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Endoplasmic Stress Inhibition during Oocyte Maturation Improves Preimplantation Development of Cloned Pig Embryos

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ABSTRACT

Mitochondrial dysfunction is found in oocytes and transmitted to offspring due to maternal obesity. Treatment of obese mothers with endoplasmic reticulum (ER) stress inhibitors such as salubrinal (SAL) can reverse the mitochondrial dysfunction and result in normal embryonic development. Pig oocytes have also shown ER stress mostly in metaphase II stage. ER stress in oocytes may hinder the *in vitro* production of pig embryos. This study investigated the effect of ER stress inhibition by SAL treatment during *in vitro* maturation (IVM) of porcine oocytes at 1, 10, 50 and 100 nM concentrations. Firstly, we tested various concentrations of SAL. SAL at 10 nM showed higher ($P < 0.05$) developmental competence to the blastocyst stage (55.6%) after parthenogenesis (PA) than control (44.2%) while not different from other concentrations (49.2, 51.6, and 50.8% for 1, 50, and 100 nM, respectively). Secondly, we performed time-dependent treatment at 10 nM of SAL for IVM of oocytes. It revealed that treatment with SAL during 22 to 44 h of IVM significantly improved PA embryonic development to the blastocyst stage compared to control (40.5, 46.3, 51.7 and 60.2% for control, 0 to 22 h, 22 to 44 h and 0 to 44 h of IVM, respectively, $P < 0.05$). Glutathione (GSH) content is an indicator of cytoplasmic maturation of oocytes. Reactive oxygen species (ROS) have a harmful effect on developmental competence of oocytes. For this, we determined the intraoocyte levels of GSH and ROS after 44 h of IVM. It was found that SAL increased intraoocyte GSH level and also decreased ROS level ($P < 0.05$). Finally, we performed somatic cell nuclear transfer (SCNT) after treating oocytes with 10 nM SAL during IVM. SAL treatment significantly improved blastocyst formation of SCNT embryos compared to control (39.6% vs. 24.7%, $P < 0.05$). Our results indicate that treatment of pig oocytes with ER stress inhibitor SAL during IVM improves preimplantation development PA and cloned pig embryos by influencing cytoplasmic maturation in terms of increased GSH content and decreased ROS level in IVM pig oocytes.

(Keywords: Oocyte maturation, Endoplasmic reticulum stress, Embryonic development, Pig)

INTRODUCTION

Unfolded or misfolded proteins accumulate in the endoplasmic reticulum (ER), triggering activation of the unfolded protein response (UPR) to allow cells to respond to stress conditions (Kaufman, 1999; Ron and Walter, 2007). The ER is a major site of synthesis of transmembrane proteins and lipids and is involved in maintenance of intracellular calcium homeostasis (Larner *et al*., 2006; Raffaello *et al*., 2016). When ER homeostasis is perturbed or the control mechanism overloaded, prolonged stress causes apoptosis. Although the exact mechanisms remain poorly described in mammals, three major sensors are thought to activate the UPR during ER stress specifically, EIF2AK3 (PERK), ERN1 (IRE1α), and ATF6 (Tang and Yang, 2015).

It has been reported that controlling ER stress influences embryonic development. In the mice, ER stress signaling was detected at the 1-cell stage and was very high at the blastocyst stage (Kim *et al.*, 1990). HSPA5 (GRP78/BiP), a stress-induced ER chaperone, was required to ensure cell proliferation and to protect the inner cell mass (ICM) from apoptosis during early mouse embryonic development (Luo *et al.*, 2006). XBP1s regulates transcription of a group of core genes that are involved in constitutive maintenance of ER function in all cell types (Acosta-Alvear *et al.*, 2007). The xbp1 gene product is essential for embryonic development in Drosophila (Souid *et al.*, 2007). A loss-of-function Xbp1 mutant caused mouse

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embryonic lethality, with liver hypoplasia as the principal symptom (Reimold *et al.*, 2000). In addition, the UPR contributed to preimplantation mouse embryonic death that was associated with inability to resolve the ER stress (Hao *et al.*, 2009).

