THE EFFECT OF RESVERATROL IN CULTURE MEDIUM AND VITRIFICATION SOLUTION ON DEVELOPMENTAL COMPETENCE AND GENE EXPRESSION OF MOUSE BLASTOCYSTS

Natchanok Puangjit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2019

ผลของสารเรสเวอราทรอลในน้ำยาเลี้ยงและน้ำยาแช่แข็งต่อความสามารถในการพัฒนาและการแสดงออกของยีนของตัวอ่อนหนูเม้าส์ระยะบลาสโตซีส

นางสาวณัฐชนก พวงจิตร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2562

THE EFFECT OF RESVERATROL IN CULTURE MEDIUM AND VITRIFICATION SOLUTION ON DEVELOPMENTAL COMPETENCE AND GENE EXPRESSION OF MOUSE BLASTOCYSTS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Apichat Boontawan)
Chairperson

(Assoc. Prof. Dr. Rangsun Parnpai)
Member (Thesis Advisor)

(Assoc. Prof. Dr. Mariena Ketudat-Cairns)
Member

(Assoc. Prof. Flt. Lt. Dr. Kontorn Chamniprasart)
Vice Rector for Academic Affairs and Internationalization

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural Technology

ณัฐชนก พวงจิตร์ : ผลของสารเรสเวอราทรอลในน้ำยาเลี้ยงและน้ำยาแช่แข็งต่อความสามารถในการพัฒนาและการแสดงออกของยีนของตัวอ่อนหนูเม้าส์ระยะบลาสโตซีส (THE EFFECT OF RESVERATROL IN CULTURE MEDIUM AND VITRIFICATION SOLUTION ON DEVELOPMENTAL COMPETENCE AND GENE EXPRESSION OF MOUSE BLASTOCYSTS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 87 หน้า.

การผลิตตัวอ่อนในหลอดแก้ว (*In vitro* embryo production) เป็นเครื่องมือที่มีประโยชน์ในการศึกษาการพัฒนาตัวอ่อนระยะก่อนฝังตัว อย่างไรก็ตามสภาวะแวดล้อมของการเลี้ยงตัวอ่อนในหลอดแก้วนั้นยังคงเป็นสภาพแวดล้อมที่ไม่เหมาะสมเพียงพอ ดังนั้นการศึกษาครั้งนี้จึงประเมินผลของสารเรสเวอราทรอล (resveratrol) ที่มีศักยภาพเป็นสารต้านอนุมูลอิสระต่อการพัฒนาของตัวอ่อนสี่เซลล์ของหนูเม้าส์ การแช่แข็งตัวอ่อนระยะบลาสโตซิสเพื่อทดสอบความสามารถในการทนทานต่อการแช่แข็ง และการเปลี่ยนแปลงของการแสดงออกของยีนอะพอพโทซิสและยีนที่เกี่ยวข้องกับการฝังตัวในมดลูกของตัวอ่อน

การทดลองที่หนึ่ง เพื่อทดสอบผลของการเติมสารเรสเวอราทรอลความเข้มข้นต่างๆ ในน้ำยาเลี้ยง (potassium simplex optimization medium, KSOM) ต่อความสามารถในการพัฒนาของตัวอ่อน ผลการศึกษาพบว่าอัตราการพัฒนาไปสู่ตัวอ่อนระยะบลาสโตซิสที่ความเข้มข้นของสารเรสเวอราทรอล 0.5 และ 1 ไมโครโมลาร์ (ร้อยละ 100 และร้อยละ 96.6 ตามลำดับ) ไม่มีความแตกต่างทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม 0 ไมโครโมลาร์ (ร้อยละ 100) นอกจากนี้ในกลุ่มที่เติมสารเรสเวอราทรอลความเข้มข้น 0.5 และ 1 ไมโครโมลาร์ (ร้อยละ 74.2 และร้อยละ 70.8 ตามลำดับ) พบว่ามีความสามารถของการพัฒนาของตัวอ่อนไปเป็นระยะบลาสโตซิสพร้อมฝังตัว (hatched blastocyst) เพิ่มขึ้นอย่างมีนัยสำคัญ ($P<0.05$) เมื่อเปรียบเทียบกับกลุ่ม 0 ไมโครโมลาร์ (ร้อยละ 52.8)

การทดลองที่สอง เพื่อตรวจสอบผลของการเติมสารเรสเวอราทรอลในน้ำยาเลี้ยง KSOM ในช่วงก่อนและหลังการแช่แข็งด้วยวิธีวิทริฟิเคชั่นต่อความสามารถในการกลับคืนสู่สภาพเดิมและความสามารถในการพัฒนาของตัวอ่อน โดยการศึกษาพบว่าไม่มีความแตกต่างในอัตราการรอดชีวิตของตัวอ่อนหลังการแช่แข็งด้วยวิธีวิทริฟิเคชั่นในทุกกลุ่มการทดลองทุกกลุ่มทดลองให้ ผลการรอดชีวิตเป็น 100% และที่น่าสนใจหลังการเติมสารเรสเวอราทรอลความเข้มข้น 0.5 ไมโครโมลาร์ และทำการแช่แข็งด้วยวิธีวิทริฟิเคชั่น (0.5 ไมโครโมลาร์ resveratrol/Vitrified) (ร้อยละ 73.7) พบว่ามีนัยสำคัญ ($P<0.05$) ในการเพิ่มความสามารถในการทนทานต่อการแช่แข็ง

โดยมีค่าอัตราการพัฒนาของตัวอ่อนไปเป็นระยะบลาสโตซิสพร้อมฝังตัวสูงที่สุด ดังนั้นการเติมสารเรสเวอราทรอลความเข้มข้น 0.5 ไมโครโมลาร์ เป็นความเข้มข้นที่เหมาะสมที่สุดสำหรับศักยภาพในการพัฒนาของตัวอ่อนทั้งตัวอ่อนหนูเม้าส์ที่ผ่านและไม่ผ่านการการแช่แข็งด้วยวิธีวิทริฟิเคชั่น

การทดลองที่สาม เพื่อประเมินประสิทธิภาพของการเติมสารเรสเวอราทรอลในน้ำยาเลี้ยงตัวอ่อน KSOM และ/หรือ น้ำยาแช่แข็งวิทริฟิเคชั่นต่อความสามารถในการอยู่รอดต่อความเย็นและความสามารถในการพัฒนาของตัวอ่อนหนูเม้าส์ พบว่าการเติมสารเรสเวอราทรอลความเข้มข้น 0.5 ไมโครโมลาร์ ในน้ำยาเลี้ยง KSOM มีอัตราการพัฒนาของตัวอ่อนไปเป็นระยะบลาสโตซิสพร้อมฝังตัวเพิ่มขึ้นอย่างมีนัยสำคัญ ($P<0.05$) เมื่อเปรียบเทียบกับทุกกลุ่มที่ไม่มีการเติมสารเรสเวอราทรอลในน้ำยาเลี้ยง KSOM

การทดลองที่สี่ การเปลี่ยนแปลงการแสดงออกของยีนโดยวิเคราะห์ระดับของการถอดรหัสของยีนอะพอพโทซิสและยีนที่เกี่ยวของกับการฝังตัวในมดลูกของตัวอ่อน ผลการศึกษาพบว่าการเติมสารเรสเวอราทรอลความเข้มข้น 0.5 ไมโครโมลาร์ ในน้ำยาเลี้ยง KSOM ไม่ได้เปลี่ยนการแสดงออกของยีน *Bax* แต่สามารถเพิ่มการแสดงออกของยีน *Bcl-2* และ *ErbB4* อย่างมีนัยสำคัญ ($P<0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุมที่ไม่มีการเติมสารเรสเวอราทรอลในน้ำยาเลี้ยง KSOM

ในการศึกษาครั้งนี้สรุปได้ว่า การเติมสารเรสเวอราทรอลในน้ำยาเลี้ยง KSOM ในความเข้มข้นต่ำ (0.5 ไมโครโมลาร์) ช่วยเพิ่มความสามารถในการพัฒนาของตัวอ่อนของหนูเม้าส์ โดยแสดงให้เห็นจากประสิทธิภาพในการพัฒนาของตัวอ่อนไปเป็นระยะบลาสโตซิสพร้อมฝังตัวและเพิ่มความสามารถในการทนทานต่อการแช่แข็ง ทั้งนี้ความสัมพันธ์ของการเติมสารเรสเวอราทรอลและกระบวนการแช่แข็งตัวอ่อนโดยวิธีวิทริฟิเคชั่นสามารถมีผลต่อการเปลี่ยนแปลงการแสดงออกของ *Bax*, *Bcl-2* และ *ErbB4* ที่เกี่ยวข้องกับการตายของเซลล์และการฝังตัวในมดลูกของตัวอ่อน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา _____

NATCHANOK PUANGJIT : THE EFFECT OF RESVERATROL IN CULTURE MEDIUM AND VITRIFICATION SOLUTION ON DEVELOPMENTAL COMPETENCE AND GENE EXPRESSION OF MOUSE BLASTOCYSTS : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 87 PP.

RESVERATROL/*IN VITRO* CULTURE (IVC)/VITRIFICATION/MOUSE EMBRYO/GENE EXPREESION

In vitro embryo production is a valuable tool to study preimplantation embryonic development. However, *in vitro* environments provide a suboptimal environment. This study, therefore, evaluated the effect of resveratrol, an antioxidant, on the development of mouse 4-cell embryo, blastocyst vitrification in terms of cryotolerance and alternation of apoptotic and implantation genes expression.

Firstly, the effects of different concentrations of resveratrol supplementation in *in vitro* culture medium (potassium simplex optimization medium, KSOM) on mouse embryo developmental competency were determined. The rate of embryos developed to the blastocyst stage in 0.5 and 1 μM resveratrol groups (100% and 96.6%, respectively) were not significantly different when compared with the 0 μM resveratrol group (100%). Additionally, the medium containing 0.5, 1 μM resveratrol (74.2% and 70.8%, respectively) significantly improved ($P < 0.05$) the rate of embryo developed to the hatched blastocyst stage, compared with the 0 μM resveratrol group (52.8%). Secondly, the effects of resveratrol supplementation in KSOM culture medium during pre- and post-vitrification on retrieval efficiency and embryo development were investigated. There were no significant differences in the post-vitrification survival

rates of mouse blastocyst embryos in all treatments. They were 100% in all treatments. Interestingly, the treatment with 0.5 μM resveratrol/Vitrified (73.7%) significantly improved ($P<0.05$) the cryotolerance, as indicated by the highest rate of embryo developed to the hatched blastocyst stage following post-warming culture. Therefore, 0.5 μM resveratrol was the most suitable for improving developmental potential of fresh and vitrified mouse embryos. Thirdly, the effects of resveratrol supplementation in KSOM culture medium and/or vitrification solution on cryo-survivability and developmental competency of vitrified mouse embryos were determined. Supplementation of 0.5 μM resveratrol in KSOM significantly enhanced ($P<0.05$) the rate of embryo developed to the hatched blastocyst stage when compared with non-supplemented resveratrol group. Lastly, expression levels of genes involved in apoptosis and implantation were analyzed. The results showed that the addition of 0.5 μM resveratrol to the KSOM medium did not alter the expression of *Bax* in fresh hatched blastocysts whereas the expression of *Bcl-2* and *ErbB4* were up significantly ($P<0.05$), compared with the control group without resveratrol supplementation in KSOM. In conclusion, these results demonstrated that low levels (0.5 μM) of resveratrol during *in vitro* culture (KSOM) improved developmental competency of mouse embryos, indicated by their effective development of mouse hatched blastocysts with enhanced cryotolerance. The association of resveratrol supplementation and the vitrification process can alter the expression of *Bax*, *Bcl-2* and *ErbB4* genes related to apoptosis and implantation in the developing embryos.

School of Biotechnology

Academic Year 2019

Student's Signature Natchanok P.

Advisor's Signature

ACKNOWLEDGEMENTS

I would like to thank Suranaree University of Technology, Nakhon Ratchasima, for providing me with a scholarship to support my Master degree study.

I would first like to express my sincere gratitude to my thesis advisor Assoc. Prof. Dr. Rangsun Parnpai for his kind, excellent supervision, precious guidance, immense knowledge and constant encouragement. His guidance helped me in all time of research and writing of this thesis. Without his precious support it would not be possible to conduct this thesis research. I am most grateful for his advice on animal biotechnology to improve my research skills for future challenges.

My deep appreciation is also to my thesis committee: Assoc. Prof. Dr. Apichat Boontawan and Assoc. Prof. Dr. Mariena Ketudat-Cairns for letting my defense be an enjoyable moment, and for their valuable comments, suggestion, and encouragement.

I wish to extend my special warmest thanks to members of Embryo Technology and Stem Cell Research Center (ESRC), Suranaree University of Technology, including Dr. Kanokwan Srirattana, Dr. Ton Yoisungnern, Dr. Sumeth Imsoontornruksa, Dr. Yuanyuan Liang, Ms. Sutatip Jirundorn, Mr. Vajira Wipassa, Ms. Sujitra Khampang, Ms. Thanya Pranomphon, Mr. Thatawat Yodrug, Ms. Praneet Wangthaisong, Ms. Chonthicha Phopan, Mrs. Nguyen Thi Hong, Ms. Nguyen Thi Thanh Nga, and Ms. Worawalan Samruan for their kindness, valuable suggestion, friendship, encouragement and all support for me in numerous ways during my course of Master study.

I would especially like to thank Laboratory animal center, Suranaree University of Technology, including Mr. Watchara Wongviriya, Dr. Rangsirat Wongsan, and Ms. Sirilak Somredngan and all cleaning staffs. They have been there to support me when I took care of

mice, the animal model in my thesis research. Words can not express how grateful I am to the mice, donating its body for this study. Especially, I would like to give my sincere apologies and forgiveness for the mice. Without their dedication, this work would not have been possible.

