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RESEARCH ARTICLE

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Effects of EGF or bFGF on the development of porcine parthenogenetic embryos *in vitro*

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Abstract Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were added into the culture medium in different culturing stages. The effects of EGF or bFGF on the development of porcine parthenogenetic embryos were studied *in vitro*. The results were as follows: The addition of EGF significantly enhanced the cleavage rate of porcine parthenogenetic embryos ($P < 0.05$). The addition of EGF or bFGF also significantly enhanced the rate of blastocysts formation of 2–4-cell porcine parthenogenetic embryos ($P < 0.05$). Additionally, the group of bFGF had more numbers of blastocysts and higher rates of blastocysts formation than the groups of EGF and the control. In conclusion, EGF and bFGF were found propitious to the development of porcine parthenogenetic embryos *in vitro*, and bFGF increased the quality of blastocysts by increasing the total cell number in porcine parthenogenetic embryos.

Keywords growth factor, porcine, *in vitro* culture

1 Introduction

Culture system of embryos *in vitro* is extremely important and directly affects the embryos development and the final pregnancy (Cutting et al., 2004). At present, the rate of development *in vitro* of porcine embryos is lower, and the rates of fertilization embryos and clone embryos are only 10%–40%. Accordingly, how to improve the embryo development rate *in vitro* and set up an ideal culture system of embryos *in vitro* is an emphasis and the hot research topic in this field constantly. However, there are few reports about the effects of growth factors on the development of porcine parthenogenetic embryos *in vitro,* especially about EGF and bFGF. Supplementing with bFGF in protein-free medium can improve the blastocyst development of rabbit, surmount the blocks and increase the development rate of embryos (Hrabe de Angelis et al., 1995). As of today, there is still no report about the effects of bFGF on the development of porcine embryos *in vitro*, so in this paper, in order to understand the effects of EGF and bFGF on the development of porcine embryos *in vitro* and to provide technique support for cloning porcine, producing transgenic porcine by somatic cell nuclear transfer (SCNT) and improving the success rate of porcine cloning, EGF and bFGF were added into the culture medium at 1-cell and 2–4-cell stages of porcine parthenogenetic embryos.

2 Materials and methods

Unless specially indicated, all chemicals were purchased from Sigma Chemical Company.

2.1 Collection of porcine oocytes

Follicular fluid and cumulus–oocyte complexes (COCs) from follicles of 3–6 mm in diameter were aspirated using a 18-gauge needle attached to a 50 mL disposable syringe (with a few of TL-HEPES-PVA or PBS-PVA) and allowed to sedimentate. The sediment was placed in TL-HEPES supplemented with 4% BSA and allowed to sediment again. After washed three times, the sediment was transferred to 60-mm Petri dishes containing TL-HEPES. Then, only those with compact, non-atretic cumulus and homogeneous cytoplasm were selected using a stereomicroscope. Cumulus oocyte complexes (COCs) selected

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were washed three times thoroughly in TL-HEPES, and cultured in the final maturation medium for use.

2.2 Culture of porcine oocytes *in vitro*

Each group of 25 COCs were transferred to a 50 μL drop of IVM medium NCSU23 (North Carolina State University 23) supplemented with 10 ng·mL^{-1} EGF (epidermal growth factor), 50 IU·mL^{-1} hCG, 50 IU·mL^{-1} PMSG, 10% FBS (fetel bovine serum) and 10% PFF (porcine follicular fluid) under mineral oil (Sigma) and cultured for 22 h at 38°C or 39°C in 5% CO_2 in humidified air in a static system. After maturation culture for 22 h, COCs were washed three times and cultured in hCG- and PMSG-free maturation medium NCSU23 for another 22 h.

2.3 Parthenogenetic activation of mature oocytes

After maturation culture, oocytes were transferred into the medium containing 1% hyaluronidase and stripped of their cumulus cells with 100 mL transferpettor and only those with one polar body, complete vitelline membrane, limpid perivitelline space, and homogeneous cytoplasm were selected for further activation.