PA embryos were normally used as model system for studying *in vitro* culture (IVC) condition or environmental stresses due to its physiological alike with fertilized embryos in early development and experimentally simple production of PA embryos with less ethical problems (Tseng *et al.*, 2006; Paffoni *et al.*, 2008). Pig oocytes and embryos are hypersensitive to various stress during *in vitro* maturation (IVM) and IVC. Interestingly, lipid content of oocytes differs in different species. Triglyceride in pig oocytes shows about three times more than both cow and sheep oocytes (McEvoy *et al.*, 2000). Palmitic, stearic and oleic acids are the most abundant in oocytes of bovine, porcine and sheep, but pig oocytes has higher palmitic acid than oleic acid whereas cow and sheep oocytes has a relatively greater oleic acid (Genicot *et al.*, 2000). Palmitic acid at high levels are known to induce ER stress, impair embryonic development in mice (Wu *et al.*, 2012). In addition, a study confirmed the ER stress in MII oocytes in pig (Zhang *et al.*, 2012). Therefore, it was hypothesized that inhibition of the ER stress during IVM would stimulate embryonic development after PA and somatic cell nuclear transfer (SCNT).

Salubrinal is a well-known ER stress inhibitor, a selective eIF2α dephosphorylation inhibitor, protect cells from lipotoxicity induced by ER stress. It maintains the high in phospho-eIF2α, which the restoration of ER function, help in protein folding and maintain cellular homeostasis (Tian *et al.*, 2011; Kuo *et al.*, 2012). This study investigated the effect of salubrinal during IVM on oocyte maturation, intra-oocytes glutathione (GSH) and reactive oxygen species (ROS) content, and embryonic development after PA and SCNT in pigs.

MATERIALS and METHODS

1. Culture media

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. The base IVM medium for oocytes was medium-199 (M199; Invitrogen, Grand Island, NY, USA). M199 was added with 0.91 mM pyruvate, 0.6 mM cysteine, 10 ng/ml epidermal growth factor, 1 μg/ml insulin and 75 μg/ml kanamycin and 10% (v/v) porcine follicular fluid (PFF). The IVC medium was porcine zygote medium-3 (PZM) (Yoshioka et al, 2002) for embryonic development after PA and SCNT, which consisted of 0.34 mM trisodium citrate, 2.77 mM myo-inositol, and 10 μM β-mercaptoethanol (You et al, 2012).

2. Oocyte collection and IVM

Pig ovaries were obtained from a local abattoir and then transported to the laboratory in warm physiological saline. The cumulus oocytes complex (COCs) were subsequently aspirated from follicles (3–8 mm in diameter) by using an 18-gauge needle connected to a 10-ml syringe. COCs with multiple layers of compact cumulus cells and uniform ooplasm were considered for after washing three times in HEPES-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (PVA). The COCs were then cultured in of IVM (500 μl) medium in the presence of 10 IU/ml hCG (Intervet International BV, Boxmeer, Holland) and 80 μg/ml FSH (Antrin R-10; Kyoritsu Seiyaku, Tokyo, Japan). COCs were matured at 39°C with 5% CO_2 at maximum humidity for 22 h. For an additional 22 h or 20 h oocytes were cultured in hormone-free IVM medium after washing in fresh hormone-free IVM medium for PA and SCNT, respectively.