Last but not the least, I would also like to give a heartfelt thank you to my family and Mr. Kitikon Promket who has been by my side throughout this journey for always believing in me and encouraging me to follow my dreams. Without them, I would not have had the courage to embark on this journey in the first place.

Natcahnok Puangjit

CONTENTS

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (continued)

Figure **Page**

LIST OF ABBREVIATIONS

ARTs	=	Assisted reproductive technologies
AI	=	Artificial insemination
ATP	=	Adenosine triphosphate
AQP3	=	Aquaporin 3
BM	=	Base medium
BSA	=	Bovine serum albumin
cDNA	=	Complementary DNA
CPA	=	Cryoprotectant
Ct	=	Cycle threshold
DMSO	=	Dimethylsulphoxide
EG	=	Ethylene glycol
ES	=	Equilibration solution
FBS	=	Fetal bovine serum
H_2O_2	=	Hydrogen peroxide
hCG	=	Human chorionic gonadotropin
ICSI	=	Intracytoplasmic sperm injection
IU	=	International unit
IP	=	Intraperitoneal
IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilization
IVM	=	*In vitro* maturation

LIST OF ABBREVIATIONS (Continued)

IVP	=	*In vitro* production
KSOM	=	Potassium simplex optimization medium
LN_2	=	Liquid nitrogen
mMTF	=	Modified mouse tubal fluid
mtDNA	=	Mitochondrial DNA
PBS	=	Phosphate-buffer saline
PMSG	=	Pregnant mare serum gonadotropin
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
RT	=	Reverse transcription
TCM-199	=	Tissue culture medium-199
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
ºC	=	Degree celsius
µM	=	Micromolar
µl	=	Microliter

CHAPTER I

INTRODUCTION

1.1 Rationale and background

Assisted reproductive technologies (ARTs) have become integral routine techniques for commercially valuable animal production and human infertility clinics over the past half century (Seidel et al., 2015; Moussa et al., 2014). ARTs are *in vitro* manipulation techniques by handling of sperms, oocytes and embryos outside the body from human or animals to achieve pregnancy such as artificial insemination (AI), *in vitro* production (IVP), intracytoplasmic sperm injection (ICSI) and cryopreservation which consists of slow freezing and vitrification (reviewed by Hafez et al., 2015; Scaravelli and Spoletini, 2015). Several studies have successfully improved ART efficiencies, resulting in increasing number of viable oocytes and transferable embryos (reviewed by Milachich and Shterev, 2016). However, ART efficiency are affected by environmental surrounding during gamete/embryo *in vitro* development which might alter health of the offspring (Padhee et al., 2015).

Cryopreservation plays a crucial role in embryo transfer technology, which enables transportation of embryos to distant places, long-term storage, widespread utilization of genetically valuable animal embryos, and increases the opportunities of pregnancy (Hayashi et al., 2018; Hara et al., 2018). Cryotop vitrification has been widely used for the cryopreservation of oocytes and embryos in different species such as bovine (Punyawai et al., 2015; Leme et al., 2016), porcine (Vallorani et al., 2012;

Wu et al., 2016), goat (Morató et al., 2011; Srirattana et al., 2013), mouse (Zhang et al., 2009; Ito et al., 2010; Almasi-turk et al., 2013; Roy et al., 2014) and human (Kuwayama et al., 2007; Lin et al., 2010). However, vitrification has detrimental effects on embryos such as inducing reduction-oxidation (redox) state alterations and decreasing glutathione (GSH) content which depend on concentration and types of cryoprotectants, times and temperature, samples containers or adding additives (Castillo-Martín et al., 2014). This imbalance between reactive oxygen species (ROS) production and cellular antioxidant defenses may cause damages of cytoskeleton structure, membrane lipids, proteins and DNA, which may induce apoptotic responses and reduce embryo viability (Castillo-Martín et al., 2014; Dehghani-Mohammadabadi et al., 2014; Hosseini et al., 2009; Gaviria et al., 2019). However, this disadvantageous effect can reasonable support with theory, known as "Embryo Cryo-treatment" by the role of hormetic-response, induces protective signals (Vladimirov et al., 2017). The current hypothesis stated that "Cryopreservation is not only a technology used for storing embryos, but also a method of embryo treatment that can potentially improve the success rate in infertile couples" (reviewed by Vladimirov et al., 2017). Thus, a major challenge in the current cryopreservation technology is the compromised quality of oocytes and embryos after warming.

In vitro embryo culture is a useful tool for preimplantation embryonic development study and necessary for the IVP of transferable embryos (Lee et al., 2010). The preimplantation embryo is highly influenced by external factors from the *in vitro* culture conditions (Rizos et al. 2002). Several studies have reported that manipulating the gametes and culturing embryos in *in vitro* conditions can cause the significant oxidative damages due to excessive reactive oxygen species (ROS) production,

resulting in abnormally embryonic development and may induce apoptosis responses (Agarwal and Majzoub, 2017; Duranthon et al., 2008; Rizos et al. 2008; Zhao et al., 2016). In order to improve the quality of IVP embryos, different strategies have been proposed, including the use of antioxidant molecules that may achieve better performance of vitrification and reduce oxidative stress during *in vitro* culture conditions (Hosseini et al., 2009).

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin produced by several plants including grapes, plums, and peanuts. Resveratrol responses to injury or when the plant is under attack by pathogens such as bacteria or fungi, moreover, it is potent antioxidant and anti-apoptotic (Baur and Sinclair, 2006; Fremont et al., 2000). Its antioxidant effects have been confirmed in various cellular models such as hepatocytes (Rubiolo et al. 2008), brain and tumor cells (Gambini et al. 2015) by reducing free radical production and inducing the expression of antioxidant genes such as catalase, manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPX1). Several studies have identified the biologic functions and numerous beneficial activities of resveratrol for mammalian reproduction (Huang et al., 2013; Liu et al., 2017; Liang et al., 2018). Supplementation of resveratrol has been used in *in vitro* maturation (IVM) (Comizzoli et al., 2009; Kwak et al., 2012; Liu et al., 2013; Mukherjee et al., 2014; Santos et al., 2018; Sprícigo et al., 2017), *in vitro* culture (IVC) (Lee et al., 2010; Salzano et al., 2014; Hayashi et al., 2018) and vitrification-warming media (Giaretta et al., 2013; Wang et al., 2018). The beneficial effects of resveratrol on *in vitro* environmental system and vitrification technique have also been widely investigated in both oocytes and embryos, resulting in improvement of survival and developmental rates in many species, including cats (Comizzoli et al., 2009), pigs

(Lee et al., 2010; Kwak et al., 2012; Giaretta et al., 2013; Santos et al., 2018), goats (Mukherjee et al., 2014), cattle (Salzano et al., 2014; Sprícigo et al., 2017; Abe et al., 2017; Hayashi et al., 2018), and mouse (Liu et al., 2013; Wang et al., 2018). For instance, supplementing porcine embryo culture media with 0.5 μM resveratrol had beneficial effects on development competency such as increasing blastocyst formation rate and improving embryo quality in terms of total cell number in the blastocysts (Lee et al., 2010). Moreover, supplementation with 2 μM resveratrol in various phases during IVM and vitrification-warming procedure could improve the resistance to cryoinjury of porcine metaphase II (MII) oocytes and reduce apoptotic process (Giaretta et al., 2013). Additionally, supplementing culture medium with 0.5 μM resveratrol increased expression levels of Surtuin family member 1 gene (*SIRT1*) which is involved in mitochondrial ATP generation, decreased lipid content, improved survival and embryo development rates following cryopreservation in bovine embryos (Abe et al., 2017). Similarly, a previous report showed that resveratrol could reduce cryopreservation-induced damage during mouse oocyte vitrification, decrease oxidative stress and moderate the abnormal mitochondrial distribution pattern, which could result in increasing blastocyst formation rate and cell number of blastocysts (Wang et al., 2018). There are limiting information about the effects of resveratrol supplementation in culture medium and/or vitrification medium, especially mouse species. Taken together, this study was to determine whether resveratrol supplementation in culture medium and/or vitrification solutions could improve *in vitro* embryo development, cryotolerance and alter gene expression profiles of mouse embryos.

1.2 Research objectives

1. To examine the effects of resveratrol supplementation in culture medium (KSOM) on mouse embryo developmental competency.

2. To investigate the effects of resveratrol supplementation in KSOM culture medium on cryo-survivability and developmental competency of vitrified mouse embryos.

3. To evaluate the effects of resveratrol supplementation in KSOM medium and/or vitrification solution on cryo-survivability and developmental competency of vitrified mouse embryos using Cryotop-vitrification method.

4. To analyze the expression levels of genes that involve in apoptotic and implantation gene on efficiency of resveratrol supplementation in KSOM medium and/or vitrification medium.

1.3 Research hypotheses

1. Resveratrol supplementation in KSOM medium could enhance embryo developmental competency.

2. Resveratrol supplementation in KSOM medium at pre- and post-vitrification could increase the survival rate and embryo developmental competency after post-warmed.

3. Resveratrol supplementation in KSOM medium and/or vitrification solution could improve the cryotolerance, enhance the survival rate and embryo developmental competency after post-warmed.

4. Resveratrol supplementation in KSOM medium and/or vitrification medium could increase the expression levels of implantation gene (*ErbB4*) as well as anti-

apoptotic activity gene (*Bcl-2*). Whereas the transcription level of pro-apoptotic activity gene (*Bax*) could be decreased after vitrification.

1.4 Scope and limitations of the study

1. The effects of various concentrations of resveratrol supplementation in KSOM medium were examined by *in vitro* culture of *in vivo* 4-cell embryos until the hatched-blastocyst stage.

2. The effects of different concentrations of resveratrol supplementation in KSOM medium at pre- and post-vitrification on survival efficiency and developmental potential of vitrified mouse embryos.

3. The effects of resveratrol supplementation in KSOM medium and/or vitrification solution on vitrification efficiency and development potential of vitrified embryos were cultured from *in vivo* 4-cell embryos until blastocyst stage and used Cryotop as a device for blastocyst mouse embryos vitrification. After warming, vitrified-warmed blastocysts were investigated survival rate and *in vitro* culture until develop to hatched-blastocyst stage.

4. The effects of resveratrol supplementation in KSOM medium and/or vitrification medium on expression level of genes (*ErbB4, Bcl-2* and *Bax*) of hatched-blastocyst embryos were analyzed using the quantitative polymerase chain reaction (qPCR).

1.5 Research methodology

1.5.1 Instrumentation

All equipment and materials were provided by Embryo Technology and Stem Cell Research Center (ESRC) and Laboratory Service Unit, The Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

1.5.2 Location of research

The experiments were carried out at Laboratory animal center and ESRC, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

CHAPTER II

LITERATURE REVIEW

2.1 The mouse as an animal model

The mouse (*Mus musculus*) is the most commonly used vertebrate species, popular because of their availability, size, low cost, ease of handling, and fast reproduction rate (Willis-Owen and Flint, 2006). Mouse is a powerful model for studying mammalian embryo development due to extensive genome similarities between mouse and human (Mandawala et al., 2016) and an especially important model organism for human biology, and human embryos during preimplantation development and implantation are scarce and difficult to obtain for both ethical and technical reasons (Minoru et al., 2006). Additionally, mouse embryos are also more readily available than those of many agriculturally important animal species (Polge et al., 1978; Mukaida et al., 1998).

The B6D2 F1 hybrid mice stain results from mating mice of two inbred strains, C57BL/6J mice and DBA/2J mice (Fig. 2.1). The C57BL/6J mice (also known as Black 6, B6, B6J and C57 Black) is the most widely used inbred strain due to strain stability and easy breeding, and the first to have its genome sequenced (Johnson, 2012). Then, the DBA/2J mice (also known as D2J, D2 and DBA2) is a widely used inbred strain. Obviously, the B6D2F1/J mice (also known as B6D2) are heterozygous for B6 and D2 alleles at all loci in their genome as they are from C57BL/6J (B6) female mice and DBA/2J (D2) male mice. This strain widely uses for transgenic/knockout creation,

as well as other deleterious mutations, research applications include radiation and behavioral research, and safety and efficacy testing, bioassays of nutrients, drugs, pathogens, and hormones. (The Jackson Laboratory Handbook on Genetically Standardized Mice, 2009). In addition, the reproductive research usually use B6D2 F1 strain because the oocytes are very translucent and the metaphase spindle is easy to see (Wakayama et al., 2010). Especially, B6D2 F1 hybrid mice stain can prevent 2-cell block during embryo development and useful for *in vitro* culture (Minoru et al., 2006). Therefore, the numerous experiments have been conducted and applied this strain on many fields of reproduction such as *in vitro* production (IVP) (Jo et al, 2011; Lee et al, 2015; Yoisungnern et al, 2015; Zhang et al, 2016; Zhou et al, 2016; Youm et al, 2017), cloning technique (Wakayama et al., 2010), cryopreservation of oocyte (Jo et al, 2011; Jo et al, 2012; Lee et al, 2015; Zhang et al, 2016) and embryos (Youm et al, 2017).

Figure 2.1 The strains of mouse. (A) C57BL/6NJc1 (B6) female mice, (B) DBA/2NJc1 (D2) male mice, (C) B6D2 F1 hybrid mice (The Jackson Laboratory, 2018).

