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Supplementation of insulin–transferrin–selenium to embryo culture medium improves the *in vitro* development of pig embryos

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Summary

Insulin, transferrin and selenium (ITS) supplementation to oocyte maturation medium improves the post-fertilization embryonic development in pigs. ITS is also commonly used as a supplement for the *in vitro* culture (IVC) of embryos and stem cells in several mammalian species. However, its use during IVC of pig embryos has not been explored. This study investigated the effect of ITS supplementation to IVC medium on the *in vitro* development ability of pig embryos produced by parthenogenetic activation (PA), *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT). We observed that ITS had no significant effect on the rate of first cleavage ($P > 0.05$). However, the rate of blastocyst formation in ITS-treated PA (45.3 ± 1.9 versus 27.1 ± 2.3%), IVF (31.6 ± 0.6 versus 23.5 ± 0.6%) and SCNT (17.6 ± 2.3 versus 10.7 ± 1.4%) embryos was significantly higher ($P < 0.05$) than those of non-treated controls. Culture of PA embryos in the presence of ITS also enhanced the expansion and hatching ability (29.1 ± 3.0 versus 18.2 ± 3.8%; $P < 0.05$) of blastocysts and increased the total number of cells per blastocyst (53 ± 2.5 versus 40.9 ± 2.6; $P < 0.05$). Furthermore, the beneficial effect of ITS on PA embryos was associated with significantly reduced level of intracellular reactive oxygen species (ROS) (20.0 ± 2.6 versus 46.9 ± 3.0). However, in contrast to PA embryos, ITS had no significant effect on the blastocyst quality of IVF and SCNT embryos ($P > 0.05$). Taken together, these data suggest that supplementation of ITS to the IVC medium exerts a beneficial but differential effect on pig embryos that varies with the method of embryo production *in vitro*.

Keywords: *In vitro* development, ITS, Pig embryo, ROS

Introduction

Pig embryos produced by parthenogenesis (PA), *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT) have important applications in agriculture and biomedicine. However, despite tremendous technical advancements in recent years, development ability of these *in vitro* produced (IVP) pig embryos remains low due to multiple factors that include inadequate or sub-optimal *in vitro* culture (IVC) conditions (Gupta *et al.*, 2009; Dang-Nguyen *et al.*, 2011; Uhm *et al.*, 2011a). Increased oxidative stress, during IVC, was also shown to be a contributing factor for the low embryonic development of IVP embryos. Accordingly, culture of embryos in the presence of anti-oxidants and/or under low partial pressure of oxygen has been recommended by several authors (Karja *et al.*, 2004; Kitagawa *et al.*, 2004).

Using a protein- and serum-free IVC medium, Uhm *et al.* (2007) recently showed that selenium improves the *in vitro* development of pig embryos and protects them against IVC-induced oxidative stress by scavenging the reactive oxygen species (ROS). This laboratory had also shown previously that insulin

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improves the *in vitro* development of pig embryos and has a synergistic effect with non-essential amino acids and vitamins (Koo *et al.*, 1997). Both insulin and selenium are two component of a commercial premix, insulin–transferrin–selenium (ITS), that also contains transferrin. Transferrin is an iron-transport protein for embryos and constitutes a major protein component in follicular and ampullary fluids (Aleshire *et al.*, 1989). This protein can also act as a chelator of highly toxic hydroxyl radical to limit oxidative stress (Nasr-Esfahani & Johnson, 1992). Several authors have shown that exogenous supplementation of ITS premix to the *in vitro* maturation (IVM) medium promotes oocyte maturation and improves subsequent early embryonic development in several mammalian species including human, mouse, cattle and pigs (Nasr-Esfahani & Johnson, 1992; Jeong *et al.*, 2008; Cordova *et al.*, 2010; Hu *et al.*, 2011). However, few studies have shown the beneficial effect of ITS premix on early embryonic development when added to the IVC medium. ITS had a synergistic action with free amino acids in promoting the development rate of IVF rat embryos (Zhang & Armstrong, 1990) and stimulated the secretion of chorionic gonadotrophin in human blastocysts (Lopata & Oliva, 1993). In oxidative stress setting, ITS minimized the negative influence of H_2O_2 and improved the quality of *in vivo* fertilized mouse embryos (Kurzawa *et al.*, 2002). Two independent studies have reported the beneficial effect of ITS supplementation to IVM medium for pig (Jeong *et al.*, 2008; Hu *et al.*, 2011). However, the utility of ITS supplementation to IVC medium for pig embryos has never been explored. Furthermore, to our knowledge, no study has evaluated the effect of ITS during IVC of SCNT embryos, which are known to differ from IVF embryos for culture requirements (Heindryckx *et al.*, 2001; Chung *et al.*, 2002; Sugimura *et al.*, 2011) and responsiveness towards hormones (Kim *et al.*, 2006), growth factors (Lee *et al.*, 2004) and mitogens (Gupta *et al.*, 2007c).

