

Effect of supplementation of different growth factors in embryo culture medium with a small number of bovine embryos on *in vitro* embryo development and quality

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When embryos are cultured individually or in small groups, blastocyst yield efficiency and quality are usually reduced. The aim of this work was to investigate the effect of supplementation of the embryo culture medium (CM) with several growth factors (GFs) on embryo development and apoptosis rate when a reduced number of embryos were *in vitro* cultured. Two experimental studies (ES) were carried out. In ES 1, five treatments were tested to study the effect of GF on embryo development: Control (~30 to 50 embryos cultured in 500 μ l of CM); Control 5 (Five embryos cultured in 50 μ l microdrops of CM), without addition of GF in either of the two control groups; epidermal GF (EGF); IGF-I; and transforming GF- α (TGF- α) (Five embryos were cultured in 50 μ l microdrops of CM with 10 ng/ml EGF, 10 ng/ml IGF-I or 10 ng/ml TGF- α , respectively). In ES 2, following the results obtained in ES 1, four different treatments were tested to study their effect on embryo development and quality (number of cells per blastocyst and apoptotic rate): Control; Control 5; EGF, all three similar to ES 1; EGF + IGF-I group (five embryos cultured in 50 μ l microdrops of CM with 10 ng/ml EGF and 10 ng/ml IGF-I). In both ESs, it was observed that a higher proportion of embryos cultured in larger groups achieved blastocyst stage than embryos cultured in reduced groups (22.6% v. 14.0%, 12.6% and 5.3% for Control v. Control 5, IGF-I, TGF- α groups in ES 1, and 24.9% v. 17.1% and 19.0% for Control v. Control 5 and EGF in ES 2, respectively; $P < 0.05$), with the exception of embryos cultured in medium supplemented with EGF (18.5%) or with EGF + IGF-I (23.5%), in ES 1 and ES 2, respectively. With regard to blastocyst quality, embryos cultured in reduced groups and supplemented with EGF, alone or combined with IGF-I, presented lower apoptosis rates than embryos cultured in reduced groups without GF supplementation (11.6% and 10.5% v. 21.9% for EGF, EGF + IGF-I and Control 5 groups, respectively; $P < 0.05$). The experimental group did not affect the total number of cells per blastocyst. In conclusion, this study showed that supplementation of the CM with EGF and IGF could partially avoid the deleterious effect of *in vitro* culture of small groups of bovine embryos, increasing blastocyst rates and decreasing apoptosis rates of these blastocysts.

Keywords: bovine embryo, reduced groups, *in vitro* culture, growth factor, apoptosis

Implications

In bovine, *in vitro* embryo production (IVEP) is especially interesting when applying the ovum pick-up technique (OPU). However, a small number of oocytes per female are usually recovered after OPU, and when embryos are cultured individually or in a reduced group blastocyst yield efficiency and quality are generally reduced. The improvement of IVEP efficiency and quality with low numbers of embryos would be of great interest for applying this technique in the dairy industry.

Introduction

In vitro embryo production (IVEP) has numerous applications in various scientific areas such as animal production or biomedicine. IVEP-related studies provide us with basic knowledge to better understand the mechanisms of oogenesis, fertilisation and embryo development. Moreover, IVEP from slaughterhouse material provides a high number of oocytes or embryos (after *in vitro* maturation and fertilisation) at a low cost to carry out other associated embryo technologies such as cryobiology. In bovine, IVEP is especially interesting to mitigate the decrease in fertility suffered by dairy cattle during environmental heat stress conditions (Ambrose *et al.*, 1999;

