Effects of fetal bovine serum on trophectoderm and primitive endoderm cell allocation of *in vitro*-produced bovine embryos

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Summary

Supplementing embryonic culture medium with fetal bovine serum (FBS) renders this medium undefined. Glucose and growth factors present in FBS may affect the results of cell differentiation studies. This study tested the hypothesis that FBS supplementation during *in vitro* culture (IVC) alters cell differentiation in early bovine embryo development. Bovine embryos were produced *in vitro* and randomly distributed into three experimental groups at 90 h post insemination (90 hpi): the KSOM-FBS group, which consisted of a 5% (v/v) FBS supplementation; the KSOM33 group, with the renewal of 33% of medium volume; and the KSOM-Zero group, without FBS supplementation nor renewal of the culture medium. The results showed that the blastocyst rate (blastocyst/oocytes) at 210 hpi in the KSOM-FBS group was higher than in the KSOM-Zero group but not different from the KSOM33 group. There were no significant changes in metabolism-related aspects, such as fluorescence intensities of CellROX Green and MitoTracker Red or reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD+). Immunofluorescence analysis of CDX2 revealed that the lack of FBS or medium supplementation reduced the number of trophectoderm (TE) cells and total cells. Immunofluorescence analysis revealed a reduction of SOX17-positive cell numbers after FBS supplementation compared with the KSOM33 group. Therefore, we concluded that FBS absence reduced blastocyst rates; however, no reduction occurred when there was a 33% volume renewal of the medium at 90 hpi. We also concluded that FBS supplementation altered TE and primitive endoderm cell allocation during early bovine embryo development.

Introduction

Recent data presented by the IETS reveal that more than 700,000 *in vitro*-produced (IVP) embryos are transferred worldwide yearly. The combination of IVP with genomic selection and sexed semen has proven to be successful in the commercial field in several countries, helping professionals and cattle producers to improve the reproductive performance, efficiency, and genetic gain of their herds (Ferré *et al.*, 2020).

However, only 40–50% of matured and *in vitro* fertilized oocytes reach the blastocyst stage (Rizos *et al.*, 2002), and the pregnancy rate may be lower than that of *in vivo*-produced embryos (de Sousa *et al.*, 2017). Fetal bovine serum (FBS) supplementation effectively maintains and develops bovine embryos (Saeki *et al.*, 1991; Gordon 2003). However, it is linked to deleterious embryonic effects (Farin *et al.*, 2004), such as alterations in compaction and blastulation, increased incidence of stillbirths and mortality after birth (Abe *et al.*, 1999), increased incidence of large offspring syndrome (Jacobsen *et al.*, 2000; van Wagtendonk-de Leeuw *et al.*, 2000; Farin *et al.*, 2004), low repeatability due to variations between FBS batches (Stringfellow *et al.*, 2004), and lower recovery rates of cryopreserved embryos after thawing (Gómez *et al.*, 2008).

In vitro culture of zygotes without adding FBS resulted in slower developing embryos. Compared with calves derived from IVP with FBS, these embryos yielded higher quality morulae that resulted in calves presenting significantly lower birth weight and a significantly easier parturition process (van Wagtendonk-de Leeuw *et al.*, 2000). These studies led to the search for alternatives to replace FBS with compounds that do not negatively interfere with embryonic quality (Mesalam *et al.*, 2017). Different culture media influence the metabolism and production of blastocysts (Krisher *et al.*, 1999), and FBS contains glucose, which is a limiting growth factor when in low concentrations; however, it is already added to culture media of different species of mammals (Barnett and Bavister, 1996).

Early stages of development require low glucose levels for intracellular signalling purposes as glucose metabolism is reduced, (Martin and Leese, 1995), but glucose consumption increases in

the morula and blastocyst stages (Thompson, 2000). The embryo changes from a relatively metabolic-inactive cell at ovulation to an active metabolic cell at the blastocyst stage (Leese, 2012)

Two distinct cell lines constitute the blastocyst, in which approximately two-thirds of the cells comprise the trophectoderm (TE), and the remainder comprises the inner cell mass (ICM). The ICM results in the embryo itself, whereas the TE cells will give rise to extraembryonic lineages, including the placenta (Frankenberg *et al.*, 2016). Diminished TE cell numbers can lead to insufficient placentation and, consequently, to embryonic losses (Koo *et al.*, 2002).

