Use of two replacements of serum during bovine embryo culture in vitro

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Abstract

This study evaluated the effect of two commercial serum replacements (Ultroser G[®] and CPSR-3[®]) on in vitro bovine embryo culture. In Experiment 1, zygotes were cultured in SOF + Ultroser G[®] (2, 4 and 6%), SOF + CPSR-3[®] (2, 4 and 6%), and SOF + 5% FCS (control). Blastocyst rates obtained after culturing with Ultroser G[®] were lower than those with FCS. However, blastocyst rates for CPSR-3[®] were similar to those for serum. In addition, embryos produced in SOF + CPSR-3[®] had the same proportion inner cell mass number and total cell number as embryos cultured with FCS. In Experiment 2, a combination of serum replacements during different periods showed that treatment before the five-to eight-cell stages had no effect on further embryo development. However, treatments up to the morula stage affected blastocyst formation. The concentration of supplement and the timing of its inclusion in culture markedly affected embryo development. The serum replacement CPSR-3[®] can supplement embryo culture with blastocyst rates and quality similar to those for serum.
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1. Introduction

Mammalian preimplantation embryos normally develop within the environment of the female reproductive tract. In the cattle embryo transfer industry, embryos are increasingly produced totally in vitro from immature oocytes collected by ovum pick-up (OPU) or from slaughterhouse ovaries. Oocytes are then matured, fertilized and cultured in vitro up to the

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blastocyst stage. The ability of embryos to develop in a particular medium does not necessarily indicate a beneficial or preferable environment but may instead simply reflect their ability to tolerate artificial conditions [1].

Although bovine embryos can be cultured *in vitro* in simple media under defined conditions [2–7], supplementation with serum or BSA has been shown to exert beneficial effects on embryo development [8]. Embryos cultured in the absence of protein exhibit differences in their metabolic activity [9–11], lower developmental capacity [12] and lower cell number [11,13–16] compared to embryos cultured in the presence of protein. Serum supplies the embryo with essential nutrients and growth factors [17], although the beneficial effects of serum may be due to its antioxidant properties [18,19]. However, serum is not a naturally occurring biological product but a pathological fluid formed by blood clotting, a process that may lead to chemical changes which have detrimental effects on embryo culture [20]. As a consequence, a replacement obtained from plasma might produce better results than serum in terms of embryonic development.

The addition of FCS to bovine embryo culture medium may be a potential cause of abnormal accumulation of cytoplasmic lipid droplets [21,22]. Embryos with large amounts of lipid content are more sensitive to cryopreservation procedures [23,24]. Moreover, use of serum is associated with large offspring syndrome (LOS) [25–27]. In addition, serum may introduce pathogenic agents, and its composition is highly variable.

Ultrosor G[®] (Invitrogen, Barcelona, Spain) is a serum substitute designed to replace FCS in the *in vitro* culture of anchorage dependent cells. It is composed of six main groups of substances necessary for eukaryotic cell growth: growth factors, binding proteins, adhesion factors, vitamins, hormones and mineral trace elements. The constancy of its composition (quantitative and qualitative) ensures a good reproducibility of biological activity from batch to batch.

Controlled Process Serum Replacement-3[®] (CPSR-3[®]) (Sigma, Madrid, Spain) is obtained by dialysis of bovine plasma and adjusted with appropriate whole fluids of bovine origin. This serum replacement has been designed to replace FCS during growth of hybrid cells. It provides chemical and functional uniformity and compares with hybridoma screened fetal bovine serum in many applications.

The aim of this study was to evaluate the effect of two commercially prepared serum replacements on *in vitro* embryo development, and the effect of a combination of the two serum replacements during different periods of development.

