPbio, which promote bovine pre-implantation embryo development. We next tested the effect of different Mg^{2+} concentrations on the development and sex ratio of bovine embryos under somatic cell-free, serum-free culture. Finally, we transferred Mg^{2+}-derived embryos into recipients to determine their full-term development.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All oocytes and resultant embryos were cultured in specified media at 39 °C in 5% CO_2 and humidified air unless otherwise noted. All animal experiments were approved by the Animal Care and Use Committee of Nanjing Normal University and complied with guidelines and standards of the Society for the Study of Reproduction.

2.1. Spectrometric mineral analysis of BSA

Magnesium content in two sources of commercially available BSA (ICPbio, Auckland, New Zealand and Sigma, A6003) and fetal bovine serum (FBS; Hyclone, Logan, UT) was determined using high-performance liquid chromatography as previously described [18]. Briefly, a 2.5-g sample was weighed into a porcelain dish and dry-ashed at 500 °C for 4 h. Three ml of 6 N HCl was added to the ash residue and evaporated to dryness on a 100–120 °C hot plate. Minerals were extracted with acid solution (1.5 N HNO_3, 0.5 N HCl) and determined using inductively coupled plasma-atomic emission spectrometry (Thermo Elemental, Franklin, MA) [18]. Due to lot-to-lot variations in BSA and FBS, the lots of same catalog BSA and FBS were tested, and only one large lot of two BSA sources and one lot of FBS were used to generate embryos in this study.

2.2. Bovine oocyte maturation in vitro

Cumulus-oocyte-complexes (COCs) (Fig. 1A) used in this study were recovered from slaughterhouse ovaries of Holstein cattle by aspirating antral follicles of 2–8-mm in diameter [2]. Briefly, COCs were selected and washed in Dulbecco phosphate-buffered saline (D-PBS; Invitrogen, Grand Island, NY) supplemented with 0.1% polyvinyl alcohol. Selected COCs were matured in groups of 20–25 in drops of 75 μl medium 199 (M199, Invitrogen) containing Earle salts, 100 mg/ml L-glutamine, 2.2 g/l sodium bicarbonate, and 25 mM HEPES supplemented with 7.5% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) along with 0.5 μg/ml ovine follicle stimulating hormone (NIDDK, Los Angeles, CA), 5.0 μg/ml ovine luteinizing hormone (NIDDK), and 1.0 μg/ml estradiol. The maturation medium was covered with mineral oil, and COCs were cultured for 22–24 h. COCs with well-expanded cumulus layers (Fig. 1B) at the completion of maturation were selected for in vitro fertilization (IVF).

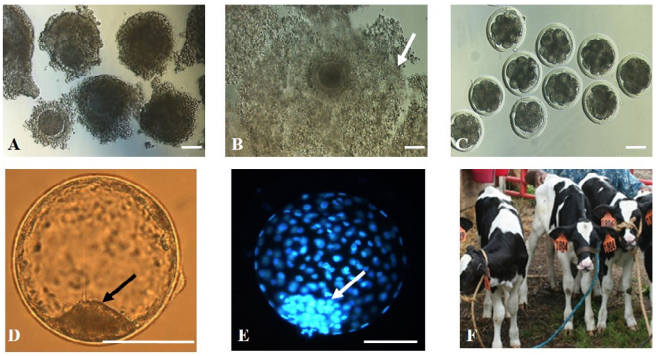

Fig. 1. Embryo development in Mg^{2+}-supplemented culture medium. (A) Bovine COCs were collected for maturation in vitro for 24 h with an expansion of cumulus complexes (arrow) (B). Cleaved embryos were cultured in (C) ICPbio BSA supplemented with 1.4 mM $MgCl_2$ for 2 days. (D) Grade C1 embryos developed into blastocysts on D7 of culture with a compacted inner cell mass (arrow) and (E) 156 nuclei (arrow) visible with Hoechst 33342 staining. (F) Calves were born after ET. Scale bars = 100 μm.

2.3. IVF

Frozen Holstein sperm were used for IVF in Brackett and Oliphant (BO) medium as previously described [2]. Briefly, semen straws were thawed for 20 s in a 37 °C water bath after 10 s of gentle shaking in air. Spermatozoa were washed in 10 ml BO medium containing 3 mg/ml BSA and 10 mM caffeine. The washed sperm pellet was re-suspended in BO washing solution at a minimum concentration of 1×10^6/ml as routinely performed in our laboratory [2]. Matured COCs were washed in BO fertilization medium containing 6 mg/ml BSA and 10 μg/ml heparin, and then 50 oocytes were allocated into a 50-μl droplet of the same fertilization medium. A 50-μl sperm suspension was added to each oocyte-containing fertilization droplet at a final sperm concentration of 0.5×10^6/ml [2]. Oocytes and washed sperm were incubated for 6 h under the culture conditions described above.

2.4. In vitro embryo culture

CR1aa medium [3,14] was used as a basal medium for embryo culture and consisted of 114.7 mM NaCl, 3.1 mM KCl, 26.2 mM $NaHCO_3$, 1 mM L-glutamine, 0.4 mM sodium pyruvate, and 5.5 mM hemicalcium L-lactate supplemented with 1 × MEM (M7145) and 1 × BME (B6766) amino acids. Presumptive zygotes were randomly allocated to modified CR1aa cultures containing 6 mg/ml BSA (ICPbio vs. Sigma) under 5% CO_2, 5% O_2, and 90% N_2 at 39 °C and incubated for 7 days. Half the volume of culture medium was replaced by fresh medium every 2 days. In the co-culture control group (named as FBS group), cumulus cells from COCs were separated and cultured in 7.5% FBS M199 for 2 days to form a confluent monolayer and were then washed three times with 10% FBS CR1aa medium; the fertilized eggs were cultured at 10% FBS CR1 for 2 days, and cleaved embryos on day 2 (D2) were co-cultured with a monolayer of cumulus cells in fresh 10% FBS CR1aa medium under 5% CO_2 humidified air at 39 °C for an additional 5 days (FBS group). Stages of embryo development were evaluated on D2 (immediately after IVF, 2–8-cell stage) (Fig. 1C), D5 (morula) and D7 (blastocyst) (Fig. 1D and E) of culture. Blastocyst-stage embryos graded as C1 according to International Embryo Transfer Society (IETS) standards were used to compare the embryo quality.