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Al-Katanani *et al.*, 2002) or to take advantage of the ovum pick-up technique (OPU). In the latter case, a small number of oocytes per female are usually recovered after OPU (approximately three to six oocytes recovered per non-stimulated cow and session; Rizos *et al.*, 2005; Chaubal *et al.*, 2006). IVEP efficiency has increased in recent years, although the quality of these embryos remains lower than in embryos produced *in vivo* (reviewed by Rizos *et al.*, 2008). In particular, when embryos were cultured individually or in reduced groups, in addition to a lower efficiency of embryo production, the obtained embryos usually presented a lower number of cells per blastocyst (Paria and Dey, 1990; O'Doherty *et al.*, 1997; Nagao *et al.*, 2008: 5 v. 25 embryos). Numerous authors have previously indicated, in several mammalian species, that IVEP efficiency is negatively affected when a single or a low number of oocytes/embryos were *in vitro* cultured in comparison with a higher number of the same (Paria and Dey, 1990; Lane and Gardner, 1992; Carolan *et al.*, 1996; O'Doherty *et al.*, 1997; Fujita *et al.*, 2006). Moreover, we have observed that post-fertilisation culture, especially the last part of the culture period, seems to be the most important period for bovine embryo development when single and/or low numbers (5 to 10) of embryos were cultured (Salvador *et al.*, 2011). Several factors could be involved in this negative effect. One of them would be the lack of growth factors (GFs) in the culture medium (CM), which could exert a beneficial effect stimulating embryo development, in a paracrine and/or autocrine manner, when embryos were *in vitro* cultured in groups (Paria and Dey, 1990; Lim and Hansel, 1996). Previously, Bavister (1995) had indicated that a rise in embryo number in culture could deplete embryotoxic substances or diminish the inhibitor concentration.

To improve the production and quality of embryos cultured in low numbers or singly, several physical modifications to optimise embryo environment have been proposed, the most simple being reducing the ratio embryo/volume in microdrops (Carolan *et al.*, 1996; Fujita *et al.*, 2006; Nagao *et al.*, 2008); other options are 'Well of Well' system (Vajta *et al.*, 2000), glass oviduct (Thouas *et al.*, 2003), woven polyester mesh (Booth *et al.*, 2007), Cell-Tak grids (Gopichandran and Leese, 2006) or the use of microfluidics (Krisher and Wheeler, 2010). Another possibility could be to modify directly the composition of culture media with the use of cell co-cultures (bovine oviduct epithelial cell, Lim and Hansel, 1996; granulosa cells, O'Doherty *et al.*, 1997; cumulus cells, Goovaerts *et al.*, 2009), conditioned media (Fujita *et al.*, 2006) or addition of GF (Paria and Dey, 1990; Carolan *et al.*, 1996; Lim and Hansel, 1996; Brison and Schultz, 1997). The review by Hardy and Spanos (2002) clearly reflects the importance of a number of GF affecting preimplantation embryo development. These authors suggested that on *in vivo* conditions, endocrine, paracrine and autocrine pathways take place during human embryo development (Hardy and Spanos, 2002). The supplementation of embryo culture media with factors such as epidermal GF (EGF), insulin GF-I and -II (IGF-I and -II, respectively), transforming GF- α (TGF- α), TGF- β 1, fibroblast GF, platelet-derived GF-AB, granulocyte-macrophage

colony-stimulating factor and stem cell factor has been used to try to increase *in vitro* embryo development (mouse: Paria and Dey, 1990; bovine: Lim and Hansel, 1996; De Moraes and Hansen, 1997; Sirisathien *et al.*, 2003). In addition, several studies indicated that the GF could protect the embryo against oxidative or thermal stress (mouse: Kurzawa *et al.*, 2004; bovine: Jousan and Hansen, 2004).

Little information is found in the literature about the effect of culture of single or reduced number of bovine embryos on apoptosis rate. Brison and Schultz (1997) observed that culturing in groups or adding 0.1 pM TGF- α to medium reduced the apoptosis rate, using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) technique, when murine embryos were cultured singly. Other studies on bovine embryos cultured singly or in reduced groups did not show significant differences on apoptotic rate per embryo (Hoelker *et al.*, 2009; Goovaerts *et al.*, 2011). In the first of them, it is possible that the number of embryos maintained in reduced culture was too high (16 embryos) for observing a significant effect in apoptosis, although a reduction in the number of *in vitro* cultured bovine embryos, from 50 to 16 embryos, significantly decreased the blastocyst rate (Hoelker *et al.*, 2009). In the second one, singly cultured embryos were cultured in the presence of a cumulus cell monolayer (Goovaerts *et al.*, 2011) and it was observed that oviductal cell coculture diminished the superoxide anion level in embryo CM (Joo *et al.*, 2001).