2.2 Cryopreservation

The cryopreservation is the most important for reproductive field nowadays. Moreover, cryopreservation is a useful tool for collection the sample to keep long time for the future or apply to a research work (Vladimirov et al., 2017). Cryopreservation is a technique which biological cells or tissues are preserved at sub-zero temperatures

(freezing), resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods, and subsequent warming of biological samples without loss of viability (Mandawala et al., 2016). The application of cryopreservation in assisted reproductive technology (ART) include the freezing of gametes, embryos, and primordial germ cells (Chian and Quinn, 2010). Cryopreservation techniques for mammalian oocytes and embryos have rapidly progressed, emphasizing their importance in various ART. Considerable progress has been achieved in the development and application of the cryopreservation of mammalian oocytes and embryos, including preservation of the reproductive potential of patients who may become infertile, establishment of cryopreserved oocyte banks, and transport of oocytes and embryos internationally for both human and veterinary medicine (Moussa et al., 2014).

Cryopreservation techniques, there are two prominent strategies that may fulfill the requirements for successful cryopreservation of mammalian oocytes and embryos: conventional slow freezing and vitrification or ultra-rapid freezing. In both methods attempt to decrease formation of ice crystals in and out of the cells during freezing and fusion in order to increase survival rate (Shoraki and Akhondi, 2016). Conventional slow freezing technique involves stepwise programmed decease in temperature. The procedure is lengthy and requires the using of expensive equipment (Michal et al., 2016). This process does not exclude ice crystal formation, which can extremely deleterious (Pegg, 2005). Limiting factor of method is ice crystal formation that drastically reduce survival of oocytes and embryos. Vitrification process produces a glass solidification of living cells, which completely avoids ice crystal formation. Interestingly, vitrification is very simple, fast and cost-effective process, but the skills

to perform require good manual training (Vajta, 2006). Vitrification requires a greater amount of cryoprotectant, toxicity effect to their environment. However, it was claimed higher survival rate after using vitrification instead of conventional slow freezing (Fadini et al., 2009). There are a number of studies that compared conventional slow freezing and vitrification. Several studies have indicated that vitrification of oocytes (Cobo et al., 2011; Levi Setti et al., 2014) and embryos of all stages has been shown to be superior to conventional slow freezing. For example, Herrero et al. (2011) established that cryopreservation of both human oocytes and blastocysts demonstrate pregnancy rates, when compared to those obtained through implantation of fresh samples and that vitrification, was preferable in terms of minimised cellular damage and higher post-warming survival rates, when compared to conventional freezing processes. The major consequences of the intracellular damage to oocytes and embryos from conventional slow freezing are decreasing survival rate and diminishing implantation potential and outcomes when compared to vitrification (Kuc et al., 2010; Li et al., 2014; Wang et al., 2012). These negative effects have limited the widespread use of conventional slow freezing techniques. While it seems that these is a gradual move toward more widespread use of vitrification, which methods is the best (Mandawala et al., 2016).

2.3 Vitrification

Vitrification is the solidification of a liquid by the extreme elevation in viscosity during cooling (Fahy et al, 1984). The method is known as a physical process by which a highly concentrated solution of cryoprotectant rapidly transforms into a glassy vitrified state, from the liquid phase by increase in the viscosity while cooling at a low

temperature (-196ºC). Thus, vitrification eradicates damage caused due to ice crystal formation during the cooling process (Almasi-turk et al., 2013). As a working model, the following equation has been proposed by Yavin and Arav (2007), which predicts the probability of successful vitrification based upon three major factors, to prevent the intracellular crystallization of water: 1) the viscosity of the sample that can be modulated by cryoprotectant (CPA) concentration; 2) cooling and warming rate; 3) sample volume:

$$\text{Probability of vitrification} = \frac{\text{Cooling and warming rate x Viscosity (CPA concentration)}}{\text{Volume}}$$

The vitrification technique can be divided into 2 parts: 1) Freezing or vitrification procedure; and 2) warming or thawing procedure (Kuwayama et al, 2005b). Freezing procedure induces precipitation of water into ice, leading to the separation of water from the dissolved substances. The presence of both intracellular ice crystals and a high concentration of solute can be lethal to the oocytes or embryos during the freezing procedure (Mazur et al, 1990). The presence of a suitable concentration of cryoprotectant in the freezing or vitrification solution usually considerably increases the cell's survival rates (Nakagata et al, 1989). The specific role of cryoprotectant takes part in lowering the freezing point and in reducing or preventing the formation of ice-crystals in aqueous solutions. The addition of cryoprotectant into solutions results in a significant increase of their osmolarity (Shaw et al, 1992). Therefore, the additional and removal of cryoprotectant from the oocytes or embryos create an osmotic imbalance across the membrane which may result in large volumetric changes (Hunter et al, 1995). The thawing or warming procedure is a rapid procedure with temperature changes. This procedure prevents the occurrence of re-crystallization, a process where

water enters the oocytes or embryos and transforms into a solid state (Hunter, 1995). During the thawing or warming procedure, the transfer of the oocytes or embryos from a solution containing a high concentration of cryoprotectant to an isotonic solution can also lead to a reverse osmotic shock or over-swelling (Fuller et al., 1984). An osmotic shock can be prevented by thawing the oocytes or embryos in a hypertonic solution containing a non-permeating cryoprotectant, such as sugar (Leibo et al., 1978). Various monosaccharides and disaccharides with sucrose being the most common sugar have been used in thawing or warming solution as an osmotic counterforce in restricting water permeation into the oocyte or embryos and in preventing a swelling injury (Nowshari et al., 1994).

The mechanism of vitrification is the use of a high concentration of cryoprotectant and an extremely rapid cooling/warming rates to pass through the glass transition temperature and avoid intracellular and extracellular ice formation (Chian et al., 2014). During vitrification step, an oocytes or embryos is exposed to equilibration Solution (ES) and undergoes osmotic change from hypertonic state to isotonic state. When osmotic pressure reaches equilibrium, the oocytes or embryos is placed in vitrification Solution (VS) containing higher concentrations to dehydrate. In the warming steps, the oocytes or embryos is rapidly warmed in thawing solution (TS) and undergo further dehydration. Stepwise reduction of sucrose concentrations, in dilution solution (DS) induces rehydration of the cell. During the incubation in wash solution (WS), the oocytes or embryos is pre-equilibrated before transferring to culture medium (Sakurai, 2017) (Fig. 2.2).

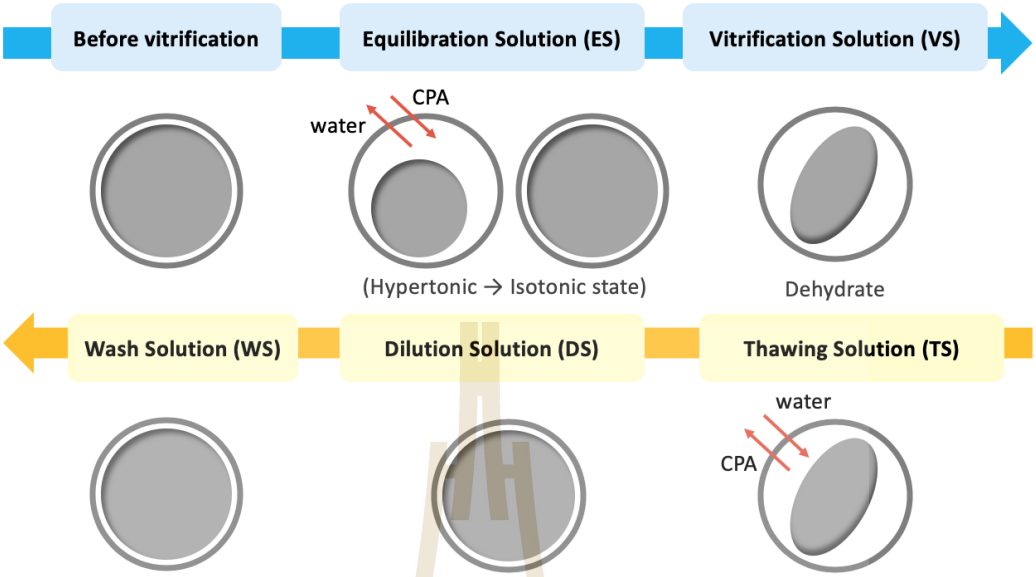

Figure 2.2 The mechanism of vitrification and warming procedure (Sakurai, 2017).

The vitrification techniques have been designed devices for vitrification to minimize the volume of CPA solution and allow the sample to be submerged quickly in liquid nitrogen (LN_2). The different carrier can generally be divided into two categories: 1) surface techniques and 2) tubing techniques (Saragusty and Arav, 2011). The surface techniques include electron microscope grid (Martino et al., 1996), Cryotop (Hamawak et al., 1999; Kuwayama et. al., 2005a), Cryoloop (Lane et al., 1999), Hemi-straw (Vanderzwalmen et al., 2000), solid surface (Dinnyes et al., 2000), nylon mesh (Matsumoto et al., 2001), Cryoleaf (Chian et al., 2005). To the tubing techniques belongs the plastic straw (Rall and Fahy, 1985), open-pulled straw (Vajta et al., 1998), closed pulled straw (Chen et al., 2001), flexipet-denuding pipette (Liebermann et al., 2002), superfine open-pulled straw (Isachenko et al., 2003), CryoTip (Kuwayama, 2007), pipette tip (Sun et. al., 2008), Cryopette (Portmann et al., 2010), Rapid-i (Larman et al., 2010). Each of carriers has its specific advantages. For

the surface methods, the size of the drop (0.1 mL) can be controlled, a high cooling rate is achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. The tubing systems have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle (Arav, 2014). Decreasing the vitrified volume and increasing the cooling rate allow a moderate decrease in cryoprotectant concentration so as to minimize its toxic and osmotic hazardous effects (Yavin et al., 2009). For example, Kuwayama et al. (2005a) showed no significant difference between blastocyst survival rates using the CryoTip^{TM} (closed system) and the Cryotop (available as either an open or closed carrier system). Comparisons were also made between conventional slow freezing and vitrification of human embryos, which indicated that vitrification was the most reliable (Kuwayama et al. 2005a). In addition, Kuwayama (2007) demonstrated similar results, cryopreservation of human blastocysts using the Cryotop method resulted in more live births when compared to any other vitrification systems. Moreover, Roy et al. (2014) showed that the new vitrification device, Gavi (Genea Biomedx) permitted the process to be further standardized and automated. Results to date demonstrated that mouse (zygotes, cleavage stage embryos and blastocysts) and human (blastocysts) vitrified samples using the Gavi technology produced similar results to those of the control samples which were vitrified using the manual Cryotop method that currently considered to be the gold standard for vitrification (Roy et al. 2014). Thus, the Cryotop method is an efficient technique for vitrification of oocytes and embryos.

Severe injuries within vitrified-warmed oocyte or embryo may occur at all phases of vitrification/warming procedures (Koutlaki et al, 2006). However, Ito, et al. (2010) evaluated the impacts of re-vitrification embryos (2-cell, 4-cell, morula and blastocyst

stages) on the same stage, resulting re-vitrification with Cryotop did not affect the developmental ability of mouse embryos (Ito, et al. 2010). Youm et al., (2017) investigated that in once, twice, or three-time vitrified mouse 8-cell stage embryos, survival rates, blastocyst-forming rates, the percentages of hatching/hatched blastocyst, and the cell counts were all similar when compared with non-vitrified control group and the mRNA expression levels of cold-inducible RNA-binding protein as cold-shock protein (*Cirbp*), caspase 3 as an apoptosis marker (*Casp3*), superoxide dismutase 1 (*Sod1*), glutathione peroxidase 3 (*Gpx3*) and catalase (*Cat*) as oxidative stress markers, were not affected. Therefore, repeatedly vitrified mouse 8-cell stage embryos well developed up to blastocyst stage without cryoinjury and without decrease of antioxidant-related genes (Youm et al., 2017). Moreover, Gharenaz, et al. (2016) reported that re-vitrification at 8-cell stage embryos had the same effect on developmental potential and re-vitrification could negative affect expressions of apoptotic and implanting genes by increasing expressions of *Bax* but decreasing expressions of *Bcl-2* and *ErbB4*. Members of *Bcl-2* gene family are known as an important role in regulating apoptosis. *Bcl-2* is known for its anti-apoptotic activity and responsibility in cell survival, whereas *Bax* is pro-apoptotic and responsible for the induction of cell death (Dhali et al., 2007; Gharenaz, et al. 2018). *ErbB4* is a gene involved in implantation which interacts with heparin binding-epithelial growth factor (HB-EGF) to mediate attachment of the blastocyst to uterine luminal epithelium (Gharenaz, et al. 2016). Therefore, several publications presumed that vitrification procedure could induce apoptosis in vitrified-warmed embryos, affect the expression of apoptosis related genes (Dhali et al, 2007; Anchamparuthy et al, 2007) and implanting genes (Gharenaz, et al. 2016).

2.4 Mouse blastocyst vitrification

Vitrification is a safe procedure for embryo cryopreservation, which has been carried out since 1990. Vitrification was successfully used for the first time to cryopreservation human cleavage-stage embryos, resulting in a live birth (Gordts et al, 1990). In development of *in vitro* techniques and together with single embryo transfer becoming greater demand for an efficient and reliable cryopreservation method for surplus embryos. Cryopreservation can be applied to the zygotes immediately after fertilization, at the pronuclear stage or embryos during early cleavage stages (2–8 cells) or at the expanded blastocyst stage. Vitrification of early stage embryo is acceptable and better alternative than slow rate freezing because of the higher survival rate and increased rates of pregnancy. Previous studies showed that the development competency of vitrified blastocysts by open-pulled straw was comparable to that of fresh embryo, which would imply that the early blastocyst stage in the most optimal stage for mouse embryo vitrification (Cuello et al, 2004; Zhou et al, 2005). However, cryopreservation of cleaved embryos is not so effective as vitrification of blastocyst (Michal et al, 2016). Blastocysts are the top embryos, have already overcome the development block associated with genomic activation, and possess relatively good development potential. The blastocyst stage is the final stage of development prior to implantation; therefore, it provides the best opportunity for screening the most developmentally competent embryos for transfer (Ling et al, 2009). Moreover, the advantage is containing numerous small cells; thus, the loss of some cells during freezing and thawing is probably less harmful for future development of the embryo. Furthermore, during extended cultivation, embryos with worse viability are arrested in

development and will not be cryopreserved. Therefore, vitrification of blastocyst stage embryos is of great importance in assisted reproduction.

