CHAPTER III

RESERCH METHODOLOGY

3.1 Chemicals:

All chemicals were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise indicated.

3.2 *In vitro* maturation (IVM)

Ovaries were collected from the slaughterhouse and stored in 0.9% NaCl at room temperature during transport to the laboratory. The ovaries were washed twice using 0.9% NaCl. The oocytes were collected by aspiration of follicle with 2-8 mm diameter using 10 ml syringe connected with 18G needle. The cumulus oocyte complexes (COCs) were examined under stereo microscope. The COCs which have at least three layers of cumulus cells and homogeneous cytoplasm were placed in modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrrolidone (PVP). The COCs were cultured in droplets of IVM medium on 60 mm culture dish (Thermo Fisher Scientific Inc, USA) covered with mineral oil (20 oocytes/100 µl of IVM medium). The composition of IVM medium consisting of TCM-199 supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 50 IU/ml human chorionic gonadotropin (HCG, Intervet, Netherlands), 0.02 IU/ml follicle stimulating hormone (FSH, Antrin®, Kyoritsu Seiyaku Co., Tokyo, Japan) and 1 µg/ml 17β-estradiol. The COCs were cultured under humidified atmosphere of 5% CO_2 in air at 38.5 ^{o}C for 23 hr.

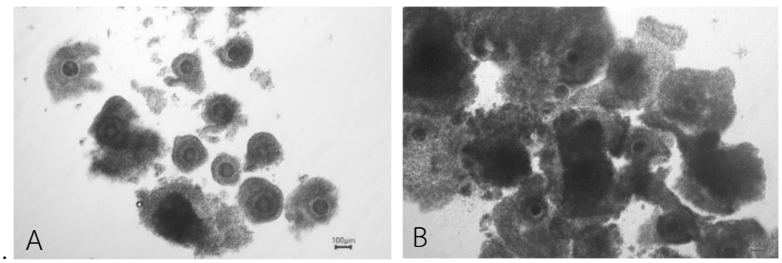

Figure 3.1 A, Oocytes before culture in IVM medium.
B, Oocytes after culture 23 hr in IVM medium.

3.3 Preparation of sperm for *in vitro* fertilization (IVF)

The frozen semen of fertile Wagyu bull was thawed in air for 10 sec and placed in water bath at 37 °C for 30 sec. Thawed semen was placed in the bottom of the snap tube (Thermo Fisher Scientific Inc, USA) containing 2 ml of TALP medium (Lu et al., 1999) and then incubated under humidified atmosphere of 5% CO_2 in air at 38.5 °C for 30 min. The 1.8 ml top layer of medium was collected and placed in 15 ml conical tube (Thermo Fisher Scientific Inc, USA) containing 5 ml TALP medium, then centrifuged at 3,000 rpm for 5 min. The supernatant was removed, and sperm pellet was adjusted to concentration of $2x10^6$ sperm/ml with TALP medium. The 100 µl drop of sperm suspension was placed on 60 mm culture dish and covered with mineral oil and then incubated under humidified atmosphere of 5% CO_2 in air at 38.5 °C.

3.4 *In vitro* fertilization (IVF)

After 23 hr of incubation in IVM medium, the COCs were washed with TALP medium for 4 times, and then 10 COCs were placed in a drop of sperm suspension prior to incubation under humidified atmosphere of 5% CO_2 in air at 38.5 °C for 10 hr.

3.5 *In vitro* embryo culture (IVC)

After sperm and COCs were co-incubated for 10 hr. The presumptive zygotes were denuded with TCM-199 Hepes supplemented with 10% FBS using a small pore pulled Pasteur pipette, and then cultured in CR1aa medium (Rosenkrans et al., 1993; Rosenkrans and First, 1994) supplemented with 5% FBS and 0.5 µM resveratrol (Lee et al., 2010). This group was considered as resveratrol-treated group (IVC+). The control group was composed of the medium without resveratrol supplement (IVC-). In each group, 1 to 50 oocysts were examined for each experimental replication. The culture system was carried out in 35mm culture dish (Thermo Fisher Scientific Inc, USA) covered with mineral oil (20 embryos/100 µl) and incubated under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C for 6-7 days. The development of embryos was examined at 2-8 cells, morulae and blastocyst stages at day 2, 5 and 6-7 respectively.