2.5. Embryo sex ratio determination by polymerase chain reaction (PCR)

Samples of embryos were digested with 20 μl K-buffer

containing 0.1 μg/μl proteinase K (Invitrogen) at 56 °C for 45 min and then held at 95–100 °C for 10 min to inactivate proteinase K. Multiplex amplification of bovine-specific autosome and Y-chromosome fragments was performed by one round of PCR [2]. A bovine-specific primer pair was designed to amplify a fragment of 219 bp from a bovine 1.715 satellite DNA as follows; forward: 5′-TGA GGC ATG GAA CTC CGC TT-3′, reverse: 5′-GGT GGT TCC ACA TTC CGT AGG AC-3'. The Y-chromosome fragment (131 bp) was amplified using male-specific primers (forward: 5′-GAT TGT TGA TCC CAC AGA AGG CAA TC-3′, reverse: 5′-GAA CTT TCA AGC AGC TGA GGC ATT TA-3′). One μl embryo lysate was used for PCR amplification in a total volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl (S-5761), 2 mM $MgCl_2$, 0.2 mM dNTPs (DNTP100A), 0.4 μM oligonucleotide primers, and 0.5 units REDTaq DNA polymerase. PCR was initiated with a cycle of 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, primer annealing at 64 °C for 30 s, primer extension at 72 °C for 30 s, and a final hold at 72 °C for 10 min. Ten μl of PCR products were analyzed on a 2% agarose gel. One band (219 bp) indicated a female embryo, and two bands (219 and 131 bp) indicated a male embryo (Fig. 2A).

2.6. Vitrification of embryos

Embryos were cryopreserved by modified droplet vitrification (Vitrification and Warming Kits, Renova Life Inc., College Park, MD) [19]. Briefly, embryos were treated with 0.25% trypsin (Gibco, Grand Island, NY) for 1 min. Embryos with intact zona pellucida were washed six times in isotonic embryo washing and holding medium (EWH, Renova Life Inc.). Vitrification solutions and warming media were prepared using basal D-PBS (Oocyte Washing Plus, Renova Life Inc.). To implement the vitrification protocol, the following solutions were prepared: (1) embryo washing solution consisting of 7.5% FBS (SH30070.03, Hyclone) in D-PBS supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, (2) embryo basal solution prepared using 15% FBS D-PBS, and (3) vitrification solution consisting of basal solution supplemented with 2.3 M dimethylsulfoxide, 2.7 M ethylene glycol, and sucrose (Vitrification Kit, Renova Life Inc.). Embryos were incubated for 5 min in washing solution followed by incubation in basal solution for 3 min at 39 °C. Embryos were then washed in a 20-μl vitrification solution drop, after which each embryo was drawn up into a separate glass pipette in a 2-μl drop of vitrification solution (Fisher Scientific, Pittsburgh, PA) and then directly dropped into liquid nitrogen. Groups of 30–50 vitrified embryo droplets were transferred into pre-labeled cryovials (Corning, Corning, NY) cooled in liquid nitrogen, sealed, and stored in liquid nitrogen.

2.7. Warming of vitrified holstein embryos and ET

Vitrified IVF embryos were warmed in 0.28 M sucrose in basal medium (i.e., warming solution) at 39 °C and rehydrated in 0.18 M sucrose basal medium (i.e., rehydration solution) for 5 min at 39 °C (Warming Kit, Renova Life Inc.). Embryos were washed in basal solution and then in EWH supplemented with 3 mg/ml BSA at 39 °C.

In the United States (Iowa and Pennsylvania), ET recipients were Simmental-angus F1 and Holstein cattle. In China, ET recipients were Native Yellow and Holstein cattle. Recipients were chosen from heifers or cows according to criteria such as age, health status, reproductive soundness (i.e., cyclic animals), breeding history, size, and weight, as well as the farm's nutritional management. Recipients were pooled and synchronized by a regimen of two injections of prostaglandin F2α (Lutalyse, Upjohn Co., Kalamazoo, MI; 25 mg/injection, i.m.) at 11-day intervals. Corpus luteum (CL) regression followed by estrus usually occurred approximately 48–72 h later. The onset of estrus in recipients was monitored twice a day, and standing heat was considered D0. On D7 after estrus, recipients were selected by palpation per rectum or

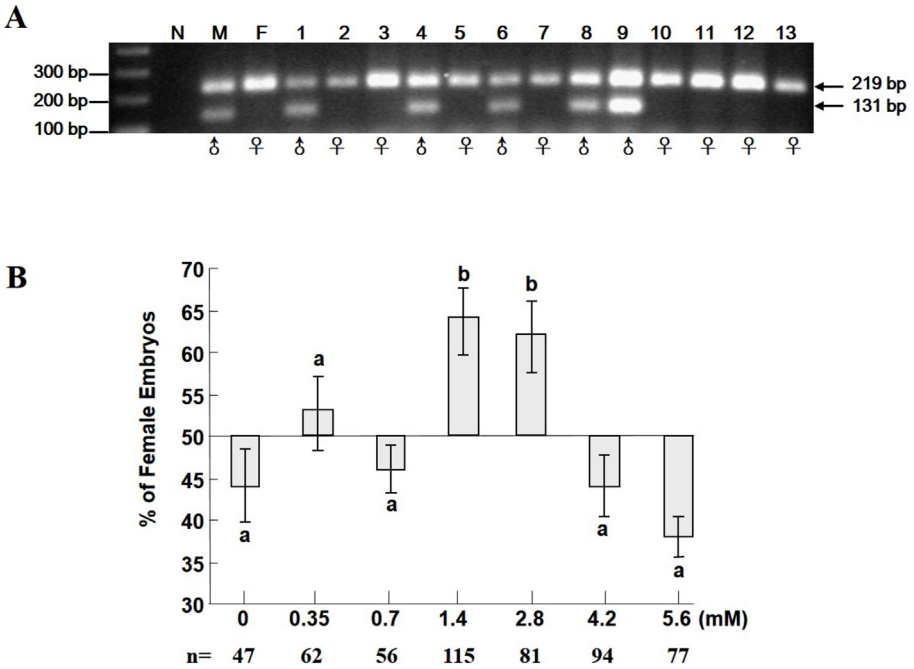

Fig. 2. Sex ratio of bovine embryos in Mg^{2+}-supplemented culture medium. (A) Representative PCR gel from bovine IVF embryos. D7 C1 grade blastocysts were derived in CR1 medium supplemented with 1.4 mM $MgCl_2$ and 6 mg/ml BSA. Amplification of bovine-specific autosome and Y-chromosome DNA fragments was carried out by multiplex PCR. PCR product sizes were 219 bp (autosome) and 131 bp (Y chromosome). N, negative control; M, positive male control; F, positive female control. Lanes 2, 3, 5, 7, and 10–13 were identified as females (♀, one band), and lanes 1, 4, 6, 8, and 9 were identified as males (♂, two bands). (B) Percentage of female embryos derived in culture medium supplemented with different concentrations of Mg^{2+}.

ultrasound monitoring to verify the presence and size of the CL. Blastocysts (1 embryo/straw) were loaded into 0.25-ml French straws containing EWH. A single embryo was deposited non-surgically into the uterine horn ipsilateral to the ovary with a functional CL.

