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A combination of bovine serum albumin with insulin–transferrin–sodium selenite and/or epidermal growth factor as alternatives to fetal bovine serum in culture medium improves bovine embryo quality and trophoblast invasion by induction of matrix metalloproteinases

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Abstract. This study investigated the use of bovine serum albumin (BSA) plus insulin–transferrin–sodium selenite (ITS) and/or epidermal growth factor (EGF) as alternatives to fetal bovine serum (FBS) in embryo culture medium. The developmental ability and quality of bovine embryos were determined by assessing their cell number, lipid content, gene expression and cryotolerance, as well as the invasion ability of trophoblasts. The percentage of embryos that underwent cleavage and formed a blastocyst was higher ($P < 0.01$) in medium containing ITS plus EGF and BSA than in medium containing FBS. Culture with ITS plus EGF and BSA also increased the hatching ability of blastocysts and the total cell number per blastocyst. Furthermore, the beneficial effects of BAS plus ITS and EGF on embryos were associated with a significantly reduced intracellular lipid content, which increased their cryotolerance. An invasion assay confirmed that culture with ITS plus EGF and BSA significantly improved the invasion ability of trophoblasts. Real-time quantitative polymerase chain reaction analysis showed that the mRNA levels of matrix metalloproteinase-2 (*MMP2*) and *MMP9*, acyl-CoA synthetase long-chain family member 3, acyl-coenzyme A dehydrogenase long-chain and hydroxymethylglutaryl-CoA reductase significantly increased upon culture with ITS plus EGF and BSA. Moreover, protein expression levels of matrix metalloproteinase-2 and -9 increased ($P < 0.01$) in medium supplemented with ITS plus EGF and BSA compared with medium supplemented with FBS. Taken together, these data suggest that supplementation of medium with ITS plus EGF and BSA improves *in vitro* bovine embryo production, cryotolerance and invasion ability of trophoblasts.

Additional keywords: cryotolerance, EGF, embryo culture, invasion ability, ITS, *MMP2*, *MMP9*.

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Introduction

In recent decades, serum has been used to supplement *in vitro* culture (IVC) media as it provides multiple beneficial factors such as macromolecules, vitamins, hormones, growth factors and amino acids (Bavister 1995). To date, serum has widely been used as a main supplement to embryo IVC media because it contains embryotrophic factors (Murakami *et al.* 2011). The use of serum in culture media bears several disadvantages, as serum can be embryo toxic and each batch of serum contains differing levels of growth factors, metabolites, protein and hormones,

making it impossible to standardise culture conditions within a laboratory (Rauch *et al.* 2011). Moreover, addition of serum to the medium may introduce detrimental as well as beneficial constituents (Bavister 1995) as serum has a biphasic effect on embryonic development; it inhibits early cleavage, but accelerates blastocyst development when it is present from the initiation of compaction (Murakami *et al.* 2011). Although serum contains substances beneficial for embryonic development, such as antioxidants, growth factors and heavy metal chelators, it can adversely affect the quality of the embryo by inducing excessive lipid droplet accumulation (Abe *et al.* 2002), altered gene expression (Fernandez-Gonzalez *et al.* 2004), premature blastulation (Holm *et al.* 2002) and by contributing to large offspring syndrome (LOS; van Wagtendonk-de Leeuw *et al.* 2000). Serum is a chemically ill-defined supplement that can be contaminated by fungi, bacteria, viruses and mycoplasma (Rauch *et al.* 2011).

Insulin, a polypeptide hormone that is present in or is added to a wide range of culture media, is an important factor in cellular differentiation and promotes the uptake of amino acids and glucose and may stimulates mitosis through its mitogenic effects (Herrler *et al.* 1998; Cordova *et al.* 2010). *In vitro* studies of preimplantation embryos from several mammalian species show that the oviduct and the uterus contain growth factors that stimulate cellular proliferation and differentiation of preimplantation embryos. Insulin and insulin-like growth factors are important for embryonic growth and metabolism (Mihalik *et al.* 2000). Transferrin is an iron-transport protein and also acts as a detoxifying protein by removing metals from the medium (George *et al.* 2008). Even iron is an essential trace element and considered as a cofactor for numerous fundamental processes in cells, but can be toxic in the free form (Smith *et al.* 2006). To overcome this problem iron should be supplied bound to transferrin in order to nourish cells in culture. Selenium (Se) is an essential trace element for several physiological processes and is usually supplied in culture media in the form of sodium selenite, which protects cells from oxidative damage by reducing free radical production and inhibiting lipid peroxidation (Cordova *et al.* 2010). Insulin–transferrin–sodium selenite (ITS) is commonly used in both complex and non-complex media as a supplement for the *in vitro* culture of embryos and stem cells in several mammalian species (Das *et al.* 2014).

The epidermal growth factor receptor (EGFR) is involved in the implantation process and enhances mitogenesis and development in several species (Chia *et al.* 1995). Seven ligands bind to and activate mammalian EGFR: four high-affinity ligands (epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF) and β-cellulin) and three low-affinity ligands (amphiregulin, epiregulin and epigen; Yang *et al.* 2017). EGF has been found in several reproductive tissues and plays an important role during pregnancy and labour (Ribeiro *et al.* 2003). It has been reported that EGF and TGF-α increase protein synthesis, the rate of cavitation, cell number and blastocyst expansion in mouse embryos (Chia *et al.* 1995). EGF enhances various steps during preimplantation development and has been implicated in the initiation of implantation in rat embryos (Johnson and Chatterjee 1993). Moreover, EGF has been shown to stimulate both cellular proliferation and differentiation as well as enhancing blastocyst formation, expansion and zona hatching in the mouse (Wei *et al.* 2001).

Ovum pick-up (OPU) combined with *in vitro* embryo production has been increasingly commercialised in the cattle-breeding industry (Isobe *et al.* 2015). Therefore, improvement of the implantation rate of OPU-derived embryos is an important goal for successful assisted reproductive technology (ART). It is a well-known fact that the earliest stages of placental development occur during implantation, when trophectoderm (TE) cells from the fetal blastocyst attach and invade the receptive maternal uterine wall (Li *et al.* 2006). Generally, migration of cells from one area to another, in response to a chemical signal, is mainly to achieve biological functions such as cell differentiation, embryonic development, wound repair and the metastasis of tumours (Hale *et al.* 2010). However, cell invasion, a similar process to cell migration, requires a cell to migrate through an extracellular matrix (ECM) by enzymatically degrading that barrier (Stetler-Stevenson *et al.* 1993). Embryo invasion, an active biochemical process, depends on the ability of TE cells to secrete protease and is one of the most important steps during implantation (Nakayama *et al.* 2002). Defects in the ability of TE cells to fully invade the maternal uterine wall are considered the main reason for implantation failure (Chaddha *et al.* 2004). EGF is expressed in the bovine placenta and likely regulates these cell properties (Dilly *et al.* 2010). It has been reported that TE cells can invade a reconstituted basement membrane *in vitro* based on their ability to secrete matrix metalloproteinases (Bischof *et al.* 2000; Nakayama *et al.* 2002; Schulz and Widmaier 2004; Pennington *et al.* 2012; Chang *et al.* 2014).

Embryo cryopreservation is an important aspect of ART and aims to ensure the high survival and viability of embryos after thawing. The success rate of cryopreservation appears to be highly correlated with the cytoplasmic lipid content because the intracellular lipid content underlies the low post-thaw survival rate of both *in vivo*- and *in vitro*-derived bovine embryos (Weathers and Prien 2014). Additionally, bovine blastocysts produced in serum-supplemented media possess fewer cryotolerant properties than those produced in serum-free media (Murakami *et al.* 2011). These findings indicate that, besides intrinsic qualities, embryo culture conditions play a crucial role in determining the quality of resultant blastocysts. Furthermore, the efficiency of embryo cryopreservation can be improved by modifying the IVC system, e.g. by removing serum from culture media (Murakami *et al.* 2011).

Defined media supplements are commercially available for some cell types. However, companies only provide limited information about the formulation and therefore these supplements are not completely defined. Moreover, conventional media supplemented with fetal bovine serum (FBS) have the disadvantage of variations in serum from lot-to-lot that can affect the reproducibility of experimental findings. Accordingly, the objective of our study was to replace the undefined medium, supplemented with FBS, by defined medium that consistently supports the development of *in vitro*-produced (IVP) bovine embryos at similar or higher rates.

Materials and methods

Ethics statement

All of the methods and experimental procedures were conducted according to the approved (Approval ID: GAR-110502-X0017) guidelines and regulations by the institutional animal care and use committee (IACUC) of the Division of Applied Life Sciences, Department of Animal Science at Gyeongsang National University, Republic of Korea.

Chemicals

Unless otherwise noted, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design

In the present study, we supplemented a type of IVC medium, modified synthetic oviduct fluid-bovine embryo 1 (SOF-BE1), with bovine serum albumin (BSA) plus ITS and/or EGF to test their beneficial effects on bovine embryo development *in vitro*. For this purpose, SOF-BE1 medium supplemented with BSA+ITS (SOF+BSA+ITS group), BSA+EGF (SOF+BSA+EGF group), BSA+ITS+EGF (SOF+BSA+ITS+EGF group) or fetal bovine serum (SOF+FBS group) was compared with SOF-BE1 medium with BSA alone (SOF+BSA group). Based on the blastocyst development data, supplementation with BSA+ITS+EGF had the largest beneficial effects on embryonic development; therefore, we further compared this medium with FBS-supplemented medium.

Oocyte collection

Ovaries obtained from Korean native cows (Hanwoo) were collected at a local abattoir, placed in physiological saline (0.9% NaCl) at ~35°C and transported to the laboratory within 2 h of slaughter. Ovaries were washed in fresh Dulbecco's phosphate-buffered saline (D-PBS) and cumulus–oocyte complexes (COCs) were recovered from follicles with a diameter of 2–8 mm using an 18-gauge needle attached to a vacuum pump. Aspirated fluid was expelled into dishes containing Tyrode lactate-HEPES (TL-HEPES) medium (114 mM sodium chloride, 3.2 mM potassium chloride, 2.0 mM sodium bicarbonate, 0.34 mM sodium biphosphate, 10 mM sodium lactate, 0.5 mM magnesium chloride, 2.0 mM calcium chloride, 10 mM HEPES, 1 μL mL^{-1} phenol red, 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin) and imaged with a stereomicroscope. Class 1 and Class 2 COCs with more than three layers of compact cumulus cells and homogenous cytoplasm were selected for *in vitro* maturation. The COCs were washed three times in TL-HEPES medium.