The most suitable procedure for the vitrification of mammalian embryos differ among the developmental stages even in the same species (Kasai and Edashige, 2010). This specificity could be caused by the difference in their cryobiological properties of embryos among the developmental stages and species, such as the sensitivity to chilling, the sensitivity to the chemical toxicity of cryoprotectants, the permeability of the plasma membrane to water and cryoprotectants, and the tolerance to osmotic swelling and shrinkage (Edashige, 2016). Similarly, the permeability of the plasma membrane of embryos to water and cryoprotectants is the most important property because this property is closely related to major causes of cell injury during vitrification (Edashige, 2017). Such damage needs to be avoided to maintain the survival of embryo after vitrification. Therefore, the permeability of the plasma membrane markedly affects whether the conditions are suitable for vitrification.

There are two pathways for the movement of water and cryoprotectants across the plasma membrane of embryos. One is the simple diffusion through the lipid bilayer. The other is facilitated diffusion via channels called aquaporins (Kasai and Edashige, 2010). The major pathway for the movement of water across the plasma membrane can be deduced from the permeability to water (L_p) and its dependence on temperature (Arrhenius activation energy, E_a). In general, a L_p-value of higher than 4.5 μm/min/atm with an E_a-value of lower than 6 kcal/mol for the permeability is suggestive of the movement of water principally by facilitated diffusion via aquaporins. In contrast, a low L_p-value with an E_a-value that is higher than 10 kcal/mol for the L_p is suggestive of movement principally by simple diffusion across the plasma membrane (Verkman

et al., 1996). This criterion may be a rough estimate but is useful for deducing the principal pathway for the movement of water in the embryo. Therefore, permeability differ among the development stage of embryo can be classified into two types; the early stages (1-to-4 cell stage, early embryos), the morulae and blastocysts. The movement of mouse embryos in the early stages, the L_P-value in a hypertonic solution containing sucrose is low at 20-25°C (0.4-0.7 μm/min/atm) and the E_a-value is high (12-13 kcal/mol) (Pfaff et al., 1998; Edashige et al., 2006; Edashige, 2016). Therefore, it is suggested that water move through the early embryos principally by simple diffusion (Fig. 2.3A). In mouse morulae and blastocysts, in contrast, the L_P- value is high at 25°C (3.1-4.5 μm/min/atm) and the E_a-value is low (5.1-6.3 kcal/mol) (Edashige et al., 2006; Edashige, 2016), suggesting that water moves through the embryos principally by facilitated diffusion via water channels (Fig. 2.3B).

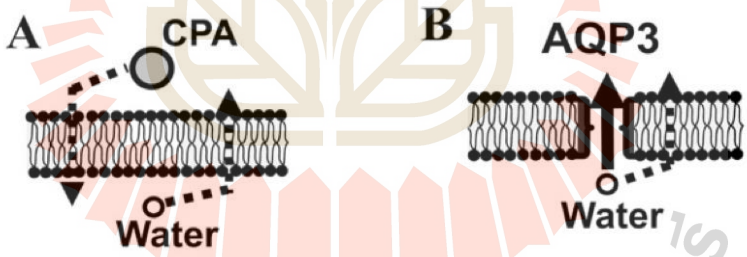

Figure 2.3 Schematic representation of the pathways for the movement of water across the plasma membrane of mouse early stages and morulae stages. A, the movement of water and cryoprotectant in an early stages by simple diffusion. B, the movement of water in a morula in a solution containing sucrose by facilitated diffusion through channel pathways. AQP3, aquaporin 3; open small circles, water molecules; shaded large circle, cryoprotectant molecules (CPA) (Edashige et. al., 2016).

A criterion for deducing the pathway for the movement of cryoprotectants in embryo has not been established, there is no quantitative value for evaluating the movement of cryoprotectants through the plasma membrane. However, considering the permeability to water, it would be reasonable to assume that a low level of permeability to cryoprotectants (P_S), with a high E_a-value for the permeability, is suggestive of the movement of cryoprotectants principally by simple diffusion across the plasma membrane and that a high P_S-value with a low E_a-value for the permeability is suggestive of the movement of cryoprotectants principally by facilitated diffusion through channels (Edashige, 2016). In this case, MII oocytes and morulae would be especially suitable for measuring change in volume, because they are a single mass and thus shrink and re-swell with less distortion (Kasai and Edashige, 2010). The permeability to ethylene glycol (P_{EG}) of oocytes is low at 25°C (0.6×10^{-3} cm/min) and the E_a-value is high (17 kcal/mol). Therefore, ethylene glycol would move through the oocytes (and probably early embryos) principally by simple diffusion. In contrast, the P_{EG}-value of morulae is quite high at 25°C (10×10^{-3} cm/min) and the E_a-value is low (9 kcal/mol). In addition, the high P_{EG}-value of morulae markedly decreases by suppressing the expression of aquaporin 3 (Edashige et al., 2007; 2016). Therefore, ethylene glycol moves through the morulae (and probably blastocysts) principally by facilitated diffusion via aquaporin 3 (Fig. 2.4A) The permeability to dimethyl sulfoxide (DMSO; P_{DMSO}) of oocytes is low at 25°C (1.0×10^{-3} cm/min) and the E_a-value is high (18 kcal/ mol). Therefore, DMSO would move through the oocytes (and probably early embryos) principally by simple diffusion. In contrast, the P_{DMSO}-value of morulae is higher at 25°C (3.0×10^{-3} cm/min) and the E_a-value is lower (12 kcal/mol), compared

to those of the oocytes (Edashige et al., 2007; 2016). Therefore, DMSO would move through the morulae principally by facilitated diffusion via channels. However, the suppression of aquaporin 3 in the morulae does not decrease the P_{DMSO}-value, suggesting that channels other than aquaporin 3 are involved in the facilitated diffusion of DMSO (Fig. 2.4B).

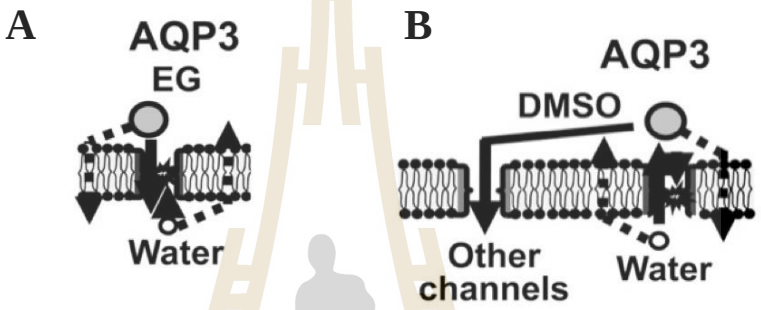

Figure 2.4 Schematic representation of the pathways for the movement of water and cryoprotectants across the plasma membrane of mouse early stages and morulae in the presence of cryoprotectants. A, the movement of water and ethylene glycol in a morula in a solution containing ethylene glycol. B, the movement of water and DMSO in a morula in a solution containing DMSO. AQP3, aquaporin 3; Other channels, DMSO-permeable channels; EG, ethylene glycol, open small circles, water molecules; shaded large circle, cryoprotectant molecules (CPA) (Edashige et. al., 2016).

To find optimal conditions for blastocyst vitrification, it is essential to identify the mechanism. Specifying the principle pathway for the movement of water and cryopreservation in oocytes and embryos at various stages will help in the selection of suitable cryoprotectants and optimal conditions for vitrification, for example the time, temperature, and number of steps for exposure of oocytes and embryos to the

cryoprotectant solution before freezing, and the procedure for removal of the cryoprotectant after warming (Kasai and Edashige, 2010). In order to design protocols for the vitrification of mammalian embryos, it would be important to consider the pathway of the movement of water and cryoprotectants for each stage. In vitrification, the time of exposure and temperature of the vitrification solution are important because the vitrification solution contains a high concentration of cryoprotectant and thus is highly toxic to oocytes and embryos. When water and cryoprotectants move through embryos principally by simple diffusion, the temperature and time of exposure to the vitrification solution are important because temperature affects the permeability to water and cryoprotectants. When water and cryoprotectants move through embryos principally by facilitated diffusion via channels, the amount of time of exposure to the vitrification solution is more important because the permeability is less affected by temperature. However, the exposure of embryos to the vitrification solution at a high temperature should be avoided because cryoprotectants are more toxic at higher temperatures.

Nowadays, many researchers are studying different methods to improve cryopreservation outcome by modification of essential factors (cryoprotectants, freezing rate, warming) and chemical supplementation (antioxidative treatment). For instance, Ling et al. (2009) reported the high survival rate and hatching rate of vitrified mouse blastocyst (96.5%, 81.0% respectively) by Cryotop as carrier, using freezing condition; equilibration solution (ES) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulphoxide (DMSO) for 10 minute, followed with vitrification solution (VS) containing 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l sucrose for 60 second at room temperature. After cryo-storage, warming condition by using a four-

step dilution procedure with sucrose (1.0, 0.5, 0 and 0 mol/l sucrose for 1, 3, 5 and 5 minutes respectively) at 37°C. In addition, Ghandy and Malekshah, (2017) showed comparison of vitrification on mouse 2-cell, 4-cell, 8-cell, morula and blastocyst using freezing condition; ES containing 7.5% (v/v) EG and 7.5% (v/v) DMSO for 8-10 minute, followed with VS containing 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l sucrose for 2 minutes at room temperature. After cryo-storage, warming condition by using a three-step dilution procedure with sucrose (1.0, 0.5, and 0 mol/l sucrose for 1, 3 and 5 minutes respectively) at 37°C. However, the vitrified blastocyst results demonstrated lower survival rate and hatching rate (60.5% and 59.8% respectively), comparing with the result of Ling et al. (2009) reporting. In this case, it indicates the different of some factors (exposure time different; from one to two minute of VS and the concentration of sucrose in the step of warming condition), can affect to optimum of condition and the future of development competency. Both of reports that mention above, have determined that gentle appropriate treatment after warming can improve survivability of embryos. Interestingly, Hosseini et al. (2009) indicated positive effect of the presence of antioxidant in cultivation medium after thawing of embryos. Similarly, supplementation of α-tocopherol in recovery culture medium resulted in a significantly higher blastocyst yield from the post-warm bovine oocytes in comparison with control oocytes (Hwang et al., 2014) Thus, further vitrification procedural improvements using post-vitrification treatment would reduce the high sensitivity of embryos to cryopreservation and provide valuable information during an advanced post-cryopreservation thawing procedure. Furthermore, the strategic placement of embryonic culture media is very important for a successful freezing/warming process.

2.5 The culture environment

In vitro embryo production is a valuable tool to study preimplantation embryonic development and is vital for the *in vitro* production of transferable embryos. During the process, the embryo is removed from the female reproductive tract, which *in vivo* provides the appropriate developmental to ensure normal development. However, *in vitro* environments provide a suboptimal environment resulting in a difference of biochemical signals that effect to the genome. In spite of significant improvements in the culture conditions, the development of *in vitro* produced embryos is still suboptimal (Rizos et al. 2008). Because the preimplantation embryo is extremely sensitive to environmental factors, deficiencies in culture conditions often lead to aberrant embryo development that manifest in lower frequency of blastocyst formation and lower cell numbers and can affect fetal as well as postnatal life (Duranthon et al., 2008). The *in vitro* embryo culture of mammalian embryos requires a suitable environment in which the early embryo can undergo a number of cleavage divisions and ultimately form a blastocyst (Thompson 1997; Rizos et al. 2002; Lonergan 2007).

Preimplantation stage embryos can develop in different media whose compositions range from simple balanced salt solutions and carbohydrates such as Charles Rosenkrans 1 (CR1), synthetic oviductal fluid (SOF) and potassium simplex optimization medium (KSOM), to very complex constituents, such as tissue culture medium (TCM)-199, with further supplementation of serum and/or a feeder layer of somatic cells (Krisher et al. 1989; Niemann and Wrenzycki 2000; Summers and Biggers 2003). However, the major reduction in development *in vitro* occurs during the last part of the *in vitro* process (post-fertilization culture), between the two-cell and

blastocyst stages, suggesting that post-fertilization embryo culture is the most critical period of the process in terms of determining the blastocyst yield (Rizos et al. 2002). In addition, another factor impairing *in vitro* embryo development is the increased oxidative stress. Therefore, it seems necessary and meaningful to further improve and optimize culture system, which can potentially improve the development of the cultured embryos by using supplementation antioxidants such as β-mercaptoethanol, cysteine, cysteamine (Hosseini et al., 2009) and resveratrol (Wang et al., 2018) to *in vitro* culture medium.