2.8. Experimental design

2.8.1. Effect of macro-molecular surfactants and $MgCl_2$ on the development of IVF embryos

Embryos were cultured in one of the following media for 7 days: (A) 6 mg/ml ICPbio BSA, (B) 6 mg/ml ICPbio BSA supplemented with 1.4 mM $MgCl_2$, (C) 6 mg/ml Sigma BSA, (D) 1 mg/ml PVA, (E) 1 mg/ml PVA supplemented with 1.4 mM $MgCl_2$, or (F) CR1-FBS cumulus cell co-culture control (FBS group), embryos were cultured in 10% FBS CR1 medium for 2 days, and then 5 days with 10% FBS CR1 under cumulus cell monolayer culture. On D2 and D5, half the volume of culture medium was replaced with freshly prepared medium. Cleavage to the 2–8 cell stage, morula stage, and C1 grade blastocyst stage (IETS standards) were assessed on D2, D5, and D7, respectively.

2.8.2. Effect of different concentration of Mg^{2+} on the development of IVF embryos

Embryo development potential was evaluated under different concentrations of Mg^{2+}. Embryos were cultured in 6 mg/ml ICPbio BSA supplemented with 0, 0.35, 0.7, 1.4, 2.1, 2.8, 4.2 and 5.6 mM $MgCl_2$ for 7 days without serum and somatic cell monolayer co-culture. On D2 and D5, half the volume of culture medium was replaced with freshly prepared medium. Cleavage to the 2–8 cell stage, morula stage, and C1 grade blastocyst stage were assessed on D2, D5, and D7, respectively.

2.8.3. Effect of Mg^{2+} and BSA sources on the development of IVF embryos

A 2 × 3 factorial experimental design was employed to compare the effects of BSA source (ICPbio vs. Sigma), Mg^{2+} source ($MgCl_2$ vs. $MgSO_4$), and Mg^{2+} concentration (0 vs. 1.4 mM) on embryo development. On D2 and D5, half the volume of culture medium was replaced with freshly prepared medium. Cleavage to the 2–8 cell stage, morula stage, and C1 grade blastocyst stage were assessed on D2, D5, and D7, respectively.

2.8.4. Term development of embryos derived in Mg^{2+}-supplemented serum-free culture

Because the best concentration of Mg^{2+} was determined as 1.4 mM for embryo development, embryos derived from 1.4 mM Mg^{2+} and 6 mg/mL BSA medium were used for ET trials. Frozen embryos were warmed and transferred into recipients on United States or Chinese farms. ET was performed with either embryos derived in 1.4 mM Mg^{2+}-supplemented serum-free culture or embryos routinely derived in serum-supplemented and cumulus cell monolayer co-culture (FBS group, controls). In the United States, pregnancy was monitored on D45 post-ET by ultrasound and terminated on D60. In China, pregnancy was determined by palpation per rectum on D70, and all pregnancies were carried to term.

2.9. Statistical analysis

The proportions of embryos that successfully reached cleavage, morula, and blastocyst stages, embryos that successfully achieved pregnancy, and that were born as live calves, sex ratio of blastocysts, and the birth weight of newborns were determined within each experiment and presented as Mean ± SEM. Embryos developing to C1 grade blastocysts (IETS standards) were compared among treatments in each experiment. Data were subjected to arc sine transformation and analyzed by one-way ANOVA (General Linear Model, SPSS 11.0, Chicago, IL). A P-value <0.05 was considered statistically significant.

3. Results

3.1. Mg^{2+} determination in BSA and FBS-supplemented medium

The Mg^{2+} in ICPbio BSA, Sigma BSA, and FBS as determined by HPLC was 4, 114, and 42 parts per million, respectively. Thus, without supplementation, Mg^{2+} concentrations in embryo culture medium were calculated as 28.4 μM for 6 mg/ml Sigma BSA medium, 0.99 μM for 6 mg/ml ICPbio BSA medium, and 0.14 mM for 10% FBS-supplemented medium. In other words, the concentration of Mg^{2+} in Sigma BSA was 28.7 times higher than that in ICPbio BSA and 1/5 of that in FBS-supplemented medium.

3.2. Effect of macro-molecular surfactants and MgCl2 on the development of IVF embryos

The rate of cleavage to the 2–8 cell stage on D2 was similar among groups ($P > 0.05$) except that it was lower in the PVA+1.4 mM $MgCl_2$ group than in the Sigma BSA and FBS groups ($P < 0.05$; Table 1). The rate of 6–8 cell embryos on D2 was lower in the ICPbio BSA, PVA, and PVA+1.4 mM $MgCl_2$ groups than in the Sigma BSA (Fig. 1C) and FBS groups ($P < 0.05$). On D7, the rate of blastocyst development was higher in the ICPbio+1.4 mM $MgCl_2$ (29.4%) (Fig. 1D and E) and FBS (33.7%) groups than in the other groups (2.2–18.9%) ($P < 0.05$). The lowest rates of blastocyst development were in the PVA groups regardless of $MgCl_2$ supplementation (2.2–5.4%). The Sigma BSA group had a higher rate of blastocyst development (18.9%) than the PVA group or the ICPbio BSA without Mg^{2+} supplementation group (2.3%) ($P < 0.05$). Importantly, supplementing 1.4 mM $MgCl_2$ into ICPbio BSA medium markedly enhanced the rate of blastocyst development (29.4%) to a degree comparable to that in the FBS group (33.7%) ($P > 0.05$).

