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Effect of growth factors on oocyte maturation and allocations of inner cell mass and trophectoderm cells of cloned bovine embryos

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Summary

This study was conducted to determine the additive effects of exogenous growth factors during *in vitro* oocyte maturation (IVM) and the sequential culture of nuclear transfer (NT) embryos. Oocyte maturation and culture of reconstructed embryos derived from bovine granulosa cells were performed in culture medium supplemented with either epidermal growth factor (EGF) alone or a combination of EGF with insulin-like growth factor-I (IGF-I). The maturation rates of oocytes matured in the presence of EGF or the EGF + IGF-I combination were significantly higher than those of oocytes matured in the presence of only fetal calf serum (FCS) ($P < 0.05$). The developing NT embryos showed no significant differences in fusion, cleavage or blastocyst rates among the culture groups ($P > 0.05$). IGF-I alone or in combination with EGF in sequential embryo culture medium significantly increased the ratio of inner cell mass (ICM) to total blastocyst cells ($P < 0.05$). Our results showed that the addition of growth factors to IVM and sequential culture media of cloned bovine embryos increased the ICM without changing the total cell number. These unknown and uncontrolled effects of growth factors can alter the allocation of ICM and trophectoderm cells (TE) in NT embryos. A decrease in TE cell numbers could be a reason for developmental abnormalities in embryos in the cloning system.

Keywords: Bovine, Cell allocation, Cloning, Embryo culture, Growth factors

Introduction

Somatic cell nuclear transfer (SCNT) is an emerging technology with many applications in animal breeding, such as from multiplying superior genotypes to making genetically engineered animals and genotyping to selecting the best genomes for animal breeding. In cattle, the efficiency of SCNT in producing both embryos and viable offsprings varies considerably among different laboratories. Some possible reasons for these variations are the cell sources used, manipulation conditions, recipient oocyte sources and embryo culture systems. The culture condition of SCNT embryos is the most important parameter affecting the efficiency of the cloning technology (Cibelli *et al.*, 1998).

Numerous studies have been performed to improve both the developmental competence of mammalian oocytes and the *in vitro* culture of embryos by supplementing culture media with components that may be advantageous for embryo development. These components include growth factors, energy substrates, amino acids, albumin and serum (Lonergan *et al.*, 1996;

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Choi *et al.*, 2002; Sirisathien *et al.*, 2003; Cevik *et al.*, 2009).

In several studies, it has been shown that the use of growth factors such as EGF and IGF-I improves the rate of blastocyst formation in IVF (Moreira *et al.*, 2002; Wasielak & Bogacki, 2007) and SCNT embryos (Wadhwa *et al.*, 2009; Wang *et al.*, 2012). EGF improves oocyte maturation and increases the developmental potential of embryos. In addition EGF might act on the cumulus cells surrounding the oocyte and/or on the oocyte itself as EGF receptor mRNA is present in bovine oocytes (Sirisathien *et al.*, 2003). IGF-I induces mitosis of granulosa cells, maturation of the oocyte and embryo development and also increases total cell numbers in blastocysts exposed to stresses such as high temperatures. IGF-I and IGF-II have a positive effect on preimplantation embryo development under detrimental culture conditions due to oxidative stress (Palma *et al.*, 1997; Wasielak & Bogacki, 2007). IGF-I and other growth factors have been implicated in many processes at different stages of follicular development, oocyte maturation and subsequent embryo development in various species (Wasielak & Bogacki, 2007). IGF-I receptor mRNA has been detected in bovine oocytes and embryos (from the zygote to the blastocyst stage) (Stefanello *et al.*, 2006). A paper by Mohan *et al.* (1999) reported the detection of EGF receptor mRNA from the two-cell stage to the blastocyst stage in cattle; the presence of this receptor provides an explanation for the positive effect exerted by EGF on early embryo development *in vitro*. In another study that analysed mRNA expression of EGF and IGF-I, EGF transcripts were not found before fertilization and IGF-I transcripts were present in immature oocytes immediately after collection and in embryos from the two-cell stage onwards (Yoshida *et al.*, 1998).

Total cell number and the proportion of TE and ICM cells in blastocysts are important parameters to evaluate embryo quality. In general, the mean ratio of ICM to total cells for bovine blastocysts produced *in vitro* or *in vivo* ranges from 20–35% (Iwasaki *et al.*, 1990; De la Fuente & King, 1997; Van Soom *et al.*, 1997; Van de Velde *et al.*, 1999). Limited information has been available concerning the structural composition of ICM and TE cells in NT bovine embryos (Koo *et al*, 2002) except for some studies with IVF, parthenogenetic and *in vivo* embryos in the cow (Iwasaki *et al.*, 1990; De la Fuente & King., 1997; Van de Velde *et al.*, 1999). Although several reports have been available regarding the effect of IGF-1 and EGF on cell proportion in IVF embryos (Makarevich & Markkula, 2002; Sirisathien *et al.*, 2003; Block *et al.*, 2008; Wadhwa *et al.*, 2009; Sakagami *et al.*, 2012), very limited data exist for a similar effect by growth factors on NT bovine embryos (Wang *et al.*, 2012). The objective of the present study was to investigate the effect of supplementing *in vitro* maturation and embryo culture media with IGF-I and EGF on oocyte maturation, embryo development and cell allocation in the NT bovine embryos.

