Beneficial effects of brain-derived neurotrophic factor on *in vitro* maturation of porcine oocytes

Eugine Lee^{1,2}, Yeon Ik Jeong^{1,2}, Seon Mi Park¹, Jong Yun Lee^{1,2}, Ji Hye Kim^{1,2}, Sun Woo Park^{1,3}, M S Hossein¹, Yeon Woo Jeong^{1,2}, Sue Kim¹, Sang Hwan Hyun^{1,3} and Woo Suk Hwang¹

¹SooAm Biotech Research Foundation, Sooambuilding 1027-4, Bangbae3-dong, Seocho-gu, Seoul 137-851, South Korea, ²Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea and ³Laboratory of Veterinary Biotechnology, College of Veterinary Medicine, Chunbuk National University, Cheongju 361-763, South Korea

Correspondence should be addressed to W-S Hwang; Email: hwangws@sooam.org

Abstract

In an effort to improve the quality of *in vitro* produced porcine embryos, we investigated the effect of brain-derived neurotrophic factor (BDNF), a neurotrophin family member, on *in vitro* maturation (IVM) of porcine oocytes. The expression of BDNF and truncated isoforms of its receptor, tyrosine kinase B (TrkB), and p75 common neurotrophin receptor was detected in both follicular cells and metaphase-I stage oocytes by RT-PCR. However, mRNA of full-length TrkB was not found in oocytes although it was detected in follicular cells. The expression pattern of BDNF and TrkB was confirmed by immunohistochemistry. Supplementation with BDNF (30 ng/ml) during IVM significantly ($P < 0.05$) increased the first polar body extrusion and glutathione levels in oocytes, whereas the effect of BDNF on nuclear maturation was diminished when gonadotropin and epidermal growth factor (EGF) were added to the culture media. However, treatment with BDNF (30 ng/ml) along with EGF (10 ng/ml) in the presence of gonadotropin significantly ($P < 0.05$) increased the developmental competence of oocytes to the blastocyst stage after both *in vitro* fertilization (IVF; 29.1% when compared with control, 15.6%) and somatic cell nuclear transfer (SCNT; 13.6% when compared with control, 3%). This appeared to reflect a stimulatory interaction between BDNF and EGF to enhance the cytoplasmic maturation of oocytes to support successful preimplantation development. In conclusion, BDNF enhanced nuclear and cytoplasmic maturation of oocytes by autocrine and/or paracrine signals. Also, when used together with EGF, BDNF increased the developmental potency of embryos after IVF and SCNT, demonstrating an improved *in vitro* production protocol for porcine oocytes.

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Introduction

Recent advances in biotechnology have enabled the production of cloned or genetically modified pigs by manipulation of *in vitro* produced embryos. Notably, however, the overall efficiency of this process is still extremely low and the quality of embryos produced *in vitro* is inferior to those produced *in vivo*. One of the major problems includes improper *in vitro* maturation (IVM) of oocytes, in both the nuclear and the cytoplasmic compartments. Although porcine IVM technology and molecular aspects of pig oocytes have been well studied and reviewed (Day 2000, Nagai 2001, Sun & Nagai 2003), there is a paucity of information on ovarian factors that may be important for oocyte maturation and subsequent development.

Neurotrophins (NTs), including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and NTs

3 and 4 (NT3/4), comprise a family of soluble polypeptide growth factors widely recognized for their essential roles in the differentiation and survival of defined neuronal populations of the central and peripheral nervous systems (Jones *et al.* 1994). However, both NTs and their receptors also play important roles in nonneuronal tissues, including the endocrine system. Several NTs and their respective receptor tyrosine kinases (Trk—) have been found to be expressed in the mammalian ovary (Dissen *et al.* 1996) and play a direct role in the regulation of early follicular development and ovulation (Dissen *et al.* 2002, Spears *et al.* 2003). BDNF is a NT family member, which is known to be important for early follicular development in mice through activation of the high-affinity TrkB receptor and the low-affinity p75 NT receptor (NTR; Ojeda *et al.* 2000, Paredes *et al.* 2004). It has been reported that BDNF was detected in human follicular fluid and the site of secretion appears to be cumulus granulosa cells. Moreover, the

authors suggested a possible role for BDNF in the regulation of oocyte nuclear maturation through mouse study (Seifer *et al.* 2002a,2002b). A recent study demonstrated that BDNF from granulosa and cumulus cells enhanced first polar body extrusion and increased the competence of mouse oocytes to complete preimplantation development *in vivo* and *in vitro* (Kawamura *et al.* 2005). Furthermore, maturation of oocytes in the presence of BDNF promoted the preimplantational development of bovine embryos (Martins da Silva *et al.* 2005).