In murine blastocysts, the TE cells are more metabolically active than ICM cells concerning oxygen consumption, lactate, and ATP production (Houghton 2006). In contrast, cattle cells isolated from TE consumed higher levels of pyruvate and produced lactate, whereas cells isolated from the ICM consumed more glucose (Gopichandran and Leese, 2003). It has been recently shown that glucose plays a decisive role in specifying the trophectoderm in murine embryos through the activation of Cdx2 during the transition from morula to blastocyst (Chi *et al.*, 2020). Also, it is known that fibroblast growth factor 4 (FGF4) is the crucial specification signal during the second cell differentiation between primitive endoderm (PE) and the epiblast (EPI) in the murine blastocyst (Yamanaka *et al.*, 2010; Frankenberg *et al.*, 2011; Kang *et al.*, 2013; Saiz *et al.*, 2016b). As FBS is not a defined supplement, it may contain growth factors such as FGF (Zheng *et al.*, 2006), acting in the differentiation of PE.

Therefore, FBS could cause distortions and biased results in studies on early cell differentiation. To verify the impacts of FBS on cell differentiation during the development of bovine embryos, we hypothesized that FBS supplementation during *in vitro* culture interferes with cell differentiation in early bovine embryo development, increasing the number of TE and PE cells. Therefore, the main goal of this study was to evaluate the effect of FBS on embryonic development *in vitro* by assessing rates, metabolic aspects that may influence cell differentiation, and the number of cells of bovine blastocysts, using specific markers of cell lineages.

Materials and methods

Ethical statement

This study was approved by the Animal Use Ethics Council of the College of Veterinary Medicine and Animal Science of the University of São Paulo, under protocol no. 7375181217.

In vitro *maturation and fertilization*

Cumulus–oophorus complexes (COCs) were obtained from ovaries at a commercial slaughterhouse. Grade I oocytes were selected based on the number of surrounding cells and cytoplasmic homogeneity. COCs were washed in HECM–HEPES (HH) medium (Bavister *et al.*, 1983) followed by *in vitro* maturation (IVM) in 199 medium (Gibco, Thermo Fisher, Waltham, MA, USA) that was supplemented with 10% FBS; Gibco), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin, 0.5 μg/ml FSH Folltropin-V (Vetrepharm, Inc. Belleville, ON, Canada), 50 μg/ml hCG (Vetecor Laboratories, Callier, Spain) and 1 μg/ml of estradiol. The COCs were placed in drops of 90 μl IVM medium that were kept under filtered mineral oil for 22 h at 38.5°C, in 5% CO_2 and high humidity. After maturation, COCs were washed in HH and transferred to 90 μl drops of IVF medium (Fert-TALP; Parrish *et al.*, 1988) containing 20 μg/ml of heparin under oil. Fertilization was performed using frozen–thawed sperm, and live cells were sorted using a 90/45% Percoll gradient (GE Healthcare, Buckinghamshire, UK) after centrifugation at 6600 *g* for 5 min. The sperm pellet formed at the bottom of the tube was then washed in IVF medium by centrifugation at 1100 *g* for 3 min. Before insemination, sperm motility was evaluated using optical microscopy, and sperm concentration was assessed using a haematocytometer. The concentration was adjusted to obtain a final concentration of 1×10^6 sperm per ml in the IVF droplets. The droplets were inseminated, and the gametes were co-incubated for 18 h at 38.5°C in 5% CO_2 and high humidity. Sperm straws from the same bull and batch were used throughout the experiment.