Materials and methods

Except when otherwise indicated, all chemicals were obtained from Sigma-Aldrich.

Isolation and culture of granulosa cells

Granulosa cells were collected by aspirating antral follicles from ovaries of an Anatolian Grey cow obtained from a slaughterhouse. Granulosa cells were cultured separately on 35 mm culture plates using Dulbecco Modified Eagle Medium (DMEM) F-12 (Gibco, Grand Island, New York, USA) supplemented with 10% FCS (Gibco, 10270–106 Batch no. 41G8370K) and 1% (v:v) penicillin/streptomycin (10,000 U/ml penicillin G, 10,000 mg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 in air. After 10 days, cells from separate 35 mm culture plates were harvested by trypsinization, counted and seeded in 100 mm tissue culture plates. When the cells reached confluency, they were collected by trypsinization and frozen in DMEM-F12 supplemented with 40% FCS and 10% dimethylsulfoxide (DMSO) (Arat *et al.*, 2001; 2002; 2011). The cells from the first culture (35 mm culture plates) that were frozen in small aliquots after one passage were considered as a separate primary cell line. Granulosa cell lines were labelled with antibodies raised against vimentin and cytokeratin (1:200). Briefly, fixed cells were treated with a blocking solution composed of 5% goat serum and Triton-X in phosphate-buffered saline (PBS) for 45 min at room temperature. After being washed three times with PBS, the cells were incubated with primary antibodies. The next day, the cells were incubated with anti-mouse IgM and IgG secondary antibody dilution (1:128) for 45 min at room temperature. Hematoxylin–eosin staining was employed to observe morphological differences among the cell cultures. One cell line was used in each replication containing all treatment groups run in parallel. Three primary cell lines having uniform morphology were used for NT during the present study.

In vitro maturation of oocytes

Bovine ovaries at various stages of their estrous cycle were obtained from a local slaughterhouse and transported to the laboratory in physiological saline solution (0.9% w/v NaCl) supplemented with

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0.1 μl/ml gentamycin sulphate at 34.0 ± 2.0°C within 2 h after slaughter.

Bovine cumulus–oocyte complexes (COCs) were recovered by the aspiration of antral follicles (3–8 mm in diameter) on the ovaries within 1–1.5 h after they arrived at the laboratory. Only COCs with a compact, nonatretic cumulus oophorus–corona radiata and a homogenously granulated ooplasm were selected. Oocytes were matured in Tissue Culture Medium 199 (TCM-199) supplemented with 10% FCS, 50 μg/ml sodium pyruvate, 1% penicillin/streptomycin (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin), 5 μg/ml luteinizing hormone (LH) and 0.5 μg/ml follicle stimulating hormone (FSH) without growth factors (maturation medium 1) or with 50 ng/ml EGF (maturation medium 2) or with 50 ng/ml EGF and 100 ng/ml IGF-I (maturation medium 3). Maturation was performed in four-well plates overlaid with mineral oil at 38.5°C in a humidified 5% CO_2 in air atmosphere for 18 h. After maturation, the cumulus cells were removed by vortexing COCs in HEPES-buffered Tyrode lactate (TL-HEPES) medium containing 100 U/ml hyaluronidase for 3 min. Mature oocytes were selected by two different researchers using stereomicroscopes. The oocytes with the first polar body extruded and a clearly expanded cumulus cell layer were considered to be mature. The average of measurements was considered the maturation rate.

Recipient cytoplasm preparation

All oocytes used for NT were matured in medium 3 giving high maturation rate. Selected oocytes were enucleated with a 15 μm (internal diameter) glass pipette (Micromanipulator-Eppendorf Transfer Man NK2, Germany) by aspirating the first polar body and metaphase II (MII) plate with a small volume of surrounding cytoplasm under an inverted microscope (Nikon Invert Microscope Eclipse Ti-FL). The oocytes were first stained in TL-HEPES containing 2 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B for 10–15 min and then kept in TL-HEPES supplemented with 7.5 μg/ml cytochalasin B during enucleation. The location of the metaphase chromosomes was determined by brief exposure (1–2 s) to ultraviolet (UV) light. Successful enucleation was confirmed by exposing all the removed cytoplasm to UV light and checking for the presence of the removed metaphase plate.