This study, therefore, investigated the effect of supplementing ITS to the IVC medium of pig embryos produced by PA, IVF or SCNT. We first evaluated the effect of ITS on PA embryos to determine the optimal concentration for pig embryos and then tested this concentration on IVF and SCNT embryos.

Materials and methods

All chemicals used were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) unless otherwise specifically indicated. The ITS premix contained 1.0 mg/ml insulin, 0.55 mg/ml transferrin and 0.5 μg/ml selenium. Each experiment consisted of at least three replicates and in each of the replication, oocytes from the same collection of abattoir-derived ovaries were randomly distributed to different groups.

Oocytes collection and IVM

Oocytes were retrieved from abattoir-derived ovaries of prepubertal pigs and matured *in vitro* as described earlier (Uhm *et al.*, 2010). Briefly, cumulus–oocyte complexes (COCs) were aspirated from follicles (3–6 mm diameter) using 10-ml syringe fitted with an 18G needle, washed three times with HEPES-buffered Tyrode's lactate (TL-HEPES) medium and matured in groups of 50 in 500 μl of Tissue Culture Medium 199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY, USA) supplemented with 25 mM $NaHCO_3$, 10% (v/v) porcine follicular fluid, 0.57 mM cysteine, 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamicin, 0.5 μg/ml follicle-stimulating hormone (FSH) (Follitropin V; Vetrepharm, Canada), 1 μg/ml estradiol-17β, and 10 ng/ml epidermal growth factor under mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air for 40–42 h (for SCNT) or 42–44 h (for PA or IVF).

Production of PA embryos

Diploid parthenogenetic embryos were produced by electro-activation as described earlier (Gupta *et al.*, 2007a). Briefly, IVM oocytes were denuded of cumulus cells using 0.1% hyaluronidase, washed three times with TL-HEPES medium and electro-activated in activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, and 1.0 mM $CaCl_2$) by a single direct current (DC) pulse of 1.0 kV/cm for 30 μs delivered by a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). Activated oocytes were then cultured in North Carolina State University Medium 23 (NCSU23) medium supplemented with 7.5 μg/ml cytochalasin B (CB) for 4 h.

Production of IVF embryos

In vitro matured oocytes were fertilized as described earlier (Gupta *et al.*, 2007b). Briefly, IVM oocytes were partial denuded of cumulus cell using 0.1% hyaluronidase, washed three times with the fertilization medium (modified Tris-buffered medium that contained 1 mM caffeine sodium benzoate and 0.1% bovine serum albumin (BSA) and were placed in groups of 15 oocytes per 50-μl droplets. Sperm were retrieved from abattoir-derived boar testis in TL-HEPES, purified by swim-up method for 10 min and deposited into the fertilization droplet to obtain a final sperm concentration of 5×10^5 cells/ml. Sperm and oocytes were co-incubated at 39°C for 6 h in a humidified atmosphere of 5% CO_2 in air.