In vitro *embryo culture*

After 18 h of IVF, the presumptive zygotes were removed from the IVF plate, washed in HH medium, vortexed for 3 min to remove excess cumulus cells, and randomly distributed in groups of 20 into 90 μl drops of KSOM (Behringer *et al.*, 2003) culture medium. KSOM was supplemented with 3 mg/ml of bovine serum albumin (BSA), 1× MEM essential amino acids and 1× MEM non-essential amino acids, and 2.5 μg/ml of gentamicin. Embryos were cultured in an incubator at 38.5°C, with 5% CO_2, 5% O_2, and high humidity.

Treatments to test FBS influence on embryo cell allocation

Embryos were allocated to the three experimental groups at 90 h post insemination (90 hpi): the KSOM-FBS group was supplemented with 5 μl of FBS (ThermoFisher). In the KSOM33 group, 33% of the medium volume was removed and replaced with fresh medium. Group KSOM-Zero received no treatment during all IVC. Embryos were collected at 186 hpi for trophectoderm cell staining or 210 hpi for all other analyses, including blastocyst rates (blastocysts/inseminated oocytes) and embryonic development (blastocysts/cleaved embryos).

Analyses of metabolic characteristics of embryos

At 210 hpi, embryos were harvested to evaluate intracellular oxidative status using the fluorescent probe CellRox (ThermoFisher), and the mitochondrial membrane potential was observed using MitoTracker Red (ThermoFisher). Blastocysts were stained with 2.5 mM CellROX Green (ThermoFisher) and 1 mM MitoTracker Red (ThermoFisher) for 30 min in KSOM medium in an incubator at 38.5°C 5% CO_2, 5% O_2, and high humidity ($n = 8$ blastocysts per group). Blastocysts were then fixed in 4% paraformaldehyde for 20 min and visualized under an epifluorescence microscope.

The activities of the oxidizing agents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD+) were measured as previously described (Dumollard *et al.*, 2007; Santos *et al.*, 2021). Briefly, embryos were imaged individually within the culture drops under an epifluorescence microscope (Olympus IX-81, Olympus Corporation, Tokyo, Japan) using a long-distance ×20 objective. For NADH autofluorescence measurement, we used a 360–385 nm excitation and 420 nm emission filter (UMWU2 filter cube), and for FAD+ autofluorescence measurement, we used 460–490 nm excitation and 520 emission filter (UMWB2 filter cube). Images were obtained with CellSens Dimension software (Olympus Corporation) using the Z-stack function, obtaining images every 10 μm. Fluorescence intensity was measured using ImageJ software (NIH) on maximum intensity
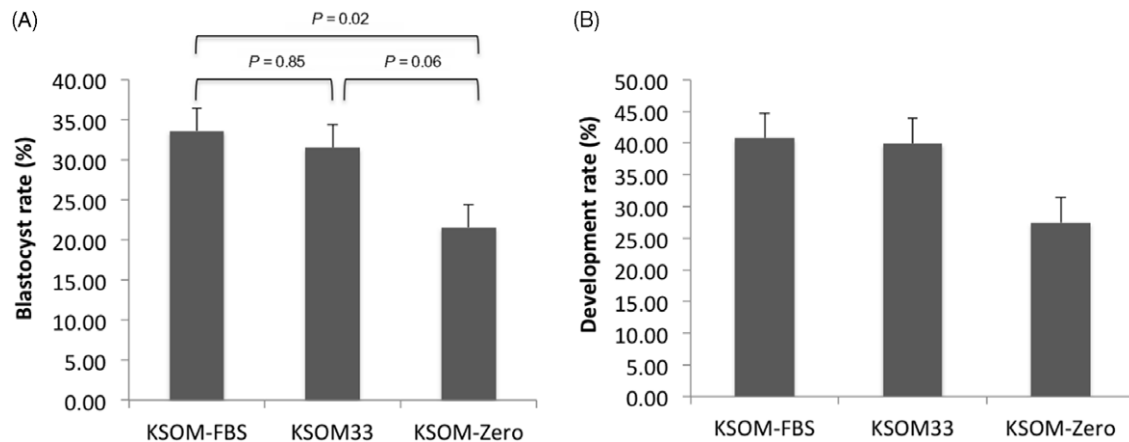
Figure 1. Results from *in vitro* embryo production in the three experimental groups. (A) Blastocyst rate (blastocysts/oocytes). *P*-values of mean comparisons are shown within the graph. (B) Development rate (blastocysts/cleaved embryos). Values are expressed as mean ± standard error of the mean (SEM). $n = 8$ replicates.