On this basis, we hypothesized that BDNF could be one of several factors secreted from follicular cells that function to enhance porcine oocyte maturation. In the present study, we investigated the effect of BDNF supplementation during IVM on oocyte maturation and subsequent embryo development after *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT).

Materials and Methods

IVM and evaluation of first polar body extrusion in pig oocytes

Ovaries were retrieved from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h. Follicular fluid and cumulus–oocyte complexes (COCs) from follicles 3–6 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. Each group of 50 COCs was selected and cultured in 500 µl tissue culture medium (TCM)-199 (Life Technologies) at 39 °C in a humidified atmosphere of 5% CO₂ and 95% air with different experimental designs. At the end of maturation culture, COCs were transferred to HEPES-buffered NCSU-23 medium containing 0.5 mg/ml hyaluronidase (Sigma–Aldrich Corp.) for 1 min and the cumulus cells were subsequently removed by gentle pipetting. Oocytes were then stained with 5 µg/ml bisbenzimidazole (Hoechst 33 342, Sigma) for 5 min and the occurrence of first polar body extrusion was observed under an inverted microscope equipped with epifluorescence.

IVF

At 44 h of IVM, oocytes freed from cumulus cells were washed in modified Tris-buffered medium (mTBM) containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate and 0.1% (w/v) BSA, and co-incubated with 2 × 10⁶ spermatozoa/ml in 50 µl droplets (20 oocytes per drop) of mTBM covered with mineral oil in 5% CO₂ in air at 39 °C for 6 h. After IVF, the oocytes were washed and transferred into *in vitro* culture (IVC) medium.

Preparation of donor cells

Fetal fibroblasts were isolated from slaughterhouse-derived fetuses at ~40 days of gestation. The head of

the fetus was removed using iris scissors and soft tissues such as liver and intestine were discarded by scooping out with two watchmaker's forceps. After washing twice with PBS, the carcass was minced with a surgical blade on a 100 mm culture dish (Becton Dickinson, Lincoln Park, NJ, USA). The minced fetal tissues were dissociated in DMEM (Life Technologies) supplemented with 0.1% (w/v) trypsin/1 mM EDTA (Life Technologies) for 1–2 h. Trypsinized cells were washed once by centrifugation at 300 g for 10 min and subsequently seeded into 100 mm plastic culture dishes. Seeded cells were subsequently cultured for 6–8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies), 1 mM sodium pyruvate, 1% (v/v) nonessential amino acids (Life Technologies), and 10 µg/ml penicillin/streptomycin solution (Life Technologies) at 39 °C in a humidified atmosphere of 5% CO₂ and 95% air. After removal of unattached clumps of cells or explants, attached cells were subcultured at intervals of 5–7 days by trypsinization for 5 min using 0.1% trypsin and 0.02% EDTA. Donor cells cultured until confluent were retrieved from the monolayer by trypsinization and subsequently used for SCNT.

SCNT

The zonae pellucidae of oocytes were partially dissected using a fine glass needle and the first polar body and adjacent cytoplasm, presumably containing the metaphase-II (MII) chromosomes, were extruded by squeezing with the same needle. Oocytes were then stained with 5 µg/ml bisbenzimidazole (Hoechst 33 342) for 5 min and observed under an inverted microscope equipped with epifluorescence. Oocytes still containing DNA material were excluded. A single donor cell with a smooth cytoplasmic membrane was introduced into the perivitelline space of an enucleated oocyte. Couplets were placed in a 0.3 M mannitol solution containing 0.5 mM HEPES, 0.1 mM CaCl₂, and 0.1 mM MgCl₂ for 4 min and transferred to a chamber consisting of two electrodes overlaid with fusion and activation solution. Couplets were fused and activated simultaneously with a single direct current pulse 2.0 kV/cm for 50 µsec using a BTX Electro-cell Manipulator 2001 (BTX Inc., San Diego, CA, USA). All treated embryos were washed thrice with NCSU-23 supplemented with 4 mg/ml BSA, transferred into IVC medium.

Embryo culture

Embryos were placed in 25 µl microdrops (five to seven oocytes per drop) of IVC medium under mineral oil and cultured at 39 °C in a 5% CO₂, 5% O₂, and 90% N₂ atmosphere. The base IVC medium for pig embryos was mNCSU-23, which contains 0.5 mM pyruvate/5 mM lactate as energy sources (instead of glucose) and also contains 0.4% BSA (Kim *et al.* 2004). The day of insemination or injection of donor cells into enucleated

oocytes was defined as day 0. The rates of cleavage and blastocyst formation were evaluated under a stereomicroscope at days 2 and 7 respectively.