Donor cell preparation and nuclear transfer

Granulosa cells from an Anatolian Grey cow on passages between 2 and 5 were used for NT. Frozen donor cells that had been preserved in the cryobank for at least 1 year were thawed and cultured with 10% FCS and allowed to grow to confluency. After confluency, they were cultured with 5% FCS for additional 4 days (G1/G0) (Arat *et al.*, 2002; 2011). The cells were dissociated by trypsinization with 0.25% trypsin–ethylenediamine tetra-acetic acid solution immediately before donor cells were transferred into the enucleated oocytes, and then the cells were pelleted and resuspended in DMEM/F-12 supplemented with 5% FCS. A single cell was inserted into the perivitelline space of the enucleated oocyte using a 15-μm (internal diameter) glass pipette. Oocyte-cell complexes were placed in TCM-199 containing 10% FCS at 38.5°C in 5% CO_2 in air until fusion.

Fusion and activation of oocyte-cell complexes

Fusion and activation of oocyte-cell complexes were performed as described previously (Arat *et al.*, 2001) after slight modification. Briefly, oocyte-cell complexes were fused by 2.66 kV/cm, 30 μs, 1 pulse in sorbitol fusion buffer (0.25 M sorbitol, 0.1 mM calcium acetate, 0.5 mM Mg acetate) in a 500-μm fusion chamber (Cell Fusion CF-150/B, Hungary). Thirty minutes or 1 h after the fusion, fused NT oocytes were exposed to 5 μM calcium ionophore for 5 min followed by incubation in TCM-199 supplemented with 10% FCS, 2.5 μg/ml cytochalasin D and 10 μg/ml cycloheximide for 1 h at 38.5°C in 5% CO_2 in air and in TCM-199 with 10% supplemented FCS and 10 μg/ml cycloheximide for 4 h at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2 to activate them.

In vitro culture of NT embryos

Following activation, reconstructed embryos were cultured in Quinn's Advantage Cleavage Medium® (QACM) (Cooke *et al.*, 2002; Arat *et al.*, 2011) supplemented with 8 mg/ml essentially fatty-acid free bovine serum albumin (FAF-BSA) for 72 h. Then developing embryos were cultured in Sequential Quinn's Advantage Blastocyst Medium (QABM) (SAGE Inc., Trumbull, Connecticut, USA) supplemented with 4 mg/ml essentially FAF-BSA + 5% FCS (culture medium 1), or with 4 mg/ml FAF-BSA+5% FCS + 100 ng/ml IGF-I (culture medium 2) or with 4 mg/ml FAF-BSA+5% FCS + 100 ng/ml IGF-I + 50 ng/ml EGF (culture medium 3) for an additional 5 days under low oxygen tension (5% CO_2, 5% O_2, 90% N_2) at 38.5°C in high humidity conditions. The culture conditions were compared and the effects of growth factors were examined. For parthenogenetic embryo production, oocytes matured in maturation medium 3 were activated by electrical pulse and chemicals and then the activated oocytes were cultured in the same culture compositions mentioned above.

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Table 1 Effects of different supplements on the maturation of bovine oocytes

Maturation media*	Maturation time (h)	No. of oocytes	No. of matured oocytes (% mean ± SD)
1. TCM-199 + FCS	18	840	536 (63.8 + 2.7[a])
2. TCM-199 + FCS + EGF	18	940	710 (75.5 + 3.2[b])
3. TCM-199 + FCS + EGF + IGF-I**	18	841	633 (75.0 + 3.0[b])

[a,b]Values within the same columns with different letters are significantly different ($P < 0.05$).
*Maturation media also contain luteinising hormone (LH) and follicle stimulating hormone (FSH).
**Before nuclear transfer (NT), only this medium was used for maturation of oocytes.
SD, standard deviation.

Differential staining of ICM and TE cells

Analysis of the distribution of ICM and TE cells by differential staining has been used as a technique to evaluate embryo quality in several species. Days 7 or 8 expanding cloned and parthenogenetic blastocysts characterized by zona pellucidae showing signs of thinning and with slightly increased embryo diameters, were stained as described by Van Soom *et al.* (1996). Briefly, zona intact blastocysts were incubated with 10 mM picrylsulphonic acid (in cold Ca^{2+}-free PBS) for 5 min in a refrigerator (+4°C). Then they were washed and incubated for 30 min at 38.5°C in anti-dinitrophenyl antibody diluted to 30% (v/v) with Ca^{2+}-free PBS. Embryos were then transferred into guinea pig complement (55852, ICN Biochemicals, Irvine, California, USA) diluted to 20% (v/v) in Ca^{2+}-free PBS containing 50 μg/ml propidium iodide, 12.5 μg/ml bisbenzimide and 50 μg/ml RNase A for 30 min at 38.5°C. Finally, the embryos were fixed in 2% paraformaldehyde in PBS for 1–2 min at room temperature, before mounting on slides with a 10-μl drop of 0.2 M 1.4 diazabicyclo-octane in 50% glycerol (v/v) in Ca^{2+}-free PBS as an anti-fading solution. Embryos were examined in whole mount under a fluorescence microscope (Nikon Invert Microscope Eclipse Ti-FL; 340–380 nm excitations and 430 nm suppression). Thus, ICM nuclei labelled with bisbenzimide appeared blue and TE nuclei labelled with both bisbenzimide and propidium iodide appeared pink to red. Numbers of ICM and TE nuclei were counted directly under an inverted microscope by two researchers blinded to treatments and the average of the measurement was taken.