3. Somatic cell nuclear transfer and parthenogenesis (PA)

As nuclei donors, porcine fetal fibroblasts were prepared as described previously (Lee *et al.*, 2013). After IVM for 41 h, the cumulus cells of COCs were dispersed by gentle pipetting in the presence of 0.1% (w/v) hyaluronidase. Oocytes having first polar bodies and uniform ooplasm were selected and stained with 5 μg/ml Hoechst 33342 for 15 min. Oocytes were then washed twice in fresh manipulation medium, transferred into a drop of this media containing 5 μg/ml cytochalasin B (CB), and overlaid with warm mineral oil. Enucleation was subsequently performed by a 17-μm beveled glass pipette (Humagen, Charlottesville, VA, USA) after aspirating the first polar body (PB) and a small amount of surrounding cytoplasm. The expelled cytoplasm was then surveyed by epifluorescence microscopy (TE300; Nikon, Tokyo, Japan) to verify that the nuclear material had been removed. A single disaggregated donor cell was injected into the perivitelline space of the enucleated oocytes, after which oocyte–cell couplets were placed on a 1 mm fusion chamber overlaid with 1 ml of 280 mM mannitol solution containing 0.001 mM

$CaCl_2$ and 0.05 mM $MgCl_2$, as previously described (Walker et al, 2002). Cell fusion was performed by using an alternating current field of 2 V cycling at 1 MHz for 2 seconds, followed by two pulses of 170 V/mm direct current (DC) for 30 µsec using a cell fusion generator (LF101; NepaGene, Chiba, Japan). The oocytes were then incubated for 1 h in TLH-BSA, after which they were assessed for confirmation of fusion under a stereomicroscope. The nuclear transferred oocytes were activated with two pulses of 120 V/mm DC for 60 µsec in a 280 mM mannitol solution containing 0.05 mM $MgCl_2$ and 0.1 mM $CaCl_2$. For PA, MII oocytes were activated as described in SCNT procedures.

4. Post-activation and embryo culture

After electrical activation, the PA were cultured with 5 µg/ml CB and SCNT embryos were treated with 0.4 µg/ml demecolcine combined with 1.9 mM 6-dimethylaminopurine in IVC medium for 4 h. Afterward, the embryos were washed three times in fresh IVC medium, cultured into 30 µl IVC medium droplets under mineral oil, at 39°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days. Cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively. The day of SCNT or PA was designated as Day 0. The total cell count in blastocysts was performed using Hoechst 33342 staining and visualized under an epifluorescence microscope.

5. Measurement of oocyte diameter

After 44 h of IVM, images of denuded oocytes in each group were captured at 200X magnification using a digital camera (DS-L3; Nikon) connected to an inverted microscope (TE 300; Nikon). The size of matured oocytes was determined by the ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD, USA).

6. Measurement of GSH and ROS contents

After 44 h of IVM, oocytes were examined for GSH and ROS levels. The GSH and ROS contents were measured as previously described (Sakatani *et al.*, 2007). Briefly, (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin, Invitrogen) Cell-Tracker Blue and (2',7'-dichlorodihydrofluorescein diacetate; Invitrogen) H2DCFDA were used to detect intraoocyte GSH and ROS with blue fluorescence and green fluorescence for GSH and ROS, respectively. A group of 7 – 10 oocytes from each treatment group were cultured for 30 min in TLH-PVA supplemented with 10 µM Cell-Tracker and 10 µM H2DCFDA and in the dark. Embryos treated with Cell-Tracker were then incubated for 30 min with PZM-3 supplemented with 0.3% (w/v) BSA at 39°C in the dark. Following incubation, the embryos were washed with Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen) containing 0.01% (w/v) PVA, placed into 2-µl droplets. Fluorescence was observed under an epifluorescence microscope (TE300; Nikon) with ultraviolet ray filters at 370 and 460 nm for GSH and ROS, respectively. The fluorescence intensities of oocytes were normalized against the untreated control.

7. Determination of mitochondrial oxidative activity

Denuded oocytes were incubated in M199 containing 200 nM Mitotracker Orange CM-H_2-TMRos (Molecular Probes, Eugene, OR, USA) for 40 min at 39°C in the dark. After washing three times in the fresh M199 medium, oocytes were examined under an inverted epifluorescence microscope (TE300; Nikon). Fluorescence signals were captured with a digital camera (DS-L3; Nikon), and normalized against the untreated control oocytes.