projection images generated by CellSens Dimension. Fluorescence intensity was corrected by a two-step approach in which we subtracted the average of two background values and adjusted the fluorescence decay along the Z-axis, as described previously (Saiz *et al.*, 2016a).

Immunofluorescence and cell counting

Immunofluorescence protocols were performed on embryos from the different groups to verify whether FBS altered cell allocation. Embryos had their zona pellucida removed by incubation in 0.5% protease in PBS (w/v) for 2 min, and they were fixed using 4% paraformaldehyde for 20 min at room temperature, followed by three washes in phosphate-buffered saline solution with 1 mg/ml polyvinylpyrrolidone (PBS–PVP) and stored at 4°C until use.

Embryos were permeabilized using 0.5% Triton X-100 solution in PBS–PVP for 15 min and then placed for 1 h at room temperature in blocking solution containing 0.1% Triton X-100, 1% BSA, and 10% fetal donkey serum in PBS–PVP. Incubation with the primary antibody occurred under gentle agitation at 4°C for 16 h. The primary antibodies used were rabbit anti-Cdx2 (1:50, ab88129, Abcam, Cambridge, MA, USA) and goat anti-SOX17 (1:100, AF1924, Novus Biologicals, Littleton, CO, USA) diluted in a solution of 0.1% Triton X-100 and 1% BSA in PBS/PVP. Embryos were then washed three times for 15 min in a washing solution (WS) containing 0.1% Triton X-100 in PBS–PVP and incubated for 1 h at room temperature in the dark with secondary donkey anti-rabbit antibody NL493 (1:200, NL006, R&D Systems, Minneapolis, MN, USA) or NL557 anti-goat donkey (1:200, NL001, R&D Systems) diluted in a solution of 0.1% Triton X-100 and 1% BSA in PBS/PVP. After this incubation with the secondary antibody, embryos were washed in WS three times for 15 min in the dark, incubated with Hoechst 33342 stain for 10 min, and mounted on slides. Embryos were then analyzed with a fluorescence microscope, and the images were obtained with CellSens Dimension software (Olympus Corporation). Using the same software, we counted trophectoderm cells labelled with anti-Cdx2 antibody, PE cells labelled with anti-SOX17 antibody, and total cells labelled with Hoechst.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), considering replicate as a random variable, treatment as an independent variable, and rates, fluorescence intensity, and cell number as dependent variables using PROC GLM from SAS 9.4. Normality of data and homogeneity of variances were tested using PROC UNIVARIATE and Guided Data Analysis of SAS. Data were transformed when necessary and back-transformed for presentation. A comparison of means was performed using Tukey's test. The level of significance was set to 0.05. We performed a power analysis using PROC POWER of SAS for each of the dependent variables, and the results varied from 0.845 to 0.999, indicating that the experimental numbers were adequate for these experiments.

Results

Analysis of blastocyst formation and development rates

Among the three groups, the KSOM-FBS group [33.61 ± 2.85%; mean ± standard error of the mean (SEM), 134/390] had the highest rate of blastocyst formation ($n = 8$ replicates) but did not differ statistically ($P = 0.85$) from the KSOM33 group (31.48 ± 2.85%, 130/460). Results showed that the blastocyst rate in the KSOM-FBS group was higher ($P = 0.02$) than in the KSOM-Zero group (21.57 ± 2.85%, 96/413), which did not differ statistically ($P = 0.06$) from the KSOM33 group (Figure 1A).