Statistical analysis

All treatment groups from oocyte maturation and embryo culture were run in parallel and replicated to avoid any seasonal bias. The experiments were repeated at least three times. Statistical data analysis was performed using the General Linear Model (GLM) procedure of Statistical Analysis System (SAS) (SAS for Windows, version 2007, SAS Inst., Inc., Cary, NC, USA). Independent samples *T*-test or one-way analysis of Variance (ANOVA) followed by Tukey's Test was used for statistical comparison of the groups. All data were expressed as mean ± standard deviation (mean ± SD). Differences were considered as significant when *P*-values were less than 0.05. Statistical data analysis was performed by an analyst who had not taken part in the experiments.

Results

Effect of culture medium combinations on *in vitro* maturation of bovine oocytes

In *in vitro* maturation of bovine oocytes, 840, 940 and 841 oocytes were cultured in maturation media 1, 2 and 3, respectively. Maturated oocytes that contained the first polar body in the perivitelline space, in media supplemented with serum, exogenous gonadotropins and growth factor combinations were evaluated at 18 h of oocytes culture. The percentages of maturation rates obtained for the three treatments were 63.8% (maturation medium 1), 75.5% (maturation medium 2) and 75.0% (maturation medium 3). As shown in Table 1, the maturation rates of oocytes in the presence of EGF alone or EGF in combination with IGF-I were significantly higher than those of oocytes matured in the presence of only FCS ($P < 0.05$).

Effects of EGF and IGF-I on *in vitro* development of parthenogenetic embryos

Parthenogenetic bovine embryos were cultured in different sequential culture media compositions (culture media 1, 2 and 3, respectively). Data on cleavage and blastocyst rates and the cell allocation of parthenogenetically activated embryos are presented in Table 2. There were no significant differences in cleavage rates and development to the blastocyst rates of parthenogenetic embryos among the culture groups ($P > 0.05$). The results of this study showed that EGF and IGF-I, even if present in the IVM medium, had no effect on *in vitro* development of

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Table 2 Effects of different supplements on *in vitro* developmental competence of parthenogenetic embryos

Culture media	No. of oocytes	Cleavage rates (mean ± SD)	*Blastocyst rates (mean ± SD)	Blastocyst cell numbers and ICM rates	
				Total cell (mean ± SD)	ICM/Total cell (mean ± SD)
1. QABM + BSA + FCS	964	57.10 ± 11.48	25.38 ± 16.13	98.27 ± 7.44	33.84 ± 3.79
2. QABM + BSA + FCS + IGF-I	173	62.58 ± 9.72	21.65 ± 13.56	97.33 ± 15.65	38.87 ± 8.85
3. QABM + BSA + FCS + IGF-I + EGF	109	53.34 ± 16.36	23.45 ± 17.63	84.00 ± 12.99	29.77 ± 9.60

*Blastocysts rates were calculated according to the number of cleaved embryos. SD, standard deviation.

Table 3 Effects of different supplements during culture of NT embryos from granulosa cells

Culture media	No. of oocytes	*Fusion rates (mean ± SD)	**Cleavage rates (mean ± SD)	***Blastocyst rates (mean ± SD)	Blastocyst cell numbers and ICM rates	
					Total cell (mean ± SD)	ICM/Total cell (mean ± SD)
1. QABM + BSA + FCS	188	53.62 ± 6.85	67.98 ± 9.44	32.65 ± 11.32	126.67 ± 41.38	29.32 ± 6.14[a]
2. QABM + BSA + FCS + IGF-I	87	53.25 ± 7.02	74.20 ± 10.10	29.47 ± 14.97	97.25 ± 21.08	46.61 ± 18.91[b]
3. QABM + BSA + FCS + IGF-I + EGF	96	57.36 ± 5.60	66.80 ± 8.98	41.77 ± 11.19	102.22 ± 25.43	41.37 ± 16.30[b]

*Fusion rates were calculated according to the number of oocytes whereas **cleavage and ***blastocysts rates were calculated according to the number of fused nuclear transfer (NT) embryos.
[a,b]Values within the same columns with different letters are significantly different ($P < 0.05$). SD, standard deviation.

bovine parthenogenetic embryos. In addition, there was no significant difference in the total blastocyst cell numbers and in the ratio of ICM to total cells among the culture groups ($P > 0.05$).