Total RNA isolation and RT-PCR amplification

MI stage oocytes and cumulus cells were obtained 22 h after IVM and mural granulosa cells were isolated from aspirated follicular fluid. Total RNA extraction from each sample and RT was carried out as reported previously (Lee *et al.* 2005). The primers for β -actin and BDNF were designed based on *Sus scrofa* β -actin mRNA (accession number: U07786) and BDNF mRNA (accession number: X16413). Because the complete mRNA sequences of *Sus scrofa* TrkB and p75 NTR are not known, primers reported in human (Anderson *et al.* 2002, Martins da Silva *et al.* 2005), and mouse studies (Kawamura *et al.* 2005) were used in this study. The primers for BDNF, full-length TrkB, truncated TrkB (trTrkB), p75 NTR and β -actin were as follows: BDNF: sense 5'-ACATGTATACGTCCCCGAGTC-3', antisense 5'-TATCCTTATGAACCGCCAGC-3'; TrkB: sense 5'-GGCCAGATGCTGTCATTAT-3', antisense 5'-TCCTGCTCAGGACAGAGGTT-3'; trTrkB: sense 5'-CATGTTACCAATCACACGGAGTA-3', antisense 5'-CCATCCAGTGGGATCTTATGAAA-3'; p75 NTR: sense 5'-GTGGAGATGGAGATGATATGGAA-3', antisense 5'-GAAGGCAATCTCCAATTAGAAGC-3'; β -actin: sense 5'-CGAAGCTGGACAAGGAGAAG-3', antisense 5'-CTCCAGGTTGCCTCTCACTC-3'.

The cDNA (5 μ l) was amplified in a 50 μ l PCR containing 1.25 units hot start *Taq* polymerase and its buffer, 1.5 mM $MgCl_2$, 2 mM dNTP, and 25 pmol specific primers. The PCR amplification was carried out for one cycle with denaturing at 95 °C for 15 min, and 35 subsequent cycles with denaturing at 95 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 15 min. Amplified PCR products (10 μ l) were fractionated on a 0.8% agarose gel, stained with ethidium bromide, and visualized with a Gel Documentation system (Gel-DocTM 2000, Bio-Rad). The PCR products were purified from the gel with an agarose gel extraction kit (Qiagen) and cloned into the pCRTopo cloning vector (Life Technologies). Sequence analysis was performed to confirm the identity of amplified PCR products using an automated DNA sequence analyzer (ABI 3100, Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Immunofluorescence-based detection of BDNF and TrkB was performed by using denuded MI stage oocytes, harvested 22 h after IVM. After removing cumulus cells, denuded oocytes were fixed with 4% paraformaldehyde for 20 min. After permeabilization with 0.1% Tween-20 and 1% Triton-X, nonspecific binding was blocked with

1% normal goat serum. Oocytes were then incubated with rabbit anti-BDNF (sc-546, Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) or rabbit anti-TrkB antibodies (sc-12, Santa Cruz) at a ratio of 1:200 dilution overnight. Oocytes incubated in the absence of primary antibody were used as a negative control. After extensive washing, oocytes were placed in goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC; Santa Cruz) at a ratio of 1:700 dilution for 3 h. After washing, oocytes were counterstained with 100 μ g/ml fluorochrome propidium iodide (PI; Sigma) for 10 min, mounted on a drop of PBS containing 0.5% FBS and examined under a confocal microscope (Radiance 2000, Bio-Rad). Cumulus cells were cultured on slides, fixed and stained for BDNF and TrkB, as outlined above.

Glutathione assay

After 44 h IVM in TCM-199 supplemented with 10% (v/v) porcine follicular fluid (pFF), with or without different doses of BDNF, the amount of glutathione (GSH) in the oocytes was assayed using a previously described microglutathione assay (Baker *et al.* 1990), with slight modifications. Briefly, denuded oocytes were washed thrice in PBS, and groups of 30–40 oocytes in 5 μ l PBS were transferred to 1.5 ml Eppendorf tubes. The samples were frozen at –70 °C and thawed at room temperature thrice and 5 μ l 1.25 M H_3PO_4 (Sigma) was added. The tubes were centrifuged at 1200 *g* for 10 min and the supernatants were transferred to a 96-well microtiter plate (50 μ l/well). A 100 μ l reaction mixture consisting of 5 ml 5,50-dithiobis(2)-nitrobenzoic acid (D8130, Sigma; 1 mM), 5 ml NADPH (N7505, Sigma; 1 mM), 5.75 ml $NaPO_4$ buffer (100 mM), and 0.1 ml GSH reductase (G3664, Sigma; 200 U/ml) was added and the plate was immediately placed in a microtiter plate reader (Bio-Rad). The formation of 5-thio-2-nitrobenzoic acid was monitored every 30 s for 3 min. Standards were prepared for each assay, and GSH content per sample was determined from a standard curve (Sigma plot/enzyme kinetics). The GSH concentrations (pmol/oocyte) were calculated by dividing the total concentration per sample by the number of oocytes present in the sample.