3.3. Effect of different concentration of Mg2+ on the development of IVF embryos

We next tested the effect of Mg^{2+} concentrations on the blastocyst development of bovine embryos. ICPbio BSA was used as a protein source, and different concentrations of $MgCl_2$ (0, 0.35, 0.7, 1.4, 2.1, 2.8, 4.2 and 5.6 mM) were supplemented into serum-free medium. There were no group differences in the rate of cleavage to the 2–8 cell stage or 6–8 cell embryos on D2 ($P > 0.05$; Table 2). However, the rate of morula development on D5 was increased when $MgCl_2$ was added to the culture medium till 2.8 mM, but reduced after $MgCl_2$ supplemented as 4.2 and 5.6 mM (Table 2). Furthermore, the rate of blastocyst development was higher in the 0.35 (38.4%) and 0.7 mM $MgCl_2$ (40.5%) groups than in the 0 mM $MgCl_2$ (2.6%) group ($P < 0.05$), higher in the 1.4 mM $MgCl_2$ group (50.2%) than in the 0 and 0.35 mM $MgCl_2$ groups ($P < 0.05$), and comparable between the 1.4 and 2.1 mM $MgCl_2$ (44.9%) groups ($P > 0.05$). With the increase of $MgCl_2$ concentration to 4.2 and 5.6 mM, blastocyst rate was decreased to 31.4 and 29.4%, respectively, compared to that in 1.4 mM $MgCl_2$ ($P < 0.05$) (Table 2). Therefore, the optimal concentration of Mg^{2+} for bovine embryo culture was determined to be 1.4 mM.

We also examined the sex ratio of blastocysts derived with different concentrations of Mg^{2+} supplementation. We observed a higher proportion of female embryos in the 1.4 and 2.8 mM $MgCl_2$ groups than in the 0–0.7 mM $MgCl_2$ groups ($P < 0.05$; Fig. 2A, B)

Table 1
Effect of macro-molecular surfactants and $MgCl_2$ on development of bovine IVF embryos in somatic cell-free culture.

Group	No. of replications	Basal medium	Treatment		No. of embryos	Cleavage (%)	6–8 Cell (%)	Morula (%)	C1 Blastocyst (%)
			Surfactant	$MgCl_2$ (mM)					
A	4	CR1aa	ICPbio BSA	0	149	$66.6 \pm 4.8^{a,b}$	44.5 ± 8.4^{a}	21.4 ± 3.9^{a}	2.3 ± 0.9^{a}
B	4	CR1aa	ICPbio BSA	1.4	153	$70.9 \pm 1.6^{a,b}$	$53.2 \pm 3.1^{a,b}$	36.4 ± 1.3^{b}	29.4 ± 2.6^{b}
C	4	CR1aa	Sigma BSA	0	151	74.3 ± 4.6^{a}	64.8 ± 5.6^{b}	$30.4 \pm 5.5^{a,b}$	18.9 ± 6.7^{c}
D	4	CR1aa	PVA	0	245	$63.6 \pm 1.3^{a,b}$	39.2 ± 0.3^{a}	5.6 ± 1.2^{c}	2.2 ± 0.9^{a}
E	4	CR1aa	PVA	1.4	219	62.5 ± 4.6^{b}	44.8 ± 6.5^{a}	8.4 ± 1.9^{c}	5.4 ± 1.5^{a}
F	4	CR1aa	FBS	0	149	74.6 ± 4.7^{a}	66.0 ± 4.7^{b}	37.3 ± 4.7^{b}	33.7 ± 4.3^{b}

[a,b,c]Values with different superscripts within columns are significantly different ($P < 0.05$). Blastocysts were compared as C1 grade according to IETS standards. FBS: FBS group with co-culture condition as controls; the fertilized eggs were cultured at 10% FBS CR1 for 2 days, subsequently transferred and cultured for 5 days into the dishes covered with cumulus cell monolayer and filled with 10% FBS CR1 medium.

Table 2
Effect of Mg^{2+} concentration on development of bovine IVF embryos in somatic/serum-free culture.

Group	No. of replications	Basal medium	Treatment		No. of embryos	Cleavage (%)	6–8 Cell (%)	Morula (%)	C1 Blastocyst (%)
			ICPbio BSA (mg/ml)	$MgCl_2$ (mM)					
A	4	CR1aa	6	0	197	92.9 ± 2.4	78.7 ± 2.6	22.3 ± 2.9^{a}	2.6 ± 1.0^{a}
B	4	CR1aa	6	0.35	193	94.3 ± 1.3	78.3 ± 2.8	47.2 ± 2.1^{b}	38.4 ± 2.8^{b}
C	4	CR1aa	6	0.7	200	95.4 ± 1.1	82.6 ± 0.7	47.2 ± 2.8^{b}	$40.5 \pm 2.9^{b,c}$
D	4	CR1aa	6	1.4	207	93.8 ± 1.1	80.7 ± 2.2	54.5 ± 2.0^{b}	50.2 ± 3.9^{c}
E	4	CR1aa	6	2.1	207	94.7 ± 1.9	81.5 ± 2.8	54.0 ± 4.6^{b}	$44.9 \pm 6.2^{b,c}$
F	4	CR1aa	6	2.8	215	92.7 ± 1.5	83.6 ± 1.5	49.2 ± 1.1^{b}	$43.4 \pm 3.1^{b,c}$
G	4	CR1aa	6	4.2	187	89.1 ± 1.1	81.2 ± 0.6	35.2 ± 1.2^{c}	31.4 ± 1.5^{b}
H	4	CR1aa	6	5.6	156	90.1 ± 1.4	78.6 ± 0.2	33.5 ± 1.5^{c}	29.4 ± 5.8^{b}

[a,b,c]Values with different superscripts within columns are significantly different ($P < 0.05$). Blastocysts were compared as C1 grade according to IETS standards.

and a lower proportion of female embryos in the 4.2 and 5.6 mM groups than in the 1.4 and 2.8 mM $MgCl_2$ groups ($P < 0.05$).