2. Materials and methods

2.1. Oocyte recovery

Ovaries recovered from slaughtered Asturiana de los Valles cows were placed in NaCl solution (9 mg/ml) containing antibiotics (penicillin, 100 IU/ml and streptomycin sulfate, 100 µg/ml) and maintained at 30–35 °C until recovery of cumulus-oocyte complexes (COCs). Ovaries were washed twice in distilled water and once in freshly prepared saline. The COCs were aspirated from 2 to 7 mm visible follicles through an 18 gauge needle connected to a syringe, and recovered in a 50 ml Corning tube. Follicular fluid and COCs

were placed in an ovum concentrator (Em-Con, Comextrade, Spain) and rinsed three times with holding medium (HM: TCM199 [Gibco, Barcelona, Spain] + 25 mM HEPES + 0.4 g/l BSA) supplemented with 2 IU/ml of heparin.

2.2. *In vitro* maturation

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected for maturation. The COCs were washed three times in HM and twice in maturation medium, which consisted of TCM199, HNaCO_3 (2.2 g/l), fetal calf serum (FCS, 10% v/v) (Sigma, Madrid, Spain; lot 88H8415), FSHp (1 $\mu\text{g/ml}$), LH (5 $\mu\text{g/ml}$), 17β -estradiol (1 $\mu\text{g/ml}$) and cysteamine (100 μM). Maturation was performed by culturing approximately 50 COCs in 500 μl of maturation medium in four-well dishes at 39 °C in 5% CO_2 in air and high humidity.

2.3. *In vitro* fertilization

Sperm separation was carried out using a swim-up procedure similar to that reported by Parrish et al. [28]. Briefly, semen from one frozen straw of a single bull was thawed in a water bath and added to a polystyrene tube containing 1 ml of pre-equilibrated Sperm-TALP. After 1 h of incubation, approximately 700 μl of the upper layer of supernatant containing the motile sperm was removed. The spermatozoa were centrifuged for 7 min at $200 \times g$ and the supernatant aspirated to leave a pellet of approximately 100 μl in volume. Sperm concentration was determined with a haemocytometer. After 22–24 h of maturation, COCs were washed two times in holding medium and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 $\mu\text{g/ml}$, Calbiochem, La Jolla, CA). Spermatozoa were then added at a concentration of 2×10^6 cells/ml in 500 μl of medium per well containing a maximum of 100 COCs. *In vitro* fertilization was accomplished by incubating oocytes and sperm cells together for 18–20 h at 39 °C in 5% CO_2 and high humidity.

2.4. *In vitro* culture

Embryo culture was performed in SOF as modified by Holm et al. [6] (285 mOsm, pH 7.2–7.3). The protein supplement was added 42 h after fertilization. The FCS batch used as a control showed high embryotrophic ability in previous experiments. Droplets of culture medium (1–2 μl /embryo) were prepared in four-well dishes under mineral oil and equilibrated in an incubator for 2 h before addition of zygotes. Culture was carried out at 39 °C, 5% CO_2 in air and high humidity.

Presumptive zygotes were vortexed for 2 min to separate cumulus cells and washed three times in HM and once in SOF before being transferred to droplets.

2.5. Cell counting

Day 8 expanded and hatched blastocysts from Experiment 1 were fixed and stained for differential cell counting as described by Van Soom et al. [29].

Briefly, expanded blastocysts were incubated in PBS (Gibco, Barcelona, Spain) + 5% pronase (Sigma, Madrid, Spain) for 1 min and in acid Tyrode's solution for 1 min for removing the zona pellucida (ZP).

Both hatched and zona-free expanded blastocysts were incubated in trinitrobenzene-sulfonic acid and in a rabbit antiserum (anti-DNP-BSA) (Sigma, Madrid, Spain) solution. Blastocysts were then incubated in guinea pig-complement serum (Sigma, Madrid, Spain) for 30 min at 39 °C. Embryos were subsequently washed in TCM199 HEPES + 10 µl/ml propidium iodide (Sigma, Madrid, Spain). Samples were fixed in ethanol and incubated in bisbenzimidazole (Hoescht 33342; 10 µl/ml ethanol). Finally, mounting on a glass slide allowed evaluation under fluorescence microscopy at magnification $\times 400$ with an excitation filter of 330–385 nm and barrier filter of 420 nm. Trophoblast cells (TFE) fluoresce red and inner mass cell (IMC) cells appear blue.

