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A comparative study of EGF effects on in vitro bovine embryo development in monoculture and sequential media

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Abstract: Different culture system conditions can affect cleavage, zygote genome activation, embryo development, and viability. The purpose of the present study was to compare a monoculture synthetic oviductal fluid medium with modified sequential G1/G2 media and also to investigate the effect of epidermal growth factor (EGF) on bovine embryo development. Oocytes were aspirated from 2- to 6-mm ovarian follicles and matured in maturation media. After in vitro fertilization of matured oocytes, presumptive zygotes were cultured in test media with or without additional exogenous EGF. Results indicated that EGF significantly increased blastocyst formation but not cleavage rate in both media. There was no significant difference between the sequential and the monoculture media.

Key words: Blastocyst, cleavage, culture system, epidermal growth factor

1. Introduction

Development of bovine embryo culture systems is very important for producing viable embryos for transfer as well as for promoting scientific research (Kim et al., 1993). The quality and survival of in vitro-produced bovine embryos has not reached the maximal rate of in vivo-derived embryos (Boni et al., 1999). In recent decades, different systems have been studied to improve in vitro production of bovine embryos (Van Blerkom, 1993; Gardner, 1998; Holm et al., 1999). Different culture system conditions can affect cleavage, zygote genome activation, and embryo development and viability (Rizos et al., 2003; Wrenzycki et al., 2004). Different factors such as oocyte quality, oxygen rate, and energy sources may alter embryo quality and development (Gordon and Lu, 1990; Menezo et al., 1992).

There are 2 different types of media for embryo culture, sequential and monoculture systems (Lane and Gardner, 2007), and comparisons have shown that in sequential media systems, developmental and implantation rates are higher (Blake et al., 2004). Sequential media are designed to meet the nutritive requirements of the growing embryos according to the changes in the reproductive tract composition. They help reduce cellular stress and provide for better adaptation for growth to the blastocyst stage, because factors supporting optimal blastocyst development inhibit development of the early-cleavage-stage embryo, and conversely, factors supporting growth of the early-stage embryo cannot guarantee optimal blastocyst development (Gardner and Lane, 1998).

It has been demonstrated that growth factors such as platelet-derived growth factor-β or transforming growth factor-α are expressed in mammalian reproductive tissues, and so growth factors could possibly have a role in normal female reproduction (Viuff et al., 1995, Akkoyunlu et al., 2000).

In addition, it has been reported that epidermal growth factor (EGF) is present in the follicular fluid of different species (Hsu et al., 1987; Hofmann et al., 1990; Das et al., 1992), and EGF receptor is expressed on granulosa cells with the highest distribution in preovulatory follicles (Feng et al., 1987). In bovines, EGF receptor has been shown to be present in ovaries, oviducts, and uteri, and in cumulus–oocyte complexes and preimplantation embryos, indicating its possible role in development (Pohland and Tiemann, 1994). Its positive effect is due to its specific binding to receptors and mitogenic activity in embryonic development and not due to nutritive effects (Lonergan et al., 1996). It was shown that supplementation of TCM199 with EGF during in vitro maturation (IVM) at physiological concentrations stimulates cumulus cell expansion and increases the percentage of oocyte maturation and the blastocyst rate. Some of these effects

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are due to an increased proportion of oocytes reaching metaphase II, and also improvement of cytoplasmic maturation. Although EGF has a stimulatory effect postfertilization, it may not completely replace serum (Lonergan et al., 1996). Fetal bovine serum (FBS) and bovine serum albumin (BSA), which are widely used as protein sources for embryo culture media, are complex and undefined mixtures of proteins, growth factors, peptides, etc. Serum and BSA can have a stimulatory effect on embryo growth (Kane and Headon, 1980; Pinyopummintr and Bavister, 1994). Of course, another report showed that supplementation of cysteine during IVM of oocytes, in conjunction with growth factors, could effectively be used as a replacement for FBS (Lott et al., 2011).

The objective of the present study was to compare 2 different media, sequential and monoculture, both supplemented with 100 ng/mL EGF, to determine the effects of this growth factor on embryo cleavage and blastocyst rates.

2. Materials and methods

All chemicals and reagents were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany), unless otherwise specified.

