Effects of Insulin, Transferrin and Selenium (ITS) on *In Vitro* Development of Porcine Parthenogenetic and Nuclear Transfer Embryos

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ABSTRACT

Insulin, transferrin and selenium (ITS) complex is reported to improve *in vitro* development of oocytes and embryos. This study was carried out to investigate the effects of ITS during *in vitro* culture (IVC) of porcine parthenogenetic and nuclear transfer (NT) embryos on subsequent developmental capacity *in vitro*. The electrically activated oocytes were cultured in Porcine Zygote Medium (PZM-3) with various concentrations (0, 0.1, 0.5, and 1.0%) of ITS for 7 days. Also, the electrically activated reconstructed embryos were cultured in PZM-3 with various concentrations (0, 0.1, 0.5, and 1.0%) of ITS for 6 days. Addition of ITS to culture medium did not affect development of porcine parthenogenetic embryos *in vitro*. To test the effect of ITS on the *in vitro* development of porcine NT embryos, factorial experiments were also performed for *in vitro* maturation (IVM) medium (TCM-199) with or without 1% ITS and culture medium (PZM-3) with or without 0.5% ITS. Addition of 0.5% ITS to culture medium increased ($p<0.05$) the proportion of NT blastocysts compared with non-treated group. In contrast, addition of 1% ITS to culture medium was ineffective or had a detrimental effect. Also, addition of ITS only to maturation medium increased ($p<0.05$) the percentage of NT blastocysts formation compared with the control group. In conclusion, addition of ITS to IVM or IVC medium could improve subsequent blastocyst development of porcine NT embryos.

(Key words : *In vitro* culture, ITS, Parthenogenetic embryo, Nuclear transfer)

INTRODUCTION

Although porcine *in vitro* maturation (IVM) and their subsequent *in vitro* culture (IVC) have been modified by many laboratories, low developmental rate of porcine embryos to the blastocyst stage and their poor qualities have been reported (Kikuchi *et al.*, 2002). The developmental potential of *in vitro* produced embryos can be affected by various factors, including the culture system, oocyte quality, the presence of serum or growth factors and an essential component for growth and development (Hong *et al.*, 2004). Addition of L-cysteine or epidermal growth factor (EGF) to modified TCM-199 medium for porcine IVM improved rates of nuclear maturation and cleaved oocytes (Kishida *et al.*, 2004). Insulin promotes the uptake of glucose and amino acids, and is beneficial for oocyte maturation *in vitro*. Transferrin is an iron-transport protein and selenium is an essential trace element (Wu *et al.*, 1973; Gutteridge *et al.*, 1986). Addition of ITS to the culture medium enhanced development of buffalo blastocysts *in vitro* (Raghu *et al.*, 2002), and the addition of amino acids and insulin to culture medium improved *in vitro* development of rat embryos (Zhang and Armstrong, 1990). Supplement of ITS in most serum-free culture medium promotes development of mouse embryos during IVC (Eppig *et al.*, 1992). ITS complex has also been used for IVM systems in many species including mouse (De La Fuente *et al.*, 1999), bovine (Gardner *et al.*, 2001) and goat (Herrick *et al.*, 2004). In addition, the presence of ITS in maturation medium has been reported to improve the developmental competence in both the defined and the porcine follicular fluid (FF) supplemented groups (Jeong *et al.*, 2007). However, beneficial effects of ITS on porcine *in vitro* culture have not been reported. Therefore, the objective of this study was to investigate the effects of ITS during IVM and IVC on *in vitro* development of porcine parthenogenetic and nuclear trans-

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fer embryos.

MATERIALS AND METHODS

Preparation of Oocytes

All regents used in our study were purchased from Sigma-Aldrich Chemicals (St, Louis, MO, USA) unless otherwise stated. Ovaries were obtained from prepubertal gilts at a local slaughter house and transported to laboratory in PBS containing 100 IU/ml penicillin and 50 μg/ml streptomycin at 30~35℃. Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles 2~6 mm in diameter using 10-ml syringe fixed with an 18-gauge needle. COCs were washed three times in TL-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA). The oocytes were matured in TCM-199 (M-4530, Sigma) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA) or 10% porcine follicular fluid (pFF), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 0.5 μg/ml LH (L-5269), 0.5 μg/ml FSH (F-2293), 10 ng/ml epidermal growth factor (E-412), 75 μg/ml penicillin, 50 μg/ml streptomycin. COCs were transferred into 500 μl of medium which had been covered with mineral oil in a 4-well multidish (Nunc, Roskide, Denmark) and incubated at 38.5℃ in an atmosphere of 5% CO_2 in air with maximum humidity. After 22 hr of IVM, the oocytes were washed three times and transferred into 500 μl of the maturation medium without hormone for additional 22 hr of culture.

