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In vitro bovine embryo production in a synthetic medium: Embryo development, cryosurvival, and establishment of pregnancy

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ABSTRACT

The aim of this study was to develop an *in vitro* embryo culture medium without either fetal calf serum or BSA, using various growth factors and cytokines (GFs-CYKs; IGF-I, IGF-II, bFGF, LIF, GM-CSF, TGF- β 1, and PDGF-BB), and other molecules with surfactant and embryotrophic properties, such as recombinant albumin (RA) and hyaluronan (HA). The first part of the study was dedicated to define the best combination of GFs-CYKs + RA + HA for optimal embryonic development. Next, we compared development rates and embryo quality (inner cell mass [ICM]-to-total cell number [TCN] ratio), and postthaw survival and hatching rates using this synthetic medium (T1) and a control medium: synthetic oviduct fluid + BSA + ITS (insulin, transferrin, and selenium). The blastocyst rates were significantly higher with T1 than those with the control at 7 and 8 days after fertilization. There was no significant difference in TCN or the ICM/TCN ratio between the two treatments. Survival and hatching rates 48 hours after thawing were similar for both treatments. Finally, nine embryo transfers were conducted using fresh and previously frozen Day-7 blastocysts to evaluate the *in vivo* viability of embryos produced in this synthetic medium; four gestations were obtained from six fresh embryos and one gestation from three frozen embryos. In conclusion, the fetal calf serum and BSA-free medium, supplemented with GFs-CYKs + RA + HA, improved embryo development and gave comparable ICM/TCN ratios and postthaw survival rates to the control with BSA. Fresh and frozen embryos produced in this medium are viable for embryo transfer. This fully synthetic method of embryo culture is a useful means of reducing the risk of disease transmission *via* embryo transfer.

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1. Introduction

The majority of embryo culture media contain fetal calf serum (FCS) or BSA; however, the former has been shown to have a negative effect on embryo quality. These embryos

have a high lipid content in the trophoblast and inner cell mass (ICM) cells [1]; this cytoplasmic accumulation of lipids can affect the cryotolerance of embryos [2]. In some cases, this resulted in large offspring syndrome, characterized by high birth weight, prolonged gestation, frequent dystocia, and elevated abortion rates [3]. In addition, the use of animal-derived proteins in culture media increases the risk of disease transmission *via in vitro*

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embryo production. There have been several attempts to develop synthetic media, with no negative impact on early embryonic development, using molecules with embryotrophic properties such as growth factors (GFs) and cytokines (CYKs), glycosaminoglycans, or recombinant proteins.

Numerous GFs and CYKs are involved in embryonic development [4]. Growth factors including insulinlike growth factors I and II (IGFs I and II), basic fibroblastic growth factor (bFGF), transforming growth factor β 1 (TGF- β 1), and platelet derived growth factor-BB (PDGF-BB) and CYKs such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) are produced by the endometrium and trophoblast during embryonic development [5]. These GFs and CYKs have been used to supplement embryonic culture media. Insulinlike growth factor I stimulates embryonic development in bovine embryos [6] and increases gestation and calving rates of dairy cows [7]. Insulinlike growth factor II decreases the apoptosis index in mouse embryos [4]. Fibroblast growth factor enhances the development of bovine embryos produced *in vitro* [8], promotes migratory activity in ovine trophoblast cells [9], and increases the production of interferon-tau in bovine trophoderm (TE), an essential component for the establishment and maintenance of pregnancy in cattle and sheep [10,11]. Leukemia inhibitory factor increases the number of ICM cells in bovine embryos produced *in vitro* [12]. Transforming growth factor β 1 increases blastocyst and implantation rates of mouse embryos *in vitro* [13]. Granulocyte-macrophage colony-stimulating factor increases blastocyst rates, the number of ICM cells, and pregnancy rates of *in vitro* bovine embryos [14]. Several GFs and CYKs have also been used in association [15].

The glycosaminoglycan, hyaluronan (HA), is found in abundance in the follicular, oviductal, and uterine fluid of cattle [16,17]. It plays an important role in embryonic development; HA receptors (CD44) are expressed on the surface of bovine embryos at different stages of development [18]. The addition of HA to embryo culture media has been shown to increase the number of bovine embryos that develop to the blastocyst stage [18,19] and improve survival rates of *in vitro* bovine embryos after vitrification [19].

