CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

All reagents described in the materials and methods were bought from Sigma-Aldrich (St. Louis, MO, USA), except otherwise noted.

3.2 Ethic statement

Ethical approval for this study was obtained from the Institutional Animal Care and Use Committee of Suranaree University of Technology, Thailand (Approval Number: IACUC-67-39).

3.3 Experimental design

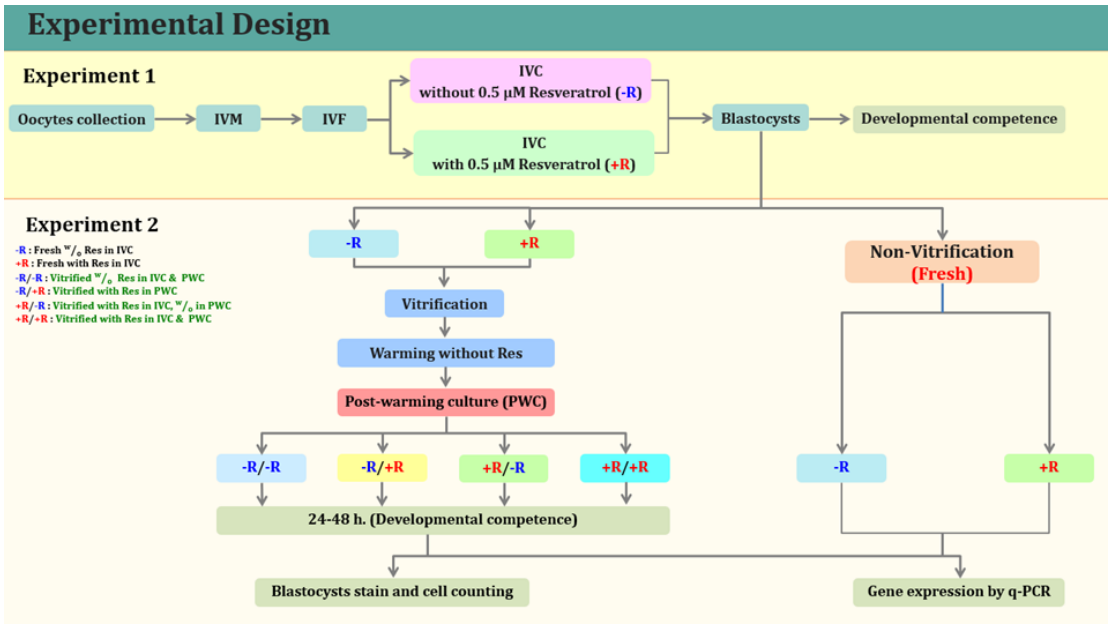

Figure 2. Diagrammatic representation of the experimental design.

3.3.1 Experiment 1: Effects of supplemented resveratrol in IVC medium on embryo development

To assess the effects of resveratrol in IVC medium on embryo development. Fresh COCs cultured in IVM medium without resveratrol were IVF and then cultured in IVC medium without and with 0.5 µM resveratrol for 7 days. The blastocyst formation rates were compared to the control embryos.

3.3.1.1 Oocyte collection and *in vitro* maturation (IVM)

Ovaries were collected from the slaughterhouse and stored in 0.9% NaCl solution at room temperature during transport to the laboratory. Subsequently, ovaries were washed with 0.9% NaCl solution and 2-8 mm diameter of follicles were collected, then the cumulus oocyte complexes (COCs) were kept in modified Dulbecco's phosphate-buffered saline (mDPBS) solution supplemented with 0.1% polyvinyl pyrrolidone (PVP). The COCs were then cultured in IVM medium (20 oocytes/100 µl of IVM medium) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 23 h. IVM medium consisting of tissue culture medium199 (TCM-199) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 50 IU/ml human chorionic gonadotropin (HCG, Intervet, Netherlands), 0.02 AU/ml follicle-stimulating hormone (FSH, Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan), and 1 µg/ml 17β-estradiol. The IVM medium was supplemented without resveratrol.