Developmental competence of NT embryos

Cloned bovine embryos that had been obtained from Anatolian Grey cow granulosa cells were cultured in a sequential medium supplemented with different combinations of growth factors (EGF and IGF-I), and their effect on the development of cloned embryos was compared. The developmental rates for cloned embryos are presented for each group in Table 3. The blastocyst developmental rates of NT embryos were not significantly different ($P > 0.05$). The results of the present study demonstrate that the addition of growth factors (EGF and IGF-I) in a sequential embryo culture medium had no effect on the development of bovine cloned embryos reconstructed using oocytes cultured in a maturation medium containing growth factors. As shown in Table 3, successive use of IGF-I alone or in combination with EGF in the sequential culture medium significantly increased the ratio of ICM to total cells in comparison with other media ($P < 0.05$). Our results revealed that the addition of growth factors in both maturation and culture media stimulated the ratio of ICM to the total blastocyst cell number of cloned bovine embryos.

Discussion

This study showed that the exogenous growth factors stimulated *in vitro* oocyte maturation in tissue culture media but had no effect on development of parthenogenetic and cloned bovine embryos and on the total cell number of blastocysts. However, supplementation of media with growth factors changed ICM and trophectoderm cell ratio in NT embryos. The improvement of developmental competence of mammalian oocytes by the supplementation of IVM media with gonadotropins, steroid hormones, serum and growth factors has been the subject of many investigations (Lonergan *et al.*, 1996; Rieger *et al.*, 1998; Choi *et al.*, 2002; Makarevich and Markkula, 2002; Cevik *et al.*, 2009). According to the results of the present study, maturation rates in the presence of EGF alone (75.5%) or in combination with IGF-I (75.0%) was significantly increased compared with the serum only supplemented group (63.8%). The maturation rates obtained in the present study were higher than in the studies of Rieger *et al.* (1998). In agreement with our results, several researchers (Lonergan *et al.*, 1996;

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Rieger *et al.*, 1998) have reported that the exposure of bovine oocytes to EGF and IGF-I accelerated the progression of meiosis in COCs. In a previous study, it was shown that the cleavage rate of porcine oocytes was markedly stimulated in a dose-dependent manner by the addition of IGF-I to the IVM medium. The increase in cleavage rate was the greatest at 50 ng/ml IGF-I, but this effect was evident only when the IVM medium contained no gonadotropins (Quetglas *et al.*, 2001).

Currently, there are different sequential media available for culturing mammalian embryos such as G1/G2 (Wang *et al.*, 2011), or Quinn's Advantage sequential medium (Cooke *et al.*, 2002; Arat *et al.*, 2009). Moreover, in a previous study, it had been reported that healthy cloned calves were obtained from cloned embryos cultured in Quinn's Advantage sequential medium (Arat *et al.*, 2011). However, there seems to be no report related to growth hormone supplementation in this medium to investigate its effect on embryo development. In the present study, two sequential stage-specific commercial culture media (QAM) (Cooke *et al.*, 2002) were used for the 72 h cleavage stage and for the blastocyst development during the following 5 days with different supplements.

According to Herrler *et al.* (1992) and Lonergan *et al.* (1996), EGF together with IGF-I increased the morula and blastocyst development rate, if they are included in both *in vitro* maturation and *in vitro* culture media. A positive effect on blastocyst development was found only when IGF-I was used in conjunction with granulosa cell co-culture (Herrler *et al.*, 1992) or only when bovine serum was included in the culture medium (Palma *et al.*, 1997). In addition, improvement in blastocyst yields after culture with EGF (Lonergan *et al.*, 1996) or IGF-I (Moreira *et al.*, 2002; Makarevich and Markkula, 2002) was determined without having any positive effect on the cleavage rates. These data contrast with our results from parthenogenetic and cloned bovine embryo development. Our results demonstrated that the addition of IGF-I and EGF during embryo culture did not have any effect on the blastocyst development of either parthenogenetic or cloned bovine embryos in our culture system when the maturation medium contained the same growth factors. Our and previous reports show that the effect of growth factor supplementation on embryo development rate mostly can be changed depending on the components of the IVM or culture medium.

Morphological observations are the most widely used in assessing the viability of the embryos but alone they are poor indicators. Therefore total cell number and the ICM/total cells ratio must be evaluated beside morphology to assess quality of embryos (Stojkovic *et al.*, 1998). The ICM cells contribute to all embryonic tissues and to a part of the extraembryonic membranes, whereas TE cells mainly form the outer layer of the placenta. Both cell lineages are vital for embryonic and fetal survival (Koo *et al.*, 2002; Stojkovic *et al.*, 1998; Van Soom *et al.*, 1996). Several reports indicated that placental abnormalities or early fetal losses in the cloning system may be due to aberrant allocations of the ICM and TE cells in NT embryos during early development. The decrease in TE cells may be related to placental abnormalities during embryonic development (Koo *et al.*, 2002; Wang *et al.*, 2011).