Recombinant proteins, such as recombinant albumin (RA), have been used to replace BSA in culture media to reduce the risk of disease transmission *via* embryo transfer. When combined with HA, RA increases the percentage of blastocysts and hatchings after cryopreservation of *in vitro* bovine embryos [20].

In a previous study in our laboratory [5], we selected six recombinant GFs and CYKs (GFs-CYKs; IGF-I, IGF-II, bFGF, LIF, TGF- β 1, and GM-CSF) on the basis of their known pleiotropic effects on embryonic development [21]. Two different concentrations of each GF and CYK (50 ng/mL and 100 ng/mL) were tested on embryonic development. Similar results were found with the majority of the GFs-CYKs; only TGF- β 1 showed a slight tendency to enhance embryo development at a concentration of 100 ng/mL in comparison with 50 ng/mL; however, the differences were not significant. Subsequent tests to determine the effect of each GF and CYK separately were therefore conducted with a concentration of 50 ng/mL.

Insulinlike growth factor I, bFGF, TGF- β 1, and GM-CSF were found to significantly improve the transition to the blastocyst stage. Finally, a combination of six GFs and CYKs (IGF-I, IGF-II, bFGF, LIF, TGF- β 1, and GM-CSF) was tested. The study reported that the use of a combination of compounds in the embryo culture medium is more effective than synthetic oviduct fluid (SOF) medium alone and as effective as FCS for *in vitro* embryo development [5]. In that particular study, each of the GF and CYK was diluted with a low concentration of BSA to promote embryo development and to reduce the risk of adherence to the plastic dishes.

The aim of the present study was to assess the effect of replacing the BSA in the GFs-CYKs medium (IGF-I, IGF-II, bFGF, TGF- β 1, GM-CSF, and LIF), with other molecules with surfactant and embryotrophic properties, such as RA and HA, on the hypothesis that the effect of the GFs-CYKs is the result of an interaction between several different molecules [21]. We then attempted to optimize this medium by supplementation with TGF- β 1 (100 ng/mL) and another growth factor (PDGF) that has shown beneficial effects on embryo development [22]. Finally, we tested the efficacy of this novel medium *in vivo*.

We used a control medium containing SOF supplemented with BSA and ITS (insulin, transferrin, and selenium) for all the experiments. This latter culture medium is commonly used as an alternative serum-free culture system [2]. For each part of the study, the media were compared in terms of development and hatching rates. For the third part, we also recorded the ICM-to-total cell number (TCN) ratio and cryosurvival. Finally, fresh and frozen Day-7 blastocysts were transferred to evaluate the *in vivo* viability of embryos produced in this synthetic medium.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), unless otherwise specified. The GFs and CYKs (IGF-I, IGF-II, bFGF, LIF, TGF- β 1, GM-CSF, and PDGF-BB) were human recombinants. Insulinlike growth factor I was solubilized using 0.1-M acetic acid at a concentration of 0.5 mg/mL. Insulinlike growth factor II was solubilized using 1-M acetic acid at a concentration of 10 μ g/mL. Basic FGF was solubilized using PBS with 0.1% polyvinylpyrrolidone (PVP) at a concentration of 25 μ g/mL. Leukemia inhibitory factor was solubilized using PBS with 0.1% PVP at a concentration of 1 μ g/mL. Transforming growth factor β 1 was solubilized using 4-mM HCl at a concentration of 1 μ g/mL. Granulocyte-macrophage colony-stimulating factor was solubilized using PBS with 0.1% PVP at a concentration of 1 μ g/mL. Platelet-derived growth factor-BB was solubilized using sterile water for embryo transfer at a concentration of 5 μ g/mL. They were all stored at -80°C .

2.2. *In vitro* embryo production

2.2.1. Oocyte collection and *in vitro* maturation

Ovaries were collected at the abattoir postmortem and stored in PBS at 37°C before transportation for 1 hour to

the laboratory. Cumulus–oocyte complexes were aspirated from follicles with a diameter of 2 to 8 mm. Cumulus–oocyte complexes with a homogeneous cytoplasm and compact cumulus oophorus and corona radiata were selected and washed in HEPES-buffered TCM-199 medium supplemented with 0.4% BSA (Fraction V). They were then matured in 500 μ L of TCM-199 medium supplemented with 60 μ g/mL of penicillin, 60 μ g/mL of streptomycin, and 10 ng/mL of EGF for 24 hours at 38.5 °C and 5% CO₂ in humidified air.