In vitro *maturation (IVM)*

The COCs were washed three times in *in vitro* maturation (IVM) medium (tissue culture medium 199 (TCM-199)) supplemented with 10% (v/v) FBS (Performance Plus; GIBCO BRL), 1 μg mL^{-1} oestradiol-17β, 10 μg mL^{-1} follicle-stimulating hormone, 10 ng mL^{-1} EGF, 0.6 mM cysteine and 0.2 mM sodium pyruvate (Mesalam *et al.* 2017*a*). Groups of up to 50 COCs were transferred to a four-well dish (Thermo Fisher Scientific) containing 500 μL IVM medium and then incubated under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 22–24 h.

In vitro *fertilisation (IVF)*

In vitro-matured COCs were fertilised with frozen–thawed bovine spermatozoa previously tested for IVF as previously described (Mesalam *et al.* 2017*a*). Frozen semen from a Hanwoo bull was thawed in a water bath at 39°C for 1 min and motile spermatozoa were obtained by centrifuging in D-PBS at 750*g* at room temperature for 5 min. The pellet, collected from the bottom, was resuspended in 500 μL 20 μg mL^{-1} heparin prepared in *in vitro* fertilisation (IVF) medium (Tyrode lactate solution supplemented with 6 mg mL^{-1} BSA, 22 μg mL^{-1} sodium pyruvate, 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin) and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 15 min to facilitate capacitation. Subsequently, spermatozoa were diluted in an appropriate volume of IVF medium to give a final concentration of 1×10^6 spermatozoa mL^{-1}. Matured COCs were transferred in groups of up to 50 to four-well dishes containing 500 μL IVF medium containing spermatozoa and incubated under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 18–20 h.

In vitro *culture*

After co-culture with spermatozoa for 20 h, cumulus cells were removed by pipetting and groups of up to 50 presumed zygotes were washed and transferred to four-well dishes containing 500 μL SOF-BE1 (Mesalam *et al.* 2017*b*) medium supplemented with (1) 4 mg mL^{-1} essentially fatty acid-free BSA and 100 ng mL^{-1} EGF (SOF+BSA+EGF group), (2) 4 mg mL^{-1} BSA, 5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin and 5 ng mL^{-1} sodium selenite (SOF+BSA+ITS group), (3) a combination of BSA, ITS and EGF (SOF+BSA+ITS+EGF group) or (4) only 4 mg mL^{-1} BSA (SOF+BSA and SOF+FBS groups) for the first 3 days. Then, 8-cell stage embryos were cultured until Day 8 of embryonic development in medium of the same composition for all groups, except that BSA was replaced with 10% (v/v) FBS in the SOF+FBS group. Day-8 blastocysts were washed three times in TL-HEPES, transferred to fixative (4% (v/v) paraformaldehyde prepared in 1 M phosphate-buffered saline (PBS)) and stored at 4°C until cells were counted. For mRNA extraction and gene expression analysis, Day-8 blastocysts were transferred to a 1.5-mL Eppendorf tube (five blastocysts per tube), immediately snap-frozen in liquid nitrogen and stored at −80°C until mRNA extraction.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed according to the manufacturer's protocol using an *In Situ* Cell Death Detection Kit (Roche Diagnostics Corp.). Briefly, fixed embryos ($n = 30$) were washed twice with 0.3% (w/v) polyvinylpyrrolidone (PVP) prepared in 1 M PBS (PVP-PBS) before being permeabilised (0.5% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate) at room temperature for 30 min. After permeabilisation, embryos were washed twice with PVP-PBS and incubated in the dark with fluorescently conjugated terminal deoxynucleotide transferase dUTP at 37°C for 1 h. TUNEL-stained embryos were then washed with PVP-PBS and incubated in PVP-PBS containing 10 μg mL^{-1} Hoechst 33342 for

10 min. After washing with PVP-PBS, blastocysts were mounted onto glass slides and their nuclear configuration was analysed. The number of cells per blastocyst was determined by counting Hoechst-stained cells under an epifluorescence microscope (Olympus IX71) equipped with a mercury lamp. TUNEL-positive cells were bright red indicating the occurrence of apoptosis. The apoptotic indices were evaluated by dividing the number of apoptotic blastomeres by the total number of blastomeres.

Counting of TE and inner cell mass (ICM) cells

Day-8 bovine embryos ($n = 30$) were fixed in freshly prepared 4% paraformaldehyde solution for 15 min at room temperature. Embryos were washed three times with ice-cold 0.2% (w/v) PVP prepared in 1 M PBS, permeabilised with 0.25% (v/v) Triton X-100 prepared in 1 M PBS at room temperature for 20 min and then washed with wash buffer (0.1% (v/v) Tween 20 and 0.1% (w/v) BSA prepared in 1 M PBS). Embryos were then incubated for 1 h at room temperature with blocking buffer (5% BSA prepared in 1 M PBS) before being incubated with an anti-CDX2 antibody (Biogenex) overnight at 4°C. Embryos were washed three times in wash buffer and then incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (1:100 in 0.1% (v/v) Tween 20 and 1% (w/v) BSA prepared in 1 M PBS; Santa Cruz Biotechnology) for 1 h at room temperature in the dark. Embryos were washed three times in wash buffer and then incubated with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI; 1:100 (v/v) in D-PBS) for 5 min. The stained embryos were mounted on glass slides with Prolong anti-fade reagent (Molecular Probes) and examined under a confocal laser-scanning Olympus Fluoview FV1000 microscope.

Cytoplasmic lipid content

Nile red (NR), a fluorescent dye specific for intracellular lipids, was used to evaluate the lipid content of Day-8 blastocysts (30 blastocysts per group). In brief, a 1 mg mL^{-1} stock solution of NR was prepared in dimethyl sulphoxide and stored in the dark at room temperature. Fixed Day-8 blastocysts were washed three times (15 min each) with D-PBS and then stained with 10 μg mL^{-1} NR dissolved in physiological saline (0.9% NaCl) containing 1 mg mL^{-1} PVP for 3 h in the dark at room temperature. Embryos were then washed with PVP-PBS and incubated in DAPI (1:100 (v/v) in D-PBS) for 10 min. Thereafter, blastocysts were washed twice with PBS, mounted onto glass slides and overlaid with coverslips. A confocal laser-scanning Olympus Fluoview FV1000 microscope was used to excite the lipophilic fluorescent dye NR (485 nm). For image analysis, the intensities of red fluorescence (lipids) were measured using ImageJ software (National Institutes of Health; https://imagej.nih.gov/ij, verified 20 July 2018) after normalisation through subtraction of the background intensity from each image of experimental groups. The software calculated the average pixel intensity. The data were normalised to an average of two background regions for each projection. The average intensity was considered for statistical analysis.

Characterisation of mitochondrial activity

Mitochondrial activity was detected with a commercial kit (MitoTracker Green FM; Invitrogen) according to the manufacturer's instructions. Day-8 blastocysts (30 blastocysts per group) were incubated with 125 nM MitoTracker Green FM for 5 min. Then, embryos were washed with PVP-PBS and incubated in DAPI (1:100 (v/v) in D-PBS) for 10 min. Embryos were then washed twice with PVP-PBS, mounted on glass slides and examined under a confocal laser-scanning Olympus Fluoview FV1000 microscope. The fluorescence excitation wavelength was 594 nm and emission was read at 608 nm. For image analysis, the intensities of green fluorescence (mitochondrial activity) were measured using ImageJ software after normalisation through subtraction of the background intensity from each image of experimental groups.

Invasion assay

To quantify invasion, Day-8 blastocysts were plated on Matrigel invasion chamber inserts (6.4 mm; Corning Inc. Life Sciences) containing polyethylene terephthalate membranes with 8 μm-diameter pores in 24-well tissue culture plates (Corning Inc. Life Sciences; Nakayama *et al.* 2002). The upper surface of the filters was coated with Matrigel (20 μg per filter; Discovery Labware Inc.) and then incubated at 37°C for 2 h to allow drying. The blastocysts were added to the filter coated with Matrigel (three blastocysts per culture insert suspended in the same medium used for embryo production) then incubated under a humidified atmosphere of 5% CO_2 in air at 37°C for 72 h and then the culture medium at the bottom of the culture (Shahbazi *et al.* 2016) was changed from IVC1 to IVC2 and refreshed on a daily basis (see Fig. S1, available as Supplementary Material to this paper). After 10 days of culture, the invasion area of trophoblasts was evaluated under a phase-contrast Olympus IX71 microscope and measured using ImageJ software. Thereafter, the upper surface of the chamber insert membrane was scrubbed three times with a cotton swab. The cells on the lower surface of the scrubbed membrane were fixed with 4% (v/v) paraformaldehyde prepared in 1 M PBS for 15 min at room temperature and then stained with 10 μg mL^{-1} Hoechst 33342 for 10 min. Cells that traversed the membrane were counted under a phase-contrast Olympus IX71 microscope at 100× total magnification.

Embryo cryopreservation and thawing

Day-7 blastocysts were washed three times with 0.5% (w/v) BSA prepared in 1 M PBS (BSA-PBS) then embryos were slowly frozen using 1.8 M ethylene glycol as a cryoprotectant together with 0.1 M sucrose and 0.5% BSA (Mesalam *et al.* 2017*b*). Embryos were kept in the freezing solution for 10 min to equilibrate and then slowly frozen from −6°C to −32°C (−0.3°C min^{-1}) in a 0.25 mL straw using the CL-8800i freeze control system (CryoLogic). The straw was placed in liquid nitrogen after slow freezing was complete. Four biological replicates were performed, with 20 embryos per replicate ($n = 80$ per group). To thaw the embryos, the straw was exposed to air for ~10 s and placed in a water bath at 37°C for 20 s. To calculate the survival rates following blastocyst re-expansion, frozen–thawed embryos were cultured in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 24 h.

Messenger RNA extraction and cDNA reverse transcription

Total RNA was extracted from Day-8 blastocysts or survived frozen–thawed blastocysts ($n = 20$ per group; four biological replicates were performed, with five embryos per replicate) using a PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer's instructions. The RNA concentration and purity were checked using a NANO DROP 2000c machine (Thermo Fisher Scientific). RNA samples were used immediately or stored at −80°C until use. The mRNA samples were reverse-transcribed into first-strand cDNA using a kit from Bio-Rad Laboratories according to the manufacturer's instructions. The cDNA samples were kept at −20°C until used for quantitative polymerase chain reaction (qPCR).