The aim of this work was to investigate the effect of supplementation in the CM of several GF on embryo development and quality (number of cells per blastocyst and apoptotic rate) when a reduced number of bovine embryos were *in vitro* cultured.

Material and methods

Unless otherwise stated, all chemical products were purchased from Sigma-Aldrich Quimica (Madrid, Spain).

In vitro maturation

Cumulus-oocyte complexes (COCs) were collected by aspiration of ~2- to 8-mm diameter follicles of bovine ovaries obtained from heifers younger than one year of age in a local slaughterhouse. After aspiration, COCs were washed three times in handling medium (HM199) based on Hepes Medium 199 (M7528) supplemented with 7.4% (v/v) heat-inactivated foetal bovine serum (FBS; 10108-157, GIBCO[®], Invitrogen, Life Technologies S.A., Madrid, Spain) and antibiotics. From aspirated COCs, those with several surrounding layers of cumulus cells were selected. After washing, selected COCs were *in vitro* matured in Medium 199 (M4530) supplemented with 10% FBS and 10 ng/ml of EGF (E1257). In addition, culture media were supplemented with 75 μ g/ml potassium penicillin G (P3032) and 50 μ g/ml streptomycin sulphate (S6051) as antibiotics. COCs were matured for 22 to 24 h at 38.5°C in an atmosphere of 5% CO₂ in humidified air. All culture media containing bicarbonate were covered with mineral oil (M8410) and equilibrated overnight in the culture conditions.

Spermatozoa preparation and in vitro fertilisation (IVF)

Three straws (0.25 ml) of frozen semen from three bulls were thawed at 37°C in a water bath for 1 min and centrifuged for 20 min at 350 × g through a Bovipure[®] gradient (Nidacon International AB, Mölndal, Sweden) with 2 ml of Bovipure bottom layer, and 2 ml of Bovipure top layer, in 15 ml centrifuge tubes. The sperm pellet was separated and resuspended in 10 ml of HM199 and separated again after centrifugation at 300 × g for 5 min. Approximately 50 µl of semen pellet remained after the last centrifugation and this was diluted with ~100 µl of HM199. For IVF, a final concentration of 1 × 10⁶ sperm/ml was used. After *in vitro* maturation, COCs were washed three times in fertilisation Fert-Talp medium (Parrish *et al.*, 1988) with 10 µg/ml of heparin (H9399) without epinephrine and hypotaurine and cocultured with the spermatozoa, in 500 µl using 4-Well Nunc[®] plates (Nunc, Fisher Scientific, Madrid, Spain), for 18 to 20 h in 5% CO₂ at 38.5°C.

In vitro culture of embryos

After IVF, presumptive zygotes were denuded from surrounding cumulus cells in HM199 and washed three times in CM, which was based on synthetic oviductal fluid aaci (Holm *et al.*, 1999) with some modifications (Salvador *et al.*, 2011) and with 3 mg/ml bovine serum albumin (A-8806) instead of bovine serum. Then, presumptive zygotes were *in vitro* cultured in groups of ~30 to 50 zygotes in 500 µl using 4-Well Nunc[®] plates, or in a number of five zygotes in 50 µl microdrops, depending on the experimental group (see below in Section 'Experimental design'), at 38.5°C in a humidified atmosphere with 5% CO₂ and 5% O₂ in air. Cleavage and blastocyst formation rates were recorded at Day 2 (~48 h post fertilisation), and at Days 7, 8 and 9 (D7, D8 and D9, respectively) of *in vitro* culture. Blastocysts were fixed and stained in ethanol with 25 µg/ml of bisbenzimidazole (B2261), and the total number of cells was counted under an epifluorescence microscope.