Development rate ($n = 8$ replicates) was not different when KSOM-FBS (40.79 ± 3.94%, 134/316, $P = 0.07$) and KSOM33 (39.93 ± 3.94%, 130/370, $P = 0.09$) groups were compared with the KSOM-Zero group (27.45 ± 3.94%, 96/323). Similarly, there was no statistically significant difference between the KSOM-FBS group and the KSOM33 group ($P = 0.98$; Figure 1B).
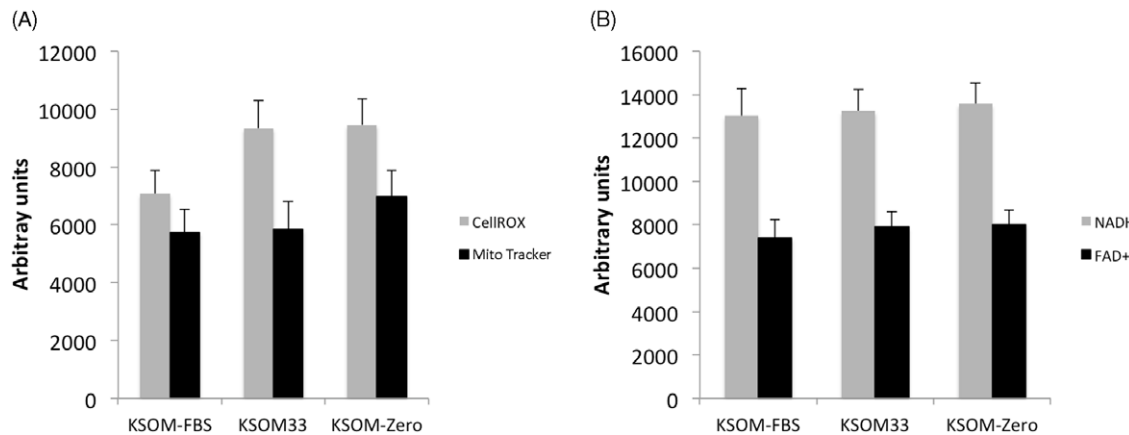
Analysis of metabolic activity

There was no significant difference in the production of reactive oxygen species (ROS), and there was also no statistical difference concerning the mitochondrial activity of the analyzed embryos from the different groups ($n = 11–16$ embryos per group), as verified with the CellROX Green and MitoTracker Red fluorescence intensities respectively (Figure 2A).

The results also did not show statistical differences in cellular energy production in the three groups ($n = 8–13$ embryos per group), demonstrated by the absence of statistical difference in autofluorescence for NADH and FAD+ (Figure 2B).

Table 1. Cell count of CDX2-positive cells (TE), CDX2-negative cells (ICM), total cells after immunostaining of 186 hpi blastocysts. Different superscript letters within columns indicate significant statistical difference ($P < 0.05$). n = 13, 10 and 8 for KSOM-FBS, KSOM33 and KSOM-Zero groups respectively. Values are expressed as mean ± standard error of the mean (SEM)

	CDX2-positive cells (TE)	CDX2-negative cells (ICM)	Total cells	Ratio (TE/total cells)
KSOM-FBS	133.77 ± 10.85[b]	75.08 ± 9.56[a]	208.85 ± 18.90[b]	0.66 ± 0.02[a]
KSOM33	131.91 ± 11.79[b]	84.91 ± 10.39[a]	216.82 ± 20.54[b]	0.60 ± 0.02[a]
KSOM-Zero	77.63 ± 13.83[a]	50.88 ± 12.19[a]	128.50 ± 24.09[a]	0.61 ± 0.02[a]