2.2.2. *In vitro* fertilization

After maturation, the oocytes were transferred into 500 μ L of IVF-Tyrode's albumin lactate pyruvate medium supplemented with 6 mg/mL of fatty acid-free BSA with 1.7 IU/mL of heparin; the oocytes were then moved to another 250 μ L of the same medium. Spermatozoa were taken from the frozen-thawed semen of a breeding bull. The straws were thawed in a water bath at 37 °C for 30 seconds, and the motile spermatozoa were selected *via* discontinuous density gradient centrifugation (40%–80%) BoviPure (Nidacon, Sweden). The semen was analyzed with a Hamilton Thorne IVOS (IMV Technologies, Saint-Ouen-Sur-Iton, France) and diluted to a final concentration of 2×10^6 spermatozoa/mL. The spermatozoa were then incubated with the oocytes in the IVF-Tyrode's albumin lactate pyruvate medium for 18 hours at 38.5 °C and 5% CO₂ in humidified air.

2.2.3. *In vitro* culture

After fertilization, the cumulus cells were removed by vortex. The presumptive zygotes were divided into groups and cultured in 30- μ L droplets of SOF supplemented according to the protocol. A mean of 23 embryos were cultured per droplet. The droplets were preserved under mineral oil in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5 °C.

2.3. Preparation of a culture medium with GFs and CYKs but without BSA and FCS

2.3.1. Experiment 1: Effect of GFs and CYKs in the absence of BSA

Presumptive zygotes obtained from 1026 oocytes were randomly assigned to one of two different treatments: (1) 50 ng/mL (IGF-I, IGF-II, bFGF, TGF- β 1, GM-CSF, and LIF) and (2) 0.4% BSA + 5 μ g/mL of insulin, 5 μ g/mL of transferrin, and 5 ng/mL of selenium (ITS; control). Development rates were compared at 7, 8, and 9 days post fertilization (dpf). Hatching rates were compared at 9 dpf.

2.3.2. Experiment 2: Effect of adding recombinant albumin to a medium containing GFs and CYKs

Presumptive zygotes obtained from 961 oocytes were randomly assigned to one of three different treatments: (1) 50 ng/mL of GFs and CYKs, (2) I + 1.5 mg/mL of RA (A9731; Sigma–Aldrich), and (3) control (BSA + ITS). Development rates were compared at 7, 8, and 9 dpf. Hatching rates were compared at 9 dpf.

2.3.3. Experiment 3: Effect of adding HA to a medium containing GFs-CYKs + RA

Presumptive zygotes obtained from 835 oocytes were randomly assigned to one of two different treatments: (1) 50 ng/mL of GFs-CYKs + RA + 0.5 mg/mL of HA (R&D Systems, Abingdon, UK) and (2) control (BSA + ITS). Development rates were compared at 7, 8, and 9 dpf. Hatching rates were compared at 9 dpf.

2.4. Optimization of the synthetic medium developed in the previous step with the addition of other GFs

2.4.1. Experiment 4: Effect of adding 100 ng/mL of TGF- β 1 to a medium containing GFs-CYKs + RA + HA

Presumptive zygotes obtained from 1043 oocytes were randomly assigned to one of two different treatments: (1) 50 ng/mL of GFs-CYKs (IGF-I, IGF-II, bFGF, LIF, GM-CSF) and 100 ng/mL of TGF- β 1 + RA + HA and (2) control (SOF + BSA). Development rates were compared at 7, 8, and 9 dpf. Hatching rates were compared at 9 dpf.

2.4.2. Experiment 5: Effect of adding PDGF-BB to a medium containing GFs-CYKs (100 ng/mL of TGF- β 1) + RA + HA

Presumptive zygotes obtained from 778 oocytes were randomly assigned to one of three different treatments: (1) GFs-CYKs (double TGF- β 1) + RA + HA; (2) I + 50 ng/mL of PDGF-BB, and (3) control (BSA + ITS). Development rates were compared at 7, 8, and 9 dpf. Hatching rates were compared at 9 dpf.