Blastocyst cell counting

Blastocysts were fixed in 4% of paraformaldehyde on day 7 of culture, and stained with 5 μ g/ml bisbenzimidazole (Hoechst 33342) for 5 min. After three washes in PBS, individual blastocysts were mounted and their cell number was assessed using epifluorescence microscopy.

Experimental design

In experiment 1, COCs were matured in TCM-199 supplemented with 0.1% polyvinyl alcohol (PVA) for

Table 1 *In vitro* maturation (IVM) conditions used in experiment 2.

	IVM ^a	
	0–22 h	22–44 h
EGF (–)	GTH	BDNF
	GTH + BDNF	BDNF
	GTH	–
EGF (+)	BDNF	BDNF
	GTH	BDNF
	GTH + BDNF	BDNF
	GTH	–
	BDNF	BDNF

^aBasically, COCs were cultured in TCM-199 supplemented with 10% pFF. The concentrations of supplements are EGF, 10 ng/ml; GTH, 4 IU/ml of PMSG and hCG; and BDNF, 30 ng/ml.

44 h with or without different doses of recombinant human BDNF (10–40 ng/ml; Sigma), NT-3 (10 ng/ml; Sigma), and NGF (10 ng/ml; Sigma). After IVM, extrusion of the first polar body was observed. The same experiments were replicated using TCM-199 supplemented with 10% (v/v) pFF instead of PVA. Some COCs were cultured with BDNF (30 ng/ml) with or without a pan-specific Trk receptor inhibitor, K252a (100 nM; Calbiochem, San Diego, CA, USA) or K252b (100 nM; Calbiochem). After IVM, extrusion of the first polar body was observed and GSH content in the oocytes was assayed to evaluate oocyte maturation.

In experiment 2, the effect of BDNF was investigated following previously reported two-step culture system (Lee *et al.* 2005) with slight modification. We assigned oocytes into two experimental groups, namely EGF-treated or nontreated. In EGF-treated group, COCs were cultured in TCM-199 supplemented with 10 ng/ml EGF, GTH, which consists of 4 IU/ml pregnant mare serum gonadotropin (PMSG; Intervet, Boxmeer, The Netherlands) and human chorionic gonadotrophin (Intervet), and 10% pFF. After culturing for 22 h, COCs were washed thrice and cultured in GTH-free TCM-199 medium for another 22 h. The same culture medium excluding EGF was used for EGF nontreated experiment. Supplementation of BDNF (30 ng/ml) is summarized in

Table 1. After IVM, extrusion of the first polar body was observed to evaluate oocyte maturation.

In experiment 3, oocytes were matured under four different conditions (Table 2), on the basis of results obtained in experiment 2. They were then subjected to IVF to examine their developmental competence.

In experiment 4, oocytes were matured under four different conditions (Table 3) on the basis of results obtained in experiment 2. They were then used for SCNT to examine their developmental competence.

Statistical analysis

All data were subjected to one-way ANOVA followed by Tukey's test to determine statistical differences among experimental groups. Statistical significance was determined when $P < 0.05$.

Results

Expression of BDNF and its receptors in porcine oocytes and follicular cells

Expression of BDNF and its receptors, TrkB, trTrkB, and p75 NTR, was analyzed in porcine oocytes and follicular cells by RT-PCR. Ovarian cDNA was used as a positive control. As shown in Fig. 1, the transcripts of BDNF, trTrkB, and p75 NTR were detected in all samples. However, the mRNA of full-length TrkB was not found in MI stage oocytes although it was detected in follicular cells. In order to confirm and localize this expression, we used immunohistochemical techniques to detect BDNF and TrkB in cultured oocytes and cumulus cells. GTH treatment induced stronger BDNF expression in cultured cumulus cells, while TrkB expression was only slightly detectable, irrespective of GTH treatment (Fig. 2A). Furthermore, expression of BDNF was localized to the chromatin, but no TrkB staining was detected in MI stage oocytes (Fig. 2B). All staining was evidenced by comparison with control experiments with secondary antibody alone, which were negative.

Table 2 Effect of brain-derived neurotrophic factor (BDNF) supplementation on the developmental potential of porcine oocytes after *in vitro* fertilization.