2.1. Collection and maturation of oocytes

Ovaries of slaughtered animals were collected from an abattoir (Karaj, Iran) and transferred to warm saline solution within 2 h. Follicles of 2 to 6 mm in diameter were aspirated and oocytes with homogeneous cytoplasm were isolated. Oocytes were washed in TCM199 (Invitrogen, GIBCO, EvoQuest Laboratory Services, Grand Island, NY, USA) modified with 3 mg/mL BSA and transferred to 100-µL droplets of maturation culture medium of TCM199 supplemented with 100 ng/mL EGF and 10% FBS. Petri plates containing droplets were incubated at 38.5 °C in 5% CO_2 for 24 h. Matured oocytes were then collected as per morphological expansion of cumulus cells.

2.2. Sperm preparation and in vitro fertilization (IVF)

Fresh epididymal sperm cells were washed in HEPES TALP medium and motile sperm cells were collected using the swim-up method. Matured oocytes with expanded cumulus cells were washed in TCM199 modified with 3 mg/mL BSA. Groups of 10 matured oocytes were transferred to 50-µL droplets of fertilization medium (IVF-TALP supplemented with 10 µg/mL heparin). Oocytes were inseminated with 10 µL of 2×10^6/mL sperm-cell concentration and incubated for 22 h at 5% CO_2 and 38.5 °C.

2.3. In vitro embryo culture

Sequential and monoculture media with and without 100 ng/mL EGF were used (G1/G2 modified media and synthetic oviductal fluid (SOF); Table 1). After cumulus removal, presumptive zygotes were divided into 4 experimental groups: 1) SOF monoculture medium

Table 1. Composition of in vitro culture media.

SOF	G2	G1	Components
1.8	1.8	1.8	$CaCl_2.2H_2O$ (mM)
7.2	5.5	5.8	KCl (mM)
1.2	0.5	0.5	NaH_2PO_4 (mM)
108	85.16	85.16	NaCl (mM)
25.0	25.0	25.0	$NaHCO_3$ (mM)
0.05	1.0	1.0	$MgSO_4.7H_2O$ (mM)
0.3	0.10	0.32	Na pyruvate (mM)
0.3	--	--	Na citrate (mM)
3.3	5.2	10.5	Na lactate (mM)
0.2	1.0	1.0	L-Glutamate (mM)
--	3.15	0.50	Glucose (mM)
--	--	0. 1	EDTA (mM)
1′	1′	1′	Nonessential amino acids (100′)
1′	1′	--	Essential amino acids (50′)
6	--	4	BSA (mg/mL)
5% of total (only in renewal medium)	5% of total	--	FBS
10	10	10	Gentamicin (µg/mL)
100	100	100	EGF (ng/mL)

without EGF, 2) SOF monoculture medium with EGF, 3) G1/G2 modified media without EGF, and 4) G1/G2 modified media with EGF. Plates were incubated at 38.5 °C with 5% CO_2 for 8 to 9 days. In the case of sequential media, 72 h after incubation, embryos were transferred from G1 to G2 medium and the SOF medium was renewed.

After 35 h of fertilization, undivided cells were removed and cleavage rate and blastocyst formation were evaluated under an inverted microscope. The experiment was implemented in 3 replicates with similar conditions, and results were recorded as rate of cleavage and rate of blastocysts per cleavage.

2.4. Statistical analysis

The analysis was performed as a completely randomized design with 2 treatments and 3 replicates for each analysis (EGF+ and EGF-, sequential and monoculture), and the comparisons were made with LSMEANS and Tukey's test with significance defined at $P < 0.05$. Statistical evaluations were carried out using the SAS Software, PROC GLM.

3. Results

Data in Table 2 show that both monoculture and sequential media supplemented with EGF showed a significant increase in blastocyst formation (Figure) compared to their controls without EGF ($P < 0.05$), although the cleavage rate was not affected by the addition of EGF.

Analysis of data showed that there was no significant difference between the sequential and the monoculture media in cleavage and blastocyst rates ($P > 0.05$).

4. Discussion

In this study, using a monoculture and sequential media supplemented with 100 ng/mL EGF, we showed that EGF has a significant effect on embryo development at the blastocyst stage by increasing the number of blastocysts per cleavage compared to the control (37.72% vs. 33% for SOF monoculture medium and 38.83% vs. 33.33% for G1/G2 sequential media), but it did not affect the cleavage rate.