2.5. Validation of the synthetic medium containing GFs-CYKs + RA + HA developed in the previous step

2.5.1. Experiment 6: Effect of the synthetic medium containing GFs-CYKs + RA + HA in comparison with the control medium

Presumptive zygotes obtained from 1070 oocytes were randomly assigned to one of two treatments: (1) GFs and CYKs (double TGF- β 1 + PDGF) + RA + HA and (2) control (BSA + ITS). Development rates were compared at 7, 8, and 9 dpf. Hatching rates were compared at 9 dpf. Inner cell mass-to-TCN ratio was compared at 7 and 8 dpf. The survival and hatching rates were compared at 12, 24, and 48 hours after thawing.

2.5.2. Differential staining of inner cell mass and trophectoderm cells

To determine the number of ICM and TE cells, hatched embryos were selected at 8 dpf. The embryos were fixed in 4% paraformaldehyde for 1 hour at room temperature. They were then washed three times in PBS containing 0.2% PVP and transferred to a solution of PBS supplemented with 10% FCS and 0.2% Triton X-100 (S1) for 1 hour at room temperature. Differential staining was performed using a goat SOX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 in S1) overnight at 4 °C. After washing once in S1 and twice in PBS-PVP for 5 minutes each, the embryos were incubated in S1 supplemented with the secondary antibody, Alexa Fluor 555 donkey antigoat IgG antibody (Life Technologies; 1:1000), and the nuclear marker, DRAQ5 (1:1000), for 2 hours at room temperature. After washing twice in PBS-PVP, labeled embryos were mounted

in Lab-Tek chambered coverglasses in PBS-PVP to preserve their structure. Confocal imaging was performed with an LSM780 confocal microscope with 561 nm and 633 nm lasers (Zeiss, Marly-le-Roi, France). Z stacks were performed throughout the thickness of the embryos; three-dimensional reconstructions were made from stacks using Fiji software [23]. Inner cell mass and TE cells were counted using the Fiji 3D nucleus counter plugin.

2.5.3. Cryopreservation of embryos

At 7 dpf, blastocysts were cryopreserved using a slow-freezing procedure. Embryos were washed three times in a holding medium (IMV Technologies) and placed in a 50- μ L droplet of freezing extender (IMV Technologies freezing extender [1.5 mol/L ethylene glycol]) at room temperature for 10 minutes. During which time, the embryos were loaded into 0.25-mL straws (IMV Technologies). The straws were placed in a programmable freezer, HUBER HS40 (Fisher Scientific, France), at -7°C for 5 minutes. Seeding was then induced, and the straws were maintained for a further 5 minutes at -7°C and then cooled at $0.3^{\circ}\text{C}/\text{min}$ to -35°C . Finally, the straws were plunged into liquid nitrogen.

For thawing, the straws were exposed to room air for 5 seconds before being plunged into a water bath at 25°C for 30 seconds and emptied into a Petri dish. The embryos were washed three times in a holding medium and then cultured in SOF medium supplemented with 5% FCS. The survival and hatching rates were evaluated at 12, 24, and 48 hours after the start of culture.

2.5.4. Estrous synchronization and embryo transfer

Heifers aged between 22 and 29 months were synchronized with a vaginal progesterone diffusion system, PRID DELTA 1.55 g (Ceva, France). The implant was inserted 16 days before embryo transfer and removed 7 days later. An injection of prostaglandin, Enzaprost (Ceva, France), was administered 24 hours before removing the implant, and an injection of PMSG (Chrono-gest 400; Intervet, France) was administered at the time of removal. The embryo transfers were performed 9 days after removal of the implant. Blastocysts cultured in the synthetic medium up to the seventh day after IVF were placed in the holding medium and mounted in 0.25-mL straws (IMV Technologies), before being transferred to the inner uterine horn of the heifers on the same side as the ovary with the CL. Gestation was diagnosed by rectal palpation and ultrasound between 30 and 65 days of gestation.