Figure 2. Quantitative image analysis of energy metabolism in the three experimental groups. (A) Measurement of mitochondrial activity using CellROX or MitoTracker. n = 16, 11 and 13 for KSOM-FBS, KSOM33 and KSOM-Zero groups, respectively. (B) Measurement of coenzymes NADH and FAD+ through autofluorescence. n = 8, 12 and 13 for KSOM-FBS, KSOM33 and KSOM-Zero groups, respectively. Values are expressed as mean ± standard error of the mean (SEM).
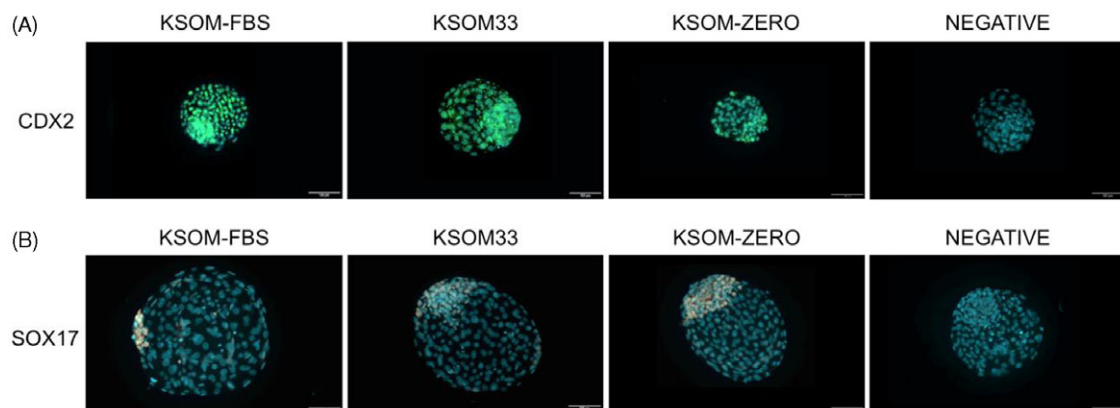
Figure 3. Immunofluorescence of blastocysts from the three experimental groups. (A) Representative images of embryos stained for CDX2 (green). Nuclei are stained with Hoechst (blue). (B) Representative images of embryos stained for SOX17 (red). Nuclei are stained with Hoechst (blue). Scale bar equals 100 μm.

Differential cell count

We collected embryos at 186 hpi and stained for CDX2 (Figure 3A) to determine TE, ICM, and total cell numbers (n = 8–13 embryos per group). KSOM-FBS and KSOM33 groups presented a higher number of TE cells and total cell number than the KSOM-Zero group (Table 1). No differences in the number of ICM cells or the ratio of TE/total cells were observed (Table 1).

Next, we analyzed PE differentiation through SOX17 immunostaining (n = 16–21 embryos per group) in 210 hpi blastocysts (Figure 3B). Surprisingly, the KSOM33 group presented more SOX17-positive cells than the KSOM-FBS group, whereas the KSOM-Zero group was not different from other groups. No differences were observed regarding the number of total cells or the ratio between SOX17-positive cells and total cells (Table 2).

Discussion

The first objective of this study was to verify whether the absence of FBS during embryo culture would reduce blastocyst and development rates in our conditions. This study's second and foremost objective was to evaluate whether FBS supplemented into embryo culture medium would have consequences on cell differentiation due to alterations in energy metabolism or the presence of growth factors present in the serum.

Table 2. Cell count of SOX17-positive and total cells after immunostaining of 210 hpi blastocysts. Different superscript letters within columns indicate significant statistical difference ($P < 0.05$). $n = 16$, 17 and 21 for KSOM-FBS, KSOM33 and KSOM-Zero groups respectively. Values are expressed as mean ± standard error of the mean (SEM)

	SOX17-positive cells (PE)	Total cells	Ratio (SOX17/total cells)
KSOM-FBS	11.22 ± 5.23[a]	215.81 ± 20.43[a]	0.07 ± 0.02[a]
KSOM33	25.95 ± 5.08[b]	248.53 ± 19.82[a]	0.12 ± 0.02[a]
KSOM-Zero	20.72 ± 4.57[a,b]	272.10 ± 17.84[a]	0.12 ± 0.02[a]