2.6 The relationship of oxidative stress by ROS and apoptosis

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues (Pizzino et al., 2017). ROS are small molecules that are short-lived and highly reactive oxidizing agents (Winterbourn, 2015). ROS are generated during intermediate steps of oxygen reduction, known as oxygen-derived free radicals such as superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$), or non-radical molecules, hydrogen peroxide (H_2O_2). In the IVP procedure, gametes and embryos which are removed from their natural microenvironments can be exposed to excessive levels of ROS as the antioxidant defense mechanisms are lost (Agarwal et al., 2006), ultimately resulting in a state of oxidative stress (Plessis et al., 2008). However, ROS can be generated by endogenous, within mitochondria and exogenous sources, when cells are exposed to variations in the culture environmental factors, such as oxygen concentration, illumination (visible light), pH and temperature (Agarwal and Majzoub, 2017).

The generation of ROS in cells or endogenous sources exists in equilibrium with a wide variety of antioxidant defences, including enzymatic scavengers such as superoxide dismutases (SOD), catalase, glutathione peroxidase and peroxiredoxins, as well as non-enzymatic scavengers such as vitamins C and E, glutathione (GSH), lipoic acid, carotenoids and iron chelators (Halliwell, 2011). The mitochondrion is a major intracellular source of ROS. The total mitochondrial O_2 consumed, 1–2% is diverted to the formation of ROS (Turrens, 2003; Circu and Aw, 2010). The production of ROS, especially $O_2^{\bullet -}$, arises mainly from leaks during mitochondrial electron transport chain activity (Dickinson and Chang, 2011). $O_2^{\bullet -}$ can react with nitric oxide ($NO^{\bullet}$), a reactive nitrogen species (RNS), to generate the powerful oxidant peroxynitrite ($ONOO^{-}$) (Winterbourn, 2015; Dickinson and Chang, 2011). Otherwise, $O_2^{\bullet -}$ is rapidly dismutated into O and H_2O_2 by SOD. H_2O_2 can be reduced by metal ions such as iron or copper to form $OH^{\bullet}$ through the Fenton reaction (Kehrer and Klotz, 2015; Halliwell, 2012). $OH^{\bullet}$ is highly reactive and harmful to biological macromolecules. To avoid the formation of $OH^{\bullet}$, H_2O_2 is detoxified by antioxidant enzymes such as catalase and glutathione peroxidases (West and Marnett, 2006) In naturally, ROS production and presence inside cells that need to be kept at a low level (Rajendran et al., 2014). In contrast, the high level of ROS can damage to biomolecules such as DNA, proteins and membrane lipids, leading to mitochondrial dysfunction, which may induce apoptotic responses and reduce embryo viability (Castillo-Martín et al. 2014; Dehghani-Mohammadabadi et al. 2014; Zhao et al. 2016) (Fig. 2.5).

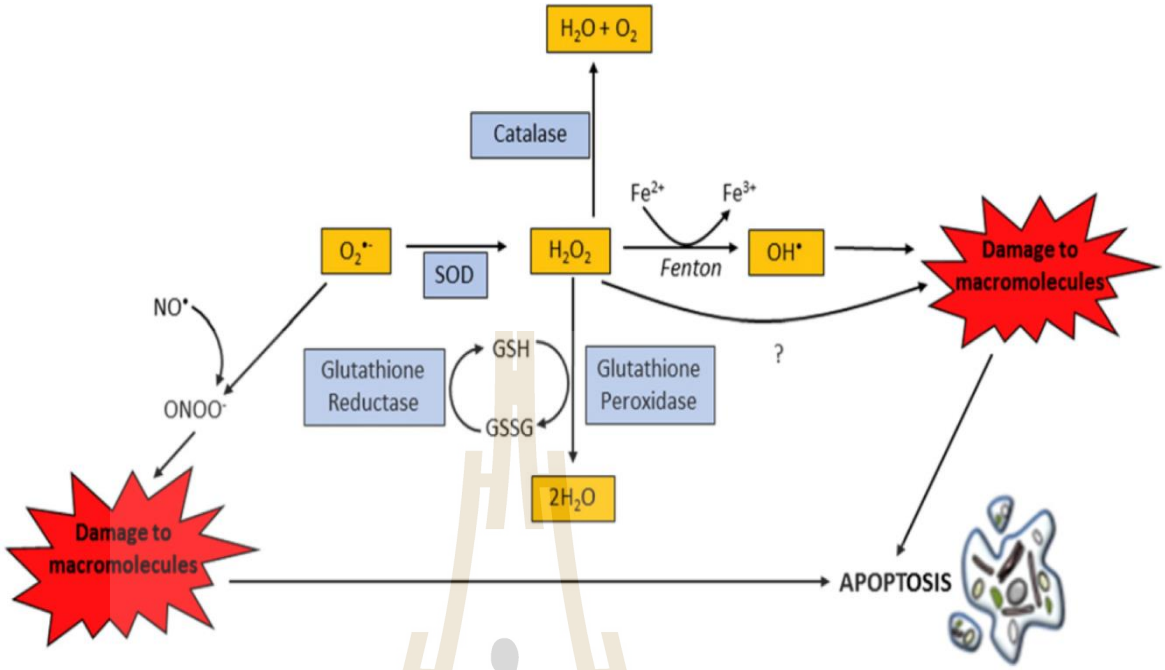

Figure 2.5 The production of ROS that cause cellular damage and apoptosis. (Redza Dutordoir and Averill-Bates, 2016).

Apoptosis or programmed cell death is a tightly regulated and highly conserved process of cell death during which a cell undergoes self-destruction (Kannan and Jain, 2000). It is an essential process in multicellular organisms that eliminates undesired or superfluous cells during development or neutralizes potentially harmful cells with DNA damage (Pallepati et al., 2012). The regulation of apoptosis is crucial for maintaining normal cellular homeostasis. Apoptosis can be triggered by a variety of extrinsic and intrinsic signals. These include different stresses such as ROS, DNA-damaging agents (radiation), heat shock, serum deprivation, viral infection and hypoxia (Fulda et al., 2010; Pallepati et al., 2012). Several publications confirmed that apoptosis contributes to the decrease in the development of oocytes and embryos after vitrification. Coutinho et al. (2007) reported that freezing could induce apoptosis in mouse embryos. Inaba et al. (2016) found that the rate of apoptotic cells in cattle

embryos increased after freezing. Vallorani et al. (2012); Niu et al. (2016) also found that vitrification pig oocytes could increase apoptosis after thawing.

2.7 Resveratrol

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a type of natural phenolic compound and phytoalexin produced in variety of plant species particularly in grapes, berries, plums, and peanuts (Baur and Sinclair, 2006). Its biological function is to protect the plant in case of a parasitic attack or environmental stress, such as UV radiation and exposure to ozone (Fremont et al., 2000; Ignatowicz and Baer-Dubowska, 2001). The plant polyphenol resveratrol is synthesis by the interaction of plants with a micro-organism, when the plant is under attack by pathogens for responding to protects the plant against fungal and bacterial infections (Pervaiz, 2003) Resveratrol exists as *cis*- and *trans*-isomer (Fig. 2.6). *Trans*-resveratrol is the preferred steric form and is relatively stable if it is protected from high pH and light (Alarcón de la Lastra and Villegas, 2007).

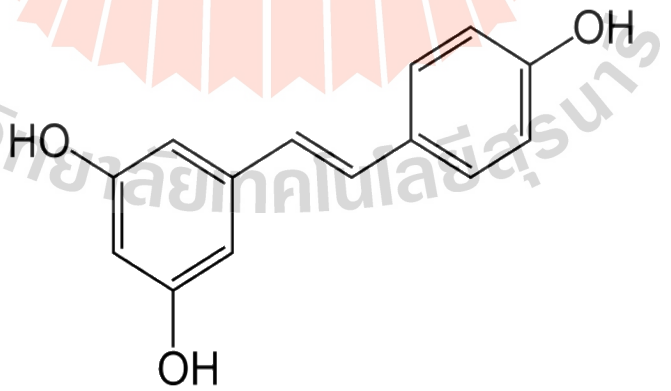

Figure 2.6 The chemical structure of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene).

Resveratrol exhibit wide variety of pharmacological properties ranging from anti-inflammatory (Conte et al., 2015), antimicrobial (Mora-Pale et al., 2015), cancer chemopreventive (Gusman et al., 2001), antioxidant (Chung et al., 2012), cardioprotective (Li et al., 2013), anticancer effects (Mikula-Pietrasik et al., 2014) and anti-aging (Smoliga et al., 2011). It is able to influence the expression of a wide range of genes that are related to DNA synthesis, cell cycle, proliferation, stress responses, and apoptosis. Several studies have been conducted to identify the biologic functions and activities of resveratrol for mammalian reproduction. For instance, Liu et al. (2013) showed that resveratrol can reduce the adverse effects of the mouse oocytes from methylglyoxal-induced oxidative damage in term of oxidative stress, preventing DNA damage, decreasing abnormal mitochondrial and chromosomal distribution and reducing lipid peroxidation (Liu et al. 2013). In addition, Liu et al. (2017) showed that resveratrol against mancozeb-induced apoptosis damage in mouse oocytes by declined fertility, decreased ovary weight and primary follicles (Liu et al. 2017). Besides, mancozeb treated oocytes displayed suboptimal developmental competence by inducing reproductive toxicity and this can also be improved by treatment of resveratrol. Interesting, Liang et al. (2018) tested the effect of resveratrol on mouse oocytes undergo a time-dependent deterioration, which is referred to as post-ovulation oocytes aging (Liang et al. 2018). Resveratrol can effectively protect against post-ovulation oocytes aging by preventing ROS production and improving mitochondrial function.

Furthermore, resveratrol has the potent to anti-apoptotic and antioxidant properties, act as antioxidative agent for *in vitro* production. The ability of the polyphenolic compounds to act as antioxidants depends on the redox properties of their

phenolic hydroxy groups and the potential for electron delocalization across the chemical structure (Alarcón de la Lastra and Villegas, 2007). Resveratrol was clarified three different antioxidant mechanisms: (1) competition with coenzyme Q and, to decrease the oxidative chain complex, the site of ROS generation, (2) scavenging $O_2^{\bullet -}$ radicals formed in the mitochondria and (3) inhibition of lipid peroxidation induced by Fenton reaction products. In fact, numerous studies have demonstrated the ability of resveratrol to scavenge both $O_2^{\bullet -}$ and $OH^{\bullet}$ radicals (Leonard et al., 2003; Losa, 2003; Martinez and Moreno, 2000). Many researches show the result that resveratrol have the positive effect to the environment of culture medium. The beneficial effects of resveratrol on reproductive system function and vitrification have been widely identified, both oocytes and embryos, resulting in improved survival and developmental rates in cats (Comizzoli et al., 2009), pigs (Lee et al., 2010; Kwak et al., 2012; Giaretta et al., 2013; Santos et al., 2018), goats (Mukherjee et al., 2014), cattle (Salzano et al., 2014; Sprícigo et al., 2017, Abe et al., 2017; Hayashi et al., 2018; Hara et al., 2018; Gaviria et al., 2018; Gaviria et al., 2019), and mouse (Liu et al., 2013; Liu et al., 2017; Liang et al., 2018; Wang et al., 2018; Chen et al., 2019). The type of resveratrol supplementation can be divided into three main phases; i*n vitro* maturation (IVM), *in vitro* culture (IVC) and vitrification/warming solution.

The positive effects of resveratrol during IVM were demonstrated in several researches, mostly pigs (Lee et al., 2010; Kwak et al., 2012; Giaretta et al., 2013; Santos et al., 2018) and some animal species such as cat (Comizzoli et al., 2009), goat (Mukherjee et al., 2014) and cattle (Salzano et al., 2014; Sprícigo et al., 2017; Abe et al., 2017; Hayashi et al., 2018; Hara et al., 2018; Gaviria et al., 2018; Gaviria et al., 2019), as concluded by resveratrol treatment beneficial microenvironment within

oocyte increases oocyte ATP content, mitochondrial DNA copy number, mitochondrial membrane potential, and glutathione (GSH) level, decreases reactive oxygen species (ROS) levels, and downregulates apoptosis-related genes such as *Bax/Bcl-2*, *Bak*, and *Caspase-3* expressions, which can lead to the significant improvement of the blastocyst formation rate and blastocyst quality after parthenogenetic activation or *in vitro* fertilization (IVF) (Kwak et al., 2012; Mukherjee et al., 2014; Sprícigo et al, 2017). Moreover, supplementation of resveratrol in IVM and vitrification/warming solution can improve the developmental potential of oocytes, increase cryotolerant and reduce the apoptotic process after vitrification. (Comizzoli et al. 2009; Giaretta et al. 2013; Santos et al., 2017). During the IVC culturation, the treatment of pig MII oocyte with 0.5 μM resveratrol in culture medium has beneficial impact on preimplantation development leading to enhanced blastocyst formation through hatching rates and improved embryo quality in terms of total cell number in the blastocysts (Lee et al., 2010). Additionally, additional resveratrol in IVC medium before vitrification increases survival rates, cryotolerance of embryos which is indicated by higher development rates, hatching rates after post-warming culture, ATP generation in mitochondrial function via Surtuin family member 1 (*SIRT1*) expression levels and decreased lipid content (Salzano et al., 2014; Sprícigo et al, 2017; Abe et al, 2017). Similarly, Wang et al. (2018) reported that supplementation resveratrol in vitrification-warming media and IVC medium after post-warming could diminish the cryopreservation injuries during the vitrification of mouse oocytes, reduce oxidative stress via ROS level and alleviate the abnormal mitochondrial distribution pattern, which could result in increased blastocyst formation and higher cell number of blastocyst (Wang et al. 2018). Interestingly, Giaretta et al. (2013) demonstrated that

positive effects of resveratrol when added various phases of IVM, vitrification/ warming procedure and IVC medium after post-warming could modulate the apoptotic process, improving the resistance of oocytes to cryopreservation-induced damage (Giaretta et al. 2013). These various effects of resveratrol are explained by the fact that resveratrol targets many components of intracellular signaling pathways by modulating a great number of enzymes including kinases, lipo- and cyclooxygenases, sirtuins, and transcription factors (Pirola and Frojdo, 2008).