Oocytes were equilibrated for 20 s in the pulse medium (0.28 mol·L^{-1} inositol, 0.1 mmol·L^{-1} Ca^{2+}, 0.1 mmol·L^{-1} Mg^{2+}, pH 7.0), and 40–50 oocytes were transferred into fusion bucket (electrode width was 500 μm, BTX, American) uniformly, then submitted to one DC pulse of 1.2 kV·cm^{-1} for 30 μs, 5 min apart. After being washed three times in culture medium containing 7.5 μg·mL^{-1} CB and cultured for 3–5 h, oocytes were transferred into culture medium droplet without 7.5 μg·mL^{-1}CB to complete the culture period.

2.4 Staining and cell counting

Blastocysts being cultured above were washed three times in D-PBS containing 3.7% paraformaldehyde and fixed for 10 min, which were then transferred into D-PBS containing 10 μg·mL^{-1} Hoechst33342 (bisbenzimide) and incubated in dark for 10 min; The stained embryos were transferred to 2 μL glycerol droplet on a microscopic slide and carefully covered with a coverslip. The number of nuclei in the parthenogenetic embryos was then counted under fluorescence microscopy after staining with Hoechst 33342 (Pan, 2005).

2.5 Experimental design

In Experiment 1, to investigate the effects of EGF and bFGF on the cleavage rate of porcine parthenogenetic embryos and the rate of blastocysts formation, NCSU-23 containing 4% BSA was supplemented with 10 μL·mL^{-1} EGF, 10 μL·mL^{-1} bFGF and their combination, respectively.

In Experiment 2, to investigate the effects of EGF and bFGF on development of porcine parthenogenetic embryos *in vitro*, 2–4-cell porcine parthenogenetic embryos were cultured in NCSU-23 (containing 4% BSA) supplemented with 10 μL·mL^{-1} EGF and 10 μL·mL^{-1} bFGF, respectively.

All the treated embryos were washed three times in NCSU-23 supplemented with 4 mg·mL^{-1} BSA and placed in 50 μL drops (20–25 oocytes per drop) of NCSU-23 medium under mineral oil and incubated at 39°C under the condition of 5% CO_2 in the air.

2.6 Statistical analysis

Experiments were replicated three times for each group. All data were analyzed by one-way ANOVA and Duncan's multiple range test, using generalized Linear Models (SAS; SAS Institute, Cary, NC, USA).

3 Results

3.1 Effects of EGF or bFGF during *in vitro* culture (IVC) on the development of porcine parthenogenetic embryos

Porcine parthenogenetic embryos were cultured in culture medium supplemented with EGF and bFGF. As can be seen from Table 1, the addition of EGF significantly improved the cleavage rate of porcine parthenogenetic embryos, but did not increase the rate of blastocyst formation. No significant difference was observed in the cleavage rate of porcine parthenogenetic embryos and the rate of blastocyst formation in the presence of bFGF or combination of EGF and bFGF.

3.2 Effects of EGF or bFGF during IVC on the development of porcine parthenogenetic embryos in 44 h

To study the effects of different growth factors on the development of porcine parthenogenetic embryos *in vitro*,

Table 1 Effect of EGF or bFGF during IVC on the development of porcine parthenogenetic embryos

	No. of oocytes used	No. of cleaved embryos (% ± *SE*)	No. of blastocysts (± *SE*)
control group	80	55(68.00 ± 2.00 ^{b})	17(21.11 ± 1.92 ^{a})
EGF	68	50(73.45 ± 1.83 ^{a})	15(22.13 ± 1.53 ^{a})
bFGF	74	52(68.49 ± 1.59 ^{b})	15(19.61 ± 2.44 ^{a})
EGF+bFGF	74	53(71.50 ± 4.76^{ab})	18(24.22 ± 2.60 ^{a})

Note: Within the same column, values with different superscripts were significantly different ($P < 0.05$). The same below.