Quantitative reverse transcription PCR (RT-qPCR) analysis

Quantitative PCR primers were designed using Primer3plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi, verified 20 July 2018) based on the mRNA sequences of selected genes available in GenBank (Table 1). Quantitative analysis of cDNA samples was performed using a CFX98 instrument (Bio-Rad Laboratories). To improve the reproducibility of the results, samples from the same cDNA source were run in duplicate for each PCR reaction. The target genes were quantified by the $^{\Delta\Delta}$CT method and results were reported as the relative expression or n-fold difference in comparison to the calibrator after normalisation of the transcript amount relative to the mean level of the reference genes. The coefficients of variation of the intra- and inter-assay variance were calculated according to the formula, s.d./mean × 100, for all genes profiled by qPCR. Reference genes used for normalisation (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin beta (*ACTB*)) were selected according to previous studies (Dhali *et al.* 2011; Mesalam *et al.* 2017*b*). RT-qPCR was performed to quantify the mRNA expression levels of several target genes, namely matrix metalloproteinase-2 (*MMP2*), matrix metalloproteinase-9 (*MMP9*),

Table 1. Primer sequences used to analyse expression of target and housekeeping genes in Day-8 blastocysts

Abbreviations: F, forward; R, reverse; *MMP2*, matrix metalloproteinase-2; *MMP9*, matrix metalloproteinase-9; *ACADL*, acyl-coenzyme A dehydrogenase long-chain; *ACSL3*, acyl-CoA synthetase long-chain family member 3; *HMGCR*, hydroxymethylglutaryl-CoA reductase; *IGF2R*, insulin-like growth factor 2 receptor; *SIRT1*, sirtuin 1; *SOD2*, superoxide dismutase 2; *TFAM*, transcription factor A mitochondrial; *BCL2*, B-cell lymphoma 2; *BAX*, BCL2 associated X apoptosis regulator; *HSP70*, heat-shock protein 70; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, actin beta

Gene	Sequence	GenBank accession number	Product size (bp)
MMP2	F: CTTCAAGGACCGATTCATCTG R: CAGTTAAAGGCAGCATCCAC	NM_174745.2	260
MMP9	F: GTTCCCAGAATGAGGTGAAT R: GAACAGATCCACTAGTTGGG	NM_174744.2	177
ACADL	F: GGGGGACTTGTATTCATCAG R:AATACATTTCCCTGCAACCA	NM_001076936.1	169
ACSL3	F: TCAAGCTGAAACGAAAAGAG R:TATTTGATCTGAGCTCCCTG	NM_001205468.2	118
HMGCR	F: AACCCTTACTGGCAGAAAAT R: TGCAGACACTCTTCATTAGG	NM_001105613.1	148
IGF2R	F: GTGAGTATGTGTTTCTCTG R: GTATCAGGTTATACAGGTTG	NM_174352.2	111
SIRT1	F: CAACGGTTTCCATTCGTGTG R: GTTCGAGGATCTGTGCCAAT	NM_001192980.2	138
SOD2	F: GGGAGAATGTAACTGCACGA R: ACAACAGAGCAGCGTACTGG	NM_201527.2	133
TFAM	F: CTGGTCAGTGCTTTGTCTGC R: CTAAAGGGATAGCGCAGTCG	NM_001034016.2	128
BCL2	F: TGGATGACCGAGTACCTGAA R: CAGCCAGGAGAAATCAAACA	NM_001166486.1	120
BAX	F: CACCAAGAAGCTGAGCGAGTGT R: TCGGAAAAAGACCTCTCGGGGA	NM_173894.1	118
HSP70	F: GAGTCGTACGCCTTCAACAT R: ACTTGTCCAGCACCTTCTTC	U09861.1	94
GAPDH	F: CCCAGAATATCATCCCTGCT R: CTGCTTCACCACCTTCTTGA	NM_001034034.2	185
ACTB	F: ATTTTGAATGGACAGCCATC R: TGTACAGGAAAGCCCTGACT	NM_173979.3	120

acyl-coenzyme A dehydrogenase long-chain (*ACADL*), acyl-CoA synthetase long-chain family member 3 (*ACSL3*), hydroxymethylglutaryl-CoA reductase (*HMGCR*), insulin-like growth factor 2 receptor (*IGF2R*), sirtuin 1 (*SIRT1*), superoxide dismutase 2 (*SOD2*), transcription factor A mitochondrial (*TFAM*), B-cell lymphoma 2 (*BCL2*), BCL2 associated X apoptosis regulator (*BAX*) and heat-shock protein 70 (*HSP70*). The levels of these genes were normalised to that of the reference genes *GAPDH* and *ACTB*.

Immunofluorescence analysis

Day-8 bovine embryos (30 blastocysts per group) were fixed in freshly prepared 4% paraformaldehyde solution for 15 min at room temperature, washed three times with PVP-PBS and permeabilised by 0.5% (v/v) Triton X-100 prepared in 1 M PBS at room temperature for 20 min and subsequently washed three times with PVP-PBS. Embryos were incubated for 4 h at room temperature with blocking buffer (10% donkey serum and 3% (w/v) BSA in PBS) before incubation overnight at 4°C with primary antibodies (1:200 in 3% (w/v) BSA and 0.1% Tween 20 in PBS) against MMP2 (mouse monoclonal; Fitzgerald) or MMP9 (rabbit polyclonal; Biorbyt). Embryos were washed three times in PVP-PBS and incubated with a fluorescein isothiocyanate (FITC)- or TRITC-conjugated secondary antibody (1:100; Santa Cruz Biotechnology) at room temperature for 90 min. The nuclei were stained with DAPI (1:100 (v/v) in D-PBS) for 10 min and then embryos were washed three times with PVP-PBS. The stained embryos were mounted on glass slides with Prolong anti-fade reagent and examined under a confocal laser-scanning Olympus Fluoview FV1000 microscope. All images were acquired in a sequential mode to avoid bleed-through of the signal. The integrated optical densities of MMP2 and MMP9 were determined using ImageJ software. The software calculated the average pixel intensity. The data were normalised to an average of two background regions for each projection. The average intensity of each protein was considered for statistical analysis.

Statistical analysis

Statistical analyses were performed using SPSS software Version 18.0 (IBM Corp.). Experiments were performed in six replicates and each replicate was performed using oocytes matured on the same day. The total cell, apoptosis cell number and immunofluorescence staining were determined using blastocysts randomly selected from each group (~5 embryos each replicate). Data were tested for a normal distribution and homogeneity of variance and underwent arcsine transformation when these criteria were not met. A one-way ANOVA was used to compare embryonic development (cleavage and blastocyst rates). Duncan's multiple-range test was used to compare the treatment groups. Data concerning blastocyst quality (i.e. TE, ICM, ICM:TE cell ratio) and expression levels of the various genes between the experimental groups were compared using the Student's *t*-test. The data are presented as the mean ± standard error of mean (s.e.m.). $P < 0.05$ was considered to be significant and $P < 0.01$ was considered to be highly significant.

Results

Determination of the ability of preimplantation embryos to undergo cleavage and develop into blastocysts

The percentage of cleaved embryos was determined on Day 3 of culture. The cleavage rate was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group (86.24 ± 0.64%) than in the SOF+BSA+ITS, SOF+BSA+EGF, SOF+FBS and SOF+BSA groups (81.12 ± 1.98%, 80.25 ± 1.60%, 80.00 ± 1.69% and 79.86 ± 1.60% respectively; Table 2). However, the percentage of Day-8 blastocysts was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group (41.42 ± 0.86%) than in the SOF+FBS and SOF+BSA+ITS groups (37.28 ± 1.41% and 36.00 ± 0.47% respectively), followed by SOF+BSA+EGF (31.28 ± 0.89%) and was lowest in the SOF+BSA group (24.70 ± 1.61%; Table 2). The hatching rate was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group (43.76 ± 2.46%) than in the SOF+BSA+EGF, SOF+FBS and SOF+BSA+ITS groups (27.73 ± 2.03%, 25.71 ± 1.46% and 25.52 ± 2.09% respectively) and was lowest in the SOF+BSA group (8.82 ± 2.66%; Table 2). To investigate whether the improvement of development by BSA+ITS+EGF supplementation affected the quality of blastocysts, the total cell number and frequency of apoptotic nuclei were determined in blastocysts. The total number of cells in Day-8 blastocysts was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group (209.87 ± 3.05) than in the SOF+FBS group (150.50 ± 2.70). By contrast, the apoptotic index did not significantly differ ($P > 0.05$) between the two groups (Fig. 1).

Counting of TE and ICM cells

The total number cells and the number of TE cells per blastocyst were higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group (210.08 ± 2.39 and 156.67 ± 3.69 respectively) than in the SOF+FBS group (169.67 ± 4.10 and 129.56 ± 2.45 respectively; Table 3 and Fig. 2). Moreover, the number of ICM cells was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group (53.42 ± 3.21 vs 40.11 ± 2.02). However, the ICM:TE cell ratio was higher in the SOF+BSA+ITS+EGF group (0.36 ± 0.03) than in the SOF+FBS group (0.31 ± 0.01) but this difference was not significant ($P > 0.05$).

Lipid content and mitochondrial activity

Confocal images of bovine embryos stained with NR showed that lipid droplets were distributed throughout the entire blastocyst. The integrated optical intensity measured by ImageJ software showed a reduction ($P < 0.01$) in the level of cytoplasmic lipid content of embryos in the SOF+BSA+ITS+EGF group compared to the SOF+FBS group (Fig. 3). However, the mitochondrial fluorescence intensity of blastocysts was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group (Fig. 4).

Post-thaw survival of IVP bovine embryos that had been cryopreserved by slow freezing

The post-thaw survival rate of blastocysts was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS

Table 2. Cleavage, developmental and hatching rates of embryos cultured in different media

[a–d]Values with different superscripts in the same column are significantly different ($P < 0.01$)

Group	Total no. of oocytes[A]	No. of presumed zygotes	No. of cleaved embryos (% ± s.e.m.)	Total no. of blastocysts (% ± s.e.m.)[B]	No. of hatched blastocysts (% ± s.e.m.)
SOF+BSA+ITS+EGF	303	285	246(86.24 ± 0.52)[a]	118(41.42 ± 0.70)[a]	52(43.76 ± 2.01)[a]
SOF+FBS	288	265	211(80.00 ± 1.38)[b]	100(37.28 ± 1.15)[b]	25(25.71 ± 1.19)[b]
SOF+BSA+ITS	266	250	203(81.12 ± 1.62)[b]	90(36.00 ± 0.38)[b]	23(25.52 ± 1.71)[b]
SOF+BSA+EGF	335	313	249(80.25 ± 1.01)[b]	98(31.28 ± 0.56)[c]	27(27.73 ± 1.28)[b]
SOF+BSA	253	232	186(79.86 ± 1.31)[b]	56(24.70 ± 1.32)[d]	5(8.82 ± 2.17)[c]

[A]Six replicates were performed.
[B]Rates are relative to the number of presumed zygote.