It has been reported that culturing bovine embryo with EGF just after the 8-cell stage aids the hatching of bovine embryos (Keefer et al., 1994). Baştan et al. (2010) used different doses of EGF in maturation media with 2 different culture systems (CR1 and G1.3/G2.3) and found that the cleavage rate was higher in EGF groups; no significant difference was observed in blastocyst development between the 2 media. EGF receptors are present on bovine cumulus–oocyte complexes and embryos at all stages of development until blastocyst (Lonergan et al., 1996). Wood and Kaye (1989) showed that EGF stimulates total uptake of [3H]-leucine into protein by mouse embryos cultured for 24 h from morulae. EGF also has a positive effect on blastocyst cell numbers (Sirisathien and Brackett, 2003) and helps to reduce oxidative stress (Kurzawa et al., 2004); its binding to EGF receptors results in DNA synthesis and cell growth (Carpenter and Cohen, 1990). These findings, along with our results, show the important role of EGF in embryo development.

Monoculture systems are single-medium formulations that support embryo development to the blastocyst stage and do not sustain any changes in the normal physiology of the embryo (Gardner and Lane, 2003; Lane et al., 2003), but sequential media are formulated to decrease cellular stress and help the embryo adapt to grow to the blastocyst stage (Gardner and Lane, 1998). Of course, a higher developmental rate does not usually happen in the sequential media compared to a single medium. For example, in a randomized human study, embryos were randomly cultured in 3 systems: monoculture (Rotterdam) medium for 5 days, Rotterdam medium for 3 days followed by medium refreshment, and G1/G2 sequential media for 5 days; no significant differences in blastulation, implantation, or pregnancy rates were observed (Macklon et al., 2002). In another human study, a sequential medium was compared with a single medium, which showed no difference in the number of good-quality embryos, although the embryo utilization rate was higher for the single medium (Paternot et al., 2010). In the present study, there was no significant difference in the cleavage rate (80.85% and 81.96% for SOF vs. 78.03% and 76.08% for G1/G2) and the rate of blastocysts per cleavage (37.72% and 33% for SOF vs. 38.83% and 33.33% for G1/G2) between the 2 media. The human reports support our finding that using single media may have the same effectiveness as sequential media.

Hosseini et al. (2008) showed that a sequential cell-free culture system can improve bovine embryo development in comparison with TCM199 co-culture. Lane et al. (2003)

Table 2. EGF treatment of SOF medium and G1/G2 modified sequential media.

Media	EGF (10 ng/mL)	Oocytes cultured	Embryos cleaved (%)	Blastocysts/cleavage (%)
SOF monoculture	+	141	114 (80.85) [a]	43 (37.72) [a]
SOF monoculture	_	122	100 (81.96) [a]	33 (33.00) [b]
G1/G2 sequential	+	132	103 (78.03) [a]	40 (38.83) [a]
G1/G2 sequential	_	138	105 (76.08) [a]	35 (33.33) [b]

Different letters in the same column show significance ($P < 0.05$).

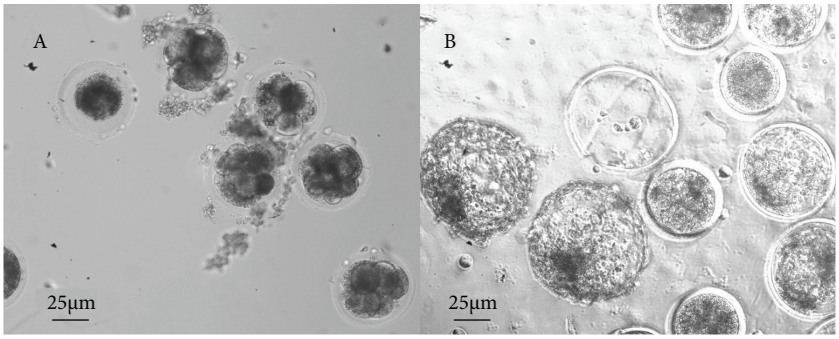

Figure. A) Embryos at 8–16 cell stage; B) expanded and hatched blastocysts.

used the sequential media G1.2/G2.2 for bovine embryo culture, which yielded equivalent blastocyst production, blastocyst cell number and inner cell mass development, and similar pregnancy rates in comparison with co-culture. However, it has been shown that sequential media systems result in higher pregnancy rates and blastocyst viability, and it is recommended to use a sequential system if a clinical blastocyst transfer is needed (Lane and Gardner, 2007). Although the sequential and monoculture media were not significantly different in this study, in order to determine which media really are more efficient for blastocyst development, the number of blastocyst cells, blastocyst viability, and pregnancy rates after transfer should be studied.

In conclusion, EGF supplementation of culture media increased embryo development to the blastocyst stage. Additionally, both the modified G1/G2 sequential culture system and the SOF monoculture system were the same in supporting bovine embryo development when supplemented with 100 ng/mL EGF.

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