2.6. Experiment 1

2.6.1. Experiment 1a

The effect of the addition of 2, 4 and 6% of Ultroser G[®] (US) at 42 h postinsemination (PI) on embryo development was tested. A group of embryos was cultured in SOF + 5% FCS (positive control). As a negative control, embryos were cultured in SOF without any supplementation.

2.6.2. Experiment 1b

This experiment tested the effect of the addition of 2, 4 and 6% of CPSR-3[®] (CP) at 42 h PI on embryo development. As a positive control embryos were cultured in SOF + 5% FCS. Embryos cultured in SOF without any supplementation acted as a negative control.

Culture media were renewed at 66 h (Day 3) and 138 h (Day 6) PI, and embryonic development was evaluated on Days 3, 6, 7 and 8.

2.7. Experiment 2

We tested a combination of two serum replacements during different periods of development.

2.7.1. Experiment 2a

Day 3, five- to eight-cell embryos produced in SOF with 2 and 6% US (Groups 2UCC and 6UCC, respectively), without protein supplement (OCC; negative control) or in SOF + 6% CP (6CCC; positive control) were cultured up to Day 8 in SOF + 6% CP.

2.7.2. Experiment 2b

Day 5 morulae and early blastocysts produced in SOF with 2 and 6% US (Groups 2UUC and 6UUC, respectively), without protein supplement (OOC; negative control) or in SOF + 6% CP (6CCC; positive control) were cultured up to Day 8 in SOF + 6% CP.

Culture media were renewed at 66 h (Day 3) and 114 h (Day 5) PI, and embryonic development was evaluated on Days 7 and 8.

2.8. Statistical analysis

Data were analyzed by ANOVA and Duncan's test ($P < 0.05$) and expressed as a mean $\pm$ standard error.

3. Results

3.1. Experiment 1a

No significant differences were found between culture groups in embryo development up to the morula stage (Table 1). However, blastocyst rates at Day 7 in groups supplemented with 4 and 6% US were lower ($P < 0.05$) than after culture in SOF + FCS. At Day 8, embryo development rates in all groups were lower than in the FCS group.

3.2. Experiment 1b

Blastocyst rates obtained after embryo culture in SOF + CP were not different from those obtained after culture in SOF + FCS (Table 2). Serum showed a tendency to accelerate the formation of blastocysts.

3.3. Cell counting

No differences were found in the number of cells of both IMC and TFE between groups (Table 3). Embryos cultured in SOF + FCS tended to show more cells than those in other groups. The IMC/total cell ratio was similar in all groups.

3.4. Experiment 2a

As shown in Table 4, developmental rates of five-to eight-cell embryos produced in SOF with 2% US (2UCC), 6% US (6UCC), 6% CP (6CCC) or without protein supplement (OCC) were similar when cultured from Day 3 to Day 8 in SOF + 6% CP.

Table 1
Development of bovine embryos cultured in SOF in the presence of commercial synthetic serum replacer Ultroser G[®] (US), FCS or no supplement

Group	c ¹	Cleavage (%)	Five-to eight-cell (%)	Morulae (%)	Blastocyst (%)	
			Day 3	Day 6	Day 7	Day 8
5% FCS	109	81.0 $\pm$ 2.0	43.1 $\pm$ 5.0	23.2 $\pm$ 7.1	20.1 $\pm$ 4.4 ^a	21.3 $\pm$ 4.4 ^a
2% US	107	83.2 $\pm$ 1.7 ^a	54.9 $\pm$ 4.3	26.5 $\pm$ 6.1	8.2 $\pm$ 3.8	8.2 $\pm$ 3.8 ^b
4% US	112	84.8 $\pm$ 1.7 ^a	56.4 $\pm$ 4.3	23.6 $\pm$ 6.1	5.5 $\pm$ 3.8 ^b	6.4 $\pm$ 3.8 ^b
6% US	99	85.3 $\pm$ 2.0 ^a	55.8 $\pm$ 4.9	13.5 $\pm$ 7.0	1.2 $\pm$ 4.4 ^b	2.0 $\pm$ 4.3 ^b
FCS(–) US(–)	65	77.3 $\pm$ 2.4 ^b	50.9 $\pm$ 4.0	19.5 $\pm$ 8.4	1.9 $\pm$ 5.2 ^b	3.2 $\pm$ 5.2 ^b

Different superscripts (a, b) within columns differ significantly ($P < 0.05$).