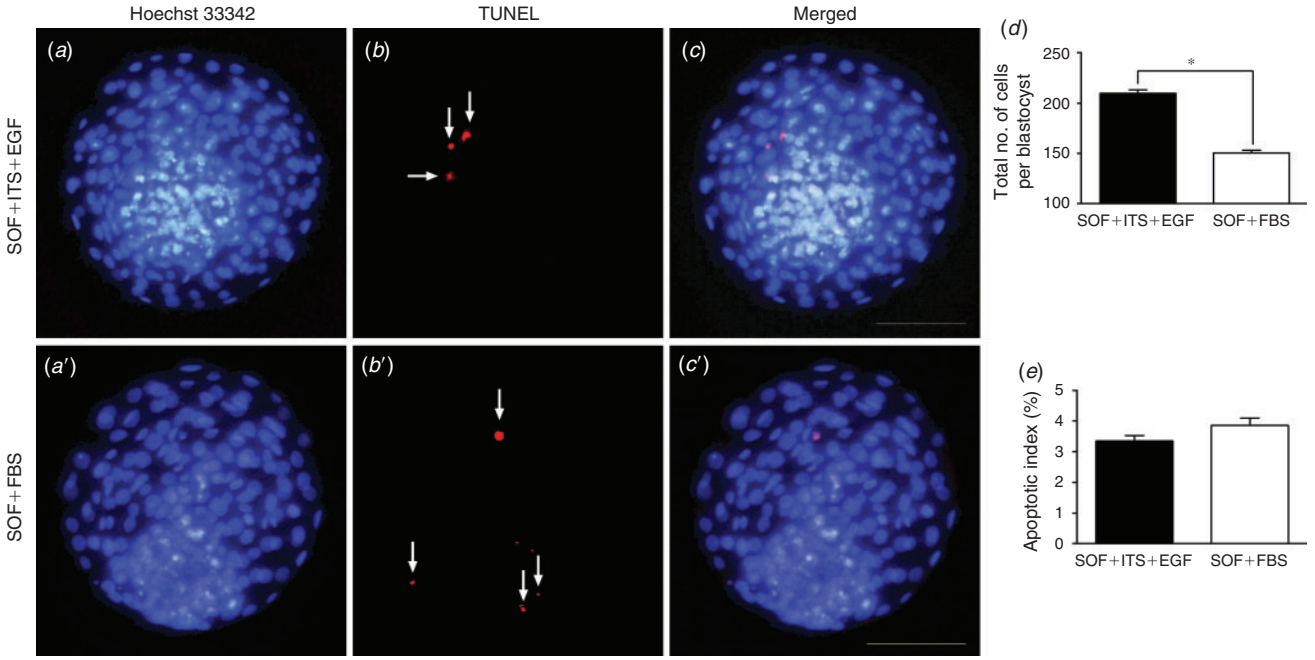

Fig. 1. Day-8 bovine embryos were stained with Hoechst 33342 to determine the total cell number and TUNEL was performed to identify apoptotic cells. The corresponding images were merged. Embryos were cultured in the (*a–c*) SOF+BSA+ITS+EGF and (*a′–c′*) SOF+FBS groups. Scale bar = 100 µm. Apoptotic cells are indicated by white arrows. (*d*) Mean total cell numbers determined by Hoechst 33342 staining. (*e*) Mean total apoptotic cell numbers determined by TUNEL. Statistically significant differences are indicated:*, $P < 0.01$.

Table 3. Total cell number and numbers of TE and ICM cells in embryos cultured in different media

TE, trophectoderm; ICM, inner cell mass. Values with different superscripts in the same column are significantly different ($P < 0.01$)

Group	Total cell no.	No. of TE cells	No. of ICM cells	ICM:TE cell ratio
SOF+BSA +ITS+EGF	210.08 ± 2.39[a]	156.67 ± 3.69[a]	53.42 ± 3.21[a]	0.36 ± 0.03
SOF+FBS	169.67 ± 4.10[b]	129.56 ± 2.45[b]	40.11 ± 2.02[b]	0.31 ± 0.01

group (96.50% ± 2.29% vs 46.00% ± 3.41%). Moreover, the hatching rate 24 h after blastocyst recovery was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group (41.00% ± 2.71%) than in the SOF+FBS group (29.50% ± 1.35%; Fig. 5).

Invasion and migration abilities of trophoblasts

The invasion area of trophoblasts measured by ImageJ software was larger ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group (242.22 ± 44.63 vs 63.01 ± 11.73 × 10^4 µm^2). Similarly, the number of migrated trophoblasts was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group (1005.80 ± 60.27 vs 262.80 ± 46.58; Fig. 6).

Messenger RNA expression of candidate genes

The mRNA levels of *MMP9* and *ACADL* in Day-8 blastocysts were higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group. Moreover, the mRNA levels of *MMP2*, *ACSL3* and *HMGCR* in Day-8 blastocysts were higher ($P < 0.05$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group. However, there was no difference

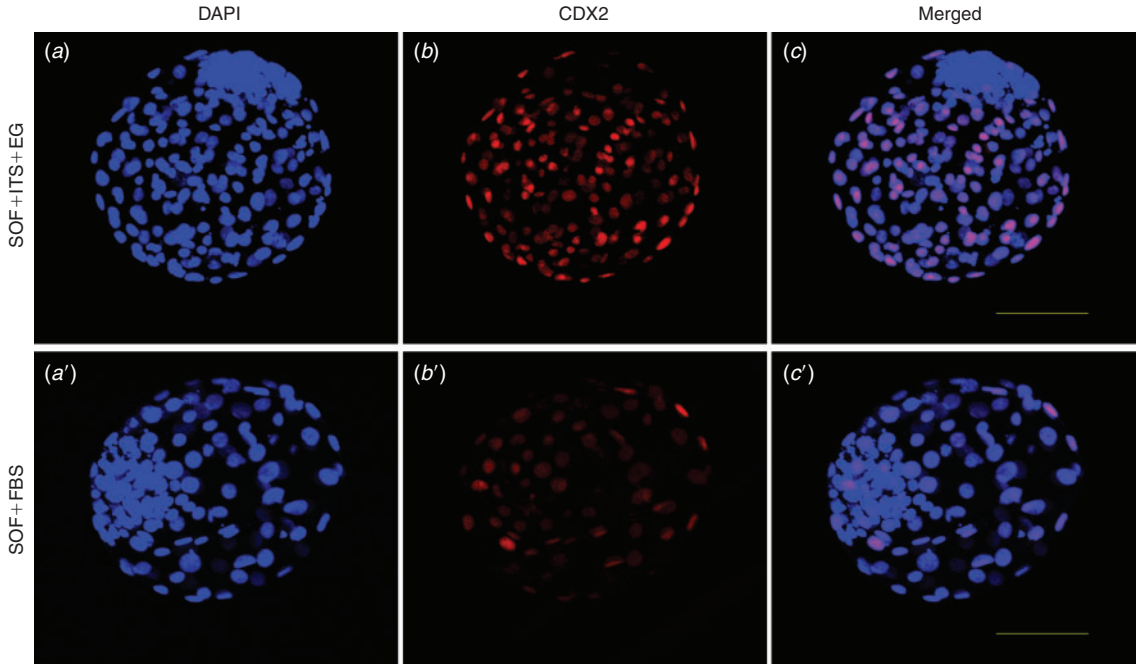

Fig. 2. Immunofluorescence staining of Day-8 blastocysts showing CDX2 protein (red) localised in the TE. Nuclei were stained blue with DAPI. Embryos were cultured in the (*a–c*) SOF+BSA+ITS+EGF and (*a′–c′*) SOF+FBS groups. Images were taken with a confocal microscope. Scale bar = 100 µm.

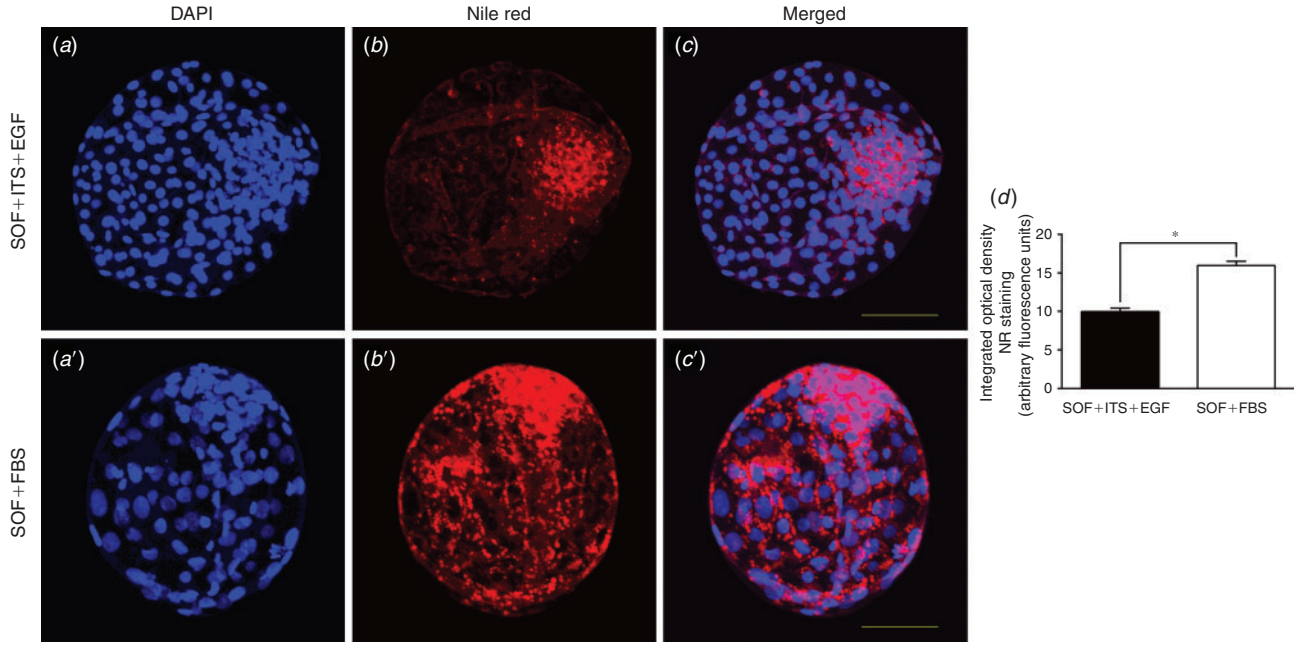

Fig. 3. Fluorescence intensity of lipid staining in Day-8 blastocysts. NR staining of Day-8 blastocysts in the (*a–c*) SOF+BSA+ITS+EGF and (*a′–c′*) SOF+FBS groups. Scale bar = 100 µm. (*d*) Mean values of the integrated optical density of lipid staining per blastocyst as analysed by ImageJ software. Statistically significant differences are indicated:*, $P < 0.01$.