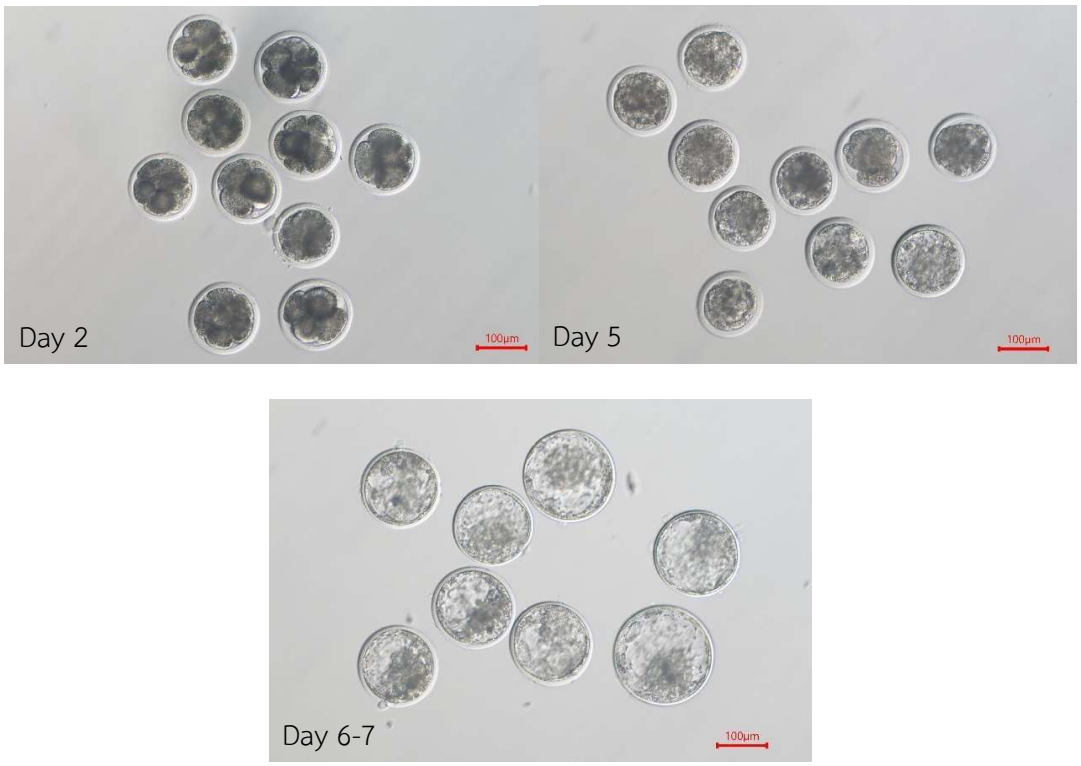

Figure 3.2 The development of the embryos at day 2, 5 and 6-7 after culture in IVC medium.

3.6 Cryopreservation of embryos using vitrification technique

3.6.1 Vitrification procedure

The blastocysts at day 6 (grade 1, IETS standard) from each treatment were vitrified after IVC as described by Martín et al. (2014). The embryo was rinsed with basal medium (BM) which consisting of TCM-199 Hepes supplemented with 20% FBS. Then embryos were placed in equilibration medium consisting of BM supplemented with 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG) at room temperature for 3 min. Then embryos were placed in vitrification medium which consisting of BM supplemented with 16.5% DMSO, 16.5% EG and 0.5 M sucrose at room temperature for 1 min. Finally, 2-3 embryos were placed on a Cryotop (Kitasato, Japan) sheet in a small volume <1 µl within 1 min in a vitrification medium. The Cryotop was directly plunged into liquid nitrogen. Vitrified embryos were stored in a liquid nitrogen tank for at least 1 week.

3.6.2 Warming technique

Vitrified embryos were warmed by directly placed the tip of Cryotop in 2.5 ml BM supplemented 1M sucrose on 35 mm culture dish at 38.5 °C for 1 min. The embryos were then transferred to a new medium drop to reduce concentrations of sucrose in BM in a stepwise warmer (0.5, 0.25 and 0 M for 3, 5 and 5 min, respectively) and then cultured in CR1aa supplemented with 5% FBS (post-warming solution) and either 1 μM resveratrol (Gaviria et al., 2019; Torres et al., 2018) for treatment group and without resveratrol for the control group. After 2 hr of incubation under humidified atmosphere 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C of embryos morphology and viability were examined under an inverted microscope. After that vitrified-warmed blastocysts from all groups were cultured under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C for 24 hr embryos were examined under inverted microscope and recorded the development to expanded, hatching and hatched blastocyst stages. In each group, 1 to 15 blastocysts were examined for each experimental replication.

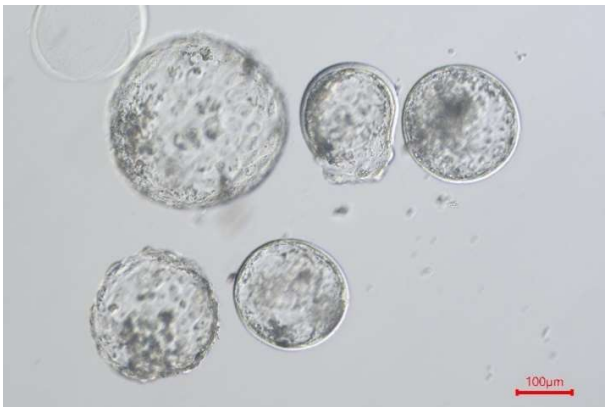

Figure 3.3 Expanded hatching and hatched embryos after 24h warm.