Proliferation and differentiation of somatic cells are regulated by autocrine and paracrine secretion of mitogenic growth factors by maternal and embryonic tissues at specific stages of development as evidenced by measurements of total cell numbers and ICM:TE ratios (Dadi *et al.*, 2007). Dadi *et al.* (2007) reported that treatment with either EGF or TGF-α enhanced the development rate of cloned mouse embryos to blastocysts, increased the total number of cells in blastocysts and the ICM/TE ratio, and decreased the number of apoptotic cells. Some reports have indicated that IGF-I can increase the number of total cells (Moreira *et al.*, 2002) and ICM (Sirisathien *et al.*, 2003) and decrease the percentage of apoptotic blastomeres (Wasielak and Bogacki, 2007). Makarevich and Markkula (2002) indicated that the addition of IGF-I to the culture medium, but not the maturation medium, increased the total cell number of blastocysts and lowered the apoptotic index by decreasing the number of apoptotic cells per embryo. According to Wang *et al.* (2012), a mixture of factors might be more beneficial than any one factor alone in the *in vitro* development of bovine SCNT embryos. As a similar sample to this observation, Sakagami *et al.* (2012) reported that EGF (100 or 200 ng/ml) or IGF-I (50–100 ng/ml) or combination of these significantly increased the blastocyst rate of *in vitro* cultured embryos and IGF-I induced a dose-dependent increase in cell numbers in both ICM and TE, whereas EGF stimulated proliferation only in the ICM. In contrast, another study reported that the addition of IGF-I to a culture medium did not have any effect on blastocyst cell number, the proportion of apoptotic blastomeres, or the number of cells in the inner cell mass (ICM) and trophectoderm (Block *et al.*, 2008). Ahumada *et al.* (2013) indicated that embryos cultured in a medium supplemented with EGF alone or combined with IGF-I presented lower apoptosis rates than those of embryos cultured without growth factor supplementation. However, supplementation of the culture medium with growth factors did not affect the total number of cells per blastocyst in the same study. Lee *et al.* (2005) reported that EGF did not affect blastocysts formation, total cell number in blastocysts or the ratio of ICM to total cell number if it was added

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to culture medium at the morula stage of porcine cloned embryos. In the present study, a single dose of EGF (50 ng/ml) and IGF-I (100 ng/ml), which gave the best result in previous studies (Sirisathien *et al.*, 2003; Lott *et al.*, 2011; Sakagami *et al.*, 2012; Sharma *et al.*, 2013) was administered in both *in vitro* maturation and embryo culture media at the morula stage. Although the ratio of ICM to the total cells of cloned embryos cultured in the medium containing IGF-I alone and in combination with EGF was significantly higher than that of those cultured in a medium without growth factors, the supplementation of growth factors did not affect total cell numbers as found in some previous studies (Lee *et al.*, 2005; Block *et al.*, 2008; Wang *et al.*, 2012; Ahumada *et al.* 2013). In addition, although NT and parthenogenetic bovine embryos revealed similar developmental competence, NT blastocysts showed a moderate increase in the ICM/total cell ratio when compared with parthenogenetic blastocysts. As all previous studies including ours were performed on different types and development stages of embryos, and by using various culture media, the allocation of ICM and TE cell in NT embryos in the present study suggests that the effect of EGF and IGF supplementation on cell proliferation and differentiation can change based not only on culture medium components but also on embryo types and development stage of embryos. It is well known that numerous growth factor studies have targeted maturation and IVF embryos but very few studies have focused on bovine NT embryos. Therefore, this study adds new information about the effect of growth factors on development of specially cloned bovine embryos, a finding that is completely different from IVF embryos.

In conclusion, the supplementation of a maturation medium containing fetal calf serum (FCS) and gonadotropins with EGF alone or combination with IGF-I significantly improves the maturation rates of bovine oocytes. The addition of exogenous EGF and IGF-I to a sequential culture medium, if included in the IVM medium as well, had no effect on the development of parthenogenetic and cloned bovine embryos. However, EGF and IGF-I supplementation of embryo culture medium can show various effects on embryo development based on embryo type, development stage of embryos and culture conditions and can change the proportion of NT embryo cells. Although several studies on IVF and limited studies on NT embryos have reported a positive or no effect of growth factors on embryo cell numbers, none of them reported that growth factor supplementation could change the cell ratio in NT embryos. These unknown and uncontrolled effects of growth factors may result in aberrant allocation of ICM and TE cells, which could be a reason for the developmental abnormalities of embryos in the cloning system.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Ahumada, C.J., Salvador, I., Cebrian-Serrano, A., Lopera, R. & Silvestre, M.A. (2013). 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. *Animal* **7**, 455–62.

Arat, S., Rzucidlo, S.J., Gibbons, J., Miyoshi, K. & Stice, S.L. (2001). Production of transgenic bovine embryos by transfer of transfected granulosa cells into enucleated oocytes. *Mol. Reprod. Dev.* **60**, 20–6.

Arat, S., Gibbons, J., Rzucidlo, S. J., Respess, D. S., Tumlin, M. & Stice, S. L. (2002). *In vitro* development of bovine nuclear transfer embryos from clonal lines of transgenic adult and fetal fibroblast cells of the same genotype. *Biol. Reprod.* **66**, 1768–74.