¹ Number of oocytes.

Table 2

Development of bovine embryos cultured in SOF in the presence of commercial serum replacer bovine plasma-derived, Controlled Process Serum Replacement-3[®] (CP), FCS or no supplement

Group	c ¹	Cleavage (%)	Five-to eight-cell (%)	Morulae (%)	Blastocyst (%)	
			Day 3	Day 6	Day 7	Day 8
5% FCS	147	82.4 ± 3.6	46.1 ± 5.2	27.6 ± 4.5	21.1 ± 3.7 ^a	22.1 ± 3.6 ^a
2% CP	138	86.9 ± 3.6	50.5 ± 5.3	26.7 ± 4.6	15.1 ± 3.8 ^a	22.8 ± 3.7 ^a
4% CP	140	76.1 ± 3.6	47.4 ± 5.3	29.8 ± 4.6	18.6 ± 3.8 ^a	21.7 ± 3.7 ^a
6% CP	159	79.7 ± 3.6	46.5 ± 5.2	28.0 ± 4.5	17.9 ± 3.7 ^a	20.6 ± 3.6 ^a
FCS(–) CP(–)	65	79.6 ± 4.9	53.4 ± 7.1	24.9 ± 6.2	6.6 ± 5.0 ^b	7.3 ± 4.9 ^b

Different superscripts (a, b) within columns differ significantly ($P < 0.05$).

¹ Number of oocytes.

Table 3

Cell numbers in embryos produced in SOF in the presence of commercial serum replacer bovine plasma-derived, Controlled Process Serum Replacement-3[®] (CP) or FCS

Group	a ^a	Inner cell mass number (ICM)	Trophectoderm cell number	Total cell number (T)	Proportion ICM/T (%)
5% FCS	5	20.9 ± 4.9	83.1 ± 9.4	104.1 ± 10.7	21.1 ± 5.3
2% CP	8	17.6 ± 3.7	66.4 ± 7.6	84.0 ± 8.6	22.6 ± 4.3
4% CP	7	25.5 ± 4.7	69.3 ± 9.7	94.8 ± 11.0	30.8 ± 5.5
6% CP	8	23.5 ± 4.5	71.1 ± 9.4	94.9 ± 10.6	27.7 ± 5.2

^a Number of blastocysts examined for cell number.

3.5. Experiment 2b

Results of this experiment (Table 5) show no differences in blastocysts and expansion rates of morulae produced with 2% US (Group 2UUC) or without protein supplement (Group OOC) after culturing in SOF + 6% CP. Morulae produced with SOF + 6% US (Group 6UUC) showed developmental rates significantly lower than other groups ($P < 0.05$).

Table 4

Development of Day 3, five-to eight-cell embryos produced in SOF + commercial serum replacers, the bovine plasma-derived Controlled Process Serum Replacement-3[®] (CP), (Group 6CCC; 6% CP) and the synthetic Ultraser G[®](US) (Group 2UCC; 2% US and 6UCC; 6% US) or no replacement (Group OCC), and subsequently developed up to Day 8 in SOF + 6% CP

Group	Treatment		a ^a	Five-to eight-cell embryos (%) Day 3	Blastocysts (%)		
	Days 2–3	Days 3–8			Day 7	Day 8	Expanded
6 CCC	6% CP	6% CP	147	65.8 ± 6.3	39.1 ± 7.6	39.1 ± 7.6	19.5 ± 6.2
2 UCC	2% US	6% CP	135	64.2 ± 3.5	34.6 ± 7.7	34.6 ± 7.7	17.6 ± 4.5
6 UCC	6% US	6% CP	140	65.0 ± 3.8	29.1 ± 3.0	29.1 ± 3.0	18.7 ± 4.0
OCC	CP(–)US(–)	6% CP	131	61.1 ± 4.1	28.4 ± 7.9	30.7 ± 6.9	14.6 ± 4.8

^a Number of oocytes.