2.8 Embryo Cryo-treatment

The current Embryo Cryo-treatment hypothesis, stating that "Cryopreservation is not only a technology used for storing embryos, but also a method of embryo treatment that can potentially improve the success rate in infertile couples" (Vladimirov et al., 2017). It is known that the procedure of cryopreservation embryo induces a stress and activates the endogenous survival and repair responses. As controlled stress, it generates responses to repair mitochondrial damage of membrane and another favorable biological response to low exposures to stress is called hormesis.

During freezing and thawing process has a therapeutic effect, concerns the increased mitochondrial activity and oxygen consumption, that occurs during the process of embryo implantation. To explanation the phenomenal of mitochondrial damaging and activity. Dalcin et al. (2013) reported that the frozen embryos maintained their quality as grade I or II were similar to those in the control group of fresh embryos. However, some ultrastructural changes, such as reduced contact between the microvilli and the zona pellucida in the blastocysts, fewer visible desmosomes, organelle-free cytoplasmic areas, and mitochondrial swelling, did not rupture of the mitochondrial

membranes (Dalcin et al. 2013). In the mammal species expression of mtDNA replication factors, was up-regulated in fresh embryos at the blastocyst stage, which leads to the re-activation of mtDNA replication (Thundathil et al., 2005; Spikings et al., 2007). At the same time, the mitochondria start to differentiate into elongated organelles that contain swollen cristae (Wilding et al., 2001; Houghton, 2006), acquire a higher membrane potential and levels of oxygen consumption, and increase oxidative phosphorylation activity, leading to ATP production (St John et al., 2010). However, these replication events are most likely specific to the trophectodermal cells, which give rise to the placenta and will mediate the process of implantation if the embryo is to continue to develop (Kaidi et al., 2001). Therefore, the increased mitochondrial activity in the process of freezing or thawing could reduce mtDNA mutations and affect not only the process of implantation, but also the formation of the placenta and the early development of embryos.

Hormesis refers to a dose response to an environmental agent which is characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect (Mattson, 2008). In the case, refer to a response that induces protective signals. In the freezing and thawing procedure induce a stress via generate alterations in protein structures (because of changes in the water amount and structure) and these alterations induce response signals (chaperones, anti-oxidant responses, and protective enzymes) that are able to repair small defects that are present in the embryo during the post-thawing period. Poor-quality embryos might therefore derive a benefit from these stress signals.

Therefore, cryo-treatment of the embryo is the role of cryopreservation in the activation of a hormetic response. There are two main components. As a result of

freezing or thawing of the embryos, there are reduced levels of ROS, detoxification of the cells is carried out, and the amount of mutated mtDNA is reduced (Fig. 2.7). Another mechanism of influence is through the rapid recovery (jumping effect) of mitochondrial activity in the trophectodermal cells of the blastocyst, which is part of the physiological process of implantation. All of the responding is the results to support the cryo-treatment of the embryo hypothesis, resulting in preserving the mitochondrial function, improving survival, and having an impact on the process of implantation, miscarriage, and the development of pregnancy.

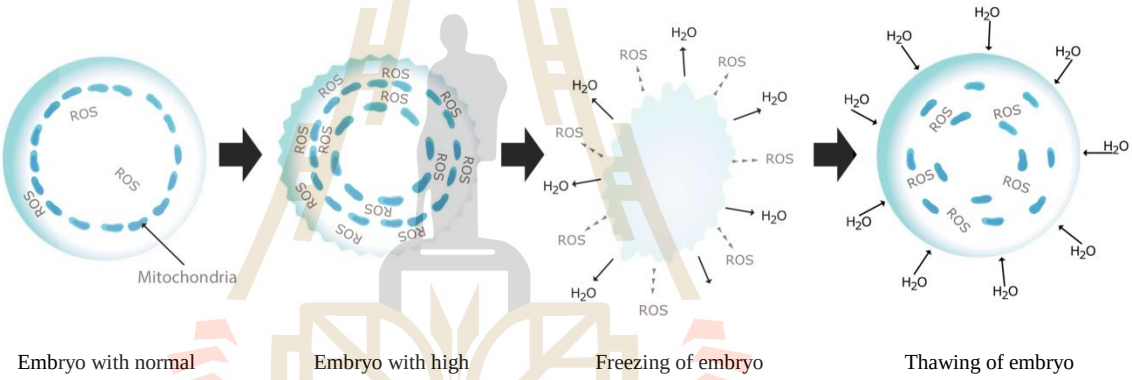

Figure 2.7 Decrease of mitochondrial activity and the levels of ROS in the embryo after freezing and thawing procedures (Vladimirov et al., 2017).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and animals

All chemicals and media used in this study were purchased from Sigma–Aldrich Chemical Company (St. Louis, Missouri, USA), unless specifically indicated.

C57BL/6NJc1 (B6) female and DBA/2NJc1 (D2) male mice were purchased from Nomura Siam International (Bangkok, Thailand) and National Laboratory Animal Center, Mahidol University (Nakhon Pathom, Thailand). Animal experiments were approved and performed under the guidelines of Animal Studies Committee and The Suranaree University of Technology Ethical Committee. (approval ID: UI-03131-2559).

3.2 Methodology

3.2.1 Breeding of B6D2 F1 hybrid mice

The B6D2 F1 hybrid mice were produced by a crossbreed between C57BL/6NJc1 (B6) female and DBA/2NJc1 (D2) male. Mice were bred starting at 8-9 weeks. After mating 19-22 days, pregnant female mice gave birth to B6D2 F1 hybrid mice.

Female B6D2 F1 hybrid mice at 8-10 weeks were used for embryo collections and male B6D2 F1 hybrid mice at 8-10 weeks were used for mating. The

mice were maintained at 25°C±1˚C and light controlled 12 hours light/12 hours dark, with lights on at 6:00 AM with free access to water and standard food.

3.2.2 Superovulation of female B6D2 mice

The female mice were superovulated by an intraperitoneal (IP) injection of 5 IU pregnant mare serum gonadotropin (PMSG, Intervet international B.V., Boxmeer, The Netherlands) followed 48 hours later by 5 IU human chorionic gonadotropin (hCG, Intervet international B.V.). A female mouse was mated with a male mouse of the same age (8-10 weeks old) and the success of mating was checked by the presence of a vaginal plug in the female mice. Mice that had vaginal plug were considered to be pregnant.

3.2.3 Collection of 4-cell embryo and *in vitro* culture (IVC)

The 4-cell embryo collection and IVC were performed as previously described, with some modification (Zhang et al., 2009). Pregnant female mice were sacrificed by cervical dislocation at 50-52 hours after the hCG injection. Embryos at 4-cell stage were flushed from the dissected oviducts using modified mouse tubal fluid (mMTF) supplemented with 0.8% (w/v) bovine serum albumin (BSA, Quinn et al., 1995). Only embryos at the 4-cell stage, with an intact zona pellucida and good morphological appearance, were selected for further experiments. After an initial wash in flushing medium, the embryos were transferred to potassium simplex optimization medium (KSOM, Lawitts and Biggers, 1993) for continuous culture under mineral oil under 37°C in a humidified atmosphere of 5% CO_2. During IVC, 4-cell embryos were cultured in KSOM media supplemented with various concentrations of resveratrol as mentioned in the experimental design. The resveratrol was dissolved in dimethyl sulfoxide (DMSO) and kept at -20°C as a stock (50 mM) until it was added to the medium. Embryo development was evaluated daily, which Day 0 for 4-cell stage, Day

1 for morula stage, Day 2 for blastocyst stage, Day 3 for hatching blastocyst stage, and Day 4-5 for hatched blastocyst stage (Fig. 3.1).

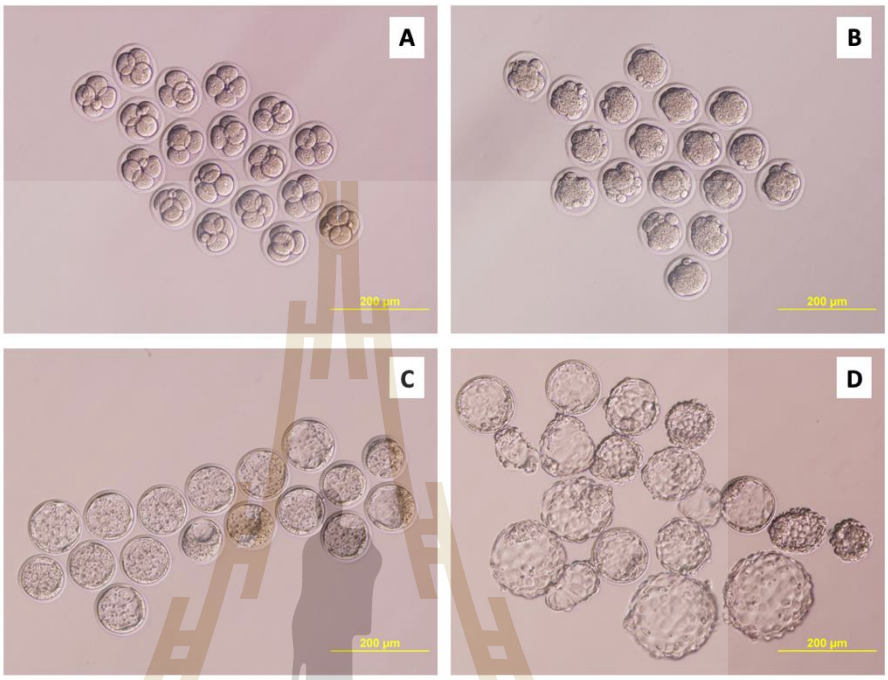

Figure 3.1 The stages of mouse embryos in culture medium (KSOM). A) 4-cell stage, B) morula stage, C) blastocyst stage, D) hatching and hatched blastocyst stage (magnification 100x).

3.2.4 Blastocyst vitrification with Cryotop and warming procedure

Blastocysts obtained on Day 2 were vitrified by a two-step procedure using Cryotop device (Kitazato Ltd., Tokyo, Japan) as previously described (Ling et al., 2009). Vitrification and warming procedures were carried out at ambient temperature (25°C). For vitrification, blastocysts were washed in base medium (BM) consisted of TCM 199-Hepes supplemented with 20% (v/v) fetal bovine serum (FBS, Gibco, USA). Then, blastocysts were initially equilibrated in equilibration solution (ES) for 10 minutes. The ES consisted of 7.5% (v/v) ethylene glycol (EG) and 7.5%

(v/v) dimethyl sulfoxide (DMSO) dissolved in BM medium. After initial shrinkage, the blastocysts regained their original volume and were transferred to vitrification solution (VS) within 1 minute. The VS consisted of 15% (v/v) EG, 15% (v/v) DMSO, and 0.5 mol/L sucrose dissolved in BM medium. Within 60 seconds, five blastocysts with minimal VS (<1.0 µl) were placed onto the tip of the Cryotop. The Cryotop was subsequently plunged vertically into liquid nitrogen and were stored at least one week. After cryo-storage, the blastocysts were warmed by using a four-step dilution procedure as previously described (Ling et al., 2009). These procedures were performed at 37°C on a warm plate. For warming, the tip of Cryotop was quickly dipped into washing solution 1 (1 mol/L sucrose in BM; WS1). After 1 minute, the blastocysts were washed in washing solution 2 (0.5 mol/L sucrose in BM; WS2) for 3 minutes. Subsequently, the blastocysts were washed twice for 5 min in BM without sucrose (0 mol/L sucrose) before being transferred in KSOM medium. During KSOM medium, blastocysts were cultured with various concentrations of resveratrol as mentioned in the experimental design.

3.2.5 Assessment of blastocyst survival and viability

The vitrified-warmed blastocysts were cultured in KSOM medium under mineral oil at 37°C under a humidified atmosphere of 5% CO_2 for 3 days after warming, in order to reach the hatched-blastocyst stage. After 24 hours post-warming, survival rates of vitrified-warmed blastocysts were evaluated on the basis of the integrity of the embryo membrane and the zona pellucida, and re-expansion of the blastocoel. Additionally, development to the hatched blastocyst stage was observed at 48 and 72 hours post-warming (Momozawa et al., 2017). The rate of hatched-blastocyst formation was evaluated by the numbers of hatched-blastocysts to blastocysts cultured *in vitro* and hatched blastocysts were subjected to the mRNA extraction.

3.2.6 Collection of hatched blastocysts

The hatched blastocysts were collected and washed three times in phosphate-buffer saline (PBS) containing 0.1% polyvinyl alcohol (PVP) and washed three times in RNase-free water. Then, three hatched blastocysts from each group were pooled and placed in microcentrifuge tube containing 5 µl of RNase-free water and stored at -80°C until further use.

3.2.7 mRNA extraction

The total mRNA was extracted from the hatched blastocysts in each group using Dynabeads® Oligo $(dT)_{25}$ nucleotide method (Dynabeads mRNA DIRECT™ kit, Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Briefly, Dynabeads® Oligo$(dT)_{25}$ was resuspended thoroughly before use and transfered the desired volume of beads from the stock tube to an RNase-free 1.5 mL microcentrifuge tube, and then the tube was placed on a magnet. After 30 seconds (or when the suspension is clear), the supernatant was removed from the tube and the tube was removed from the magnet. The beads were washed by resuspending in an equivalent volume of fresh Lysis/Binding Buffer. The pre-washed beads were mixed with sample and incubated with continuous mixing for 3–5 minutes at room temperature to allow the poly-A tails of the mRNA hybridize to the oligo $(dT)_{25}$ on the beads. Then, the tube was placed on the magnet for 2 minutes and the supernatant was removed from the tube. The beads/mRNA complex were washed twice with washing buffer A and washing buffer B at room temperature. To separate of mRNA from the beads, 20 µL of 10 mM Tris-HCl pH 7.5 (elution buffer) was added into the tube and the tube was incubated at 75°C for 2 minutes. After that, the tube was immediately placed on the magnet and the supernatant containing the mRNA was transferred to a

new RNase-free tube. Then, the tube was placed on ice and stored at -80°C until further use.