8. Differential count of inner cell mass and trophectoderm cells

Differential staining of blastocysts to determine cell number of inner cell mass and trophectoderm was performed as described previously (Thouas *et al.*, 2001). Briefly, blastocysts were stained with 5 µg/ml Hoechst 33342 for 1 h, treated with 0.04% (v/v) Triton X-100 for 3 min and then with 0.005% (w/v) propidium iodide for 10 min. Stained blastocysts were observed for fluorescence. The propidium iodide and Hoechst 33342-labeled trophectoderm nuclei appeared pink or red and bisbenzimide-labeled ICM nuclei appeared blue.

9. Experimental design

In the first experiment, oocytes were exposed to salubrinal for 44 h during IVM at 0 (control) 1, 10, 50, and 100 nM concentrations to determine the optimal concentration for pig IVM system. Based on the result from the first experiment, oocytes were treated with salubrinal at 10 nM for 0 to 22 h, 22 to 44 h, or 0 to 44 h of IVM (designated as 022h, 2244h, and 044h, respectively) to determine the time-dependent effect in the second and further experiments. The intra-oocytes GSH and ROS contents were evaluated in the third experiment.

Mitochondrial oxidative activity and diameter of IVM oocytes were measured the fourth experiment and the effect on SCNT embryonic development was determined in the fifth experiment.

10. Statistical analyses

Statistical analyses were executed by using the Statistical Analysis System (version 9.3; SAS Institute, Cary, NC, USA). The general linear model procedure followed by the least significant difference mean separation procedure was used for data analysis. When treatments differed at $p < 0.05$. The percentage data were considered to arcsine transformation before analysis to maintain homogeneity of variance. The results are present as the mean ± standard error of the mean (SEM).

RESULTS

1. Dose-dependent effects of salubrinal during IVM on embryonic development after PA

To determine the optimal concentration of salubrinal for pig oocyte maturation, oocytes were treated for 0-44 h of IVM with salubrinal at various concentrations. Salubrinal did not influence nuclear maturation of oocytes after IVM. Among the concentrations tested, 10 nM salubrinal showed significant effect ($P < 0.05$) on embryo cleavage (94.0 ± 1.7% vs. 86.0 ± 2.3%) and blastocyst development (55.6 ± 2.8% vs. 44.2 ± 3.4%) compared to control. Mean cell number in blastocyst was not affected by salubrinal treatment (Table 1).

2. Effects of salubrinal during various stages of IVM on PA embryonic development

Oocytes were untreated or exposed to 10 nM salubrinal for 0 to 22 h, 22 to 44 h and 0 to 44 h of IVM. Cleavage (95.9 ± 1.5% vs. 84.4 ± 3.1%) and blastocyst (60.2 ± 4.5% vs. 40.5 ± 4.2%) development were significantly ($P < 0.05$) increased in 044h than the control. Oocyte maturation and average cell number per blastocyst were not influenced by the salubrinal treatment (Table 2).

3. Effect of salubrinal treatment during IVM on the quality of PA blastocysts

Effect of salubrinal treatment during IVM on blastocyst quality in terms of ICM and trophectoderm cell numbers was evaluated. Salubrinal treatment in 2244h (9.7 ± 1.0 cells) and

Table 1. Effect of salubrinal treatment during *in vitro* maturation (IVM) on oocyte maturation and parthenogenesis (PA) embryonic development

Salubrinal treatment*	% of oocytes that reached metaphase II	No of PA oocytes cultured	% of embryos developed to		No of cells in blastocyst
			≥ 2-cells	Blastocyst	
Control	93.7 ± 1.4	226	86.0 ± 2.3[a]	44.2 ± 3.4[a]	37.7 ± 1.2
1 nM	95.8 ± 0.9	217	91.7 ± 1.7[b]	49.2 ± 4.4[ab]	35.9 ± 1.2
10 nM	95.0 ± 1.4	212	94.0 ± 1.7[b]	55.6 ± 2.8[b]	36.9 ± 1.3
50 nM	97.1 ± 1.2	217	90.8 ± 2.2[ab]	51.6 ± 3.8[ab]	37.7 ± 1.2
100 nM	96.3 ± 1.1	225	91.1 ± 1.2[ab]	50.8 ± 2.5[ab]	35.3 ± 1.3

Six replicates.
*Oocytes were untreated (control) or treated with various concentrations of salubrinal during 0-44 h of IVM.
[ab]Values with different superscripts denote difference within the same column ($P < 0.05$).