Table 5

Development of Day 5 morulae and early blastocysts produced in SOF + commercial serum replacers, the bovine plasma-derived Controlled Process Serum Replacement-3[®] (CP), (Group 6CCC; 6% CP), and the synthetic Ultrosor G[®](US) (Group 2UUC; 2% US and 6UUC; 6% US) or no replacement (Group OOC), and subsequently developed up to Day 8 in SOF + 6% CP

Group	Treatment		<i>d</i> ¹	Morulae + early blastocyst (%) Day 5	Blastocysts (%)		
	Days 2–5	Days 5–8			Day 7	Day 8	Expanded
6 CCC	6% CP	6% CP	146	35.7 ± 3.7	71.7 ± 13.0 ^a	71.7 ± 13.0 ^a	34.9 ± 10.6 ^a
2 UUC	2% US	6% CP	134	39.1 ± 4.1	51.5 ± 11.3 ^b	51.5 ± 11.3 ^b	22.3 ± 8.2 ^b
6 UUC	6% US	6% CP	132	35.4 ± 2.1	34.0 ± 9.0 ^c	43.2 ± 10.6 ^c	15.4 ± 5.2 ^c
OOC	CP(–)US(–)	6% CP	126	35.9 ± 3.7	56.2 ± 14.1 ^b	59.5 ± 14.0 ^b	29.9 ± 9.6 ^b

Different superscripts (a, b, c) within columns differ significantly ($P < 0.05$).

¹ Number of oocytes.

4. Discussion

In the present study, it was demonstrated that supplementation of SOF with a serum replacement (CPSR-3[®]) allowed the production of blastocysts rates similar to SOF medium with serum.

Results obtained after supplementation with CPSR-3[®] are not surprising, considering the origin of the replacement. Serum contains growth factors with embryotrophic effects [1], and the clotting process used to obtain serum from blood involves the increase of several factors which could have embryotrophic activity. One of these, vascular endothelial growth factor (VEGF), is a highly specific mitogen secreted by the placental endothelial cells; its secretion is correlated with endothelial cell growth [30]. Serum VEGF levels increase during blood clotting as a result of its release from platelets [31]. Although in this study we did not find significant differences between embryo development in the presence of FCS or CPSR-3[®], serum tended to accelerate blastocyst formation. The CPSR-3[®], obtained from plasma, contains cholesterol, triglycerides, glucose, iron, sodium and proteins as albumin and globulin, with a total protein content similar to that of FCS. However, it is likely that during dialysis, some factors with embryotrophic activity are eliminated, resulting finally in a replacement similar to serum but without the accelerator effect of serum. Moreover, the use of CPSR-3[®] as a replacement for FCS did not represent additional costs and is easy to use.

Ultrosor G[®], however, was not a good FCS replacement for in vitro bovine embryo culture. It yielded morulae at rates similar to serum, but was unable to produce good blastocyst development. It is likely that the composition of Ultrosor G[®] is lacking some factor essential for blastocyst formation. Although the exact formulation of Ultrosor G[®] is protected by commercial copyright, growth factors, binding proteins, adhesion factors, vitamins, hormones and mineral trace elements are included in it. Notably, protein content is five times less than that of FCS [32].

Several authors have reported the use of serum replacements with satisfactory results. Pope et al. [32] found that Ultrosor G[®] supported in vivo two-cell mouse embryo development up to the hatching stage at rates similar to human serum. Broussard et al.