3.7 Blastocyst staining

Fresh and vitrified-warmed blastocysts were stained with 0.1 mg/ml propidium iodide (PI) and 0.2% Triton X-100 in mDPBS supplemented with 0.1%PVP for 1 minute, then transferred to 25 μg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) in 99.5% ethanol (EtOH) for 5 min, then mounted on glass slide with glycerol. The embryos were observed trophectoderm (TE) and inner cell mass (ICM) under UV light with excitation at 330-385 nm and emission at 420 nm using an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan). ICM cells were stained with blue-colored Hoechst 33342 while TE cells

exhibited red or pink color of PI. Digital images of each embryo were captured using a Nikon Eclipse E600 camera (Tokyo, Japan), and cell numbers were subsequently counted using NIH ImageJ software (v. 1.52). In each group, 1 to 7 blastocysts were examined for each experimental replication.

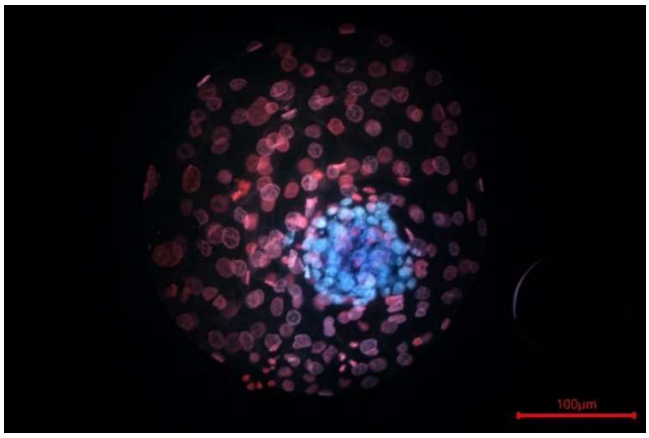

Figure 3.4 TE and ICM cells of blastocyst after PI and Hoechst 33342 staining.

3.8 Evaluation of gene expression in blastocysts

Five blastocysts were collected from each group and washed three times in PBS + 1 mg/ml polyvinyl alcohol (PVA). Then, the blastocysts were washed three times in sterilized milli-Q water. The blastocysts from each group were placed in a microcentrifuge tube containing 5 µl of sterilized milli-Q water and stored at -80 ^{O}C until use.

Total RNA from blastocysts was isolated using the FavorPrep Tissue Total RNA Mini Kit (Favorgen Biotech Corp., PingTung, Taiwan), following the manufacturer's instructions. The RNAs were subsequently reverse transcribed using an iScriptTM reverse transcription (RT) supermix kit (Biorad, Hercules, California, USA) for cDNA synthesis. Gene expressions of blastocysts were evaluated using KAPA SYBR FAST qPCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Gene expression was examined with a QuantStudio 5 real-time PCR system (QuantStudio 5, Applied Biosystems, Carlsbad, CA, USA). Melting curve analysis was also performed to specificity of the specific primers (Table 3.1). *GAPDH* was used as a reference gene to normalize the target genes. The expression fold change was calculated relative to the control group (IVC-). qPCR was performed in triplicate and statistical analysis was performed using the $2^{-\Delta\Delta CT}$ method.

Table 3.1 Primer for qPCR

Gene	Accession no.	Primer sequences	Product size (bp)	References
*GAPDH**	NM_001034034.2	F-CTCCCAACGTGTCTGTTGTG R-TGAGCTTGACAAAGTGGTCG	222	Sprícigo et al. (2017)
BCL2	NM_001166486.1	F-ACTATAAGCTGTCGCAGCG R-TACAGCTCCACAAAGGCGTC	517	Torres et al. (2018)
BAX	NM_173894.1	F-TCTGACGGCAACTTCAACTG R-TCGAAGGAAGTCCAATGTCC	135	Torres et al. (2018)
SIRT1	NM_001192980.3	F-AGAGGCGGTTGAAAGATGGC R-CTGGATAGGCCCTGTAAGCC	314	Gaviria et al. (2019)
FOXO3A	NM_001206083.1	F-CTGCTGACTCCATGATCCCC R- AGTGAGCCGTTTGTCTGGAG	380	Gaviria et al. (2019)
PNPLA2	NM_001046005.2	F-TGCCAGTACCTGATGATACGC R-CCTCCCATTTGGCCAGTAC	193	Gaviria et al. (2019)

*Endogenous reference gene. Abbreviations: F, forward primer; R, reverse primer.

3.8 Statistical analysis

Results were analyzed using t-test and one-way analysis of variance (ANOVA), followed by Tukey-Kramer Honest Significant Difference (HSD) as a post hoc test using SPSS software (version 26.0; SPSS, Inc., Chicago, Illinois, USA). $P < 0.05$ was defined as the significance level.