3.4. Effect of Mg2+ and BSA sources on the development of IVF embryos

We further examined the effects of BSA source (ICPbio vs. Sigma), Mg^{2+} source ($MgCl_2$ vs. $MgSO_4$), and Mg^{2+} concentration (0 vs. 1.4 mM) on embryo development. Rates of cleavage to the 2–8 cell stage on D2, percentage of 6–8 cell embryos on D2, and morula development on D5 were similar among groups ($P > 0.05$; Table 3). However, the Sigma BSA group without Mg^{2+} supplementation (26.4%) had a higher rate of blastocyst development on D7 than the ICPbio BSA group without Mg^{2+} supplementation (6.8%) ($P < 0.05$). Supplementation of 1.4 mM Mg^{2+} into the culture medium regardless of its source ($MgCl_2$, 30.0–38.7% vs. $MgSO_4$, 33.1–37.4%) increased blastocyst rates to comparable levels ($P > 0.05$). The lowest blastocyst rate occurred in the ICPbio BSA without Mg^{2+} supplementation group (6.8%) ($P < 0.05$).

3.5. Term development of embryos derived in Mg2+-supplemented serum-free culture

In one trial conducted on farms in the United States, pregnancy rate after ET of vitrified blastocysts derived in serum- and co-culture free medium containing Sigma BSA supplemented with 1.4 mM $MgCl_2$ (CR1-Mg^{2+}-BSA group, $n = 31$) was compared with that of vitrified blastocysts derived in FBS-containing cumulus cell co-culture (FBS group, $n = 71$). On D45, the pregnancy rate as determined by ultrasound monitoring was higher for CR1-Mg^{2+}-BSA group (70.1%) than for FBS group (57.1%) ($P < 0.05$). Due to the cost of ET services, all pregnancies in this trial were terminated.

A second ET trial on Chinese farms was conducted to evaluate term development potential. A total of 230 vitrified CR1-Mg^{2+}-BSA group embryos and 154 vitrified FBS group embryos were singly transferred into recipients. On D70 post-ET, pregnancy rate as determined by rectal palpation was higher for CR1-Mg^{2+}-BSA group (54.3%, 125/230) than for FBS embryos (41.5%, 64/154; $P < 0.05$). Likewise, a higher percentage of live Holstein calves were born from CR1-Mg^{2+}-BSA group (44.3%, 102/230) (Fig. 1F) than from FBS embryos (32.5%, 50/154; $P < 0.05$). The proportion of females among the live calves was similar between CR1-Mg^{2+}-BSA group (53.9%, 55/102) and FBS embryos (48.0%, 24/50; $P > 0.05$).

Table 3
Effect of Mg^{2+} source on development of bovine IVF embryos in somatic/serum-free culture.

Group	No. of replications	Basal medium	Treatment			No. of embryos	Cleavage (%)	6–8 Cell (%)	Morula (%)	C1 Blastocyst (%)
			BSA (6 mg/ml)	Mg^{2+} source						
				$MgSO_4$ (mM)	$MgCl_2$ (mM)					
A	4	CR1aa	Sigma	0	0	208	84.5 ± 3.8	61.5 ± 3.7	42.7 ± 7.2	26.4 ± 2.8^{a}
B	4	CR1aa	Sigma	0	1.4	178	84.95 ± 5.4	68.5 ± 6.6	48.0 ± 3.6	38.7 ± 1.1^{b}
C	4	CR1aa	Sigma	1.4	0	170	80.5 ± 2.2	65.3 ± 5.8	43.2 ± 6.1	37.4 ± 7.9^{b}
D	4	CR1aa	ICPbio	0	0	203	72.6 ± 6.4	52.4 ± 6.6	35.9 ± 9.4	6.8 ± 2.4^{c}
E	4	CR1aa	ICPbio	0	1.4	183	86.3 ± 4.7	66.4 ± 10.3	36.2 ± 7.3	$30.0 \pm 6.9^{a,b}$
F	4	CR1aa	ICPbio	1.4	0	186	84.7 ± 4.9	67.8 ± 5.0	49.4 ± 8.3	33.1 ± 8.6^{b}

[a,b,c]Values with different superscripts within columns are significantly different ($P < 0.05$). Blastocysts were compared as C1 grade according to IETS standards.

The average birth weight of calves in CR1-Mg^{2+}-BSA group was 39.4 kg for females (n = 45) and 42.5 kg for males (n = 44) while that in FBS group was 41.5 kg for females (n = 22), and 43.5 kg (n = 24), respectively. There was not different in average birth weight in corresponding male and female calves between CR1-Mg^{2+}-BSA group and FBS groups ($P > 0.05$), although it was heavier in FBS group than in CR1-Mg^{2+}-BSA group. The rate of miscarriage after D70 post-ET was similar between CR1-Mg^{2+}-BSA (10.0%) and FBS (9.0%) groups ($P > 0.05$).

4. Discussion

Our study demonstrates that Mg^{2+} is important for bovine early embryo development, particularly in our CR1aa BSA cell-free culture system (CR1-Mg^{2+}-BSA). Specifically, a blastocyst development rate of 50.2% was achieved when 1.4 mM $MgCl_2$ was added to ICPbio BSA culture medium, perhaps due to a positive direct effect on the post-compaction stage, leading to higher quality blastocysts. Although cleavage to the 2–8 cell stage was not affected, the rate of 6–8-cell embryos was higher in the Sigma BSA and FBS groups than in the ICPbio BSA groups (Table 1). HPLC analysis showed that Mg^{2+} residue was 28.7 times higher in Sigma BSA than in ICPbio BSA, resulting in Mg^{2+} concentrations of 28.4 μM in Sigma BSA medium and 0.99 μM in ICPbio BSA medium. Interestingly, although Mg^{2+} at lower concentrations markedly affected embryo development in culture, ICPbio BSA supplemented with 1.4 mM Mg^{2+} rescued more 6–8-cell embryos and improved blastocyst development comparable to FBS. Therefore, the beneficial effect of Mg^{2+} on embryonic development may become more pronounced under serum-free and co-culture-free conditions. Furthermore, the CR1-Mg^{2+}-BSA culture system could eliminate potentially harmful metabolites and toxicants secreted by somatic cells or present in FBS, as embryos derived in serum and/or co-culture have been reported to exhibit abnormal fetal development, such as large offspring syndrome [20] and fetal loss after conception [21].