Assessment of cellular apoptosis of embryos

Apoptosis rate was evaluated by cellular DNA fragmentation using the TUNEL procedure. The TUNEL procedure described by Schwarz *et al.* (2008) was followed, with some modifications. The *In situ* Cell Death Detection Kit Fluorescein (11684795910, Roche Diagnostics SL, Barcelona, Spain) was used. Blastocysts of D7 and D8 were fixed in 4% paraformaldehyde in phosphate buffered-saline solution (PBS) for at least 1 h at room temperature. After that, embryos were washed in PBS plus 1 mg/ml of polyvinyl alcohol (PVA) (P8136) (PBS-PVA) and either processed immediately (for D8 blastocysts) or kept at 4°C for 24 h in PBS-PVA (for D7 blastocysts). Blastocysts were treated in a solution consisting of 1 µl/ml of Triton X-100 (T9284) and 1 mg/ml sodium citrate (S4641) in PBS during 30 min at 37°C in humidified atmosphere. Some embryos were used for positive and negative control groups. Positive control blastocysts were incubated in PBS-PVA with 50 IU/ml of DNase I (04536282001, Roche Diagnostics SL, Barcelona, Spain) for 30 min at 37°C.

After washing, embryos from positive control and experimental groups were incubated in 30 µl of TUNEL reagent from the kit, composed of 10% enzyme solution and 90% staining solution in 0.2 ml PCR Eppendorf tube (Bio-Rad Laboratories, S.A., Madrid, Spain), for 1 h at 37°C in humidified atmosphere. Negative control blastocysts were only treated with the staining solution in the absence of TdT (enzyme solution). After this treatment, embryos were washed and stained with ethanol and bisbenzimidazole, mounted on Polysine[®] slides (Thermo Fisher Scientific S.L.U., Alcobendas, Spain) and evaluated by epifluorescence microscopy (Nikon Eclipse E-400, Izasa Distribuciones Técnicas S.A., Barcelona, Spain) using different filters. Blastocyst cells with nuclei showing green fluorescence were considered as TUNEL positive (DNA fragmented), and those showing blue fluorescence (bisbenzimidazole) indicated the total number of cells (Schwarz *et al.*, 2008).

Experimental design

Two experimental studies (ES) were carried out.

In ES 1, five different treatments were tested:

1. Control group (Control), ~30 to 50 embryos were cultured in 500 µl of CM, without addition of any GF in the medium.
2. Microdrop control group (Control 5), five embryos were cultured in 50 µl microdrops of CM, without addition of any GF in the medium.
3. EGF group, five embryos were cultured in 50 µl microdrops of CM with 10 ng/ml EGF (G502A, Promega Biotech Ibérica, Madrid, Spain).
4. IGF-I group, five embryos were cultured in 50 µl microdrops of CM with 10 ng/ml IGF-I (I3769).
5. TGF-α group, five embryos were cultured in 50 µl microdrops of CM with 10 ng/ml TGF-α (T7924).

Assessments of blastocyst formation were done at D7 and D8 of culture.

In ES 2, following the results obtained in ES 1, four different treatments were tested: Control; Control 5; EGF group, all three similar to ES 1; and EGF + IGF-I group, where five embryos were cultured in 50 µl microdrops of CM with 10 ng/ml EGF and 10 ng/ml IGF-I. Assessments of blastocyst formation rate and number of cells were done at D7, D8 and D9 of culture, and cell apoptosis rate per blastocyst were done at D7 and D8 of culture.

Statistical analysis

Six and eight replicates were performed for ES 1 and 2, respectively. Results for cleavage and blastocyst rates were analysed using the χ^2 -test. Results of blastocyst cell number (ES 1 and ES 2) and cellular apoptosis rate (ES 2) were analysed using a multifactor ANOVA. In ES 1, two factors and their interaction were analysed: experimental group (Control, Control 5, EGF, IGF-I, TGF-α) and days of culture (D7, D8). In ES 2, factors analysed were: experimental group (Control, Control 5, EGF, EGF + IGF-I) and days of culture (D7, D8, D9) and their interaction.