2.6. Statistical analysis

Blastocyst and hatching rates and survival after cryopreservation were analyzed using a linear mixed-effects model. The ICM/TCN ratios were analyzed using a one-way ANOVA; all tests were performed with R software [24]. The residuals for each model were identically and independently distributed as $N(0, \sigma^2)$; the random effects were distributed as $N(0, \Sigma^2)$ (Σ^2 : variance covariance matrix of a random effect) [25]. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Preparation of a culture medium with growth factors and cytokines but without BSA and FCS

3.1.1. Experiment 1: (1) growth factors and cytokines; control (BSA + ITS)

After seven replicates of IVF, blastocyst rates obtained with treatment 1 were significantly lower than those with the control at 7, 8, and 9 dpf (at 7 dpf: $4.3 \pm 1.7\%$ vs. $17.1 \pm 2.3\%$; at 8 dpf: $11.5 \pm 2.1\%$ vs. $24.4 \pm 2.9\%$; and at 9 dpf: $21.9 \pm 2.4\%$ vs. $39.5 \pm 2.9\%$, respectively). Hatching rates were lower with treatment 1 than those with the control ($4.9 \pm 1.6\%$ and $13.9 \pm 1.7\%$, respectively).

3.1.2. Experiment 2: (1) GFs and CYKs; (2) GFs-CYKs + RA; control

After five replicates of IVF, blastocyst rates obtained with treatments 1 and 2 were significantly lower than those with the control at 7 and 8 dpf (at 7 dpf [1]: $16.0 \pm 2.1\%$; [2]: $16.7 \pm 1.8\%$; and [control]: $23.6 \pm 1.5\%$; and at 8 dpf [1]: $21.4 \pm 2.9\%$; [2]: $19.1 \pm 2.3\%$; and (control): $30.9 \pm 2.2\%$; $P < 0.05$). There were no significant differences in blastocyst and hatching rates between the treatments at 9 dpf: (1): $30.3 \pm 3.1\%$ and $9.2\% \pm 1.8\%$; (2): $29.2 \pm 2.9\%$ and $8.2 \pm 2.1\%$; and (control): $31.8 \pm 3.2\%$ and $10.7 \pm 3.0\%$, respectively, ($P > 0.05$).

3.1.3. Experiment 3: (1) GFs-CYKs + RA + HA; control

After five replicates of IVF, there were no significant differences in blastocyst rates between the treatments at 7 and 8 dpf (at 7 dpf [1]: $31.3 \pm 3.4\%$ and control: $33.5 \pm 4.0\%$ and at 8 dpf [1]: $38.1 \pm 3.2\%$ and [control]: $40.8 \pm 4.4\%$). At 9 dpf, blastocyst and hatching rates were comparable: (1): $40.4 \pm 2.9\%$ and $15.2 \pm 1.9\%$; (control): $41.7 \pm 4.1\%$ and $16.7 \pm 1.6\%$, respectively, ($P > 0.05$).

3.2. Optimization of the synthetic medium developed in the previous step with the addition of other GFs

3.2.1. Experiment 4: (1) GFs-CYKs (double TGF- β 1) + RA + HA; control

After six replicates of IVF, the blastocyst rates were not significantly different between treatment 1 and the control: at 7 dpf ($30.2 \pm 3.0\%$ vs. $22.9 \pm 4.8\%$), 8 dpf ($37.4 \pm 2.0\%$ vs. $29.6 \pm 5.1\%$), and 9 dpf ($39.2 \pm 2.3\%$ vs. $32.6 \pm 4.1\%$, respectively; $P > 0.05$). No significant differences were observed in the hatching rates at 9 dpf between treatment 1 ($15.1 \pm 2.6\%$) and the control ($13.8 \pm 2.9\%$).

3.2.2. Experiment 5: (1) GFs-CYKs (double TGF- β 1) + RA + HA; (2) I + PDGF-BB; control

After four replicates of IVF, there was a significant difference in blastocyst rates between the three treatments at 7 dpf: (1): $24.9 \pm 1.6\%$; (2): $30.8 \pm 1.9\%$, and (control): $17.6 \pm 2.6\%$ ($P < 0.05$). Blastocyst rates were significantly higher at 8 dpf with treatment 2, containing PDGF-BB: $34.6 \pm 1.4\%$, compared with treatment 1: $29.3 \pm 2.0\%$ and with the control: $25.4 \pm 4.1\%$. At 9 dpf, blastocyst and hatching rates were higher with treatment 2: $36.9 \pm 1.7\%$ and $17.5 \pm 1.3\%$ compared with treatment 1: $30.5 \pm 1.9\%$

Table 1

Effect of two treatments on blastocyst rates and hatched blastocyst yield at 7 and 8 days after fertilization.