Production of Porcine Parthenogenetic Embryos

Cumulus cells were removed from the oocytes by pipetting in TL-Hepes supplemented with 0.1% PVA and 0.1% hyaluronidase. Cumulus-free oocytes were transferred to activation solution consisting of 0.3 M D-mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.01% PVA, and washed three times. Oocytes were activated by a direct current pulse of 1.5 kV/cm for duration of 100 μsec using a BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA, USA). Porcine Zygote Medium-3 (PZM-3) containing 0.3% BSA (Yoshida et al., 2002) was used for culture medium. After stimulation, oocytes were washed and transferred into 500 μl of PZM-3 covered with mineral oil in a 4-well multidish. The oocytes were then cultured in groups of 500 μl of PZM-3 under 38.5℃ in an atmosphere of 5% CO_2 in air for 7 days.

Preparation of Fetal Fibroblasts

Fetuses were obtained from a pregnant sow at day 35 after insemination, and the tissue was cut into small pieces with fine scissors. The tissues were washed three times and incubated for 30 min at 37℃ in PBS containing 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA), and this suspension was centrifuged at 500 × g for 10 min. The cell pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 75 μg/ml penicillin G, 50 μg/ml streptomycin, 5% (v/v) fetal bovine serum (FBS; 16000-044, Gibco), and 5% (v/v) fetal calf serum (FCS; 26010-074, Gibco) and cultured at 38.5℃. All cells were cryopreserved upon reaching confluence. Fetal fibroblast cells were cultured until confluent in DMEM with 5% FBS, 5% FCS in 12-well plates before use, and the cells were treated with 0.25% trypsin and 0.5 mM EDTA in a 38.5℃ incubator for single-cell isolation for 3 min before NT.

Production of Porcine Nuclear Transfer Embryos

Porcine NT was carried out as outlined previously described by Park et al. (2001). Cumulus cells were removed by repeated pipetting in TL-Hepes medium supplemented with 0.1% PVA and 0.1% hyaluronidase. Cumulus-free oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm with a fine glass pipette in PZM-3 with 7.5 μg/ml cytochalasin B. A donor cell was placed in the perivitelline space of the enucleated recipient oocyte to contact the oocyte membrane. The reconstructed embryos were washed once in cell fusion medium composed of 0.3 M mannitol, 1.0 mM $CaCl_2 \cdot H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM Hepes, and then they were placed between the two wire electrodes (1 mm apart) of a fusion chamber slide in the cell fusion medium. Cell fusion was induced with two DC pulses of 1.2 kV/cm for 30 μsec on a BTX Elector-Cell Manipulator 2001. The oocytes were then cultured in groups of 500 μl of PZM-3 medium under 38.5℃ in an atmosphere of 5% CO_2 in air for 6 days.

Evaluation of Developmental Ability of Parthenogenetic and Reconstructed Embryos

Cleavage and blastocyst rates of the activated oocytes were monitored and evaluated on the day 3 and the day 6 to 7 of IVC respectively under the stereo microscope. Blastocyst cell numbers were counted under fluorescent microscope (Olympus, Japan) after Hoechest 33342 staining (2 mg/ml in 2.2% sodium citrate solution).

Experimental Design

Experiment 1 was conducted to determine the effects of ITS during IVC of parthenogenetic embryos on subsequent developmental capacity *in vitro*. After electrical activation, oocytes were transferred into a 500 μl of drop of PZM-3 medium with different concentrations

of ITS (I-3146, Sigma) [0, 0.1, 0.5, and 1.0% (v/v)] covered with mineral oil in a 4-well multidish (Nunc, Roskide, Denmark) and were held in 5% CO_2 in air at 38.5℃ for 7 days following parthenogenetic activation.

Experiment 2 was conducted to determine the effects of ITS during IVC of the reconstructed embryos on subsequent developmental capacity *in vitro*. After fusion and activation, the reconstructed embryos were transferred into a 500 μ l of drop of PZM-3 with different concentrations of ITS [0, 0.1, 0.5, and 1.0% (v/v)] covered with mineral oil in a 4-well multidish and were held in 5% CO_2 in air at 38.5℃ for 6 days following nuclear transfer.

Experiment 3 was conducted to determine the effects of ITS in presence or absence of ITS during IVM/IVC of the reconstructed embryos on subsequent developmental capacity *in vitro*. The COCs were matured in TCM-199 media (with 10% porcine FF) supplemented with or without 1.0% ITS for 44 hr and then cultured in PZM-3 with or without 0.5% ITS for 6 days following NT.

Statistical Analysis

Data were expressed as mean ± standard error (SE). The significant difference among treatment groups was determined by ANOVA using the GLM procedures of SAS (SAS Institute Inc., Cary, NC) and Duncan's multiple range after analysis of variance. Differences were considered to be statistically significant when a value was less than 0.05.

RESULTS

In experiment 1 where different concentrations of ITS were added to culture medium for parthenogenetic embryos, ITS treatment tended to support *in vitro* development of parthenogenetic porcine blastocysts although the data were not statistically significant (Table 1).

In experiment 2 where different concentrations of ITS were supplemented with culture medium for NT embryos, there was no difference in cleavage rates and total cell numbers between the ITS-treated groups and the control group. However, the blastocyst formation rate of NT embryos cultured with 0.5 % ITS was significantly improved compared with that of the other groups (Table 2). In contrast, NT embryos treated with 1% ITS had significantly lower percentage of blastocyst development compared to the control group.