Results

In ES 1, we studied the effect of several GF in CM on IVEP efficiency when embryos were cultured in reduced groups. Results of this first experimental study are shown in Table 1. No differences in cleavage rates were detected between the different experimental groups. However, significant differences were observed between treatments in the ability of the cleaved embryos to reach blastocyst stage. A higher proportion of embryos cultured in larger groups achieved blastocyst stage at D7 post-fertilisation than embryos cultured in reduced groups (21.8% v. 13.6%, 11.6% and 4.6% for Control v. Control 5, IGF-I, TGF- α respectively; $P < 0.05$), except for embryos cultured in medium supplemented with EGF (18.4%). However, the EGF group did not present significant differences in blastocyst rates in comparison with Control 5 group. Similar effects were observed when total blastocyst rates (putting blastocyst at D7 and D8 together) were analysed relative to cleaved embryos or COCs.

ES 2 consisted of a more in-depth study of the effect of EGF and its combination with IGF-I in the CM on IVEP efficiency and apoptosis when embryos were cultured in reduced groups. Results of embryo development are shown in Table 2. No significant differences in cleavage rates were

found between experimental groups. However, it was observed that a higher proportion of embryos cultured in large groups achieved blastocyst stage than embryos cultured in reduced groups (24.9% v. 17.1% and 19.0% for Control v. Control 5 and EGF, respectively; $P < 0.05$), except for embryos supplemented with EGF + IGF-I (23.5%). Moreover, EGF + IGF-I group presented significantly higher total blastocyst rates (blastocysts/oocytes) than Control 5 group ($P < 0.05$). With regard to quality of obtained blastocysts, the total number of cells per blastocyst was significantly affected by the factor 'days of culture' ($P < 0.01$), but not by the 'experimental group' factor or their interaction. The number of cells per blastocyst depending on different experimental groups ranged from 107.3 to 132.5. In this sense, blastocysts at D7 presented the highest number of cells, and blastocysts at D8 a higher number of cells than blastocysts at D9 (134.2 v. 118.9 v. 98.3 for blastocysts at D7, D8 and D9, respectively; $P < 0.01$, Table 3). Both 'days of culture' and 'experimental group' factors and their interaction significantly affected apoptosis rate of blastocysts ($P < 0.05$). In this way, blastocysts at D7 presented a lower apoptosis rate than blastocyst at D8 (12.5 v. 15.2% of cells from blastocysts were apoptotic for blastocyst at D7 and D8, respectively; $P < 0.05$, Table 3). Embryos cultured in larger

Table 1 Effect of EGF, IGF-I and TGF- α in culture medium on in vitro embryo production efficiency when embryos were cultured in reduced groups (Experimental study 1)

Treatment	Total number of COC	Cleaved embryos (%)	Blastocysts at D7 (%) [*]	Blastocysts at D8 (%) [*]	Total blastocysts (%) [*]	Total blastocyst rate ^{**}
Control	265	202 (76.2)	44 (21.8) ^a	16 (7.9)	60 (29.7) ^a	22.6 ^a
Control 5	221	176 (79.6)	24 (13.6) ^b	7 (4.0)	31 (17.6) ^b	14.0 ^b
EGF	205	152 (74.1)	28 (18.4) ^{ab}	10 (6.6)	38 (25.0) ^{ab}	18.5 ^{ab}
IGF-I	207	147 (71.0)	17 (11.6) ^b	9 (6.1)	26 (17.7) ^b	12.6 ^b
TGF- α	209	151 (72.2)	7 (4.6) ^c	4 (2.6)	11 (7.3) ^c	5.3 ^c

EGF = epidermal growth factor; TGF- α = transforming growth factor- α ; COC = Cumulus-oocyte complexes. Control: ~30 to 50 embryos in 500 μ l of culture medium; Control 5: five embryos in 50 μ l of culture medium; EGF: five embryos in 50 μ l of culture medium supplemented with EGF (10 ng/ml). IGF-I: five embryos in 50 μ l of culture medium supplemented with IGF-I (10 ng/ml). TGF- α : five embryos in 50 μ l of culture medium supplemented with TGF- α (10 ng/ml).

^{a,b,c}Different superscripts in the same column indicate a statistical difference ($P < 0.05$).

^{*}Percentage of blastocyst is calculated from cleaved embryos.

^{**}Percentage of blastocyst is calculated from COCs.