| Treatment | n | Blastocyst rates (% mean \pm SEM) from 10 replicates | | | |
|---------------------------|-----|--|-----------------------------|-----------------------------|-----------------------------|
| | | Day 7 | Day 8 | Day 9 | HB/Day 9 |
| SOF + BSA + ITS (control) | 563 | 19.1 \pm 1.6 ^a | 28.1 \pm 2.6 ^a | 30.3 \pm 2.9 ^a | 19.3 \pm 5.7 ^a |
| SOF + GFs-CYKs + RA + HA | 507 | 30.9 \pm 2.6 ^b | 36.0 \pm 2.4 ^b | 38.2 \pm 2.7 ^b | 24.6 \pm 5.4 ^a |

n = number of oocytes.

^{a,b}Columns with different letters are significantly different ($P < 0.05$).

Abbreviations: GFs-CYKs, growth factors-cytokines; HA, hyaluronan; HB, hatched blastocyst; ITS, insulin, transferrin, and selenium; RA, recombinant albumin; SOF, synthetic oviduct fluid.

and $12.5 \pm 1.6\%$ and with the control: $27.5 \pm 3.7\%$ and $13.1 \pm 1.7\%$, respectively.

3.3. Validation of the synthetic medium containing GFs-CYKs (double TGF- β 1 + PDGF-BB) + RA + HA, developed in the previous step

3.3.1. Assessment of blastocyst development

After 10 replicates of IVF, at 7, 8, and 9 dpf, the blastocyst rates were higher with treatment 1 than with the control; however, hatching rates at 9 dpf were similar between the mediums; the results are summarized in Table 1.

3.3.2. Assessment of total cell number and ICM/TCN ratio

No significant differences were observed in the TCN and ICM/TCN ratio at 8 dpf, between treatments (Fig. 1, Table 2).

3.3.3. Assessment of survival and hatching rates after cryopreservation

There were no significant differences in survival and hatching rates after cryopreservation between treatments ($P > 0.05$; Table 3).

3.3.4. Assessment of *in vivo* viability of embryos produced in the synthetic medium

Preliminary trials, based on the transfer of fresh and frozen embryos at the blastocyst stage produced in the synthetic medium, resulted in four gestations from six fresh embryos and one gestation from three frozen embryos.

4. Discussion

A previous study, in our laboratory, reported that the synergistic use of several GFs and CYKs (IGF-I, IGF-II, bFGF, LIF, TGF- β 1, and GM-CSF) at 50 ng/mL each [5] has beneficial effects on the development of bovine embryos. However, in that study, all the GFs and CYKs were solubilized in the presence of a low concentration of BSA.

The aim of this study was to develop an *in vitro* embryo culture medium that is completely BSA-free, with the addition of various GFs and CYKs and other molecules with surfactant and embryotrophic properties, such as RA and HA, to reduce the risk of disease transmission *via* embryo transfer. The GFs and CYKs were therefore solubilized with synthetic products.

Growth factors and CYKs are typically found in FCS [26]. Their expression has been reported in preimplanted embryos in cattle [27], and they are generally added to the basal culture medium to increase cell proliferation and stimulate specific cell functions *in vitro* [28]. The beneficial effects of various GFs and CYKs in embryo culture media have been reported in different species [4,8,9,29,30].

The mechanisms by which GFs and CYKs enhance embryo development are not known [6]. Possible mechanisms include stimulation of cell multiplication and the formation of the blastocoele, inhibition of apoptosis, stimulation of interferon- τ production, and reduced interferon- γ production [4,6,11–14]. The GFs and CYKs may act in synergy on the same cellular function and may have identical effects on embryo development [31]. For example, bFGF stimulates the expression of α receptors,