Table 2. Effect of salubrinal treatment during various stages of *in vitro* maturation (IVM) on oocyte maturation and parthenogenesis (PA) embryonic development

Salubrinal treatment*	% of oocytes that reached metaphase II	No of PA oocytes cultured	% of embryos developed to		No of cells in blastocyst
			≥ 2-cells	Blastocyst	
Control	96.8 ± 1.6	128	84.4 ± 3.1[a]	40.5 ± 4.2[a]	32.9 ± 1.4
0-22 h	97.3 ± 2.7	131	90.8 ± 2.1[ab]	46.3 ± 4.9[ab]	34.8 ± 1.6
22-44 h	96.8 ± 0.6	121	91.5 ± 3.1[ab]	51.7 ± 4.4[ab]	37.4 ± 1.5
0-44 h	96.1 ± 1.3	118	95.9 ± 1.5[b]	60.2 ± 4.5[b]	37.0 ± 1.8

Four replicates.
* Oocytes were untreated (control) or treated with 10 nM salubrinal during 0-22, 22-44 and 0-44 h of IVM.
[ab] Values with different superscripts denote difference within the same column ($P < 0.05$).

Table 3. Effect of salubrinal treatment during *in vitro* maturation on inner cell mass (ICM) and trophectoderm (TE) cell numbers of parthenogenesis (PA) blastocysts

Salubrinal treatment*	No of PA blastocysts examined	Cell number			ICM/total (%)
		ICM	TE	Total	
Control	17	6.4 ± 0.5^a	31.8 ± 3.4	38.1 ± 4.1	17.8 ± 1.3
22-44 h	20	9.7 ± 1.0^b	27.1 ± 2.2	36.1 ± 2.7	25.3 ± 2.0
0-44 h	17	9.8 ± 1.3^b	35.6 ± 4.0	45.4 ± 4.2	23.3 ± 3.1

Three replicates.
* Oocytes were untreated (control) or treated with 10 nM salubrinal during 22-44 and 0-44 h of IVM.
[ab] Values with different superscripts denote difference within the same column ($P < 0.05$).

044h (9.8 ± 1.3 cells) significantly increased ($P < 0.05$) the number of ICM cells compared to control (6.4 ± 0.5 cells). The number of trophectoderm cells, total cell number, and the ratio of ICM to total cell number was not altered by the treatment (Table 3).

4. Effects of salubrinal on intra-oocyte GSH and ROS contents

The intra-oocyte GSH and ROS contents were evaluated after 44 h of IVM. The result revealed that salubrinal increased ($P < 0.05$) the GSH content of oocytes in 044h compared to those in control and 2244h. In contrast, salubrinal decreased ($P < 0.05$) the ROS level of oocytes in 044h and 2244h compared to that of oocytes in control (Table 4).

5. Effects of salubrinal on mitochondrial oxidative activity and oocytes diameter

The mitochondrial oxidative activity was determined in the MII oocytes by Mitotracker Orange CMTMRos, which only stains the respiring mitochondria based on the oxidative activity of oocytes. The results indicated that the respiring mitochondria was significantly higher in control oocytes than in salubrinal-treated oocytes (1.00, 0.91, and 0.81 pixels/oocyte for control, 044h, and 2244h, respectively). Salubrinal treatment significantly increased oocyte diameter in 044h (118.7 μm) compared to control (115.8 μm) while there was no difference between 044h and 2244h (117.2 μm) (Table 5).