Arat, S., Tas, A., Bagis, H., Sagirkaya, H., Nak, Y., Nak, D., Akkoc, T. & Cetinkaya, G. (2009). Cloning of Anatolian Grey bull. *Reprod. Fertil. Dev.* **21**, 126–7.

Arat, S., Caputcu, A., Akkoc, T., Pabuccuoglu, S., Sagirkaya, H., Cirit, U., Nak, Y., Koban, E., Bagis, H., Demir, K., Nak, D., Senunver, A., Kilicaslan, R., Tuna, B., Cetinkaya, G., Denizci, M. & Aslan, O. (2011). Using cell banks as a tool in conservation programs of native domestic breeds: the production of the first cloned Anatolian Grey cattle. *Reprod. Fertil. Dev.* **23**, 1012–23.

Block, J., Wrenzycki, C., Niemann, H., Herrmann, D. & Hansen, P.J. (2008). Effects of insulin-like growth factor-1 on cellular and molecular characteristics of bovine blastocysts produced *in vitro*. *Mol. Reprod. Dev.* **75**, 895–903.

Cevik, M., Tas, A., Akkoc, T., Bagis, H. & Arat, S. (2009). A comparative study of parthenogenetic activation and *in vitro* fertilization of *in vitro* matured bovine oocytes. *Turk. J. Vet. Anim. Sci.* **33**, 393–9.

Choi, Y.H., Lee, B.C., Lim, J.M., Kang, S.K. & Hwang, W.S. (2002). Optimization of culture medium for cloned bovine embryos and its influence on pregnancy and delivery outcome. *Theriogenology* **58**, 1187–97.

https://doi.org/10.1017/S0967199415000519 Published online by Cambridge University Press

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, F. A. & Robl, J.M. (1998). Cloned transgenic calves from nonquiescent fetal fibroblasts. *Science* **280**, 1256–8.

Cooke, S., Quinn, P., Kime, L., Ayres, C., Tyler, J.P. & Driscoll, G. L. (2002). Improvement in early human embryo development using new formulation sequential stage-specific culture media. *Fertil. Steril.* **78**, 254–60.

Dadi, T.D., Li, M-W. & Lloyd, K.C.K. (2007). EGF and TGF-α supplementation enhances development of cloned mouse embryos. *Cloning Stem Cells* **9**, 315–26.

De la Fuente, R. & Allan King, W. (1997). Use of a chemically defined system for the direct comparison of inner cell mass and trophectoderm distribution in murine, porcine and bovine embryos. *Zygote* **5**, 309–20.

Herrler, A., Lucas-Hahn, A. & Niemann, H. (1992). Effects of insulin-like growth factor-I on *in vitro* production of bovine embryos. *Theriogenology* **37**, 1213–24.

Iwasaki, S., Yoshiba, N., Ushijima, H., Watanabe, S. & Nakahara, T. (1990). Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *J. Reprod. Fertil.* **90**, 279–84.

Koo, D-B., Kang, Y-K., Choi, Y-H., Park, J.S., Kim, H-N., Oh, K.B., Son, D-S., Park, H., Lee, K-K. & Han, Y-M. (2002). Aberrant allocations of inner cell mass and trophectoderm cells in bovine nuclear transfer blastocysts. *Biol. Reprod.* **67**, 487–92.

Lee, G.C., Kim, H.S., Hyun, S.H., Jeon, H.Y., Nam, D.H., Jeong, Y.W., Kim, S., Kim, J.H., Kang, S.K., Lee, B.C., Hwang, W.S. (2005). Effect of epidermal growth factor in preimplantation development of porcine cloned embryos. *Mol. Reprod. Dev.* **71**, 45–51.

Lonergan, P., Carolan, C., Van Langendonckt, A., Donnay, I., Khatir, H. & Mermillod, M. (1996). Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. *Biol. Reprod.* **54**, 1420–9.

Lott, WM., Anchamparuthy, , V.M. McGilliard, M.L. Mullarky, & I.K. Gwazdauskas, FC. (2011). Influence of cysteine in conjunction with growth factors on the development of *in vitro*-produced bovine embryos. *Reprod. Domest. Anim.* **46**, 585–94.

Makarevich, A.V. & Markkula, M. (2002). Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during *in vitro* maturation and culture. *Biol. Reprod.* **66**, 386–92.

Mohan, M., Malayer, J., Geisert, R. & Morgan, G. (1999). Detection of transcripts for retinoic acid receptors., retinol-binding protein and epidermal growth factor receptor in bovine preimplantation embryos. *Biol. Reprod.* **60** (Supp1), 192–3.

Moreira, F., Paula-Lopes, F.F., Hansen, P.J., Badinga, L. & Thatcher, W.W. (2002). Effects of growth hormone and insulin-like growth factor-I on development of *in vitro* derived bovine embryos. *Theriogenology* **57**, 895–907.