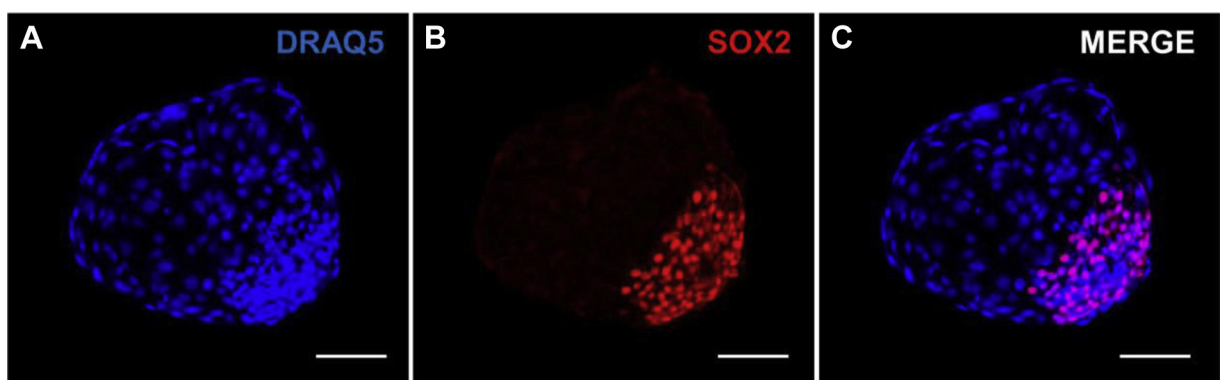


Fig. 1. Differential staining of inner cell mass (ICM) and trophectoderm (TE) cells of hatching bovine embryos at 8 days post fertilization. (A) DRAQ5: TCN; (B) SOX2: ICM; (C) overlay. Scale bar: 50 μ m.

Table 2

Effect of two treatments on inner cell mass (ICM) and trophectoderm (TE) cell number at 8 days after fertilization.

| Treatment | n | Total | ICM | TE | ICM/total (%) |
|---------------------------|----|-------------|-------------|-------------|---------------|
| | | Cell number | Cell number | Cell number | |
| SOF + BSA + ITS (control) | 9 | 203.8 ± 7.1 | 60.9 ± 3.5 | 142.9 ± 4.2 | 29.9 |
| SOF + GFs-CYKs + RA + HA | 13 | 214.4 ± 7.7 | 67.1 ± 2.8 | 147.3 ± 5.8 | 31.3 |

n = number of embryos stained.

Abbreviations: GFs-CYKs, growth factors-cytokines; HA, hyaluronan; ITS, insulin, transferrin, and selenium; RA, recombinant albumin; SOF, synthetic oviduct fluid.

which are required for interaction with PDGF-BB [32]. Platelet-derived growth factor-BB and hyaluronic acid act in synergy to stimulate cellular differentiation [33].

In experiment 1, we used the same association of GFs-CYKs as that defined by Neira et al. [5] but without the addition of BSA. This culture medium gave lower blastocyst rates than the control medium (SOF + BSA + ITS) at 7, 8, and 9 dpf. The viscosity of the medium was suboptimal without BSA, and embryo manipulation became difficult. Recombinant albumin was added to resolve this issue. The latter is free from any potential biological contamination [20], and it plays a role in the transport of steroids, fatty acids, and thyroid hormones. Lane et al. [20] reported equivalent blastocyst rates using a medium supplemented with 2.5 mg/mL of RA and 0.125 mg/mL of HA when compared with a medium supplemented with BSA. After previous experiments, a minimal concentration of RA (1.5 mg/mL) was chosen to obtain optimal viscosity.

In experiment 2, we therefore added RA (1.5 mg/mL) to the medium with the GFs and CYKs; this resolved the viscosity problems, and we observed a comparable rate of development at 9 dpf. However, development between 7 and 8 dpf was delayed compared with the control medium (SOF + BSA + ITS). Hyaluronan was added for its beneficial effects in bovine embryo culture in association with RA [20].

Hyaluronan is one of the most abundant substances in follicular, oviduct, and uterine fluid [17,34]. It is involved in several processes mediated through interaction with matrix components, cell proliferation, and cell differentiation [35]. Hyaluronan and its receptors are thought to be involved in the regulation of GFs [36]. Various concentrations of HA have been tested in previous studies. For example, it has been shown that implantation rates and fetal development are increased by the addition of a low concentration of HA (0.5 mg/mL) in the culture media during embryo transfer in mice [37]. Stojkovic et al. [35] reported that a high concentration (6 mg/mL) increased the viscosity of the culture medium and improved development rates and cell numbers in bovine embryos

produced *in vitro*. Block et al. [19] found that the addition of 1 mg/mL of HA to the culture medium increased blastocyst rates at 7 dpf compared to a culture medium without HA, but no effect was reported at lower concentrations (0.1 and 0.5 mg/mL). In our study, HA was added at a concentration of 0.5 mg/mL.