Table 4. Effect of salubrinal treatment during *in vitro* maturation (IVM) on intra-oocyte glutathione (GSH) and reactive oxygen species (ROS) contents

Salubrinal treatment*	No of oocytes	GSH content (pixels/oocyte)	No of oocytes	ROS content (pixels/oocyte)
Control	60	1.00 ± 0.12^a	40	1.00 ± 0.18^a
22-44 h	60	1.12 ± 0.13^a	40	0.59 ± 0.08^b
0-44 h	60	1.37 ± 0.12^b	40	0.58 ± 0.08^b

Three replicates.
* Oocytes were untreated (control) or treated with 10 nM salubrinal during 22-44 and 0-44 h of IVM.
[ab] Values with different superscripts denote difference within the same column ($P < 0.05$).

Table 5. Effect of salubrinal on mitochondrial oxidative activity and oocyte diameter after *in vitro* maturation

Salubrinal treatment*	No of oocytes examined	Mitochondrial oxidative activity (pixels/oocyte)	No of oocytes examined	Oocytes diameter (μμm)
Control	38	1.00 ± 0.04^a	58	115.8 ± 0.8^a
22-44 h	41	0.91 ± 0.03^b	62	117.2 ± 0.5^{ab}
0-44 h	39	0.81 ± 0.03^c	59	118.7 ± 0.5^b

Three replicates.
* Oocytes were untreated (control) or treated with 10 nM salubrinal during 22-44 and 0-44 h of IVM.
[a-c] Values with different superscripts denote difference within the same column ($P < 0.05$).

6. Effects of salubrinal during IVM on SCNT embryonic development

To determine the developmental competence of SCNT oocytes after inhibition of ER stress, oocytes were untreated (control) or exposed to salubrinal during 0 to 44 h and 22 to 44 h of IVM. The results revealed that salubrinal treatment during 0 to 44 h (35.9 ± 3.4%) and 22 to 44 h of IVM (39.6 ± 2.8%) significantly ($P < 0.05$) improved the SCNT embryonic development compared to control (24.7 ± 1.9%). Oocyte-cell fusion, cleavage, and mean cell number of SCNT blastocysts were not influenced by the salubrinal treatment (Table 6).

DISCUSSION

IVM oocytes are less competent than *in vivo*-matured oocytes. Oocytes are prone to various stress during IVM that decrease the oocyte quality to embryo development. ER stress is one of these stress, which is present in MII oocytes and blastocysts in pig. For this reason, this study investigated the effect of ER stress inhibition during IVM by salubrinal on embryonic development after PA and SCNT. Firstly, it was evaluated the optimal concentration for IVM of pig, oocytes were exposed to 1, 10, 50 and 100 nM of salubrinal for the first 22 h of IVM. The result revealed that 10 nM of salubrinal exhibited a higher embryo development than control and other concentration after PA. Then it was performed time depend treatment for oocytes maturation and embryo development in various period of IVM. It was found that 044 h significantly improved the embryonic development than control, 022 h and 2244 h. Overall, this result was consistent with previous result of (Wu *et al.*, 2012; Wu *et al.*, 2015). Bovine COCs exposed to palmitic, stearic and oleic acid during IVM increased the genes regarding energy oxidative stress and metabolism in oocytes (Van Hoeck *et al.*, 2013). Palmitc acid increased ER stress and mitochondria dysfunction in mouse oocytes during IVM (Wu *et al.* 2012). COCs treated with lipid-rich follicular fluid shown similarly augmented expression of ER stress genes and disrupt nuclear maturation (Yang *et al.*, 2012). ER stress inhibitor recuperated oocyte mitochondrial activity and poor embryonic development induced by high doses of palmitic acid (Wu *et al.*, 2012).