between the mRNA level of *IGF2R* in Day-8 blastocysts ($P > 0.05$) in the SOF+BSA+ITS+EGF group and in the SOF+FBS group. Similarly, the mRNA level of *SOD2* in frozen–thawed blastocysts was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group. Furthermore, the mRNA levels of *SIRT1*, *TFAM* and *BCL2* in frozen–thawed blastocysts were higher ($P < 0.05$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group,

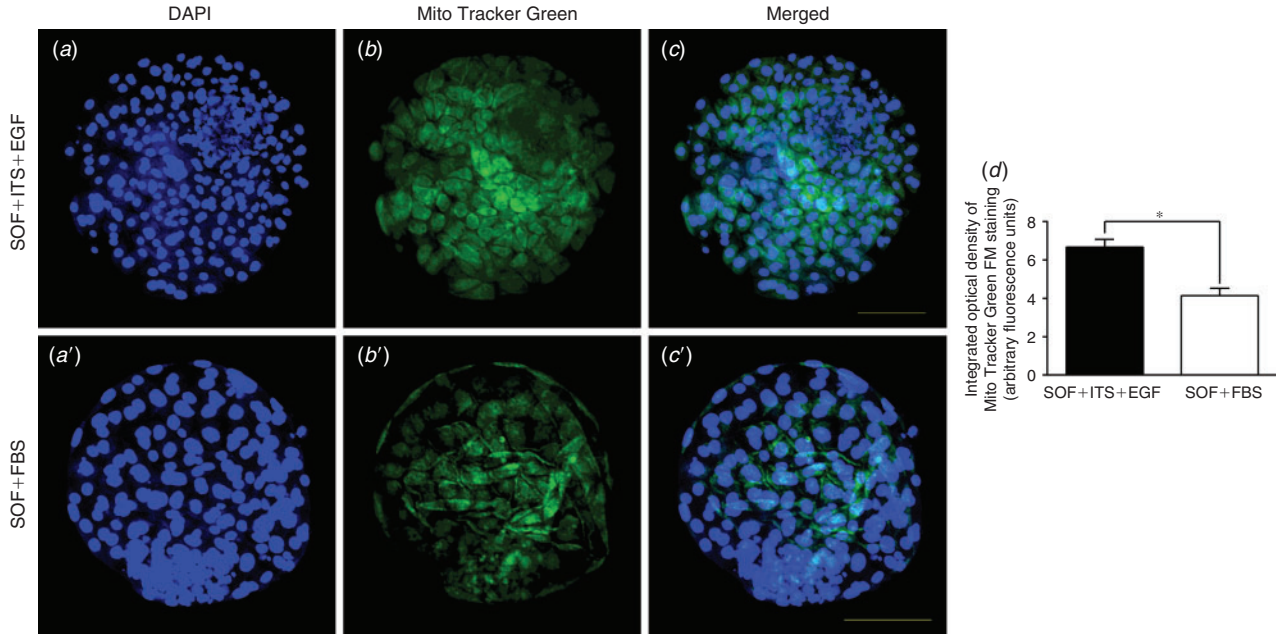

Fig. 4. Fluorescence intensity of mitochondrial staining in blastocysts. MitoTracker Green staining of Day-8 blastocysts in the (*a*–*c*) SOF+BSA+ITS+EGF and (*a*′–*c*′) SOF+FBS groups. Scale bar = 100 µm. (*d*) Mean values of the integrated optical density of mitochondrial staining per blastocyst as analysed by ImageJ software. Statistically significant differences are indicated:*, $P < 0.01$.

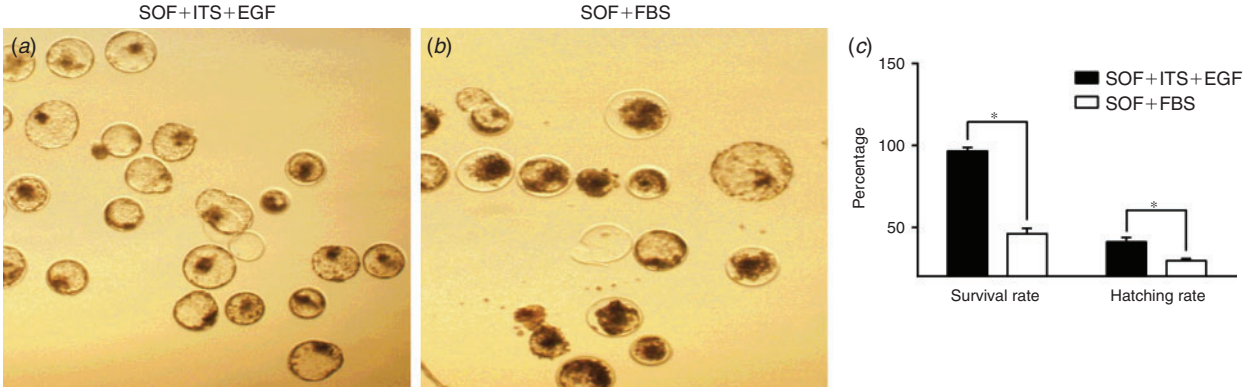

Fig. 5. Survival and hatching rates of embryos 24 h after thawing. Brightfield images showing the survival and hatching rates in the (*a*) SOF+BSA+ITS+EGF and (*b*) SOF+FBS groups. (*c*) Mean values of the survival and hatching rates. Statistically significant differences are indicated: *, $P < 0.01$.

whereas that of *BAX* was significantly lower. However, there was no difference between the mRNA level of *HSP70* in frozen–thawed blastocysts ($P > 0.05$) in the SOF+BSA+ITS+EGF group and in the SOF+FBS group (Fig. 7).

Immunofluorescence analysis of MMP2 and MMP9

Immunofluorescence analysis was performed using monospecific antibodies to quantify the protein expression levels of MMP2 and MMP9. The integrated optical intensity measured by ImageJ software showed an increase ($P < 0.01$) in the level of MMP2 in the SOF+BSA+ITS+EGF group compared with the SOF+FBS group (Fig. 8). Furthermore, the expression level of MMP9 was higher ($P < 0.01$) in the SOF+BSA+ITS+EGF group than in the SOF+FBS group (Fig. 9).

Discussion

Recently, there has been a shift from the use of FBS to serum-free defined media in IVP embryo culture systems. Efforts to develop a substitute for FBS are primarily based on the rapidly expanding field of IVC, which requires defined, safe and animal component-free culture conditions (Rauch *et al.* 2011). Although it is poorly understood how serum-containing culture medium improves embryonic development, it is linked with embryotrophic factors and inactivation of embryotoxic agents

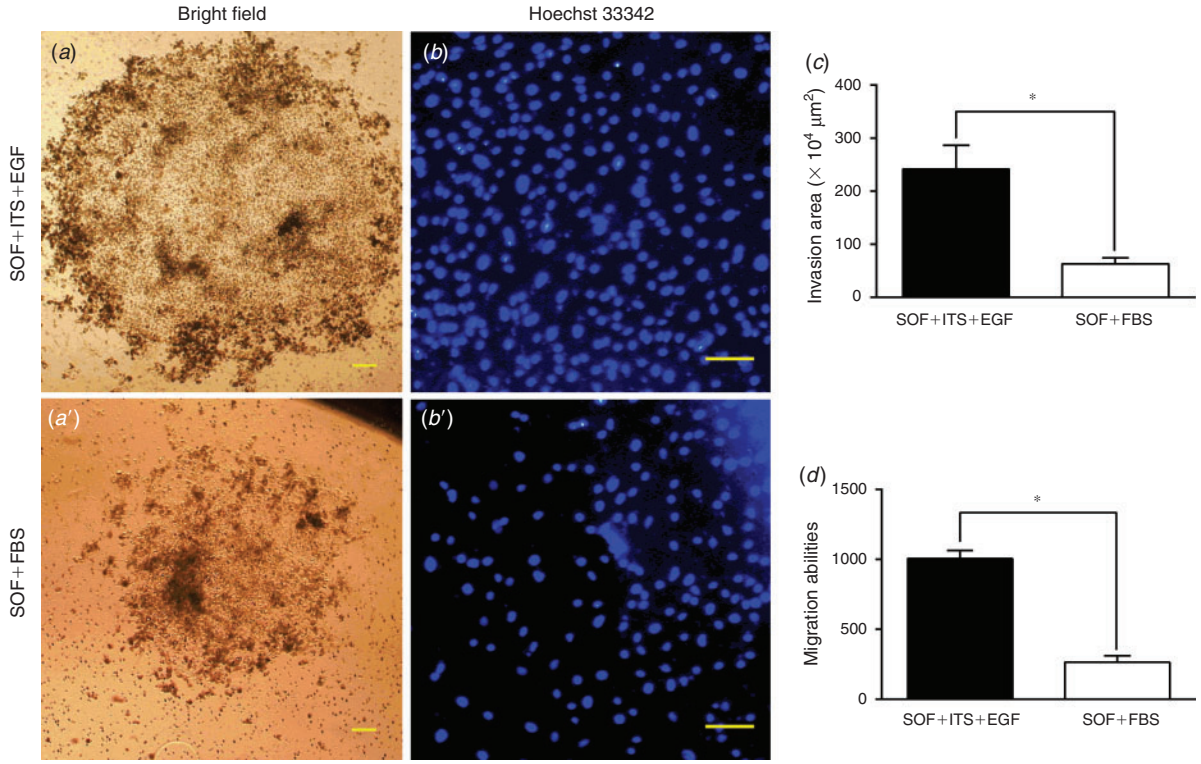

Fig. 6. Invasion and migration abilities of trophoblasts. Brightfield images showing the invasion area in the (*a*) SOF+BSA+ITS+EGF and (*a*′) SOF+FBS groups. Migrated cells stained with Hoechst 33342 in the (*b*) SOF+BSA+ITS+EGF and (*b*′) SOF+FBS groups. Scale bar = 100 μm. (*c*) Mean invasion areas as analysed by ImageJ software. (*d*) Mean numbers of migrated cells. Statistically significant differences are indicated: *, $P < 0.01$.