Intracellular Mg^{2+} has many physiological functions in cells [22]. For instance, Mg^{2+} is a cofactor of many important enzymes such as DNA polymerase, RNA stability enzymes, protein kinases, ATPases, and ATP-binding proteins and regulates calcium homeostasis [11]. Within cells, Mg^{2+} is stored in mitochondria, and its homeostasis is maintained by plasma membrane Mg^{2+} transporters including SLC41A1, MRS2P, CNNM3, and TRPM7 [23–27]. Nagashima et al. found that Mg^{2+} significantly enhances sperm hyperactivation and acrosome exocytosis for the fertilization of canine eggs [28]. Different species of animals have different requirements for Mg^{2+} during the oocyte-to-embryo transition (i.e., 0.2, 1.0, and 1.2 mM for mice, cats, and humans, respectively) [29]. Moreover, an increase in the concentration of Mg^{2+} in culture medium significantly reduces intracellular calcium levels in hamster embryos [30], which is necessary for pre-implantation development, and specific doses of Mg^{2+} reduce toxicity and teratogenicity in Xenopus embryos [31]. In the present study, we believe that the effect of Mg^{2+} was not obvious at the cleavage stage because oocyte mitochondria may store a large amount of Mg^{2+} at fertilization, which is necessary for early cleavage at the 2–8 cell stage. However, during the course of development to morula and blastocyst stages, intracellular Mg^{2+} is depleted or consumed, especially when zygotic genome activation (ZGA) begins at the 8-cell stage [32,33]. As Mg^{2+} serves many functions in cells, including embryos, we propose that a deficiency in Mg^{2+} affects a global range of biological processes (e.g., metabolism and DNA/RNA synthesis, repair, and maintenance) rather than causing a single enzymatic blockage. We found that most blastocysts (50.2%) developed from culture medium with a concentration of 1.4 mM Mg^{2+}, although there was not a significant difference of blastocyst development among the groups supplemented with Mg^{2+} concentration from 0.7 to 2.8 mM (40.5–44.9%) (Table 2). That is, serum is not necessary for bovine embryo culture if BSA (6 mg/ml) and a sufficient amount of Mg^{2+} (i.e., 1.4 mM) are supplemented in simple CR1aa medium. We also found that $MgCl_2$ and $MgSO_4$ similarly promote bovine early embryo development. Furthermore, we observed that an increase in Mg^{2+} concentration not only improved blastocyst development but also changed the sex ratio of embryos, with a higher female-to-male ratio at 1.4–2.8 mM than at 4.2–5.6 mM. It was observed that, with an increase of Mg^{2+} concentration at 4.2–5.6 mM, blastocyst rate was decreased to 29.4–31.4% (Table 2). A previous study reports that different culture conditions can change the sex ratio of embryos, with male embryos developing fast in IVF [34] or intravaginal culture [35–37]. There was one possibility that an increase of Mg^{2+} concentration affected development speed, some female embryos derived from 4.2 to 5.6 mM Mg^{2+} might not reach the blastocyst stage on Day 7 because of slow development speed. Therefore, our results indicate that the sex ratio of embryos can be controlled by the concentration of Mg^{2+} in the culture medium.

Serum albumin, such as BSA, is found in high concentrations within mammalian reproductive tracts [16]. We tested BSA from two commercial sources, Sigma and ICPbio, and unexpectedly found that Sigma BSA promoted blastocyst formation to a greater degree than ICPbio BSA [14]. Both ICPbio BSA and Sigma BSA are free of fatty acids. However, ICPbio BSA is chromatographically fractionated, resulting in minimal denaturation of the albumin structure, followed by charcoal treatment to remove endotoxin whereas Sigma BSA is isolated by alcohol precipitation and charcoal treatment [14]. Thus, as organic solvent precipitation and charcoal treatment may not completely remove impurities [38], varying amounts of impurity may remain in BSA. Although chromatographical fractionation can give rise to much purer albumin, some associated molecules can also be removed during this process. We confirmed using HPLC analysis that Mg^{2+} content in Sigma BSA was 28.7 times higher than that in ICPbio BSA, but the amount of Mg^{2+} present in medium containing 6 mg/ml Sigma BSA was still not sufficient to promote further embryo compacting and development into blastocysts (Table 1). However, when 1.4 mM Mg^{2+} (either $MgCl_2$ or $MgSO_4$) was added to ICPbio BSA, embryo development to the blastocyst stage was enhanced. We therefore hypothesize that Mg^{2+} is necessary for the development of morulae to blastocysts in embryo culture medium containing BSA and that ICPbio BSA alone may not be as supportive of blastocyst formation due to greater removal of Mg^{2+} residue during its preparation.

We also found that Mg^{2+} and BSA have a synergistic effect on early embryo development. BSA can serve as a replacement for FBS to eliminate many undefined factors present in complex FBS and cell co-culture medium. BSA is a protein source supplemented in embryo flushing and transfer medium [19] and in culture medium for in vitro embryo development [2]. BSA acts as a protein surfactant and can also be replaced by other macromolecules, such as PVA [39]. In the present study, we found that BSA-supplemented medium was more supportive of embryo development than PVA-supplemented medium, suggesting that BSA contains not only Mg^{2+} residue, but also a protein source that can be consumed by embryos. Even when 1.4 mM Mg^{2+} was supplemented into PVA, embryos did not develop well into blastocysts, and embryo quality was inferior to that observed in BSA-containing medium. We propose that embryos utilize endocytosis to intake and digest BSA protein into small peptides or amino acids as a nutrient source promoting metabolism and growth [40–43]. To reveal the underlying mechanisms of this process, BSA could be labeled with a radioactive or fluorescent marker during embryo culture in future experiments, allowing its endocytosis, subsequent molecular

breakdown, and route of distribution to be traced during embryo development. Although FBS and co-culture have beneficial effects on bovine embryo development [44,45], ideal culture conditions remain largely undefined. The variability and complexity of the co-culture system makes it problematic for defining the specific nutritional requirements of embryos. In addition, employing serum or co-culture may introduce unknown toxic components or pathogens [44,45]. For instance, significant accumulation of intracellular granules and lipid vesicles are observed in blastocysts when serum is included in the culture medium [35,44,46]. Thus, serum may have adverse effects on the morphology of embryos and may be associated with abnormal fetal development, such as large offspring syndrome or fetal loss after conception [20,47,48].