GSH is an important non-enzymatic antioxidants in cells of mammal and is a requisite for maintenance, formation, and protection of the meiotic spindle against oxidative stress (Luberda, 2005). GSH is synthesized during oocyte maturation in the cytosol and stored as a separate redox pools in mitochondria, nucleus and ER. MII oocytes has higher level of GSH, decrease during the preimplantation development and is synthesized during oocyte maturation, reaches its lowest concentration in the blastocyst (Hansen and Harris, 2015). Alterations in intracellular GSH content or glutathione-related EGSH can increase apoptosis and halt embryonic development (Furnus *et al.*, 2008; Li *et al.*, 2011). GSH content or EGSH was significantly decrease in different species after IVM (Somfai *et al.*, 2007; Curnow *et al.*, 2010). Since GSH is also synthesized by cumulus and transferred to the oocyte during maturation, removal of cumulus can influence intra-oocyte GSH levels (Curnow *et al.*, 2010; 2011). However, salubrinal increased the GSH content after 44 h of IVM than control. It indicates the inhibition of ER stress, improved the intra-oocytes GSH level as well as embryonic development through redox potentiality and homeostasis.

ROS can influence oxidative stress, which is closely related to ER stress. For instance, lipid oxidation can induce ER Stress and recapture for ER stress can itself generate ROS. Inhibition of ER stress can diminish ROS in embryos. These are agreed

Table 6. Effect of salubrinal treatment during various stages of *in vitro* maturation (IVM) on somatic cell nuclear transfer (SCNT) embryonic development

Salubrinal treatment*	% of fused oocytes	No of SCNT oocytes cultured	% of embryos developed to		No of cells in blastocyst
			≥ 2-cells	Blastocyst	
Control	72.2 ± 9.1	103	80.0 ± 3.4	24.7 ± 1.9[a]	33.1 ± 2.5
22-44 h	74.2 ± 5.6	100	84.8 ± 1.3	39.6 ± 2.8[b]	39.0 ± 2.4
0-44 h	80.2 ± 4.9	109	87.5 ± 2.5	35.9 ± 3.4[b]	37.6 ± 2.4

Four replicates.

* Oocytes were untreated (control) or treated with 10 nM salubrinal during 22-44 and 0-44 h of IVM.

[ab] Values with different superscripts denote difference within the same column ($P < 0.05$).

with the present result because salubrinal treatment decreased the ROS level in oocytes after IVM. The activity of mitochondria has a relation with embryo development in both oocytes and pre-implanted embryo, it is a more vulnerable target of ROS. Free oxygen radicals can impair the mitochondrial DNA of oocytes, ultimately loss the mitochondrial function. As a source of ROS generated from the 'leakage' of high-energy electrons along the electron transport channels (Crompton, 1999; Agarwal and Allamaneni, 2004). Attack originated from free oxygen radicals can damage the mitochondrial DNA of quiescent oocytes and lead to the loss of their intrinsic mitochondrial function (Barritt, 1999; Hsieh *et al.*, 2004). The mitochondrial function is suggested to play a major role in controlling aging of fertility aging (Gottlieb, 2001) and the activity of mitochondria in both oocytes and pre-implanted embryos appears to be inversely correlated with maternal aging and embryo development (Wilding *et al.*, 2001). Mitotracker Orange staining the respiring mitochondria and fluorescence intensity define the respiratory activity per oocyte. It proves that higher levels of mitochondrial activities in oocytes indicates an apoptosis in surrounding cumulus cells (Torner *et al.,* 2004). In addition, a higher mitochondrial activities are related to increase a level of ROS. Interestingly, ER stress inhibitor recuperated oocyte mitochondrial activity and poor embryo development induced by high doses of palmitic acid (Wu *et al.*, 2012). A similar effect was found by treating oocytes with salubrinal during 44 h of IVM. Interestingly, the oocyte diameter was increased by salubrinal treatment during IVM. It has been reported oocytes with larger diameter shows higher developmental competence than smaller ones (Kim *et al.*, 2010). Although it was now known how salubrinal altered oocyte size, the improved blastocyst formation in this study could be associated with increased oocyte diameter in salubrinal treated group. In conclusion, the present result demonstrated that treatment of oocytes with ER stress inhibitor salubrinal during IVM improved embryonic development probably by maintaining the redox homeostasis and prevents oxidative stress after IVM.

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