IVM			Number of oocytes studied	Number (%) of embryos cleaved	Number (% of total) of embryos developed to blastocyst	Cell number in blastocyst (mean $\pm$ S.E.M.)	Replicate
	0–22 h	22–44 h					
EGF (–)	GTH	–	218	131 (60.1)	34 (15.6)*	70.1 $\pm$ 6.2	6
	GTH + BDNF	BDNF	232	140 (60.3)	28 (12.1)*	56.3 $\pm$ 10.0	6
EGF (+)	GTH	–	371	225 (60.7)	60 (16.2)*	61.9 $\pm$ 10.1	6
	GTH + BDNF	BDNF	244	155 (63.5)	71 (29.1) [†]	67.4 $\pm$ 11.7	6

*[†]Within the same column, values with different superscripts were significantly different ($P < 0.05$).

Table 3 Effect of brain-derived neurotrophic factor (BDNF) supplementation on developmental potential of porcine oocytes after somatic cell nuclear transfer.

IVM								
	0–22 h	22–44 h	Number of oocytes injected	Number (%) of oocytes fused	Number (%) of embryos cleaved	Number (%) of embryos developed to blastocyst	Cell number in blastocyst (mean ± S.E.M.)	Replicate
EGF (–)	GTH	–	251	168 (66.9)	95 (56.6)	5 (3.0)*	46.0 ± 14.0	6
	GTH + BDNF	BDNF	297	191 (64.3)	125 (65.5)	17 (9.0) [†]	61.0 ± 16.7	6
EGF (+)	GTH	–	255	173 (67.8)	93 (53.8)	15 (8.7) [†]	61.3 ± 8.6	6
	GTH + BDNF	BDNF	293	199 (67.9)	132 (66.3)	27 (13.6) [‡]	69.0 ± 9.6	6

,[†],[‡] Within the same column, values with different superscripts were significantly different (^{*} P <0.01; [†] P <0.01; [‡] P <0.001).

Effect of BDNF supplementation on nuclear and cytoplasmic maturation of porcine oocytes

Initially, COCs were cultured in basic TCM-199 media supplemented with 0.1% PVA and various concentrations of BDNF or other NTs. Under these conditions, only 30 ng/ml BDNF significantly (P <0.05) increased first polar body extrusion when compared with control (control, 24.0%; 10 ng/ml BDNF, 23.2%; 20 ng/ml BDNF, 29.2%; 30 ng/ml BDNF, 37.1%; 40 ng/ml BDNF, 29.9%; NGF, 25.1%; NT-3, 25.6%; Fig. 3A). We next examined the effect of BDNF on COCs cultured in TCM-199 supplemented with 10% pFF and added two treatment groups (25, 35 ng/ml BDNF) to provide confidence in the dose–response data. As shown in Fig. 3B, similar results were obtained where only 30 ng/ml BDNF significantly (P <0.05) increased first polar body extrusion when compared with control (control, 28.7%; 10 ng/ml BDNF, 28.6%; 20 ng/ml BDNF, 34.1%; 25 ng/ml BDNF, 34.6%; 30 ng/ml BDNF, 46.1%; 35 ng/ml BDNF, 37.7%; 40 ng/ml BDNF, 33.3%; NGF, 28.9%; NT-3, 30.4%). The effect of BDNF was further confirmed by GSH assay and by using the TrkB receptor inhibitors, K252a, and K252b. During IVM, concurrent treatment of 30 ng/ml BDNF with K252a, but not the membrane nonsoluble K252b, blocked the effect of BDNF on first polar body extrusion (control, 28.5%; BDNF, 51.3%; BDNF + K252a, 27.5%; BDNF + K252b, 48.3%; K252a, 26.9%; K252b, 30.8%; P <0.05; Fig. 4). Moreover, when compared with the control, BDNF (20, 30, and 40 ng/ml) significantly (P <0.01) increased the GSH content in oocytes (control, 5.8 pmol; 10 ng/ml BDNF, 8.6 pmol; 20 ng/ml BDNF, 11.0 pmol; 30 ng/ml BDNF, 12.7 pmol; 40 ng/ml BDNF, 10.0 pmol; Fig. 5).

When BDNF was applied to the two-step culture system, 44-h treatment of BDNF in the absence of EGF significantly (P <0.05) promoted the meiotic progression of oocytes when compared with BDNF untreated oocytes amongst those groups supplemented with GTH for the first 22 h of culture (Fig. 6A). However, the effect was diminished with the addition of EGF and no differences were found between groups supplemented with GTH for the first 22 h of culture (Fig. 6B).