Table 2 Effect of EGF and its combination with IGF-I in culture medium on in vitro embryo production efficiency when embryos were cultured in reduced groups (Experimental study 2)

Treatment	Total number of COC	Cleaved embryos (%)	Blastocysts at D7 (%) [*]	Blastocysts at D8 (%) [*]	Blastocysts at D9 (%) [*]	Total blastocysts (%) [*]	Total blastocyst rate ^{**}
Control	393	351 (89.3)	41 (11.7)	42 (12.0) ^a	15 (4.3)	98 (27.9)	24.9 ^a
Control 5	398	339 (85.2)	32 (9.4)	26 (7.7) ^{ab}	10 (2.9)	68 (20.1)	17.1 ^c
EGF	395	333 (84.3)	48 (14.4)	20 (6.0) ^b	7 (2.1)	75 (22.5)	19.0 ^{bc}
EGF + IGF-I	391	344 (88.0)	46 (13.4)	33 (9.6) ^{ab}	13 (3.8)	92 (26.7)	23.5 ^{ab}

EGF = epidermal growth factor; COC = Cumulus-oocyte complexes. Control: ~30 to 50 embryos in 500 μ l of culture medium; Control 5: five embryos in 50 μ l of culture medium supplemented with EGF (10 ng/ml). EGF + IGF-I: five embryos in 50 μ l of culture medium supplemented with EGF (10 ng/ml) + IGF-I (10 ng/ml).

^{a,b,c}Different superscripts in the same column indicate a statistical difference ($P < 0.05$).

^{*}Percentage of blastocyst is calculated from cleaved embryos.

^{**}Percentage of blastocyst is calculated from COCs.

Table 3 Average number of cells and apoptosis percentage per blastocyst depending on days of embryo culture (Experimental study 2)

Factors	Blastocysts at D7 \pm s.e. (n)	Blastocysts at D8 \pm s.e. (n)	Blastocysts at D9 \pm s.e. (n)
Cells/blastocyst	134.15 ^a \pm 3.90 (169)	118.89 ^b \pm 4.22 (124)	98.32 ^c \pm 6.51 (37)
Apoptosis percentage/blastocyst	12.5 ^a \pm 0.6 (163)	15.2 ^b \pm 1.0 (121)	ne

s.e. = standard error; n = number of embryos studied; ne = non-evaluated.

^{a,b,c}Different superscripts in the same row indicate a statistical difference ($P < 0.05$).

Table 4 Average apoptosis percentage per blastocyst depending on experimental group (Experimental study 2)

Experimental group	Average of apoptosis percentage/blastocyst \pm s.e. (n)
Control	12.5 ^a \pm 1.0 (81)
Control 5	21.9 ^b \pm 1.6 (58)
EGF	11.6 ^a \pm 0.7 (66)
EGF + IGF-I	10.5 ^a \pm 0.7 (79)

s.e. = standard error; n = number of embryos studied; EGF = epidermal growth factor.

^{a,b}Different superscripts in the same column indicate a statistical difference ($P < 0.05$).

groups (~30 to 50 embryos in culture) presented a lower apoptosis rate than embryos cultured in reduced groups (five embryos in culture). Moreover, embryos cultured in reduced groups and supplemented with GF, EGF alone or combined with IGF-I, presented a lower apoptosis rate than embryos cultured in reduced group without GF supplementation (11.6 and 10.5 v. 21.9% for EGF, EGF + IGF-I and Control 5 groups, respectively; $P < 0.05$, Table 4).

Discussion

In the present work, we studied the effect of embryo *in vitro* CM supplementation of different GF on embryo development and quality when a reduced number of embryos were cultured. Generally, it was observed that a greater proportion of embryos cultured in large groups achieved blastocyst stage than embryos cultured in reduced groups. In a previous work, in our conditions, we observed that culture of 10 embryos or less, maintaining the ratio 1/10 embryo/ μ L, impaired the efficiency of blastocyst yield (Salvador *et al.*, 2011). In the ES 1 of the present work, we observed that the addition of 10 ng/ml EGF in the CM reduced the negative effect of the culture in reduced numbers, as IVEP efficiency was not significantly different compared with embryos cultured in larger groups. However, this slight improvement in the blastocyst rate was not observed in ES 2. This controversial result was also observed by Sirisathien *et al.* (2003); in their third experiment, the EGF group (5 ng/ml) significantly increased the rate of bovine blastocysts from oocytes, but this fact did not happen in their first experiment (20 embryos/50 μ L). In this sense, some authors observed no beneficial effects of EGF in the embryo CM on the blastocyst rate either in individual (mouse: O'Neill, 1997, 0.2 to 2000 ng/ml; bovine: Keefer *et al.*, 1994, 10 ng/ml) or in group culture (bovine: Flood *et al.*, 1993, 10 ng/ml, Shamsuddin, 1994, 10 to