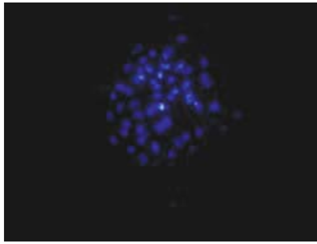

Fig. 1 Numbers of blastocysts cells cultured in the medium supplemented with bFGF at 2–4-cells stage, stained with Hoechst 33342

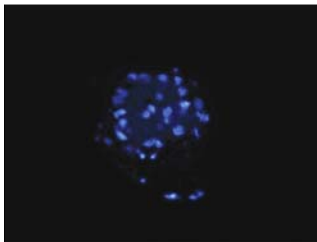

Fig. 2 Numbers of blastocysts cells cultured in the medium supplemented with EGF at 2–4-cells stage, stained with Hoechst 33342

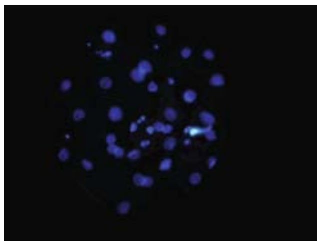

Fig. 3 Numbers of blastocysts cells cultured in the medium no growth factors supplemented, stained with Hoechst 33342

2–4-cells porcine parthenogenetic embryos were cultured in culture medium supplemented with EGF or bFGF respectively. As shown in Table 2, the rate of blastocysts formation in the group supplemented with 10 ng·mL^{-1} EGF was significantly higher ($P < 0.05$) than that in the control group, whereas no significant difference in the number of nuclei in the parthenogenetic embryos was observed among experimental groups. However, the rate of blastocysts formation and the number of nuclei in the parthenogenetic embryos in bFGF supplemented groups were higher than those in EGF supplemented groups (Figs. 1–3).

4 Discussion

During the embryos development *in vivo*, growth factors that genital duct secretes, such as insulin, insulin-like growth factors, EGF and bFGF and so on, play extremely important roles in the preimplantation embryos development (Kaye, 1997). Therefore, many studies have been conducted to observe if these growth factors can promote embryos development during embryos culture *in vitro*, and there have been a lot of achievements. Our study was mainly to investigate whether supplementing culture medium with EGF and bFGF at different culture stages was beneficial for improving the development of porcine parthenogenetic embryos. The results showed that EGF can significantly enhance the cleavage rate of porcine parthenogenetic embryos and the rate of blastocyst formation, and bFGF can significantly enhance the rate of blastocyst formation.

EGF is a sort of single strand polypeptide containing amino acids, which is separated from submaxillary gland of mouse first (Cohn, 1960), and thereafter discovered in many animals like rabbit, sheep and pig, etc. EGF from the genital duct of female animals can excite embryo cell proliferation and improve the cleaver rate of embryos and the rate of blastocyst formation in form of paracrine.

At present, there have been many reports about the effect of EGF on development of porcine embryos *in vitro*. For example, Abeydeera et al. (1998) and Wei et al. (2001) reported the effect of EGF on development of *in vitro* fertilization (IVF) embryos, Lee et al. (2005) reported the effect of EGF on the development of porcine somatic cell nuclear transfer (SCNT) embryos and IVF embryos. And they gained different results. Lee et al. (2005) made an intimate report about expression of EGF and its receptor (*EGFr*) gene in preimplantation embryos. The expression of EGF and *EGFr* mRNA was determined using reverse transcription-polymerase chain reaction (RT-PCR). In SCNT and IVF embryos, the EGF mRNA was detected at the stages of oocytes, 2-cell, 4-cell, 8-cell, morulae, and blastocysts, and the expression of which was decreasing gradually. Therefore, supplementing with EGF, EGFr mRNA was detected at the stages of oocytes, 2-cell, morulae, and blastocysts. SCNT embryos at 1-cell stage were cultured in North Carolina State University (NCSU)-23 medium supplemented with EGF. Supplementing with 10 ng·mL^{-1} EGF improved cleavage rate, but not the rate of blastocyst formation compared to the control. Supplementing with 10 ng·mL^{-1} EGF increased the total number of cells in blastocysts, but

Table 2 Effects of EGF or bFGF during IVC on the development of porcine parthenogenetic embryos after 44 h of culture

	No. of oocytes used	No. of blastocysts (% ± *SE*)	No. of blastocysts cells (± *SE*)
control group	156	30(19.01 ± 1.72 ^{b})	40.80 ± 8.23 ^{a}
EGF	130	47(36.20 ± 5.37 ^{a})	31.40 ± 9.96 ^{a}
bFGF	147	65(44.25 ± 5.83 ^{a})	43.25 ± 9.74 ^{a}

not the rate of blastocyst formation compared to the control. However, we drew a contrary conclusion. EGF improved the rate of blastocyst formation, but decreased the total cell number in blastocysts. Supplementing with EGF at 1-cell stage improved the cleavage rate of parthenogenetic embryos, and degraded the rate of blastocyst formation slightly. This proved that EGF combined with *EGFr* existed in mature oocytes and 2-cell stage, and reacted.

Basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF) belong to the FGF family. FGF was first combined with acetyl heparin sulfate proteoglycan of target cell, whose compounds are then combined with trans- membrane tyrosine receptor and react. BFGF mRNA was proved to exist in preimplantation blastocysts of mouse (Campbell et al., 1992). And, bFGF mRNA was decreasing from fertilization embryos to blastocysts of sheep preimplantation embryos gradually (Watson et al., 1994). Preimplantation embryos of rabbits could secrete and combine with bFGF to promote gastrula form of embryos *in vitro* (Hrabe de Angelis et al., 1995). Anupma et al. (1997) suggested that although bFGF and aFGF belong to the same proteinum family, they might have different effects on porcine embryos development. But, there was no report that bFGF and bFGF exist in porcine embryos. In our study, 2-cell embryos were cultured in NCSU-23 supplementing with 10 ng·mL^{-1} bFGF, which significantly improved the rate of blastocyst formation and slightly increased the total cell number in blastocysts compared to EGF. No effect on the cleavage rate and the rate of blastocyst formation was observed in bFGF supplemented group at 1-cell stage. Therefore, from this result, we can presume that bFGF receptor might exist in embryos after 2–4-cell stage and had the same effect as EGF, which should be further proved using RT-PCR.

In a word, supplementing with 10 ng·mL^{-1} EGF or 10 ng·mL^{-1} bFGF in NCSU-23 can improve parthenogenetic embryos development of oocytes in puberty ovaries.

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References

Abeydeera L R, Wang W H, Cantley T C, Rieke A, Prather R S, Day B N (1998). Presence of epidermal growth factor during *in vitro* maturation of pig oocytes and embryo cuture can modulate blastocyst development after *in vitro* fertilization. Mol Reprod Dev, 51(4): 395–401

Anupma G, Fuller W B, Laurie A J (1997). Immunolocalization of acidic and basic fibroblast growth factors in porcine uterine and conceptus tissues. Biol Reprod, 56(6): 1527–1536

Campbell W J, Miller K A, Anderson T M, Shull J D, Rizzino A (1992). Expression of fibroblast growth factor receptors by embryonal carcinoma cells and early mouse embryos. *In Vitro* Cell Dev Biol, 28(1): 61–66

Cohn S (1960). Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. Proc Natl Acad Sci USA, 46(3): 302–311

Cutting R C, Pritchard J, Clarke H S, Martin K L (2004). Establishing quality control in the new IVF laboratory. Hum Fertil (Camb), 7(2): 119–125

Hrabe de Angelis M, Grundker C, Herrmann B G, Kispert A, Kirchner C (1995). Promotion of gastrulation by maternall growth factor in cultured rabbit blastocysts. Cell Tissue Res, 282(1): 147–154

Kaye P L (1997). Preimpantation growth factor physiology. Rev Reprod, 2: 373–386

Lee G S, 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(1): 45–51

Pan D K (2005). Factors affecting developmental competence of porcine cloned embryos by somatic cell nuclear transfer. Dissertation for the Doctoral Degree. Beijing: China Agricultural University, 57–59 (in Chinese)

Watson A J, Watson P H, Arcellana-Panlilio M, Warnes D, Walker S K, Schultz G A, Armstrong D T, Seamark R F (1994). A growth factor phenotype map for ovine preimplantation development. Biol Reprod, 50: 725–733

Wei Z, park K W, Day B N, Prather R S (2001). Effect of epidermal growth factor on preimplantation development and its receptor expression in porcine embryos. Mol Reprod Dev, 60(4): 457–462