Effect of BDNF supplementation on developmental potential of porcine oocytes after IVF

Four groups were designed based on the results of Fig. 6A and B, to examine the effect of BDNF on the developmental potency of oocytes in the presence or absence of EGF. As shown in Table 2, 30 ng/ml BDNF in the presence of 10 ng/ml EGF significantly (P <0.05) increased the rate of blastocyst formation, although no differences in cleavage rates and average blastocyst cell number were observed between the four groups.

Effect of BDNF supplementation on the developmental potential of porcine oocytes after SCNT

As shown in Table 3, both 30 ng/ml BDNF and 10 ng/ml EGF significantly (P <0.01) increased the rate of blastocyst formation of SCNT embryos. When BDNF was added in combination with EGF, blastocyst development was significantly (P <0.01) higher than in the groups treated with BDNF or EGF alone. However, no differences were observed between the four groups in terms of cleavage rates or average cell numbers in blastocysts.

Figure 1 Expression of BDNF, full-length TrkB, truncated TrkB (trTrkB), and p75 NTR mRNAs in porcine isolated ovarian cells and MI stage oocytes was determined by RT-PCR. β -actin served as a ubiquitously expressed control. Total ovarian mRNAs was used for positive control tests and reactions lacking cDNA showed no PCR amplification products (con).

Figure 2 Immunostaining for BDNF and TrkB in porcine cumulus cells (A) and MI stage oocyte (B). Cumulus cells were stained with anti-BDNF or TrkB antibody (green) both before (b and d) and after (c and e) treatment with GTH. MI stage oocyte was obtained 22 h after IVF and stained with anti-BDNF or TrkB antibody (green). The DNA was counterstained with PI (red). Samples incubated without primary antibody were used as a negative control (control). Scale bar = 10 μm.

Discussion

IVM studies of porcine oocytes are of strategic value for research and commercial use. In order to improve the existing porcine IVM system, the present study examined the effect of BDNF on porcine oocyte IVM, since it is

thought to be one of several intra-follicular paracrine factors. The main finding of this study is that BDNF enhances nuclear and cytoplasmic maturation by autocrine and/or paracrine signals and increases the developmental potency of embryos after IVF and SCNT.

Although oocyte maturation is triggered by the GTH surge, GTH receptor expression was not detected in either the oocyte itself or its surrounding cumulus cells, but these receptors were expressed in the follicular cells of both theca and granulosa cells (Peng *et al.* 1991). In this context, Ackland *et al.* (1992) previously reported that some paracrine factors, which accumulate in follicular fluid in response to GTH, may affect oocyte maturation. Thus, we propose that these factors could be used in an IVM system, and would be expected to improve its efficiency. However, with the exception of EGF, IGF-I, and IGF-II there is only limited information on the nature of ovarian factors which could have a stimulating effect on porcine oocyte IVM (Ding & Foxcroft 1994, Sirotkin *et al.* 2000).

Based on their expression and substantial roles in the mammalian ovary (Dissen *et al.* 1996, 2002, Spears *et al.* 2003), several investigations have reported that various NTs and their respective receptor tyrosine kinases could enhance the IVM of oocytes from different species. For example, NGF treatment promoted ovine oocyte GVBD (Barboni *et al.* 2002) and both NT-4/5 and BDNF were shown to play a paracrine role in enhancing polar body extrusion in mouse oocytes (Seifer *et al.* 2002a, 2002b). Furthermore, the maturation of oocytes in the presence of BDNF promoted the preimplantational development of mouse (Kawamura *et al.* 2005) and bovine (Martins da Silva *et al.* 2005) embryos.

In the mouse study, BDNF transcripts were expressed in follicular cells, while TrkB and p75 NTR expression was detected exclusively in oocytes (Kawamura *et al.* 2005). However, in the bovine study, BDNF and p75 NTR mRNA was detected in both cumulus cells and oocytes, whereas TrkB showed a similar pattern of expression to that of mouse (Martins da Silva *et al.* 2005). As shown in Fig. 1, our current study provided evidence that the transcripts of BDNF, trTrkB, and p75 NTR were detected in all samples, whereas the mRNA of full-length TrkB was not found in MI stage oocyte but detected in follicular cells. Immunohistochemistry, which was used to confirm the localization of BDNF and TrkB proteins, showed immunopositivity for both proteins in cultured cumulus cells. TrkB expression was only slightly detectable in cumulus cells, regardless of GTH treatment, but stronger BDNF expression was induced after GTH treatment (Fig. 2A). This suggests the stimulated expression of BDNF by GTH in porcine ovarian follicles. In addition, BDNF expression was confirmed in the chromatin of oocytes, but no TrkB staining was detected in MI stage oocytes (Fig. 2B). The functions of truncated isoforms of the TrkB receptor, which lack the intra-cellular domain, but retain the ligand-binding domain

Figure 3 Effect of BDNF supplementation on first polar body extrusion of porcine oocytes (mean $\pm$ s.e.m.). COCs were cultured without (control, c) or with different doses of BDNF. Some COCs were cultured with NGF (10 ng/ml) and NT-3 (10 ng/ml). COCs were cultured in TCM-199 supplemented with 0.1% PVA (A) or TCM-199 supplemented with 10% pFF (B). Five replications and between 280 and 350 oocytes for each of the seven treatment groups (A) or between 280 and 450 oocytes for each of the nine treatment groups (B) were used for each experiment. Different letters denote significant differences ($P < 0.05$).