Finally, we found that the D45 pregnancy/implantation rate of embryos derived in CR1-Mg^{2+}-BSA culture was higher than that of embryos derived in FBS group on United States farms. Likewise, on Chinese farms, both D70 pregnancy and term calving rates were higher in the CR1-Mg^{2+}-BSA group than in the FBS group. These findings are in agreement with previous proposals that Mg^{2+} plays a role in early and late pregnancy [49]. Based on the results of our ET trials, CR1-Mg^{2+}-BSA embryos may have greater potential for implantation and term development. In addition, consistent with findings that the droplet vitrification technique developed by our group produces satisfactory and reliable outcome in sexed IVF bovine embryos [2], Wagyu beef embryos in vivo [19], cloned bovine embryos [50], and mouse embryos [51], the present study further confirms that droplet vitrification can effectively cryopreserve IVF embryos derived in serum-free CR1-Mg^{2+}-BSA medium with minimal embryonic damage, resulting in higher in vivo developmental competence after warming and ET. It is important to notice that embryo transfer results were interesting, but not definitive with respect to the effects of Mg^{2+}. The higher pregnancy rates could reflect any of the differences between the two culture conditions (FBS group with co-culture vs CR1-Mg^{2+}-BSA). Our finding that the term development potential of embryos derived in CR1-Mg^{2+}-BSA culture was higher than that of embryos derived in FBS group suggests that a simple and efficient defined culture medium (CR1-Mg^{2+}-BSA) can replace a complex serum and co-culture system (FBS group) for bovine embryo production to reduce the risk of miscarriage and embryo loss after ET.

5. Conclusions

In summary, we found that Mg^{2+} has a profound stimulatory effect on bovine embryo development, not only during pre-implantation to blastocyst stages but also during post-ET development to term. The optimal concentration of Mg^{2+} appears to be 1.4 mM, which promotes a blastocyst rate of 50.2% in CR1 medium supplemented with 6 mg/ml BSA. In CR1 medium, which is a relatively simple culture medium, the addition of BSA (regardless of Sigma or ICPbio preparation) and Mg^{2+} has a synergistic effect on embryo development in vitro and increases pregnancy and birth rates after ET. Therefore, a simple defined CR1-Mg^{2+}-BSA medium can eliminate the need for serum and cell co-culture for competent bovine embryo development.

Conflicts of interest

The authors declare no conflict of interest in this study.

Acknowledgments

This study was supported by the USDA Cooperative State Research, Education, and Extension Service (2006-03069), Premium Science and Technology Foundation of Jiangsu Province of China, National Natural Science Foundation of China (31701285, 31471388), China Postdoctoral Science Foundation (2018M632330), Priority Academic Program Development of Jiangsu Higher Education Institutions, and Major Program of Natural Science Research of Jiangsu Higher Education Institutions of China (14KJA180003) to FD.

References

[1] Wan PC, Hao ZD, Zhou P, Wu Y, Yang L, Cui MS, et al. Effects of SOF and CR1 media on developmental competence and cell apoptosis of ovine in vitro fertilization embryos. Anim Reprod Sci 2009;114:279–88.
[2] Xu J, Guo Z, Su L, Nedambale TL, Zhang J, Schenk J, et al. Developmental potential of vitrified holstein cattle embryos fertilized in vitro with sex-sorted sperm. J Dairy Sci 2006;89:2510–8.
[3] Rosenkrans Jr CF, Zeng GQ, GT MC, Schoff PK, First NL. Development of bovine embryos in vitro as affected by energy substrates. Biol Reprod 1993;49:459–62.
[4] Seidel Jr GE. Sexing mammalian spermatozoa and embryos–state of the art. J Reprod Fertil Suppl 1999;54:477–87.
[5] Pontes JH, Silva KC, Basso AC, Rigo AG, Ferreira CR, Santos GM, et al. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. Theriogenology 2010;74:1349–55.
[6] Senatore EM, Xu J, Suarez Novoa MV, Gong G, Lin T, Bella A, et al. Improved in vitro development of OPU-derived bovine (Bos taurus) embryos by group culture with agarose-embedded helper embryos. Theriogenology 2010;74:1643–51.
[7] Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972;30:493–7.
[8] Liu Z, Foote RH. Effects of amino acids on the development of in-vitro matured/in-vitro fertilization bovine embryos in a simple protein-free medium. Hum Reprod 1995;10:2985–91.
[9] Nedambale TL, Dinnyes A, Groen W, Dobrinsky JR, Tian XC, Yang X. Comparison on. in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. Theriogenology 2004;62:437–49.
[10] Lane M, Gardner DK, Hasler MJ, Hasler JF. Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. Theriogenology 2003;60:407–19.
[11] Pilchova I, Klacanova K, Tatarkova Z, Kaplan P, Racay P. The involvement of Mg(2+) in regulation of cellular and mitochondrial functions. Oxid Med Cell Longev 2017;2017:6797460.
[12] Lebbink JHG, Fish A, Reumer A, Natrajan G, Winterwerp HHK, Sixma TK. Magnesium coordination controls the molecular switch function of DNA mismatch repair protein MutS. J Biol Chem 2010;285:13131–41.
[13] Adhikari S, Toretsky JA, Yuan LS, Roy R. Magnesium, essential for base excision repair enzymes, inhibits substrate binding of N-methylpurine-DNA glycosylase. J Biol Chem 2006;281:29525–32.
[14] Sung LY, Du F, Xu J, Chang W, Nedambale TL, Zhang J, et al. The differential requirement of albumin and sodium citrate on the development of in vitro produced bovine embryos. Reprod Nutr Dev 2004;44:551–64.
[15] Lim KT, Lee BC, Kang SK, Hwang WS. Effects of protein source and energy substrates on the in vitro development of bovine embryos in a two-step culture system. J Vet Sci 2003;4:73–8.
[16] Eckert J, Pugh PA, Thompson JG, Niemann H, Tervit HR. Exogenous protein affects developmental competence and metabolic activity of bovine pre-implantation embryos in vitro. Reprod Fertil Dev 1998;10:327–32.
[17] Olson SE, Seidel Jr GE. Reduced oxygen tension and EDTA improve bovine zygote development in a chemically defined medium. J Anim Sci 2000;78:152–7.
[18] Siitonen PH, Thompson Jr HC. Analysis of calcium and lead in calcium supplements by inductively coupled plasma-atomic emission spectrometry and graphite furnace atomic absorption spectrophotometry. J AOAC Int 1994;77:1299–304.
[19] An L, Ling PP, Zhu X, Liu Y, Zhang F, Ma X, et al. Successful vitrification of in vivo embryos collected from superovulated Japanese black cattle (Wagyu). Reprod Domest Anim 2016;51:255–61.
[20] Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998;3:155–63.
[21] Hewitt MJ, Pratten MK, Regan L, Quenby SM, Baker PN. The use of whole rat embryo culture as a technique for investigating potential serum toxicity in recurrent miscarriage patients. Hum Reprod 2000;15:2200–4.
[22] Wolf FI, Trapani V. Cell (patho)physiology of magnesium. Clin Sci (Lond) 2008;114:27–35.
[23] Kolisek M, Launay P, Beck A, Sponder G, Serafini N, Brenkus M, et al. SLC41A1 is a novel mammalian Mg2+ carrier. J Biol Chem 2008;283:16235–47.
[24] Kolisek M, Nestler A, Vormann J, Schweigel-Rontgen M. Human gene SLC41A1 encodes for the Na+/Mg2+ exchanger. Am J Physiol-Cell Ph 2012;302:C318–26.
[25] Kolisek M, Zsurka G, Samaj J, Weghuber J, Schweyen RJ, Schweigel M. Mrs2p is an essential component of the major electrophoretic Mg2+ influx system in