50 ng/ml, Lee and Fukui, 1995, 10 ng/ml). However, other authors observed differences both in single (mouse: Paria and Dey, 1990, 10 ng/ml; bovine: Lim *et al.*, 2007, 10 ng/ml) and group culture (bovine: Lonergan *et al.*, 1996, 100 ng/ml; Mtango *et al.*, 2003, 10 ng/ml; Sirisathien and Brackett, 2003, 5 ng/ml).

On the other hand, the effect of TGF- α was also evaluated in the present work, and no beneficial effects, but rather negative, of this GF on embryo development were found, confirming results previously showed by other authors observing no beneficial effects (Brison and Schultz, 1997; Zhou *et al.*, 2008). Regarding IGF-I, in agreement with other studies (mouse: Paria and Dey, 1990, 10 ng/ml; bovine: Flood *et al.*, 1993, 10 ng/ml; mouse: Byrne *et al.*, 2002, 13 nM), we did not find a positive effect of this GF on embryo development when it was supplemented alone. In contrast with our results, other authors assaying with higher concentrations than our concentration (10 ng/ml) observed an increase in the blastocyst rate after IGF-I supplementation on embryo CM (O'Neill, 1997; Block *et al.*, 2003). In this sense, both O'Neill (1997) and Sirisathien *et al.* (2003), in mouse and bovine, respectively, only found significant effects on embryo development when IGF-I concentration was higher than 30 ng/ml and 50 ng/ml, respectively. In particular, Sirisathien *et al.* (2003), in agreement with our results, did not find a significant effect of IGF-I with 10 ng/ml on embryo development (blastocyst from oocytes) when bovine embryos were cultured in larger groups (20 embryos/50 μ L) in comparison with non-IGF-supplemented group. Recently, Sakagami *et al.* (2012) found similar results to Sirisathien *et al.* (2003). Despite this, even using a high concentration, Block *et al.* (2008) did not detect any significant effect of the supplementation of 100 ng/ml IGF-I on the blastocyst rate (25 embryos/50 μ L) in comparison with the non-supplemented group.

In ES 2, we studied the effect of culture of small numbers of embryos on embryo production and quality, in particular the number of cells and apoptosis rate per blastocyst. In bovine, in disagreement with our results, some authors observed that blastocysts obtained after culture individually contained a lower number of cells than embryos cultured in large groups (O'Doherty *et al.*, 1997; Larson and Kubisch, 1999). However, other authors did not find a significant decrease on blastocyst cell number of the embryos cultured singly (Goovaerts *et al.*, 2009 and 2011) or in reduced groups (Hoelker *et al.*, 2009). However, in the experiments of Goovaerts *et al.* (2009 and 2011), embryos were cultured with a cumulus cell coculture.

Apoptotic rates obtained in this work for IVEP blastocysts were similar to those obtained by other authors (~7% to