(Klein *et al.* 1990), are unclear. Consistent with our findings, in mouse, low levels of full-length TrkB receptors are detected in oocytes at all phases of follicular development, but they can be detected in granulosa cells only in growing follicles (Paredes *et al.* 2004). Furthermore, the finding that the majority of the TrkB receptors detected in the growing oocyte's cell membrane are truncated suggests that truncated TrkB receptors might be important for oocyte survival (Paredes *et al.* 2004). On the basis of these results, we can hypothesize that the mechanism of action of BDNF in porcine could be bidirectional with autocrine and/or paracrine signals being relayed between cumulus cells and oocytes. Their complex interactions and functional differences remain to be resolved.

Based upon the expression patterns, we analyzed the effect of BDNF on IVM of porcine oocytes to determine whether it could improve *in vitro* production (IVP)

Figure 4 Effect of Trk receptor inhibitor K252a or K252b on first polar body extrusion of porcine oocytes (mean $\pm$ s.e.m.). COCs were cultured in TCM-199 supplemented with 10% pFF with or without BDNF (30 ng/ml), K252a (100 nM) or K252b (100 nM). Four replications and between 249 and 303 oocytes for each of the six treatment groups were used. Different letters denote significant differences ($P < 0.05$).

efficiency. Extrusion of first polar bodies and oocyte GSH levels were used to evaluate nuclear and cytoplasmic maturation of oocytes respectively. GSH is known to be an important intra-oocyte factor for supporting events after fertilization, such as decondensation of sperm nuclei and male pronuclear formation (Perreault *et al.* 1988, Yoshida *et al.* 1993). As shown in Fig. 3A, 30 ng/ml BDNF significantly ($P < 0.05$, when compared with control) increased the rate of first polar body extrusion in oocytes cultured in TCM-199 media supplemented with 0.1% PVA, whereas other BDNF concentrations and the NTs, NGF, and NT-3 were without effect. There were no

Figure 5 Effect of BDNF supplementation on GSH content in porcine oocytes (mean $\pm$ s.e.m.). COCs were cultured in TCM-199 supplemented with 10% pFF with or without (control, c) different doses of BDNF. Five replications and between 150 and 200 oocytes for each of the five treatment groups were used. Different letters denote significant differences ($P < 0.01$).

Figure 6 Effect of BDNF supplementation on first polar body extrusion of porcine oocytes (mean $\pm$ S.E.M.). COCs were cultured in two-step culture system without (A) or with (B) EGF. Effect of BDNF on the latter 22 h (vertical striped bar) or entire 44 h (gray bar) of IVM was investigated. One-step culture group with BDNF (black bar) was used as control. Five replications and between 181 and 237 oocytes for each of the four treatment groups were used for each experiment (A and B). Different letters denote significant differences (^{a,b} $P < 0.05$; ^{a,c} $P < 0.001$; ^{b,c} $P < 0.001$).

significant differences observed at either 20 or 40 ng/ml of BDNF supplementation. In order to clarify if only such a precise concentration of BDNF has an effect on nuclear maturation, we added two treatment groups (25 and 35 ng/ml) in the next study. In addition, although PVA was used for initial study to exclude the effect of inherent NTs in pFF, supplementation of PVA was insufficient to substitute the known benefit of pFF in oocyte maturation. Thus, we next examined the effect of BDNF on COCs cultured in TCM-199 supplemented with 10% pFF. As shown in Fig. 3B, similar results were obtained where only 30 ng/ml BDNF significantly ($P < 0.05$) increased first polar body extrusion when compared with control with no effect on other tested concentrations. Further investigation including determination of the physiological level of BDNF in porcine species would be required to explain this phenomenon. The increase rate of meiotic maturation with 30 ng/ml BDNF is comparable between the two experiments (from 24 to 37.1%, 155% increase, + PVA; from 28.7 to 46.1%, 161% increase, + pFF).