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Production of SCNT embryos

Cloned embryos were produced by SCNT as described earlier (Gupta *et al.*, 2008a). Briefly, IVM oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled borosilicate pipette (25 μm internal diameter). Successful enucleation was confirmed by ultraviolet (UV) light-assisted visualization of fluorescent metaphase plate in the aspirated cytoplasm contained within the enucleation pipette. Enucleated oocytes were subsequently reconstructed by inserting a small sized (~15 μm in diameter), smooth bordered fibroblast cell into the peri-vitelline space using the same pipette used for enucleation. Donor cells for SCNT were prepared essentially the same as we described earlier (Das *et al.*, 2010b). Membrane fusion of donor cell with recipient cytoplast was achieved by a single DC pulse of 2.1 kV/cm for 30 μs delivered by the BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). The fused couplets were then cultured in NCSU23 supplemented with 7.5 mg/ml CB for 4 h.

IVC of embryos

Embryos were cultured in groups of 12–15 embryos per 50-μl microdroplets of NCSU23 medium supplemented with 0.4% (w/v) essential fatty acid-free BSA and 1% (v/v) non-essential amino acids under mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air for 7 days as described earlier (Gupta *et al.*, 2008b). The IVC medium was further supplemented with different concentrations of ITS (0, 0.5, 1.0, 2.0 % v/v) to evaluate its effect on the embryos. The rates of cleavage (percentage of embryos that cleaved) and blastocyst formation and expansion were recorded on Day 2 and Day 7 of IVC, respectively.

Fluorescent staining for assessment of blastocyst cell number

Total nuclei number of blastocysts was counted on Day 7 of IVC using Hoechst 33342 staining as described earlier (Das *et al.*, 2010a). Briefly, Day 7 blastocysts were fixed for 5 min in a fixative solution that contained 2% formalin and 0.25% gluteraldehyde, mounted on clean glass slides and stained with a glycerol-based Hoechst 33342 (12.5 μg/ml) staining solution for 10 min. Total number of stained nuclei, which appeared blue when visualized under UV light illumination were then counted in each blastocyst and digital images were taken (Nikon Coolpix 990; Nikon Corporation, Tokyo, Japan).

Measurement of intracellular ROS level

Intracellular ROS level in embryos was measured using 2′,7′-dichlorofluorescene (DCF) fluorescence assay as described earlier (Gupta *et al.*, 2010). The procedure uses 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA), which readily permeates cellular membranes and in the presence of intracellular esterases forms 2′,7′-dichlorodihydrofluorescein (DCHF). DCHF in turn is oxidizes by ROS to form the fluorescent DCF molecule. Briefly, 2–4-cell stage embryos were incubated with 10 mM 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) for 20 min at 39°C, washed three times in NCSU23 medium to remove the traces of the dye and immediately analyzed for intracellular fluorescent DCF under epifluorescence microscope (Nikon Eclipse T*i*, Tokyo, Japan) fitted with green filter (excitation: 450–490 nm; emission: 520 nm; dichromatic: 500 nm). A digital camera (Nikon Digital Sight, Tokyo, Japan) attached to the microscope acquired the images and mean grey value of fluorescent embryos were measured using ImageJ software (NIH, Bethesda, Maryland, USA). Background fluorescence values were subtracted from the final values before analysing the statistical difference among the groups. The experiment was replicated three times with 15–20 oocytes in each replicate.

Statistical analyses

Data were analyzed by SPSS Statistical software version 17.0 (SPSS Inc., Chicago, IL, USA) for chi-squared test or analysis of variance (ANOVA) as appropriate. Data are presented as mean ± standard error of the mean (SEM). Differences at $P < 0.05$ were considered to be significant.

Results

Effect of ITS on PA embryos

In the first set of experiments, we examined the dose-dependent effect of ITS on PA embryos. Presumptive diploid PA zygotes were produced by electro-activation and cultured for 7 days in IVC medium supplemented with 0, 0.5, 1 and 2% (v/v) ITS. Results showed that ITS had no significant effect on the occurrence of first cleavage (Table 1) but significantly improved the rate of blastocyst formation in a dose-dependent manner. The highest rate of blastocyst formation was observed when 1% ITS was added to the NCSU23 medium ($P < 0.05$). Blastocysts cultured in the presence of 1% ITS also had a significantly increased ability to expand and hatch (29.1 ± 3.0 versus 18.2 ± 3.8%; Fig. 1) and contained significantly more number of cells (53 ± 2.5 versus 40.9 ± 2.6) than