[33] reported that SerXtend[®], a commercially-prepared serum extender containing fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, BSA, transferrin, ethanolamine, thiotic acid, selenium and hydrocortisone, stimulated proliferation of bovine granulosa cells during in vitro culture. SerXtend[®] increased the development of bovine IMV/IFV embryos into morulae or blastocysts by acting on cumulus cells [34]. In order to compare the efficacy of a globulin-containing protein source (Synthetic Serum Substitute[®]) with human serum albumin (HSA), Graham et al. [5] cultured human embryos in Human Tubal Fluid (HTF) and found that there were no differences in morphological quality of the embryos. Desai et al. [3] reported that Synthetic Serum Substitute[®] with alphaMEM medium may be a good basal medium for culture of human embryos to the blastocyst stage. Tucker et al. [35] demonstrated that Synthetic Serum Substitute[®] is a good protein source for the culture of mouse embryos.

Although serum provides beneficial factors to the embryo culture medium, its deletion from the culture medium is essential to analyze the role of specific molecules in embryo culture [14]. Serum itself contains a variety of growth factors, which can affect embryonic development. Platelet-derived growth factor (PDGF), exerts regulatory roles during the fourth cycle in bovine embryos [36–38] whereas transforming growth factor β_1 (TGF β_1) and FGF are required during the fifth cycle and for blastocyst formation [39]. In fact, the production of PDGF is one of the beneficial effects observed following coculture with bovine oviductal epithelial cells [38]. Other reports indicate that growth factors such as EGF and FGF may synergistically act on bovine embryo development in vitro [40]. In addition, several growth factor genes have been detected in preimplantation bovine embryos and bovine oviductal cells such as TGF, PDGF and insulin growth factor (IGF) [1,41].

The concentration of supplement and the timing of its inclusion in culture markedly affect embryo development. Results of Experiment 2a showed that different treatments in the first stages of early embryo culture (up to the five-to eight-cell stage) have no effect on further developmental ability. Several authors found that although high molecular weight factors may be necessary during later stages [42], they are unnecessary during the earlier stages [16,42–44]. In the current study, both morulae produced in SOF + 2% US and those produced without serum replacement showed similar rates of development. In these groups, blastocyst rates were lower than those obtained with serum and indicate that Ultrosers G[®] at 2% has no effect on embryo development. Blastocyst rates for groups cultured in SOF + 6% US up to Day 5 were lower than those of all experimental groups, showing a possible negative effect of Ultrosers G[®] at 6% concentration. This damage was not reversible; embryos moved to SOF + 6% CP on Day 5 showed no increase in blastocyst rates. Negative effects of high concentrations of Ultrosers G[®] (5 and 10% versus 1, 2 and 3%) on in vivo two-cell mouse embryo development have been described [32].

In the present work, the negative effect of Ultrosers G[®] occurred after the five-to eight-cell stage; the different treatments until this stage had no effect on further embryo developmental ability. The 8-to 16-cell stage may be crucial since embryonic genome activation occurs at this period [45].

The best method of assessing embryo quality is embryo transfer to recipients and the establishment of pregnancies. However, there are some tests which can partially predict embryo quality, such as inner cell mass/trophectoderm cells ratio [46], changes in cellular

metabolism [47], ultrastructural modifications [48] or cell membrane integrity [49]. In this work we have used a double nuclei staining technique to accomplish differential counting. Embryonic cells start to differentiate in to inner cell mass and trophoblast cells at the morula stage, in a process mediated by growth factors which play a key regulatory role [38]. Serum increases the blastocyst cell number [11,13,15,17] and high molecular mass components of serum could be responsible for this cell proliferation in blastocysts [17,50]. In our study, embryos produced with CPSR-3[®] showed comparable cell numbers and the same ratio inner cell mass number/total cell number to those of embryos cultured with serum, which suggests that CPSR-3[®] contains the factors necessary for embryonic cell differentiation, similar to FCS.

In conclusion, Ultroser G[®] produces morulae rates similar to FCS, but the blastocyst rates are lower than those obtained with FCS. The serum replacement CPSR-3[®] yields blastocyst rates and blastocyst quality similar to those obtained with FCS. Therefore, in our conditions CPSR-3[®] is an appropriate replacement of serum for in vitro bovine embryo culture. As seen in the present work, the concentration of supplement and the timing of its inclusion in culture markedly affect embryo development.

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