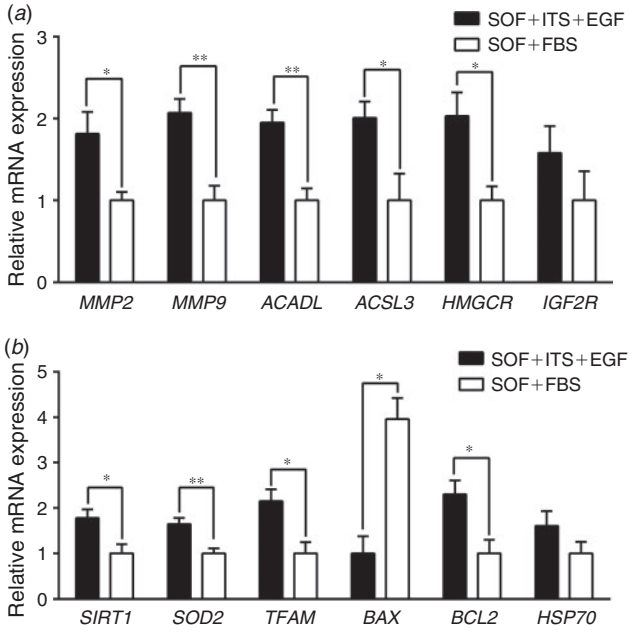

Fig. 7. Relative mRNA levels of various genes in blastocysts determined by RT-qPCR. (*a*) Relative mRNA levels of *MMP2*, *MMP9*, *ACADL*, *ACSL3*, *HMGCR* and *IGF2R* in Day-8 blastocysts. (*b*) Relative mRNA levels of *SIRT1*, *SOD2*, *TFAM*, *BAX*, *BCL2* and *HSP70* in frozen–thawed blastocysts. Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$.

(Bavister 1995). In previous studies, a serum-free culture system supplemented with BSA and ITS resulted in an acceptable number of embryos; however, the presence of serum ensured the highest blastocyst development rates (Goovaerts *et al.* 2012). Moreover, developmental rates to the blastocyst stage of embryos cultured in-group did not increase by adding BSA to SOF medium supplemented with ITS (George *et al.* 2008). Addition of several growth factors, including EGF, produced no positive effects on blastocyst development when TCM-199 was employed as the chemically defined culture medium (Sirisathien *et al.* 2003). In the present study, BSA, ITS and EGF were used either alone or in combination as alternatives to serum for bovine embryo culture. The cleavage, blastocyst formation and hatching rates of embryos were significantly higher in the SOF+BSA+ITS+EGF group than in the SOF+FBS group, suggesting that the combination of ITS and EGF served either as competence factors or acted synergistically to enhance the developmental ability and quality of bovine embryos, which is in agreement with previous a study that reported that EGF could possibly increase blastocyst formation and hatching in the mouse (Wei *et al.* 2001).

It has previously been reported that embryo quality is a key factor for successful embryo implantation in ART, whereas cell number plays important roles in embryo quality (Yu *et al.* 2007). Moreover, total cell number and ICM:TE cell ratio offer important additional information for the evaluation of the quality of individual embryos (Wydooghe *et al.* 2011). Embryos

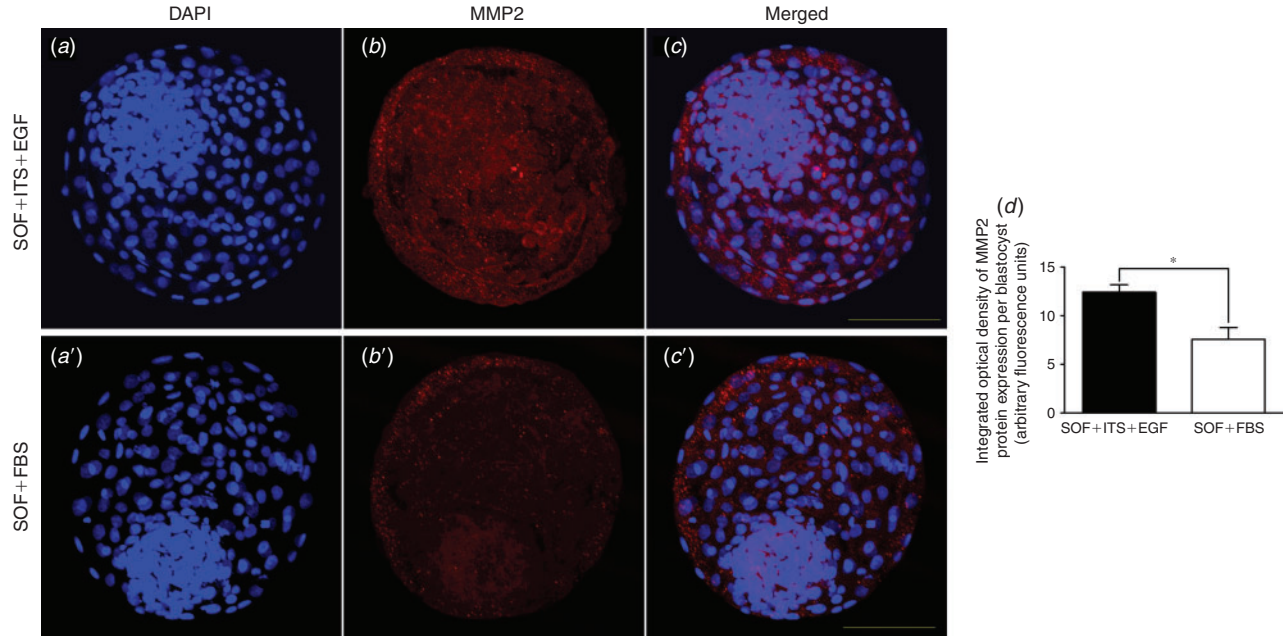

Fig. 8. Fluorescence intensity of MMP2 protein expression in blastocysts. MMP2 protein expression of Day-8 blastocysts in the (*a*–*c*) SOF+BSA+ITS+EGF and (*a*′–*c*′) SOF+FBS groups. Scale bar = 100 µm. (*d*) Mean values of the integrated optical density of MMP2 protein expression per blastocyst as analysed by ImageJ software. Statistically significant differences are indicated: *, $P < 0.01$.

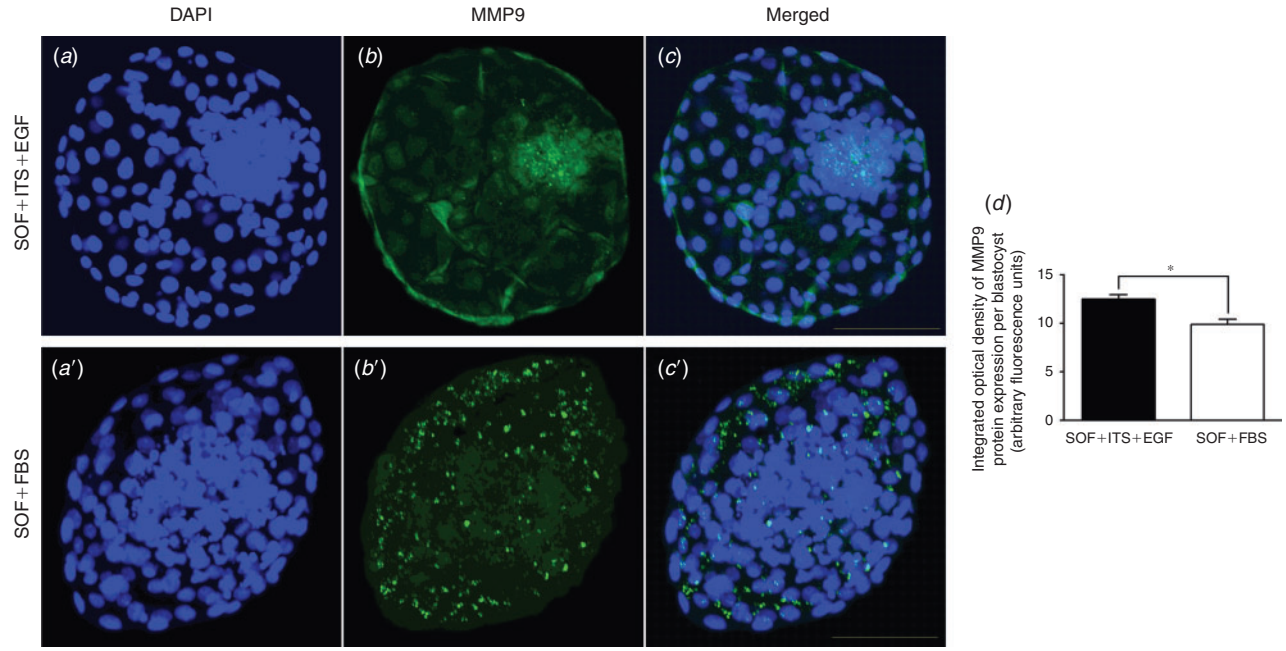

Fig. 9. Fluorescence intensity of MMP9 protein expression in blastocysts. MMP9 protein expression of Day-8 blastocysts in the (*a*–*c*) SOF+BSA+ITS+EGF and (*a*′–*c*′) SOF+FBS groups. Scale bar = 100 µm. (*d*) Mean values of the integrated optical density of MMP9 protein expression per blastocyst as analysed by ImageJ software. Statistically significant differences are indicated: *, $P < 0.01$.

containing a greater number of cells are more likely to implant and develop into live offspring (Van Soom *et al.* 2007). In the present study, the number of TE cells, ICM cells and the total cell number per blastocyst were significantly higher in the SOF+BSA+ITS+EGF group than in the SOF+FBS group. A previous study observed that a group culture in serum-free medium based on BSA and ITS typically generated blastocysts with higher total cell numbers (Wydooghe *et al.* 2014).

An alternative approach to improve the cryotolerance of IVP embryos is to modify the cells themselves to make them more cryopreservable. Reduction of the cytoplasmic lipid content (delipation) of bovine embryos improves their cryotolerance (Seidel 2006). Slow freezing using a standard 0.25 mL plastic straw allows post-thawed embryos to be directly transferred into cattle. Delipation represents an efficient way to improve the cryosurvival rate of pig embryos by removing cytoplasmic lipid droplets (Wang *et al.* 2015). Development of the delipation technique confirmed that intracellular lipids are linked to cold sensitivity (Nagashima *et al.* 1994). We also investigated the effects of ITS and EGF, as alternatives to FBS in culture medium, on cryotolerance. Culture with ITS plus EGF and BSA effectively reduced the intracellular lipid content of embryos, which increased their post-thaw survival and hatching rates when compared with embryos cultured with FBS.