14% embryo cells; Van Soom *et al.*, 2002; Vandaele *et al.*, 2006), but higher than others (around 2% to 3%; Goovaerts *et al.*, 2011). We observed that the *in vitro* culture of a reduced number of embryos impaired blastocyst production and also quality, in particular by increasing the apoptotic rate per blastocyst. In bovine, few works were found in the literature related with the effect of *in vitro* culture of individual or small groups of embryos on their quality, and information about apoptosis is particularly scarce. Programmed cell death or apoptosis may be necessary, as this mechanism would be involved in the elimination of injured embryo cells and also, to help to the preservation of a proper number of cells and inner cell mass (ICM)/trophoblast (TE) ratio (reviewed by Leese *et al.*, 2008). A non-adequate environment such as *in vitro* culture increases apoptotic rate of mouse embryos (Brison and Schultz, 1997). In the case of embryo culture in small groups, Nagao *et al.* (2008) found that the effect of oxygen concentration of incubator atmosphere was related with the embryo number in culture, and that a reduction in oxygen concentration improved bovine embryo development mainly of the smallest groups. Although we do not have evidence, it might be possible that the surrounding microenvironment could be different when fewer embryos are cultured than in large groups. Exogenous reactive oxygen species are produced by many sources as high oxygen tension, exposure to visible light or excess of glucose in the medium among others (reviewed by Guérin *et al.*, 2001) and it is known that oxidative stress induces DNA fragmentation in embryos (Kitagawa *et al.*, 2004). In any case, the present work showed that supplementation of EGF plus IGF-I or EGF alone when embryos were cultured in small groups diminished apoptosis rate at similar levels to those of larger groups. In several works, it has been observed that GF reduced apoptosis rate in embryos in 'standard' *in vitro* conditions (IGF-I: Byrne *et al.*, 2002; Makarevich and Markkula, 2002) or artificially induced stressful conditions (hydrogen peroxide: EGF: Kurzawa *et al.*, 2004). However, this effect was not always evident; Sirisathien and Brackett (2003) observed a slight positive effect of IGF-I reducing apoptosis rates only on D8 bovine blastocyst but not on D7 blastocyst or after EGF treatment. In contrast, Block *et al.* (2008) did not observe any effect of IGF-I on apoptosis.

In our case, the supplementation of EGF alone reduced the apoptotic rate of obtained blastocysts, although it had no effect on the total cells per blastocyst. Moreover, a significant effect of combination EGF + IGF-I on both embryo development and apoptotic rate was found, but without increasing the total cell number per blastocyst. Our results are consistent with several studies that did not show significant effects of EGF on total cells per blastocyst (Keefer *et al.*, 1994; Sirisathien and Brackett, 2003) or on ICM and trophoblast cells (Sirisathien *et al.*, 2003). In contrast, other studies found a beneficial effect of EGF, increasing the number of cells per blastocyst cultured singly (Paria and Dey, 1990) or in groups (Lee and Fukui, 1995; Mtango *et al.*, 2003). In line with our results, Brice *et al.* (1993) hypothesised that EGF receptor seemed to be more linked to the

regulation of TE differentiation rather than cell proliferation in mouse preimplantation embryos. In agreement with our results but with larger groups of bovine embryos in culture (20 embryos/100 μ l), a recent work found a significant effect of the combination IGF-I and EGF on embryo development in comparison with non-GF-supplemented (Sakagami *et al.*, 2012). With regard to total cells per embryo, supplementation of IGF-I and EGF did not increase total cell number per blastocyst, only the ICM cell number (Sakagami *et al.*, 2012).

On the other hand, the quality of embryos obtained at different days of culture was studied in the present work. We found that blastocyst at D7 had better quality, more cells per blastocyst and a lower apoptosis rate, than the rest of the blastocysts obtained later. In line with our results, Larson and Kubisch (1999) observed that blastocysts at D7 had higher numbers of cells than blastocysts at D8 or D9. Moreover, Van Soom *et al.* (1997) observed that timing of blastocyst formation was linked with cell numbers per blastocyst, detecting that blastocysts at D8 had lower numbers of cells than blastocysts at D6 or D7. In turn, the apoptotic cell index increased significantly in blastocysts at D9 in comparison with the previous days (Van Soom *et al.*, 2002). Vandaele *et al.* (2006) also observed significantly higher apoptosis rates in blastocysts at D8 than at D7. Similar results were observed, but not in all experiments, by Sirisathien and Brackett (2003).

In conclusion, this study showed that supplementation of the CM with EGF and IGF could avoid partially the deleterious effect of *in vitro* culture of small groups of bovine embryos, increasing blastocyst rates and decreasing apoptosis rates of these blastocysts.

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