mitochondria. EMBO J 2003;22:1235–44.
[26] Mastrototaro L, Smorodchenko A, Aschenbach JR, Kolisek M, Sponder G. Solute carrier 41A3 encodes for a mitochondrial Mg(2+) efflux system. Sci Rep 2016;6:27999.
[27] Schmitz C, Deason F, Perraud AL. Molecular components of vertebrate Mg2+-homeostasis regulation. Magnes Res 2007;20:6–18.
[28] Nagashima JB, Sylvester SR, Nelson JL, Cheong SH, Mukai C, Lambo C, et al. Live births from domestic dog (Canis familiaris) embryos produced by in vitro fertilization. PLoS One 2015;10:e0143930.
[29] Herrick JR, Strauss KJ, Schneiderman A, Rawlins M, Stevens J, Schoolcraft WB, et al. The beneficial effects of reduced magnesium during the oocyte-to-embryo transition are conserved in mice, domestic cats and humans. Reprod Fertil Dev 2015;27:323–31.
[30] Lane M, Gardner DK. Regulation of ionic homeostasis by mammalian embryos. Semin Reprod Med 2000;18:195–204.
[31] Boga A, Erdogan S, Sertdemir Y. Effects of specific dosages of magnesium and zinc on the teratogenicity of cadmium, nickel, and cobalt in Xenopus embryos, as assessed by the FETAX test. Dose-Response 2008;6:16–29.
[32] Wei Q, Zhong L, Zhang S, Mu H, Xiang J, Yue L, et al. Bovine lineage specification revealed by single-cell gene expression analysis from zygote to blastocyst. Biol Reprod 2017;97:5–17.
[33] Benesova V, Kinterova V, Kanka J, Toralova T. Potential involvement of SCF-complex in zygotic genome activation during early bovine embryo development. Methods Mol Biol 2017;1605:245–57.
[34] Avery B, Jorgensen CB, Madison V, Greve T. Morphological development and sex of bovine in vitro-fertilized embryos. Mol Reprod Dev 1992;32:265–70.
[35] Heras S, De Coninck DIM, Van Poucke M, Goossens K, Pascottini OB, Van Nieuwerburgh F, et al. Suboptimal culture conditions induce more deviations in gene expression in male than female bovine blastocysts. BMC Genomics 2016;17:72.
[36] Hansen D, Moller H, Olsen J. Severe periconceptional life events and the sex ratio in offspring: follow up study based on five national registers. BMJ 1999;319:548–9.
[37] Perez-Cerezales S, Ramos-Ibeas P, Rizos D, Lonergan P, Bermejo-Alvarez P, Gutierrez-Adan A. Early sex-dependent differences in response to environmental stress. Reproduction 2018;155:R39–51.
[38] Stone MT, Kozlov M. Separating proteins with activated carbon. Langmuir 2014;30:8046–55.
[39] Gomez E, Correia-Alvarez E, Caamano JN, Diez C, Carrocera S, Peynot N, et al. Hepatoma-derived growth factor: from the bovine uterus to the in vitro embryo culture. Reproduction 2014;148:353–65.
[40] Dunglison GF, Kaye PL. Endocytosis in mouse blastocysts: characterization and quantification of the fluid phase component. Mol Reprod Dev 1995;41:225–31.
[41] Moriyama T, Sasaki K, Karasawa K, Uchida K, Nitta K. Intracellular transcytosis of albumin in glomerular endothelial cells after endocytosis through caveolae. J Cell Physiol 2017;232:3565–73.
[42] Lotti LV, Hand AR. Endocytosis of native and glycosylated bovine serum albumin by duct cells of the rat parotid gland. Cell Tissue Res 1989;255:333–42.
[43] Li HH, Li J, Wasserloos KJ, Wallace C, Sullivan MG, Bauer PM, et al. Caveolae-dependent and -independent uptake of albumin in cultured rodent pulmonary endothelial cells. PLoS One 2013;8:e81903.
[44] Fukui Y, Ono H. Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. J Reprod Fertil 1989;86:501–6.
[45] Thompson JG, Peterson AJ. Bovine embryo culture in vitro: new developments and post-transfer consequences. Hum Reprod 2000;15:59–67.
[46] Abe H, Yamashita S, Satoh T, Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Mol Reprod Dev 2002;61:57–66.
[47] van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B, et al. Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. Theriogenology 2000;53:575–97.
[48] Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, et al. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002;67:767–75.
[49] Stuefer S, Moncayo H, Moncayo R. The role of magnesium and thyroid function in early pregnancy after in-vitro fertilization (IVF): new aspects in endocrine physiology. Bba Clin 2015;3:196–204.
[50] Du F, Shen PC, Xu J, Sung LY, Jeong BS, Lucky Nedambale T, et al. The cell agglutination agent, phytohemagglutinin-L, improves the efficiency of somatic nuclear transfer cloning in cattle (Bos taurus). Theriogenology 2006;65:642–57.
[51] An LY, Chang SW, Hu YS, Li Y, Xu BW, Zhang FL, et al. Efficient cryopreservation of mouse embryos by modified droplet vitrification (MDV). Cryobiology 2015;71:70–6.