The role of the TrkB receptor in mediating this effect was demonstrated using the Trk receptor inhibitor K252a. Supplementing BDNF treatment with K252a, but not the membrane nonsoluble K252b, during IVM blocked the effect of BDNF on first polar body extrusion (Fig. 4). In agreement with the nuclear maturation observations, 30 ng/ml BDNF significantly ($P < 0.01$) increased GSH content in oocytes, when compared with controls (Fig. 5). Thus, we used 30 ng/ml BDNF for further studies.

Next, BDNF was applied to the two-step culture IVM system, in which oocytes are cultured in GTH for an initial 22 h and then in GTH-free medium for a further 22 h. We assigned oocytes into two experimental groups, namely EGF-treated or nontreated, because both BDNF and EGF activate receptor tyrosine kinases. This raises the possibility that they could act either competitively or synergistically. We investigated the effect of BDNF on both the entire 44 h and latter 22 h of IVM in the presence and absence of EGF (Table 1). When EGF was omitted from culture medium, 44-h treatment of BDNF significantly ($P < 0.05$) promoted the nuclear maturation of oocytes when compared with BDNF untreated oocytes amongst groups supplemented with GTH for the first 22 h of culture (Fig. 6A). However, the effect was diminished with the addition of EGF such that no differences were found between groups supplemented with GTH for the first 22 h of culture (Fig. 6B).

Although no differences were observed between BDNF-treated groups in the presence of GTH, whether EGF was added or not, we choose those groups treated with BDNF for the entire duration of two-step culture for further study, because their nuclear maturation was slightly better than that of the groups treated with BDNF for only the latter period. We next evaluated the effect of BDNF on developmental competence of oocytes after IVF and SCNT.

In the presence of EGF, treatment with BDNF during IVM significantly ($P < 0.05$) increased the developmental competence of embryos to blastocyst stage after IVF. Moreover, since this was not observed with either BDNF or EGF alone, it must reflect a stimulatory interaction between BDNF and EGF. Notably, we failed to observe the previously reported promoting effect of EGF on the developmental competence of porcine IVF embryos (Abeydeera *et al.* 1998). However, in the case of SCNT, a single treatment of BDNF or EGF did significantly ($P < 0.01$) enhance the developmental potency of embryos to blastocyst stage (Table 3). This result indicates that the addition of EGF during IVM can influence the blastocyst formation of porcine SCNT embryos and that BDNF can substitute for this effect. Also, combined treatment with BDNF and EGF significantly ($P < 0.01$) increased blastocyst development of SCNT embryos when compared with the single treatment groups, similar to the stimulatory effect observed in the IVF experiment. Meanwhile, in both IVF and SCNT studies, no differences

were observed between the four groups in either cleavage rates or the number of blastocyst cells.

Although TrkB and the EGF receptor have different ligand-binding domains on the outer surface of the plasma membrane, both are receptor tyrosine kinases and act through similar intracellular signaling pathways (Alberts *et al.* 2002). In fact, stimulatory crosstalk between EGF and IGF-I signaling, which is also mediated through receptor tyrosine kinase activity, has been well described. In certain cell types, the EGF receptor can transactivate the IGF-I receptor (Burgaud & Baserga 1996), and the IGF-I receptor was able to activate some important members of the EGF-signaling pathway (Roudabush *et al.* 2000). Thus, the observed effects of combined BDNF and EGF treatment on blastocyst development in IVF and SCNT embryos suggest the existence of a synergetic interaction between BDNF and EGF.

In the present study, concurrent treatment of BDNF with EGF increased the rate of embryos developed to the blastocyst stage after both IVF and SCNT. However, no differences were observed when we examined the kinetics of meiotic maturation under the same culture conditions (Fig. 6B). Similarly, it has been reported that the addition of EGF did not increase the proportion of oocytes maturing to MII stage but did increase developmental ability of embryos after IVF in mouse (Merriman *et al.* 1998) and pig (Abeydeera *et al.* 1998). In addition, a recent study using bovine oocytes demonstrated that BDNF may benefit oocyte competence for embryo development without necessarily benefiting nuclear maturation (Martins da Silva *et al.* 2005). Cytoplasmic maturation more generally refers to those other maturational events that occur in the oocyte, necessary for the acquisition of developmental competence but not directly related to meiotic progression, and can probably occur independent of nuclear maturation (Eppig 1996). This possibly explains why only 30 ng/ml BDNF was effective in increasing nuclear maturation, whereas 20–40 ng/ml increased GSH content in oocytes.

In conclusion, when administered during IVM, BDNF increased both nuclear and cytoplasmic maturation of porcine oocytes via autocrine and/or paracrine signals between the cumulus and oocytes. Furthermore, BDNF, together with EGF, enhanced the blastocyst development of oocytes after IVF and SCNT, demonstrating an improved IVP protocol for porcine oocytes.

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