3.2.8 cDNA synthesis

The mRNA was immediately synthesized complementary DNA (cDNA) by using iScript™ reverse transcription (RT) supermix kit (Biorad, Hercules, California, USA) following the manufacturer's instructions. Briefly, the reactions were assembled on ice at total volume of 20 µL containing of 4 µL iScriptTM RT supermix, 4 µL mRNA sample and 12 µL RNase-free water. Then, the reaction mixture was incubated in Eppendorf Mastercycler DNA Engine Thermal Cycler PCR (Eppendorf, Hamburg, Germany). The cDNA synthesis was carried out at 25 °C for 5 minutes (pre-heated priming), following by 42 °C for 30 minutes (reverse transcription, RT) and at 85 °C for 5 minutes (RT inactivation). All cDNA samples were stored at -20°C until further use.

3.2.9 Quantitative polymerase chain reaction (qPCR)

The quantification of *Bax*, *Bcl2* and *ErbB4* gene transcripts was carried out by quantitative polymerase chain reaction (qPCR). The qPCR was performed with QuantStudio 5 Real-Time PCR Systems (Applied Biosystems™, Waltham, Massachusetts, USA) using KAPA SYBR® FAST qPCR kit master mix universal (KAPA biosystems, Wilmington, Massachusetts, USA). The qPCR was carried out in a 20 µL reaction volume containing 10 µL KAPA SYBR® master mix, 2 µL cDNA, 0.4 µL 50X Rox low, 6.8 µL RNase-free water and 0.4 µL of each of forward/reverse primers. *GAPDH* was used as the reference gene. Sequences for primers and conditions are listed in Table 3.1.

Table 3.1 The primer sequences for qPCR.

Genes	Primer sequences 5'-3'	Product size (bp)	Accession number	References
GAPDH	F:5′-GACTTCAACAGCAACTCCCAC-3′ R:5′- TCCACCACCCTGTTGCTGTA-3′	125	NM_001289726.1	Gharenaz el al., 2016, 2018
Bax	F:5′- CGGCGAATTGGAGATGAACTG-3′ R:5′-GCAAAGTAGAAGAGGGCAA-3′	161	XM_006540584.1	Gharenaz el al., 2016, 2018
Bcl2	F:5′- ACCGTCGTGACTTCGCAGAG -3′ R:5′- GGTGTGCAGATGCCGGTTCA -3′	239	NM_009741.1	Gharenaz el al., 2016, 2018
ErbB4	F:5′-TACGAGCCTGCCCAAGTTC- 3′ R:5′- GTGCCGATTCCATCACATCCT- 3′	103	XM_006536907.1	Gharenaz el al., 2016

The qPCR program started with an initial melting cycle for 5 minutes at 95°C to activate the polymerase, followed by 45 cycles of melting (15 sec at 95 °C), annealing (30 sec at 58 °C) and extension (15 sec at 72 °C). At the end of each phase, the amount of fluorescence was measured to quantitate objectives. The no-template control was used to check contamination in the PCR reagents. The quality of the PCR reactions was determined by melting curve analyses (Gharenaz el al., 2016, 2018). All samples were repeatedly analyzed three times and values were averaged. Measured values were obtained as the cycle threshold (Ct) and ΔCt was calculated (Ct of target gene minus Ct of *GAPDH* as a control). ΔΔCt value was calculated by ΔCt minus the mean value of each group and then the real mRNA level was expressed as $2^{-\Delta\Delta Ct}$ (Youm et al., 2017).

3.2.10 Experimental designs

Experiment 1: This experiment was to determine the effect of different concentrations of resveratrol supplementation in culture medium (KSOM) on embryo developmental competency. *In vivo* mouse embryos at 4-cell stage were randomly selected and allocated to culture medium (KSOM) containing either 0 (control), 0.5, 1,

2.5 and 5 µM resveratrol for five days. The development to hatched blastocyst stage in each group were examined. Experiments were conducted seven times (Fig. 3.2).

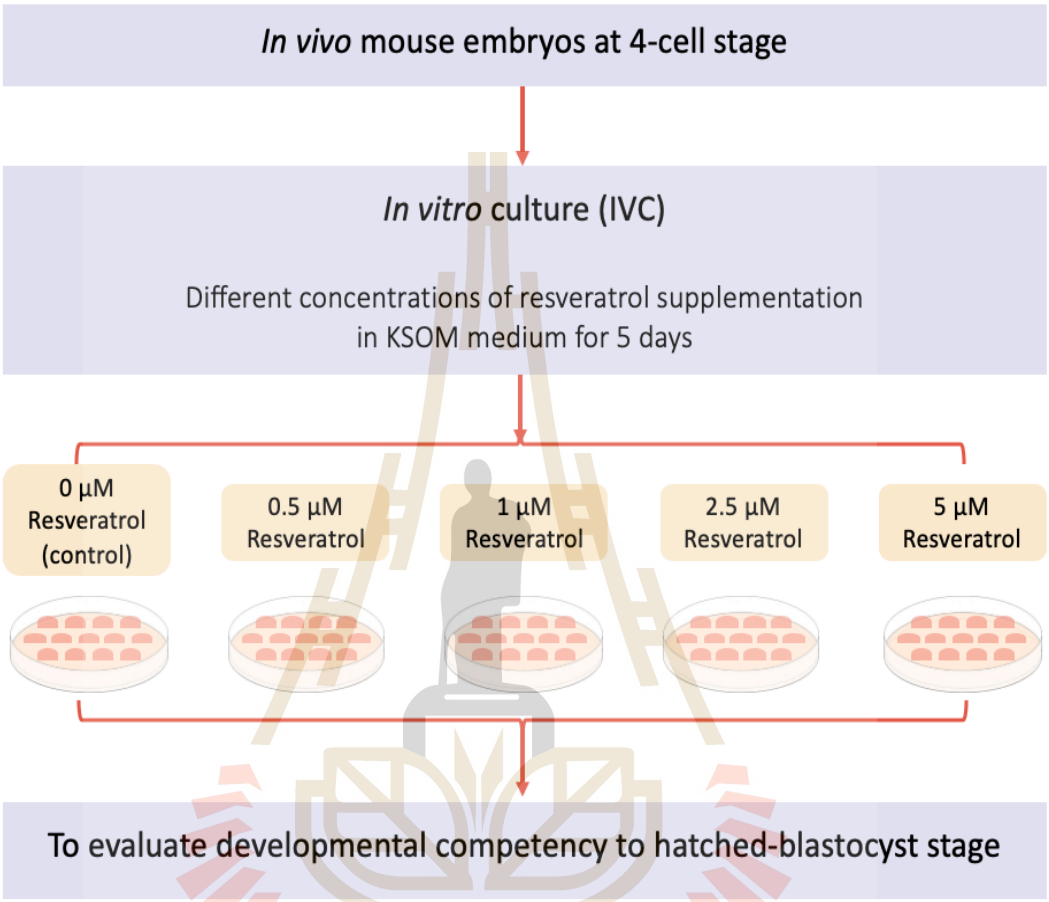

Figure 3.2 Experimental design of experiment 1. To examine the effects of resveratrol supplementation in culture medium (KSOM) on mouse embryo developmental competency.

Experiment 2: This experiment was to investigate the optimal concentrations of resveratrol supplementation in KSOM medium during pre- and post-vitrification on the cryo-survivability and developmental potential of vitrified-embryos. *In vivo* 4-cell embryos were randomly selected and allocated to KSOM medium containing either 0, 0.5, 1, and 2.5 µM resveratrol (based on the results from

Experiment 1) for two days until reach the blastocyst stage. Blastocysts from each group were divided into two groups, fresh and vitrified groups. For fresh group, the blastocysts were continuous cultured at the same concentration of resveratrol for three days until the hatched blastocysts stage. For vitrified group, the blastocysts were vitrified by Cryotop method. After warming, vitrified-warmed blastocysts were cultured at the same concentration of resveratrol for three days. Percentages of survival and embryo developmental competence in each group were observed. Experiments were conducted nine times (Fig. 3.3).

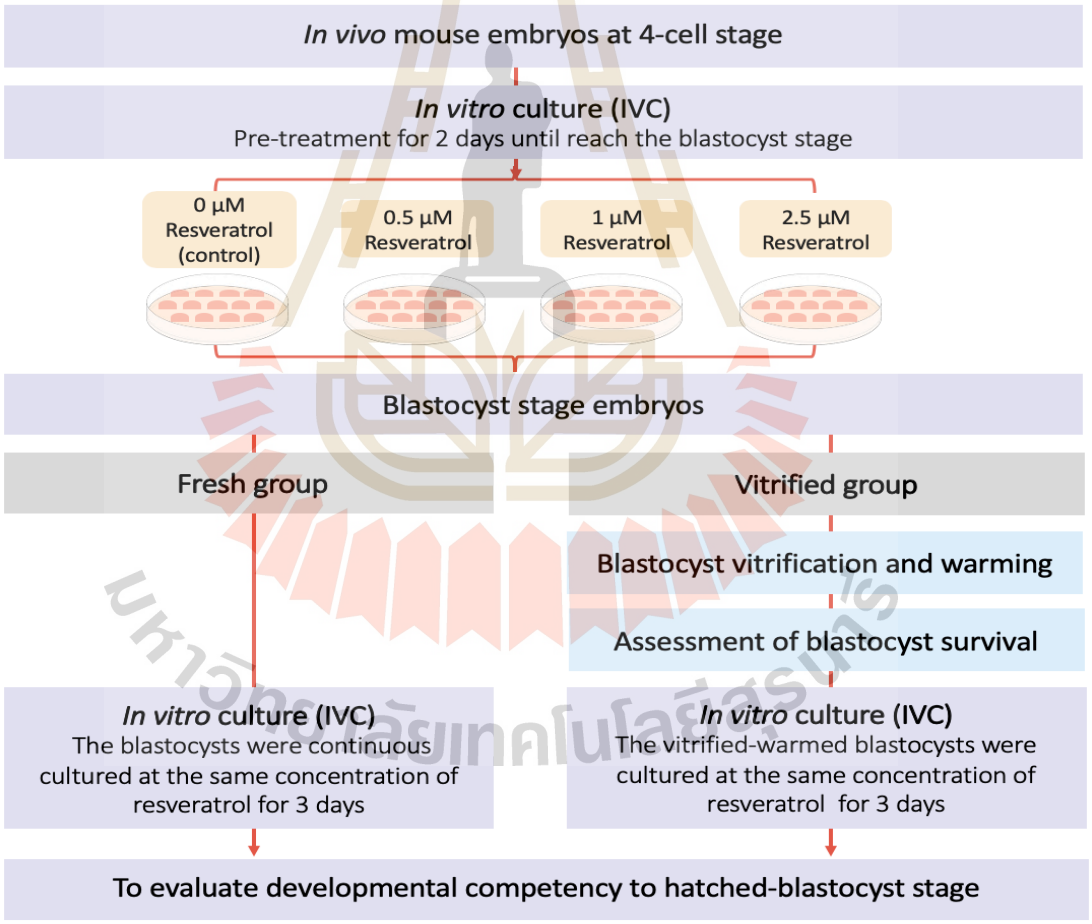

Figure 3.3 Experimental design of experiment 2. To investigate the effects of resveratrol supplementation in KSOM culture medium on cryo-survivability and developmental competency of vitrified mouse embryos.

Experiment 3: This experiment was to evaluate the effect of resveratrol supplementation in KSOM medium and/or vitrification solution on vitrified embryos. *In vivo* 4-cell embryos were randomly selected and allocated to KSOM medium containing either 0 or 0.5 μM resveratrol (based on the results from Experiment 2) for two days until reach the blastocyst stage. Blastocysts in each group were divided into three groups, fresh group, vitrified group without resveratrol supplementation in ES and VS (vitrified-), and vitrified group with 0.5 μM resveratrol supplementation in ES and VS (vitrified+). For fresh group, the blastocysts were continuous cultured at the same concentration of resveratrol either 0 or 0.5 μM for three days until hatched blastocysts stage. For vitrified groups, the blastocysts were vitrified by Cryotop method in the ES and VS solution with or without 0.5 μM resveratrol supplementation. After warming, vitrified-warmed blastocysts were cultured at the same concentration of resveratrol either 0 or 0.5 μM for three days. Percentages of survival and embryo developmental competence in each group were examined. Experiments were conducted five times (Fig. 3.4).