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Table 1 Effect of insulin, transferrin and selenium (ITS) premix on *in vitro* development (mean ± SEM) of pig embryos produced by parthenogenetic activation

Conc. of ITS (% v/v)	Total no. of oocytes	Percentage (n) of embryos developing to				Total cell no. per blastocyst
		2–4 cells	8–16 cells	Morula	Blastocyst	
0.0	186	$71.2^{a} \pm 2.2$ (133)	$34.9^{a} \pm 2.1$ (65)	$27.1^{a} \pm 3.2$ (51)	$27.1^{a} \pm 2.3$ (51)	$40.9^{a} \pm 2.6$ (51)
0.5	182	$76.7^{a} \pm 3.8$ (141)	$40.3^{a,b} \pm 4.5$ (75)	$27.5^{a} \pm 1.4$ (50)	$30.5^{a} \pm 3.4$ (56)	$50.6^{a,b} \pm 2.6$ (56)
1.0	181	$78.8^{a} \pm 3.0$ (144)	$53.2^{b} \pm 5.2$ (94)	$40.2^{b} \pm 4.5$ (71)	$45.3^{b} \pm 5.0$ (79)	$53.6^{b} \pm 2.5$ (79)
2.0	203	$75.3^{a} \pm 3.8$ (155)	$39.3^{a,b} \pm 2.5$ (80)	$30.0^{a} \pm 3.2$ (60)	$28.7^{a} \pm 1.9$ (57)	$46.3^{a,b} \pm 2.7$ (57)

Values in the parentheses indicate the number of embryos.
^{a,b}Values with different superscripts within the column differ significantly ($P < 0.05$).

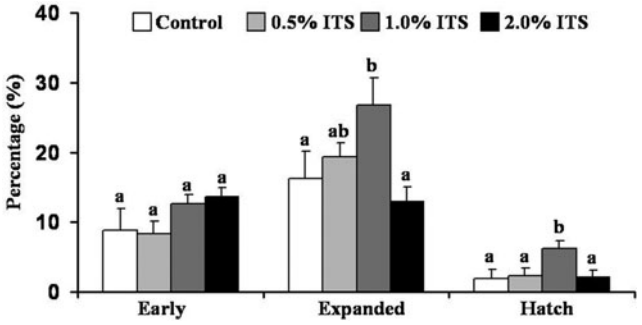

Figure 1 Effect of insulin–transferrin–selenium premix (ITS) on expansion and hatching ability of pig blastocysts produced by parthenogenetic activation (PA). Bars with different superscript (a,b) indicate significant difference ($P < 0.05$) within same group. Early: early non-expanded blastocysts; Expanded: expanded blastocysts; Hatch: hatching or hatched blastocysts.

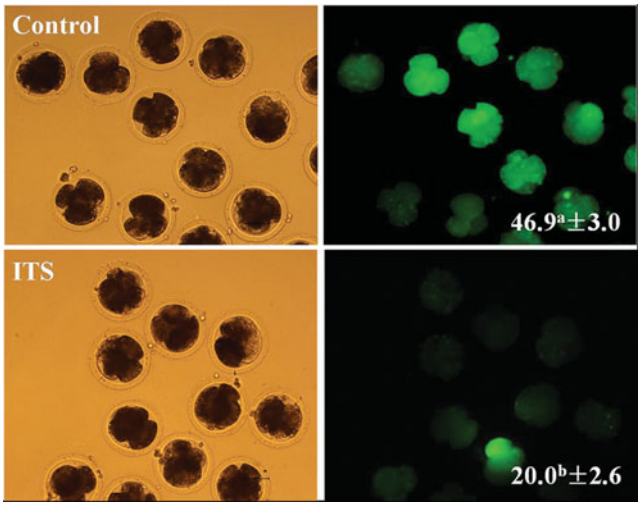

Figure 2 Effect of insulin- transferrin-selenium premix (ITS) on the level of reactive oxygen species (ROS) in 2–4 cell stage pig embryos produced by parthenogenetic activation (PA). Panel 1: Bright field; Panel 2: Green fluorescence indicates the ROS activity. Values within the figures represent mean ± standard error of the mean (SEM) of mean grey value of fluorescence intensity. Values with different superscript (a,b) differ significantly ($P < 0.05$).

those of non-treated controls (Table 1). Thus, a 1% concentration of ITS was considered optimal and used for all further experiments.