To determine the quality of the frozen–thawed blastocysts, the expression profiles of the pro-survival gene *SIRT1*, which plays a protective role for cell survival under oxidative stress conditions (Ou *et al.* 2014), was analysed by RT-qPCR. Our results demonstrated that ITS plus EGF and BSA significantly increased the expression of *SIRT1*, compared with FBS, which in turn improved the survival rate of frozen–thawed embryos. The expression of the pro-apoptotic gene *BAX* can activate the release of cytochrome c from the mitochondria into the cytoplasm leading to apoptosis (Zhao *et al.* 2016). We found that the addition of ITS plus EGF and BSA to IVC medium improved frozen–thawed blastocyst quality by reducing *BAX* expression. By contrast, the mRNA level of the anti-apoptotic gene *BCL2* was significantly higher in the ITS plus EGF and BSA group than in the FBS group.

Additionally, *SOD2* is located in the mitochondrial matrix and is involved in protecting embryos from oxidative stress (Holley *et al.* 2010). It has been reported that *SOD2* can be used as an indicator of mitochondrial activity and an informative marker of high-quality embryos (Ramalho-Santos *et al.* 2009). Moreover, *TFAM* plays a crucial role in stability, maintenance and transcriptional control of mitochondrial DNA (Antelman *et al.* 2008). In the present study, we found that the mRNA levels of *SOD2* and *TFAM* were significantly increased in frozen–thawed blastocysts in the ITS plus EGF and BSA supplemented group.

One of the basic factors that determines developmental potential in preimplantation embryos is the availability of energy in the form of ATP produced from aerobic mitochondrial metabolism (Wilding *et al.* 2009). Since embryo development requires a huge amount of energy, the developing zygote is dependent on the existing pool of mitochondria until blastocyst implantation (Chappel 2013). Our findings showed that supplementation of medium with ITS plus EGF and BSA significantly increased mitochondrial activity compared with FBS.

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent proteinases. These enzymes can degrade most components of the extracellular matrix. MMPs play important roles in trophoblast invasion and extracellular matrix remodelling (Gao *et al.* 2002). Successful implantation is closely linked to the expression of MMPs, which greatly influences the ability of an embryo to degrade the basement membrane of the uterine epithelium and invade the uterine stroma (Bernhard *et al.* 1994). Our results indicate that levels of MMP2 (gelatinase A) and MMP9 (gelatinase B) were significantly increased after ITS plus EGF and BSA supplementation at both transcriptional and translation levels as confirmed by RT-qPCR and immunofluorescence analysis respectively. Moreover, invasion of trophoblasts, which can be considered as a critical marker for implantation, was stimulated by ITS plus EGF and BSA supplementation, which is consistent with a previous report (Dilly *et al.* 2010) that found that EGF is involved in the upregulation of *MMP9* in bovine trophoblast cells and thus could influence trophoblast migration and may be involved in bovine placental tissue remodelling.

The increased lipid contents of embryos cultured with FBS could be related to the following aspects: (1) tendency of embryonic cells to absorb lipoproteins in serum, (2) ability of serum to change the function of β-oxidation in mitochondria and (3) neo-synthesis of triglycerides in embryos triggered by serum (Sudano *et al.* 2011). Lipids are metabolised to generate ATP via fatty acid oxidation, which is an important energy source for embryos (Dunning *et al.* 2014). Lipid β-oxidation is essential for early embryonic development (Dunning *et al.* 2010). From this angle, the serum can affect mitochondrial metabolism and result in an imbalance in the oxidation–reduction (redox) state that can impair lipid metabolism, leading to excess accumulation of intracellular lipids (Sudano *et al.* 2011). *ACSL3* and *ACADL* encode two important enzymes in the lipid β-oxidation pathway (Yuan *et al.* 2011). In the present study, the mRNA levels of these genes were significantly increased after ITS plus EGF and BSA supplementation.

Cholesterol is present in cell membranes of the majority of eukaryotes where it plays a distinct role in membrane structure and stabilises the lipid bilayer due to its rigid ring structure (Pratt 1982). Increasing the cholesterol content of membranes in spermatozoa and oocytes appears to improve their cryotolerance (Seidel 2006). Cholesterol is important for preimplantation development (Yuan *et al.* 2011) because treatment of zygotes with a cholesterol-depleting drug prevents embryonic development (Comiskey and Warner 2007). HMGCR is the rate-limiting enzyme in the cholesterol biosynthesis pathway (Brown and Goldstein 1990). The mRNA level of *HMGCR* was significantly increased after ITS plus EGF and BSA supplementation.

Conclusion

Our results show that supplementation of IVC medium with ITS plus EGF and BSA can be used as a semi-defined medium that consistently supports the development of IVP bovine embryos at a similar rate to that achieved using a conventional medium supplemented with FBS. Furthermore, culture with ITS plus EGF and BSA can effectively improve the invasion ability of trophoblasts via induction of MMPs at both the transcriptional and translation levels.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Abe, H., Yamashita, S., Satoh, T., and Hoshi, H. (2002). 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.* **61**, 57–66. doi:10.1002/MRD.1131

Antelman, J., Manandhar, G., Yi, Y. J., Li, R., Whitworth, K. M., Sutovsky, M., Agca, C., Prather, R. S., and Sutovsky, P. (2008). Expression of mitochondrial transcription factor A (TFAM) during porcine gametogenesis and preimplantation embryo development. *J. Cell. Physiol.* **217**, 529–543. doi:10.1002/JCP.21528

Bavister, B. D. (1995). Culture of preimplantation embryos: facts and artifacts. *Hum. Reprod. Update* **1**, 91–148. doi:10.1093/HUMUPD/1.2.91

Bernhard, E. J., Gruber, S. B., and Muschel, R. J. (1994). Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc. Natl. Acad. Sci. USA* **91**, 4293–4297. doi:10.1073/PNAS.91.10.4293

Bischof, P., Meisser, A., and Campana, A. (2000). Paracrine and autocrine regulators of trophoblast invasion – a review. *Placenta* **21**, S55–60.

Brown, M. S., and Goldstein, J. L. (1990). Atherosclerosis. Scavenging for receptors. *Nature* **343**, 508–509. doi:10.1038/343508A0

Chaddha, V., Viero, S., Huppertz, B., and Kingdom, J. (2004). Developmental biology of the placenta and the origins of placental insufficiency. *Semin. Fetal Neonatal Med.* **9**, 357–369. doi:10.1016/J.SINY.2004.03.006

Chang, W. L., Yang, Q., Zhang, H., Lin, H. Y., Zhou, Z., Lu, X., Zhu, C., Xue, L. Q., and Wang, H. (2014). Role of placenta-specific protein 1 in trophoblast invasion and migration. *Reproduction* **148**, 343–352. doi:10.1530/REP-14-0052

Chappel, S. (2013). The role of mitochondria from mature oocyte to viable blastocyst. *Obstet. Gynecol. Int.* **2013**, 183024. doi:10.1155/2013/183024

Chia, C. M., Winston, R. M., and Handyside, A. H. (1995). EGF, TGF-alpha and EGFR expression in human preimplantation embryos. *Development* **121**, 299–307.

Comiskey, M., and Warner, C. M. (2007). Spatio-temporal localization of membrane lipid rafts in mouse oocytes and cleaving preimplantation embryos. *Dev. Biol.* **303**, 727–739. doi:10.1016/J.YDBIO.2006.12.009

Cordova, B., Morato, R., Izquierdo, D., Paramio, T., and Mogas, T. (2010). Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the *in vitro* maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development. *Theriogenology* **74**, 1341–1348. doi:10.1016/J.THERIOGENOLOGY.2010.06.003

Das, Z. C., Gupta, M. K., Uhm, S. J., and Lee, H. T. (2014). Supplementation of insulin-transferrin-selenium to embryo culture medium improves the *in vitro* development of pig embryos. *Zygote* **22**, 411–418. doi:10.1017/S0967199412000731

Dhali, A., Anchamparuthy, V. M., Butler, S. P., Mullarky, I. K., Pearson, R. E., and Gwazdauskas, F. C. (2011). Development and quality of bovine embryos produced *in vitro* using growth factor supplemented serum-free system. *Open J. Anim. Sci.* **1**, 97–105. doi:10.4236/OJAS.2011.13013

Dilly, M., Hambruch, N., Haeger, J. D., and Pfarrer, C. (2010). Epidermal growth factor (EGF) induces motility and upregulates MMP-9 and TIMP-1 in bovine trophoblast cells. *Mol. Reprod. Dev.* **77**, 622–629. doi:10.1002/MRD.21197

Dunning, K. R., Cashman, K., Russell, D. L., Thompson, J. G., Norman, R. J., and Robker, R. L. (2010). Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol. Reprod.* **83**, 909–918. doi:10.1095/BIOLREPROD.110.084145

Dunning, K. R., Anastasi, M. R., Zhang, V. J., Russell, D. L., and Robker, R. L. (2014). Regulation of fatty acid oxidation in mouse cumulus-oocyte complexes during maturation and modulation by PPAR agonists. *PLoS One* **9**, e87327. doi:10.1371/JOURNAL.PONE.0087327

Fernandez-Gonzalez, R., Moreira, P., Bilbao, A., Jimenez, A., Perez-Crespo, M., Ramirez, M. A., Rodriguez De Fonseca, F., Pintado, B., and Gutierrez-Adan, A. (2004). Long-term effect of *in vitro* culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proc. Natl. Acad. Sci. USA* **101**, 5880–5885. doi:10.1073/PNAS.0308560101

Gao, F., Wei, P., Chen, X., Zhang, Z., and Liu, Y. (2002). Relationship between uterine expression of matrix metalloproteinases and their inhibitors and endometrial receptivity. *Sci. China C Life Sci.* **45**, 406–411. doi:10.1360/02YC9045

George, F., Daniaux, C., Genicot, G., Verhaeghe, B., Lambert, P., and Donnay, I. (2008). Set up of a serum-free culture system for bovine embryos: embryo development and quality before and after transient transfer. *Theriogenology* **69**, 612–623. doi:10.1016/J.THERIOGENOLOGY.2007.11.008

Goovaerts, I. G., Leroy, J. L., Langbeen, A., Jorssen, E. P., Bosmans, E., and Bols, P. E. (2012). Unravelling the needs of singly *in vitro*-produced bovine embryos: from cumulus cell co-culture to semi-defined, oil-free culture conditions. *Reprod. Fertil. Dev.* **24**, 1084–1092. doi:10.1071/RD11286