3.3.1.2 *In vitro* fertilization (IVF)

Frozen fertile semen of Wagyu bull (Pornchai Intertrade Ltd., Ratchaburi, Thailand) in 0.25 ml straws was thawed in the air for 10 seconds, followed by immersion in a water bath at 37.5°C for 1 min. The semen was then placed at the bottom of a 5 ml snap tube (Corning, Glendale, AZ, USA) containing 2 ml of TALP medium (Lu et al., 1987). The snap tube was incubated under a humidified atmosphere of 5% CO_2 in air at 38°C for 30 min. After incubation, the top 1.8 ml layer of the medium was gently collected and transferred to a 15 ml conical tube (SPL Life Sciences) containing 5 ml of TALP medium. The sperm suspension was centrifuged at 400 g for 5 min, and the supernatant was removed. The sperm pellet was diluted and adjusted to a concentration of 2×10^6 sperm/ml using a TALP medium. Then, 50 µl droplets of the sperm suspension were transferred to a 35 mm culture dish covered with

mineral oil. At 23 h of IVM, COCs were washed three times with TALP medium and placed in droplets of sperm suspension (10 COCs/drop) and incubated under a humidified atmosphere of 5% CO_2 in the air at 38°C for 10 h.

3.3.1.3 *In vitro* embryo culture (IVC)

Following sperm-oocyte co-incubation, the presumptive zygotes were denuded of cumulus cells and excess sperm. The presumptive zygotes were subsequently cultured in an IVC medium (CR1aa medium, Rosenkrans et al., 1993) supplemented with 5% FBS with or without 0.5 µM resveratrol in a 35 mm culture dish covered with mineral oil (10 oocytes/50 µl of IVC medium) under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 ºC for 7 days. The development of the embryos was examined on days 2 and 7 to record cleavage and blastocyst rate.

3.3.2 Experiment 2: Effects of supplemented resveratrol in IVC and post-warming culture (PWC) media on the developmental potential of vitrified blastocysts, ICM, TE, and total cell numbers of fresh and gene expression

We investigated the effect of adding resveratrol to the culture medium during culture up to the blastocyst stage or during PWC of blastocysts after freezing and thawing on the developmental ability of vitrified blastocysts, two experimental groups were established: **1) -R**: fresh embryos cultured in IVC medium without resveratrol and **2) +R**: fresh embryos cultured in IVC medium supplemented with 0.5 µM resveratrol. The embryos were subsequently vitrified, warmed, and divided into four post-warm culture groups: **1) -R/-R**: embryos cultured in IVC medium without 0.5 µM resveratrol, vitrified, warmed, and then cultured in medium without 0.5 µM resveratrol, **2) -R/+R**: embryos cultured in IVC medium without 0.5 µM resveratrol, vitrified, warmed, and then cultured in medium supplemented with 0.5 µM resveratrol, **3) +R/-R**: embryos cultured in IVC medium supplemented with 0.5 µM resveratrol, vitrified, warmed, and then cultured in medium without 0.5 µM resveratrol, and **4) +R/+R**: embryos cultured in IVC medium supplemented with 0.5 µM resveratrol, vitrified, thawed, and then cultured in medium supplemented with 0.5 µM resveratrol.

Their developmental abilities to hatching and hatched blastocyst stage at 48 h were compared among four groups. The blastocysts from this experiment were stained with PI and Hoechst 33342 before examining the number of ICM and TE cells. The blastocysts from all four groups were analyzed for gene expression by q-PCR and compared with fresh blastocysts derived from IVC without and with 0.5 μM resveratrol.

3.3.2.1 Vitrification and warming of blastocysts

Grade 1 and 2 blastocysts were washed with BM. Subsequently, the blastocysts were incubated at 24-25°C in ES composed of BM supplemented with 7.5% (v/v) EG and 7.5% (v/v) DMSO for 3 min. Following this, the blastocysts were transferred to VS consisting of BM supplemented with 15% (v/v) DMSO, 15% (v/v) EG, and 0.5 M sucrose for 1 min. Finally, 2-3 blastocysts were placed onto a Cryotop® (Kitazato BioPharma Co. Ltd., Fujinomiya, Japan) which was immediately immersed in liquid nitrogen at 1 min after exposure to the vitrification solution. The blastocysts were stored in liquid nitrogen for at least one week before use. For warming, the Cryotop® tip was directly placed into a 35 mm culture dish containing 2.5 ml of warming solution composed of BM supplemented with 0.5 M, 0.25 M, and 0 M sucrose for 3, 5, and 5 min, respectively. After warming, all thawed blastocysts were cultured in IVC medium (with or without resveratrol) under a humidified atmosphere of CO_2 5% O_2 and 90 % N_2 in air at 38.5°C and their developmental stages were observed after 24 and 48 h. of culture.