In a separate set of experiments, the intracellular level of ROS in 2–4-cell stage PA embryos cultured in the absence (control) or presence of 1% ITS in the medium was evaluated. The results indicate that, embryos cultured in the presence of 1% ITS had significantly lower ($P < 0.05$) level of ROS than those of non-treated control embryos (20.0 ± 2.6 versus 46.9 ± 3.0; Fig. 2).

Effect of ITS on IVF embryos

We next examined the effect of 1% ITS on *in vitro* development and embryo quality of IVF embryos. Presumptive diploid zygotes, produced by IVF, were cultured in the absence (control) or presence of 1% ITS for 7 days and evaluated for embryonic development and embryo quality. As shown in Table 2, embryos in the ITS-treated and non-treated control groups did not differ ($P > 0.05$) with respect to the rate of first cleavage. However, on Day 7, significantly more number of blastocysts was observed in ITS-treated group than in non-treated control group (31.6 ± 0.6 versus 23.5 ± 0.6%; $P < 0.05$). The blastocysts in ITS-treated and control groups did not differ ($P > 0.05$) with respect to their expansion and hatching ability (22.4 ± 0.5 versus 16.1 ± 0.2%; $P = 0.35$) or total cell number per blastocyst (69.3 ± 15.4 versus 62.2 ± 16.1; $P = 0.24$) (Fig. 3).

Effect of ITS on SCNT embryos

Finally, we evaluated the effect of 1% ITS on *in vitro* development and embryo quality of SCNT embryos. Cloned embryos, produced by SCNT of fibroblast cells into enucleated oocytes, were cultured in the

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Table 2 Effect of insulin, transferrin and selenium (ITS) premix on *in vitro* development (mean ± SEM) of pig embryos produced by *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT)

Groups	Conc. of ITS (% v/v)	Total no. embryos	Percentage (*n*) of embryos developing to		Total cell no. per blastocyst
			2–4 cells	Blastocyst	
IVF	0.0	220	67.5^{a} ± 0.1 (149)	23.5^{a} ± 0.6 (50)	62.2^{a} ± 16.1 (50)
	1.0	203	73.9^{a} ± 0.3 (148)	31.6^{b} ± 0.6 (61)	69.3^{a} ± 15.4 (61)
SCNT	0.0	151	72.7^{a} ± 3.8 (109)	10.7^{a} ± 1.4 (16)	34.2^{a} ± 6.1 (16)
	1.0	161	73.8^{a} ± 2.0 (161)	17.6^{b} ± 2.3 (27)	39.5^{a} ± 5.8 (27)

Values in the parentheses indicate the number of embryos.
^{a,b}Values with different superscripts within the column differ significantly ($P < 0.05$) among respective IVF and SCNT groups.