In experiment 3, when the effect of ITS were tested during IVM and IVC by factorial experiments, blastocyst formation rates of NT embryos which were matured in 1.0% ITS and cultured without ITS, or matured without ITS and cultured in 0.5% ITS were sig-

Table 1. Effects of ITS during *in vitro* culture on development of porcine oocytes following parthenogenetic activation

ITS concentration (%)	N[1]	Cleavage rates (No.)	Blastocyst rates (No.)	Cell no. (mean±S.E.)
Control	359	76.9±1.0(276)	17.8±3.2(64)	38.1±4.2
0.1	136	69.9±3.8(95)	16.9±0.9(23)	36.7±7.5
0.5	120	80.8±4.4(97)	16.7±2.2(20)	36.5±4.6
1.0	115	77.3±2.8(89)	21.7±3.0(25)	35.0±5.1

[1] Number of activated oocytes cultured in 4 replicate experiments.

Table 2. Effects of ITS during *in vitro* culture on development of porcine NT embryos

ITS concentration (%)	N[1]	Cleavage rates (No.)	Blastocyst rates (No.)	Cell no. (mean±S.E.)
Control	225	67.6±2.8(173)	18.7±0.3(42)[b]	28.4±1.1
0.1	83	68.9±4.3(57)	16.7±1.6(14)[b]	28.3±1.0
0.5	90	82.1±2.8(70)	26.7±0.8(24)[a]	28.8±1.0
1.0	89	65.7±5.4(67)	7.9±1.5(7)[c]	31.5±4.5

[a,b,c] Values within a columns with different superscripts are significantly different ($p<0.05$).

[1] Number of reconstructed embryos cultured in 4 replicate experiments.

Table 3. Effects of ITS during IVM and IVC on *in vitro* development of porcine NT embryos following nuclear transfer

Treatment IVM / IVC*	N[1]	Fusion rates (No.)	Cleavage rates (No.)	Blastocyst rates (No.)	Cell no. (mean±S.E.)
− / −	42	85.7±2.8(36)	64.3±3.1(27)	9.5±0.6(4)[b]	27.3±0.9
− / +	43	88.4±2.7(38)	65.1±2.1(28)	20.9±1.5(9)[a]	30.3±1.5
+ / −	40	82.5±3.3(33)	67.5±6.4(27)	22.5±2.9(9)[a]	30.8±2.0
+ / +	41	80.5±4.3(33)	70.3±8.2(29)	14.6±3.0(6)[ab]	29.8±2.3

[a,b] Within a column, means with different superscripts are significantly different ($p<0.05$).

* IVM medium (TCM-199) with (+) or without (-) 1.0% ITS and IVC medium (PZM-3) with (+) or without (-) 0.5% ITS.

[1] Number of activated oocytes cultured in 4 replicate experiments.

nificantly improved compared with control group (Table 3). However, addition of ITS to both IVM and IVC media could not enhance subsequent development of NT embryos compared with control group. Total cell number of blastocysts did not differ among ITS-treated groups. The maturation condition used in this experiment was based on recently report in which 1% ITS was added TCM-199 with 10% porcine FF for porcine oocyte maturation (Jeong *et al.*, 2007).

DISCUSSION

The medium for IVM of porcine oocytes and culture of porcine embryos have been modified with significant improvements in *in vitro* development (Written, 1977; Menino *et al.*, 1982). Developmental capacity of embryos could be affected by culture conditions during *in vitro* maturation and culture. North Carolina State University 23 (NCSU-23) and PZM-3 with serum, amino acids or growth factors have been widely used for porcine IVC, to reduce level of oxidative stress during culture for development of embryos (Petters *et al.*, 1993; Yoshioka *et al.*, 2002: Im *et al.*, 2004; Iwamoto *et al.*, 2005).

Insulin stimulates the uptake of glucose and amino acids and may have mitogenic effects (Spicer and Echternkamp, 1995). Insulin and insulin-like growth factors enhance the developmental potential of porcine oocytes during IVM and IVC (Kim et al., 2005 and *Lee et al.*, 2005). The presence of ITS in maturation medium improved the developmental competence of porcine oocytes (Jeong *et al.*, 2007). Transferrin and selenium are the trace elements that have antioxidant activity (Wu *et at.*, 1973; Gutteridge *et al.*, 1986). ITS treatment tended to support *in vitro* development of parthenogenetic porcine blastocysts although the data were not statistically significant (see Table 1).

Our results indicated that addition of ITS to IVC medium could improve subsequent blastocyst development of porcine NT (see Table 2). The addition of ITS to maturation medium produced higher blastocyst rate following NT. The present study has demonstrated that the presence of ITS in culture medium is beneficial for NT embryos development *in vitro*. Supplementation of ITS to both IVM and IVC could not support blastocyst development in porcine NT (see Table 3). The ITS during IVC may suppress *in vitro* developmental potential of oocytes matured in ITS. Effects of ITS treatment during IVM may be maintained through IVC and therefore additive effect of ITS in IVC medium could not be achieved. In conclusion, porcine IVM or IVC medium supplemented with insulin- transferrin-selenium complex can promote subsequent blastocyst development of porcine NT embryos.

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