Hale, N. A., Yang, Y., and Rajagopalan, P. (2010). Cell migration at the interface of a dual chemical-mechanical gradient. *ACS Appl. Mater. Interfaces* **2**, 2317–2324. doi:10.1021/AM100346K

Herrler, A., Krusche, C. A., and Beier, H. M. (1998). Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis. *Biol. Reprod.* **59**, 1302–1310. doi:10.1095/BIOLREPROD59.6.1302

Holley, A. K., Dhar, S. K., and St Clair, D. K. (2010). Manganese superoxide dismutase vs. p53: regulation of mitochondrial ROS. *Mitochondrion* **10**, 649–661. doi:10.1016/J.MITO.2010.06.003

Holm, P., Booth, P. J., and Callesen, H. (2002). Kinetics of early *in vitro* development of bovine *in vivo*- and *in vitro*-derived zygotes produced and/or cultured in chemically defined or serum-containing media. *Reproduction* **123**, 553–565. doi:10.1530/REP.0.1230553

Isobe, T., Ikebata, Y., Do, L. T., Tanihara, F., Taniguchi, M., and Otoi, T. (2015). *In vitro* development of OPU-derived bovine embryos cultured either individually or in groups with the silk protein sericin and the viability of frozen–thawed embryos after transfer. *Anim. Sci. J.* **86**, 661–665. doi:10.1111/ASJ.12341

Johnson, D. C., and Chatterjee, S. (1993). Epidermal growth factor (EGF) replaces estradiol for the initiation of embryo implantation in the hypophysectomized rat. *Placenta* **14**, 429–438. doi:10.1016/S0143-4004(05)80463-1

Li, M., Yee, D., Magnuson, T. R., Smithies, O., and Caron, K. M. (2006). Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. *J. Clin. Invest.* **116**, 2653–2662. doi:10.1172/JCI28462

Mesalam, A., Khan, I., Lee, K. L., Song, S. H., Chowdhury, M. M. R., Uddin, Z., Park, K. H., and Kong, I. K. (2017*a*). 2-Methoxystypandrone improves *in vitro*-produced bovine embryo quality through inhibition of IKBKB. *Theriogenology* **99**, 10–20. doi:10.1016/J.THERIOGENOLOGY.2017.05.012

Mesalam, A., Kong, R., Khan, I., Chowdhury, M., Choi, B. H., Kim, S. W., Cho, K. W., Jin, J. I., and Kong, I. K. (2017*b*). Effect of charcoal: dextran stripped fetal bovine serum on *in vitro* development of bovine embryos. *Reprod. Biol.* **17**, 312–319. doi:10.1016/J.REPBIO.2017.09.002

Mihalik, J., Rehak, P., and Koppel, J. (2000). The influence of insulin on the *in vitro* development of mouse and bovine embryos. *Physiol. Res.* **49**, 347–354.

Murakami, M., Dong, Y. J., Suzuki, T., Taniguchi, M., Kaedei, Y., Sato, Y., Tanihara, F., and Otoi, T. (2011). Development and subsequent cryotolerance of domestic cat embryos cultured in serum-free and serum-containing media. *Cryobiology* **63**, 170–174. doi:10.1016/J.CRYOBIOL.2011.06.002

Nagashima, H., Kashiwazaki, N., Ashman, R. J., Grupen, C. G., Seamark, R. F., and Nottle, M. B. (1994). Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol. Reprod.* **51**, 618–622. doi:10.1095/BIOLREPROD51.4.618

Nakayama, T., Fujiwara, H., Maeda, M., Inoue, T., Yoshioka, S., Mori, T., and Fujii, S. (2002). Human peripheral blood mononuclear cells (PBMC) in early pregnancy promote embryo invasion *in vitro*: HCG enhances the effects of PBMC. *Hum. Reprod.* **17**, 207–212. doi:10.1093/HUMREP/17.1.207

Ou, X., Lee, M. R., Huang, X., Messina-Graham, S., and Broxmeyer, H. E. (2014). SIRT1 positively regulates autophagy and mitochondria function in embryonic stem cells under oxidative stress. *Stem Cells* **32**, 1183–1194. doi:10.1002/STEM.1641

Pennington, K. A., Schlitt, J. M., and Schulz, L. C. (2012). Isolation of primary mouse trophoblast cells and trophoblast invasion assay. *J. Vis. Exp.* **3202**, . doi:10.3791/3202

Pratt, H. P. (1982). Preimplantation mouse embryos synthesize membrane sterols. *Dev. Biol.* **89**, 101–110. doi:10.1016/0012-1606(82)90298-6

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P., and Amaral, A. (2009). Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum. Reprod. Update* **15**, 553–572. doi:10.1093/HUMUPD/DMP016

Rauch, C., Feifel, E., Amann, E. M., Spotl, H. P., Schennach, H., Pfaller, W., and Gstraunthaler, G. (2011). Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media. *ALTEX* **28**, 305–316. doi:10.14573/ALTEX.2011.4.305

Ribeiro, M. L., Farina, M., Aisemberg, J., and Franchi, A. (2003). Effects of *in vivo* administration of epidermal growth factor (EGF) on uterine contractility, prostaglandin production and timing of parturition in rats. *Reproduction* **126**, 459–468. doi:10.1530/REP.0.1260459

Schulz, L. C., and Widmaier, E. P. (2004). The effect of leptin on mouse trophoblast cell invasion. *Biol. Reprod.* **71**, 1963–1967. doi:10.1095/BIOLREPROD.104.032722

Seidel, G. E., Jr (2006). Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology* **65**, 228–235. doi:10.1016/J.THERIOGENOLOGY.2005.09.025

Shahbazi, M. N., Jedrusik, A., Vuoristo, S., and Recher, G. (2016). Self-organization of the human embryo in the absence of maternal tissues. *Nat. Cell Biol.* **18**, 700–708. doi:10.1038/NCB3347

Sirisathien, S., Hernandez-Fonseca, H. J., and Brackett, B. G. (2003). Influences of epidermal growth factor and insulin-like growth factor-I on bovine blastocyst development *in vitro*. *Anim. Reprod. Sci.* **77**, 21–32. doi:10.1016/S0378-4320(02)00272-5

Smith, S. R., Ghosh, M. C., Ollivierre-Wilson, H., Hang Tong, W., and Rouault, T. A. (2006). Complete loss of iron regulatory proteins 1 and 2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development. *Blood Cells Mol. Dis.* **36**, 283–287. doi:10.1016/J.BCMD.2005.12.006

Stetler-Stevenson, W. G., Liotta, L. A., and Kleiner, D. E., Jr (1993). Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. *FASEB J.* **7**, 1434–1441. doi:10.1096/FASEBJ.7.15.8262328

Sudano, M. J., Paschoal, D. M., Rascado Tda, S., Magalhaes, L. C., Crocomo, L. F., de Lima-Neto, J. F., and Landim-Alvarenga Fda, C. (2011). Lipid content and apoptosis of *in vitro*-produced bovine embryos as determinants of susceptibility to vitrification. *Theriogenology* **75**, 1211–1220. doi:10.1016/J.THERIOGENOLOGY.2010.11.033

Van Soom, A., Vandaele, L., Goossens, K., de Kruif, A., and Peelman, L. (2007). Gamete origin in relation to early embryo development. *Theriogenology* **68**, S131–S137. doi:10.1016/J.THERIOGENOLOGY.2007.03.019

van Wagtendonk-de Leeuw, A. M., Mullaart, E., de Roos, A. P., Merton, J. S., den Daas, J. H., Kemp, B., and de Ruigh, L. (2000). Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. *Theriogenology* **53**, 575–597. doi:10.1016/S0093-691X(99)00259-9

Wang, C., Niu, Y., Chi, D., Zeng, Y., Liu, H., Dai, Y., and Li, J. (2015). Influence of delipation on the energy metabolism in pig parthenogenetically activated embryos. *Reprod. Domest. Anim.* **50**, 826–833. doi:10.1111/RDA.12596

Weathers, J. D., and Prien, S. D. (2014). Estimation of weight and lipid composition in preimplantation embryos from Jersey and beef breeds of cattle. *Open Journal of Veterinary Medicine* **4**, 51301. doi:10.4236/OJVM.2014.411031

Wei, Z., Park, K. W., Day, B. N., and Prather, R. S. (2001). Effect of epidermal growth factor on preimplantation development and its receptor expression in porcine embryos. *Mol. Reprod. Dev.* **60**, 457–462. doi:10.1002/MRD.1110

Wilding, M., Coppola, G., Dale, B., and Di Matteo, L. (2009). Mitochondria and human preimplantation embryo development. *Reproduction* **137**, 619–624. doi:10.1530/REP-08-0444

Wydooghe, E., Vandaele, L., Beek, J., Favoreel, H., Heindryckx, B., De Sutter, P., and Van Soom, A. (2011). Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Anal. Biochem.* **416**, 228–230. doi:10.1016/J.AB.2011.05.033

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2014). Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reprod. Fertil. Dev.* **26**, 717–724. doi:10.1071/RD13043

Yang, Y. P., Ma, H., Starchenko, A., Huh, W. J., Li, W., Hickman, F. E., Zhang, Q., Franklin, J. L., Mortlock, D. P., Fuhrmann, S., Carter, B. D., Ihrie, R. A., and Coffey, R. J. (2017). A chimeric Egfr protein reporter mouse reveals Egfr localization and trafficking *in vivo*. *Cell Reports* **19**, 1257–1267. doi:10.1016/J.CELREP.2017.04.048

Yu, Y., Ding, C., Wang, E., Chen, X., Li, X., Zhao, C., Fan, Y., Wang, L., Beaujean, N., Zhou, Q., Jouneau, A., and Ji, W. (2007). Piezo-assisted nuclear transfer affects cloning efficiency and may cause apoptosis. *Reproduction* **133**, 947–954. doi:10.1530/REP-06-0358

Yuan, Y., Ida, J. M., Paczkowski, M., and Krisher, R. L. (2011). Identification of developmental competence-related genes in mature porcine oocytes. *Mol. Reprod. Dev.* **78**, 565–575. doi:10.1002/MRD.21351

Zhao, X. M., Hao, H. S., Du, W. H., Zhao, S. J., Wang, H. Y., Wang, N., Wang, D., Liu, Y., Qin, T., and Zhu, H. B. (2016). Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes. *J. Pineal Res.* **60**, 132–141. doi:10.1111/JPI.12290