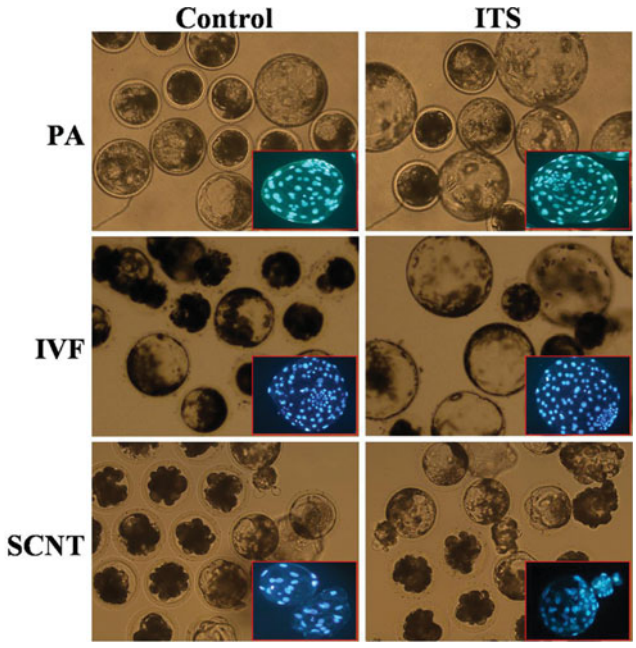

Figure 3 Pig blastocysts produced by parthenogenetic activation (PA), *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT) and cultured in the absence (Control) or presence of 1% insulin–transferrin–selenium (ITS). Inset figures represent fluorescent images following Hoechst 33342 staining to visualize total nuclei within each blastocyst. Magnification: ×200.

absence (control) or presence of 1% ITS for 7 days and evaluated for embryonic development and embryo quality as described above. Similar to both PA and IVF embryos, there was no significant effect of ITS on the rate of first cleavage (Table 2) but the rate of blastocyst formation was significantly improved (17.6 ± 2.3 versus 10.7 ± 1.4%; $P < 0.05$). However, similar to the findings for IVF embryos, there was no significant difference ($P = 0.28$) between the ITS-treated and non-treated control groups for the total number of cells per blastocyst (39.5 ± 5.8 versus 34.2 ± 6.1; Fig. 3).

Discussion

Embryos require a strictly defined culture medium to sustain their viability and developmental ability *in vitro*. We had shown previously that the supplementation of a chemically defined IVC medium with non-essential amino acids (Gupta *et al.*, 2008b), vitamins (Koo *et al.*, 1997), insulin (Koo *et al.*, 1997) and selenium (Uhm *et al.*, 2007) improves the *in vitro* developmental ability of pig embryos. Here, we extended our previous studies to further show the beneficial effect of ITS, a commercially available premix of insulin, selenium and transferrin, on *in vitro* development of PA, IVF and SCNT pig embryos. The beneficial effect was associated with reduction in the intracellular ROS level in the embryos.

As no previous studies have evaluated the effect of ITS supplementation in IVC medium for pig embryos, the optimal concentration of ITS required in this species was evaluated. Parthenogenetic embryos were utilized for this purpose because they obviate the need of sperm for the embryo production and hence, avoid the possible confounding variation due to male factors such as polyspermy. Consistent with previous reports on mouse (Kurzawa *et al.*, 2002), rat (Zhang & Armstrong, 1990), cattle (Lim & Hansel, 2000) and buffalo (Raghu *et al.*, 2002) embryos produced by *in vivo* or *in vitro* fertilization, our results indicate that pig embryos responded positively to exogenous ITS supplementation and an optimal effect was seen at 1% ITS medium supplementation. We also evaluated the effect of ITS on blastocyst quality in terms of their expansion and hatching ability and total cell number per blastocyst, which are valuable indicators of embryo viability (Yoshioka *et al.*, 2002; Uhm *et al.*, 2011a,b). Results showed that, both the total cell number per blastocyst and the expansion and hatching ability was significantly improved in the 1% ITS group than in the non-treated controls thereby, a result that suggested they were of higher quality. Thus, for all further experiments, ITS was used at a 1% concentration.