In experiment 3, with this completely synthetic medium containing GFs and CYKs (IGF-I, IGF-II, bFGF, LIF, TGF-β1, and GM-CSF) 50 ng/mL + RA + HA, we obtained comparable development rates to the control medium (SOF + BSA + ITS) at 7, 8, and 9 dpf [38]. These results confirm a previous study in which the association of RA and HA gave similar blastocyst rates to a medium with BSA [20].

In experiment 4, we tried to optimize the synthetic medium (GFs-CYKs + RA + HA) by doubling the concentration of TGF-β1 (100 ng/mL), which confirmed previous findings in which this GF showed a slight tendency to enhance embryo development at a concentration of 100 ng/mL compared with 50 ng/mL. A preliminary report of these results was presented at the International Embryo Transfer Society Meeting 2014 [39].

In experiment 5, we tested the effects of supplementing this last medium with another growth factor with embryotrophic properties (PDGF-BB). The resulting development rates were higher than those of the same medium without PDGF-BB and of the control. These results confirm previous studies on the beneficial effect of this GF on embryonic development [22]. A composition that gave the best development rates compared to the control medium (BSA + ITS) was finally reached.

The final study, experiment 6, tested this last completely synthetic medium on embryo development, cellular proliferation and differentiation, cryosurvival, and pregnancy rates. Superior blastocyst development rates were obtained in comparison with the control (BSA-ITS) at 7, 8, and 9 dpf. These results confirm previous findings [5], in which the association of GFs and CYKs (IGF-I, IGF-II, bFGF, LIF, TGF-β1, and GM-CSF) improved the potential for embryo development in comparison with SOF alone or SOF and FCS

Table 3

Effect of two treatments on survival and hatching rates after thawing.

| Treatment | n | 12 h After thawing | | 24 h After thawing | | 48 h After thawing | |
|---------------------------|----|--------------------|----------------|--------------------|----------------|--------------------|----------------|
| | | Surviving n (%) | Hatching n (%) | Surviving n (%) | Hatching n (%) | Surviving n (%) | Hatching n (%) |
| SOF + BSA + ITS (control) | 32 | 21 (65.6) | 6 (18.7) | 21 (65.6) | 6 (18.7) | 21 (65.6) | 8 (25.0) |
| SOF + GFs-CYKs + RA + HA | 34 | 21 (61.7) | 4 (11.8) | 21 (61.7) | 4 (11.8) | 21 (61.7) | 7 (20.5) |

n = number of embryos freeze.

Abbreviations: GFs-CYKs, growth factors-cytokines; HA, hyaluronan; ITS, insulin, transferrin, and selenium; RA, recombinant albumin; SOF, synthetic oviduct fluid.

combined. In the latter study, all the GFs and CYKs were diluted in the presence of BSA to reduce the risk of adherence to the plastic dishes. In our study, we used the same combination of GFs and CYKs but replaced the BSA with RA and HA; we also added a double concentration of TGF- β 1 (100 ng/mL) and a new growth factor with embryotrophic properties (PDGF-BB). The results obtained confirm that TGF- β 1 has a superior effect on embryo development at a concentration of 100 ng/mL, as suggested in a previous study [5]. They also confirm the beneficial effect of PDGF-BB on embryo development [22] and a possible synergy between PDGF-BB and HA [33].

This association gave comparable results to the control for TCN, ICM/TCN ratios, and postthaw survival rates. With the synthetic medium, cell proliferation and differentiation were similar to the control medium. And the embryos produced in this synthetic medium tolerate cryopreservation as well as those produced in the presence of BSA.

Finally, four gestations (66%) were obtained from six fresh embryo transfers and one gestation (33%) was obtained from three frozen embryo transfers. These rates are comparable to previous studies that obtained average gestation rates of 50.5% [40] and 41.8% [41] with fresh IVF embryos, and 27.7% [40] with frozen embryos.

This is a fully synthetic method of embryo culture; it presents a valuable tool for reducing the risk of disease transmission *via* embryo transfer. This medium promotes embryo development and has no negative effects on embryo quality and cryosurvival. The next step will be to replace the BSA in the fertilization medium to achieve a completely synthetic means of producing bovine embryos *in vitro*.

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Competing Interests

None of the authors have any conflicts of interest to declare.

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