Three experimental groups were defined: 5% supplementation (v/v) with FBS (KSOM-FBS group), removal and refreshment of 33% of the medium volume (KSOM33), or without any supplementation (KSOM-Zero) at 90 hpi. The values observed for blastocyst rate showed that the absence of FBS reduced the blastocyst and development rates; however, this was not observed when there was a 33% renewal of the medium at 90 hpi. Half-renewal of culture medium reduced ROS and improved blastocyst formation during rabbit embryos *in vitro* culture (Wang *et al.*, 2022). In addition, the renewal of medium can remove amino acid degradation products such as ammonium, which can impair embryo development (Gardner and Lane, 1993). Similar to our results, embryos cultured in synthetic oviduct fluid (SOF) in the absence of FBS presented reduced blastocyst formation (Van Langendonckt *et al.*, 1997).

Cell count results showed that supplementation with FBS or medium renewal at 90 hpi increased the total number of cells compared with lack of supplementation. Corroborating our findings, a more significant amount of total cells was found in D7 blastocysts cultured with serum or BSA compared with non-supplemented medium (Lazzari *et al.*, 2002). The increased total cell number in these groups could be related to an amplified activity of the pentose phosphate pathway, which generates ribose for DNA synthesis, which led to an increase in TE cell number within these same groups.

The increased proliferation of these cells could be due to the renewal of amino acids in the medium. Supplementation with essential and non-essential amino acids increased the numbers of total cells, ICM cells, and TE cells compared with supplementation with fewer amino acids during bovine embryo culture (Steeves and Gardner, 1999; Lee *et al.*, 2004). In addition, it was shown that glucose and amino acids enhance the proliferation of porcine and ovine trophoblasts *in vitro* through activation of the mTOR pathway (Kim *et al.*, 2011, 2012), which was shown to be activated by glucose in mouse embryos (Chi *et al.*, 2020).

Interestingly, even though TE cells were increased and the number of ICM cells was not statistically different, the ratio of TE:Total cells was not different, similar to that observed in a recent study that compared sequential medium with reduced concentrations of glucose and amino acids (Santos *et al.*, 2021). Interestingly, in this referred study, medium that reduced 50% of nutrient concentration increased total and TE cells. Conversely, when all nutrients were reduced to 25%, the total, TE and ICM cell counts were diminished (Herrick *et al.*, 2020).

Contrary to our hypothesis, FBS supplementation decreased the number of PE cells, as observed by SOX17 staining, compared with KSOM replacement. In the mouse, FGF4 signalling through the ERK pathway is responsible for PE specification (Yamanaka *et al.*, 2010; Kang *et al.*, 2013), and similar results were observed

in bovine embryos (Kuijk *et al.*, 2012; Canizo *et al.*, 2019). FGF4 requires endogenous heparan sulfate for proper signalling (Lanner *et al.*, 2010), and heparin is often supplemented with FGF4 in exogenous treatments (Yamanaka *et al.*, 2010; Kang *et al.*, 2013). Therefore, if an excess of FGF was present after FBS supplementation, the lack of an excess of heparan sulfate or heparin might not have led to proper FGF4 signalling. However, as the serum is an undefined component, there could be several other possibilities to justify the decrease in SOX17-positive cells after FBS treatment; for example, the ERK pathway is inhibited by protein kinase B (Galetic *et al.*, 2003), which can be activated by insulin or IGF signalling (Navarrete Santos *et al.*, 2008).

In conclusion, the results of this study confirm that it is possible to produce bovine blastocysts using KSOM medium without the use of FBS, as long as the replacement of 33% of the volume of the culture medium is performed at 90 hpi. It was also observed that the supplementation of FBS did not change the studied metabolic variables, but that the total and TE cell numbers were reduced when no supplementation with FBS or medium was added at 90 hpi, and FBS decreased PE cell number. In conclusion, FBS did not increase the number of TE or PE cells as hypothesized, but an absence of embryo feeding at 90 hpi negatively influenced the number of total cells or cells allocated to the TE.

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