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The mechanism by which ITS improves the embryonic development is not yet clear. Previous studies have reported that selenium (Sneddon *et al.*, 2003; Uhm *et al.*, 2007) and transferrin (Aleshire *et al.*, 1989; Nasr-Esfahani & Johnson, 1992) are required to guarantee the adequate protein synthesis and cellular metabolism as well as to prevent oxidative damage by ROS. In contrast, insulin facilitates glucose and amino acid transport across the cell membranes (Augustin *et al.*, 2003; Kahn, 1985) and stimulates protein synthesis (Harvey & Kaye, 1988; Lewis *et al.*, 1992) to exert an embryotrophic action. In certain culture conditions, selenium mimics the effects of insulin on several processes to also act as insulin mimetics (Zhu *et al.*, 1994). Thus, components of ITS appear to assist cellular metabolism as well as protect the embryos from oxidative stress induced by ROS and generated during IVC. Indeed, quantification of intracellular ROS level by measurement of DCF fluorescence revealed that ITS-treated PA embryos had a significantly reduced level of ROS compared with those of non-treated embryos. Interestingly, these effects were seen in the presence of non-essential amino acids and BSA in the IVC medium. These data are in contrast to our previous report (Uhm *et al.*, 2007), which showed that the beneficial effect of selenium was masked in the presence of BSA. Thus, insulin and transferrin appears to have an additive effect with selenium to further augment the anti-oxidant-like action from BSA. A synergistic effect of insulin and selenium with non-essential amino acids has also been shown previously (Koo *et al.*, 1997; Zhang & Armstrong, 1990). Zhang & Armstrong (1990) reported that, in the presence of amino acids, the beneficial effect of ITS premix was primarily due to insulin. However, together with our previous study (Uhm *et al.*, 2007) and previously reported insulin-mimetic action of selenium (Zhu *et al.*, 1994; Uhm *et al.*, 2007), it seems possible that the effect of selenium was concealed in the presence of insulin and could not be observed by Zhang & Armstrong (Zhu *et al.*, 1994).

Next, we evaluated the effect of ITS on preimplantation development of IVF and SCNT embryos. This and other laboratories have shown previously that PA and SCNT embryos may have different culture requirements (Heindryckx *et al.*, 2001; Chung *et al.*, 2002; Sugimura *et al.*, 2011) or different degree of responsiveness to IVC components such as hormones (Kim *et al.*, 2006), growth factors (Lee *et al.*, 2004) and mitogens (Gupta *et al.*, 2007c). However, no previous studies have evaluated the requirement of ITS for IVC of SCNT embryos. The results indicate that, similar to PA embryos, both IVF and SCNT embryos responded positively to ITS and showed improved rate of blastocyst formation compared with those of non-treated controls. However, blastocyst quality, in terms of their expansion and hatching ability and the total cell number per blastocyst, did not differ between ITS-treated and non-treated control groups ($P < 0.35$). The cause for the difference between PA and IVF/SCNT embryos is interesting but elusive. Previous studies have shown that mitogens and growth factors that alter the expression of imprinted genes may have differential effect on PA and IVF/SCNT embryos owing to maternal origin of PA embryos and bi-parental origin of IVF/SCNT embryos (Rappolee *et al.*, 1992; Gupta *et al.*, 2007c). Of note, members of insulin growth factor (IGF) system such as IGF2, which represents one of the mechanisms to regulate the cellular proliferation, is maternally imprinted to express at very low level in PA embryos and is aberrantly expressed in SCNT embryos (Han *et al.*, 2003, 2008; Hofmann *et al.*, 2002). Thus, it is plausible that insulin and insulin-mimetic selenium may be one possible cause for the differential but improved cell numbers in embryos (Zhang & Armstrong, 1990; Koo *et al.*, 1997). A recent study also reported that pig SCNT blastocysts have anomalous oxygen consumption, which might reflect their limited hatchability (Sugimura *et al.*, 2011) and hence differential response to ITS. It is also likely that IVF and SCNT embryos may need a higher dose of ITS than PA-derived embryos. However, further studies are required to confirm these hypotheses.

In summary, supplementation of ITS to IVC medium for pig embryos enhanced their *in vitro* development, increased the expansion and hatching ability of blastocysts, and increased the total cell number per blastocyst in PA embryos. Culture in the presence of ITS, also increased the *in vitro* development of IVF and SCNT embryos but did not have a significant effect on the blastocyst quality in terms of their expansion and hatching ability and the total cell number per blastocyst. Supplementation of ITS to the IVC medium also reduced the level of intracellular ROS in pig embryos. Taken together, these data suggest that supplementation of ITS to IVC medium exerts an embryotrophic action and reduces oxidative stress on pig PA embryos. However, it had beneficial but differential effect on IVF and SCNT embryos.

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