Palma, G.A., Müller, M. & Brem, G. (1997). Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced *in vitro*. *J. Reprod. Fertil.* **110**, 347–53.

Quetglas, M.D., Coelho, L.A., Garcia, J.M., Oliveira, F.E.B. & Esper, C.R. (2001). Effect of insulin-like growth factor-I during *in vitro* oocyte maturation and *in vitro* culture of bovine embryos. *Arq. Bras. Med. Vet. Zootec.* **53**, 1–5.

Rieger, D., Luciano, A. M., Modina, S., Pocar, P., Lauria, A. & Gandolfi, F. (1998). The effect of epidermal growth factor and insulin-like growth factor I on the metabolic activity, nuclear maturation and subsequent development of cattle oocytes *in vitro*. *J. Reprod. Fertil.* **112**, 123–30.

Sakagami, N., Umeki, H., Nishino, O., Uchiyama, H., Ichikawa, K., Takeshita, K., Kaneko, E., Akiyama, K., Kobayashi, S. & Tamada, H. (2012). Normal calves produced after transfer of embryos cultured in a chemically defined medium supplemented with epidermal growth factor and insulin-like growth factor-I following ovum pick-up and *in vitro* fertilization in Japanese Black cows. *J. Reprod. Dev.* **58**, 140–6.

SAS (2007). Statistical Software Program. SAS User Guide, Ver. 9.01, SAS Campus Drive, Cary NC 27513, USA.

Sharma, G.T., Dubey, PK., Nath, A. & Saikumar, G. (2013). Co-culture of buffalo (Bubalus bubalis) preantral follicles with antral follicles: a comparative study of developmental competence of oocytes derived from *in vivo* developed and *in vitro* cultured antral follicles. *Zygote*, **21**, 286–94.

Sirisathien, S., Hernandez-Fonseca, H.J. & Brackett, B.G. (2003). Influences of epidermal growth and insulin-like growth factor-I on bovine blastocyst development *in vitro*. *Anim. Reprod. Sci.* **77**, 21–32.

Stefanello, J.R., Barreta, M.H., Porciuncula, P.M., Arruda, J.N., Oliveira, J.F., Oliveira, M.A. & Gonçalves, P.B. (2006). Effect of angiotensin II with follicle cells and insuline-like growth factor-I or insulin on bovine oocyte maturation and embryo development. *Theriogenology* **66**, 2068–76.

Stojkovic, M., Büttner, M., Zakhartchenko, V., Brem, G. & Wolf, E. (1998). A reliable procedure for differential staining of *in vitro* produced bovine blastocysts: comparison of tissue culture medium 199 and Menezo's B2 medium. *Anim. Reprod. Sci.* **50**, 1–9.

Van de Velde, A., Liu, L., Bols, P.E., Ysebaert, M.T. & Yang, X. (1999). Cell allocation and chromosomal complement of parthenogenetic and IVF bovine embryos. *Mol. Reprod. Dev.* **54**, 57–62.

Van Soom, A., Boerjan, M., Ysebaert, M-T. & Kruif, A. (1996). Cell Allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. *Mol. Reprod. Dev.* **45**, 171–82.

Van Soom, A., Boerjan, M.L., Bols, P.E.J., Vanroose, G., Lein, A., Coryn, M. & Kruif, A.D. (1997). Timing of compaction and inner cell allocation in bovine embryos produced *in vivo* after super ovulation. *Biol. Reprod.* **57**, 1041–9.

Wadhwa, N., Kunj, N., Tiwari, S., Saraiya, M. & Majumdar, S.S. (2009). Optimization of embryo culture conditions for increasing efficiency of cloning in buffalo (*Bubalus bubalis*) and generation of transgenic embryos via cloning. *Cloning Stem Cells* **11**, 387–95.

Wang, Y.S., Tang, S., An, Z.X., Li, W.Z., Liu, J., Quan, F.S., Hua, S. & Zhang, Y. (2011). Effect of mSOF and G1.1/G2.2 media on the developmental competence of

SCNT-derived bovine embryos. *Reprod. Dom. Anim.* **46**, 404–9.

Wang, L-J., Xiong, X-R., Zhang, H., Li, Y-Y., Li, Q., Wang, Y-S., Xu, W-B., Hua, S. & Zhang, Y. (2012). Defined media optimization for *in vitro* culture of bovine somatic cell nuclear transfer (SCNT) embryos. *Theriogenology* **78**, 2110–9.

Wasielak, M. & Bogacki, M. (2007). Apoptosis inhibition by insulin-like growth factor (IGF)-I during *in vitro* maturation of bovine oocytes. *J. Reprod. Dev.* **53**, 419–26.

Yoshida, Y., Miyamura, M., Hamano, S. & Yoshida, M. (1998). Expression of growth factor ligand and their receptor mRNAs in bovine ova during *in vitro* maturation and after fertilization *in vitro*. *J. Vet. Med. Sci.* **60**, 549–54.