3.3.2.2 Evaluation of TE and ICM cell numbers

Ten blastocysts from each group were stained by immersing them in a solution containing 1 mg/ml propidium iodide (PI) and 0.2% Triton X-100, prepared in mDPBS supplemented with 0.1% PVP, for 1 min. Subsequently, the blastocysts were transferred to a solution of 25 μg/ml Hoechst 33342 in 99.5% ethanol for 5 min. Finally, the stained blastocysts were mounted on glass slides using glycerol. TE and ICM cells of the blastocysts were then counted under an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan).

3.3.2.3 Quantitative real-time PCR (q-PCR)

Twenty blastocysts from each group were washed three times with PBS (-) and stored at -80°C until further use. The manufacturer extracted Total

mRNA using the FavorPrep™ Tissue Total RNA Mini Kit (Favorgen Biotech Crop., Pingtung, Taiwan). cDNA synthesis was performed using biotechrabbit™ cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany), and the expression of specific genes was assessed using the KAPA SYBR FAST qPCR Master Mix (Applied Biosystems) on the CFX Opus 96 real-time PCR system (Biorad, Hercules, California, USA). Melting curve analysis was performed for all primers, which were optimized. The primer sequences are provided in Table 1. GAPDH was used as a housekeeping gene to normalize the expression of target genes. qPCR was performed in triplicate, and statistical analysis was conducted using the 2^-ΔΔCt method.

Table 1. Primer sequences used for real-time qPCR of blastocysts

Genes	Primer sequences	Product length (bp)	Accession numbers
Bax	F:(5´–3´) TCTGACGGCAACTTCAACTG R:(5´–3´) TCGAAGGAAGTCCAATGTCC	135	NM_173894.1
BCL2	F:(5´–3´) ATGCGGCCCCTGTTTGATTT R:(5´–3´) GCCTGTGGGCTTCACTTATG	116	NM_001166486.1
SIRT1	F:(5´–3´) TTACAGGGCCTATCCAGGGAG R:(5´–3´) GCATGCGAGGCTCTATCATCT	185	NM_001192980.3
TFAM	F:(5´–3´) TTTGTCTGCGGATGCAATGG R:(5´–3´) AGATCCGCTCCTGACTTTCC	78	NM_001034016.2
CAT	F:(5´–3´) GAGGAAACGCCTGTGTGAGA R:(5´–3´) GGATGCGGGAGCCATATTCA	116	NM_001035386.2
GPX4	F:(5´–3´) GTGCTCGCTCCATGCACGA R:(5´–3´) CCTGGCTCCTGCCTCCCA	223	NM_001346430.1
SOD1	F:(5´–3´) GTTGGAGACCTGGGCAATGT R:(5´–3´) CTCTGCCCAAGTCATCTGGTT	145	NM_174615.2
IFN-tua	F:(5´–3´) CTGGCCCGAATGAACAGACT R:(5´–3´) AGAGGTTGAAGCACTGCTGG	151	XM_024989143.2
Oct4	F:(5´–3´) GAGTGTGGTTTTGCAAGCGT R:(5´–3´) ATACGGGTCCCCCTGTGAA	108	NM_174580.3
Hsp70/HSPA8	F:(5´–3´) GGCCCTTCATGGTGGTGAAT R:(5´–3´) ACAGCGTTGGTAACCGTCTTC	160	NM_174345.4
DNMT1	F:(5´–3´) AAGAGTAAGACCAGGAACACAC R:(5´–3´) CTAGCTAGATCTTTGGGTTGAC	213	XM_015471993.1
DNMT3A	F:(5´–3´) TTCGACAGTGCAGTTCCTCC R:(5´–3´) TTTCTAGCAACTGCGCCTCA	135	NM_001206502.2
*GAPDH**	F: (5´–3´) CTCCCAACGTGTCTGTTGTG R: (5´–3´) TGAGCTTGACAAAGTGGTCG	222	NM_001034034.2

3.4 Statistical analysis

The results were analyzed using a t-test and one-way analysis of variance (ANOVA), followed by Tukey-Kramer Honest Significant Difference (HSD) as a post hoc test using GraphPad Software (version 5; San Diego, CA, USA). $P < 0.05$ was defined as the significance level.