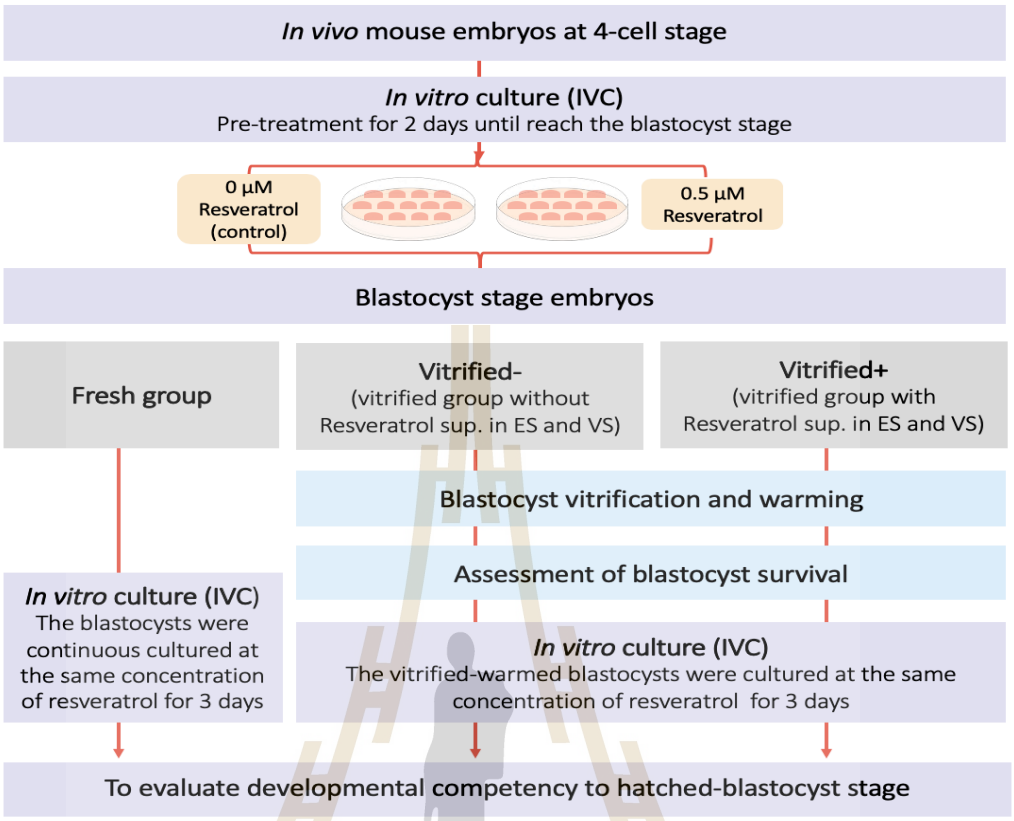

Figure 3.4 Experimental design of experiment 3. To evaluate the effects of resveratrol supplementation in KSOM medium and/or vitrification solution on cryo-survivability and developmental competency of vitrified mouse embryos using Cryotop-vitrification method.

Experiment 4: This experiment was to determine the effect of resveratrol supplementation in KSOM medium and/or vitrification solution on expression levels of genes involved in apoptotic (*Bax*, *Bcl2*) and implantation (*ErbB4*) genes of fresh and vitrified mouse embryos. Embryos at the hatched blastocyst stage from experiment 3 were collected (three embryos per tube, three tubes per each group). mRNA extraction and cDNA synthesis were performed. Gene expression levels of each sample were assessed using qPCR technique. Experiments were conducted three times (Fig. 3.5).

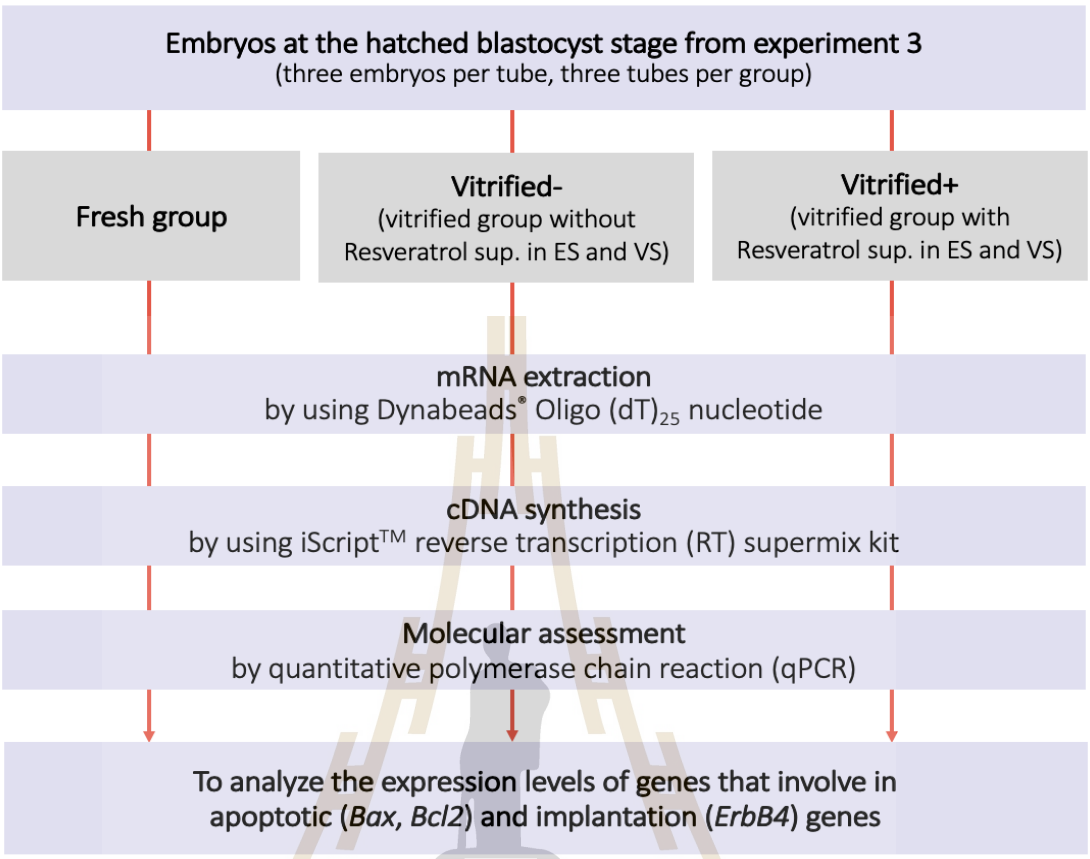

Figure 3.5 Experimental design of experiment 4. To analyze the expression levels of genes that involve in apoptotic and implantation gene on efficiency of resveratrol supplementation in KSOM medium and/or vitrification medium.

3.2.11 Statistical analysis

Data were evaluated by One-way Analysis of Variance (ANOVA) using SPSS software (version 17.0; SPSS, Inc., Chicago, Illinois, USA) and comparison of means by Duncan's Multiple Range Test (DMRT) were analyzed. P value of less than 0.05 was considered a statistically significant difference.

CHAPTER IV

RESULTS

4.1 Effects of resveratrol supplementation in KSOM culture medium on mouse embryo development

To determine the effects of resveratrol on mouse embryo development, *in vivo*-produced mouse embryos at 4-cell stage were *in vitro* cultured in KSOM medium supplemented with 0 (control group), 0.5, 1, 2.5 and 5 μM resveratrol for 5 days. The results are summarized in Table 4.1. Morula and blastocyst rates of embryos cultured in KSOM supplemented with 0.5 μM and 1 μM resveratrol were no significant difference when compared with those of control group (100% and 100%, respectively). Moreover, embryos cultured in 0.5 μM (74.2%) and 1 μM (70.8%) resveratrol had significantly higher rates of hatched blastocyst when compared with control group (52.8%, $P<0.05$). There was no significant difference on hatched blastocyst rate (54.6%) of embryos cultured with 2.5 μM resveratrol, however, morula (92.1%) and blastocyst rates (86.4%) were significantly lower than those of control group ($P<0.05$). The lowest morula, blastocyst and hatched blastocyst rates were found in 5 μM resveratrol group (83.3%, 41.1% and 22.2%, respectively).

Supplementing KSOM culture media with resveratrol could improve hatched blastocyst formation rate of mouse embryos. Embryos cultured in KSOM supplemented with 0.5 μM resveratrol had the highest hatched blastocyst rate.

However, high concentration of resveratrol had detrimental effects on mouse embryo development.

Table 4.1 Effects of resveratrol supplementation in KSOM culture media on mouse embryo development.

Resveratrol in KSOM (μM)	No. of *in vivo* 4-cell embryo cultured	No. (%) of embryo developed to		
		Morula	Blastocyst	Hatched blastocyst
0	89	89 (100)[a]	89 (100)[a]	47 (52.8)[b]
0.5	89	89 (100)[a]	89 (100)[a]	66 (74.2)[a]
1	89	89 (100)[a]	86 (96.6)[a]	63 (70.8)[a]
2.5	88	81 (92.1)[b]	76 (86.4)[b]	48 (54.6)[b]
5	90	75 (83.3)[c]	37 (41.1)[c]	20 (22.2)[c]

Seven independent replicates were performed.

Different superscripts within columns indicate significant differences ($P < 0.05$, ANOVA).

4.2 Effects of resveratrol supplementation in KSOM culture medium on cryo-survivability and developmental competency of vitrified mouse embryos

This experiment was to determine the effects of resveratrol on survival rate and developmental competency of vitrified embryos, *in vivo* 4-cell embryos were *in vitro* cultured in KSOM medium supplemented with 0 (control), 0.5, 1, and 2.5 μM

resveratrol for 2 days, then blastocyst stage embryos were vitrified and warmed. After that, vitrified-warmed embryos were cultured in KSOM media at the concentration of their counterpart of another 3 days.

The results were summarized in Table 4.2. At pre-vitrification, the blastocyst rates of embryos cultured in KSOM supplemented with 0.5 and 1 μM resveratrol were not significant different (99.6% and 97.8%, respectively) when compared with 0 μM control group (97.8%), whereas 2.5 μM resveratrol had significantly lower blastocyst rate than other groups (89.1%, $P<0.05$). The survival rates after warming were no significant difference among all groups (100% survival rate). Without resveratrol supplementation in KSOM culture media, vitrified embryos had significantly lower hatched blastocyst rate than that of fresh embryos (58.2% and 66.8%, respectively, $P<0.05$). Interestingly, hatched blastocyst rate of vitrified embryos cultured in 0.5 μM resveratrol (73.7%) was significantly higher than that of other vitrified groups (55-68%, $P<0.05$). Moreover, culturing vitrified embryos in KSOM media supplemented with 0.5 μM resveratrol after warming could increase hatched blastocyst rate (73.7%) to the same level of that of fresh embryo counterpart (75.0%). In 1 μM resveratrol group, vitrified embryos had significantly lower hatched blastocyst rate (67.8%) when compared with that of fresh counterpart (74.6%, $P<0.05$). High concentration of resveratrol (2.5 μM) had detrimental effect on embryo development of both fresh and vitrified embryos as low hatched blastocyst rates were found (58.1% and 55.0%, respectively).

Therefore, 0.5 μM resveratrol was the most suitable for improving developmental potential of fresh and vitrified mouse embryos.

Table 4.2 Effects of pre- and post-treatment of 4-cell embryos with various concentrations of resveratrol supplementation in KSOM medium on developmental competency of embryo after post-warmed.

Resveratrol in KSOM (μM)	No. of *in vivo* 4-cell cultured	Morulae	Blastocysts	Group	No. of Blastocysts	Survival rate (%)	Hatched blastocyst (%)
0	228	225 (98.7)	223 (97.8)[a]	Fresh	113	-	75 (66.8)[b]
				Vitrified	110	110 (100)	64 (58.2)[c]
0.5	227	227 (100)	226 (99.6)[a]	Fresh	112	-	84 (75.0)[a]
				Vitrified	114	114 (100)	84 (73.7)[a]
1	228	227 (99.6)	223 (97.8)[a]	Fresh	110	-	82 (74.6)[a]
				Vitrified	113	113 (100)	78 (67.8)[b]
2.5	230	228 (99.1)	205 (89.1)[b]	Fresh	105	-	61 (58.1)[c]
				Vitrified	100	100 (100)	55 (55.0)[c]

Nine independent replicates were performed.

Different superscripts within columns indicate significant differences ($P < 0.05$, ANOVA).

4.3 Effects of resveratrol supplementation in vitrification solution on cryo-survivability and developmental competency of vitrified mouse embryos

From results of experiment 4.1 and 4.2, supplementing KSOM culture media with 0.5 μM resveratrol could increase hatched blastocyst rate of both fresh and vitrified mouse embryos. In this experiment, 0.5 μM resveratrol was also supplemented to vitrification solution (ES and VS) to determine whether resveratrol could improve survivability and developmental potential of vitrified embryos.

Without resveratrol supplementation in KSOM, supplementing vitrification solution with 0.5 μM resveratrol significantly increased hatched blastocyst rate of vitrified embryos (68.9%) when compared with non-supplemented group (57.9%), moreover, vitrified embryos in 0.5 μM resveratrol had same hatched blastocyst rate (68.9%) as fresh embryos (66.7%). When embryos cultured in KSOM supplemented with 0.5 μM resveratrol, supplementing vitrification solution with 0.5 μM resveratrol did not have beneficial effect on development of vitrified embryos. The blastocyst rate was no significant difference between embryos vitrified in vitrification solution with or without resveratrol supplementation (76-77%). Notably, higher hatched blastocysts were found in embryos cultured in KSOM supplemented with 0.5 μM resveratrol either from fresh (72.4%) or vitrified groups (76-77%).

These results showed beneficial effects of resveratrol supplementation in culture media over supplementation in vitrification solution.

Table 4.3 Effects of resveratrol supplementation in KSOM medium and/or vitrification solution on developmental competency of embryo after post-warmed.

Resveratrol in KSOM (μM)	No. of *in vivo* 4-cell cultured	Morulae	Blastocysts	Group	No. of Blastocysts	Survival rate (%)	Hatched blastocyt (%)
0	321	321 (100)	321 (100)	Fresh	108	-	72 (66.7)[b]
				Vitrified –	107	107 (100)	62 (57.9)[c]
				Vitrified +	106	106 (100)	73 (68.9)[b]
0.5	322	322 (100)	322 (100)	Fresh	105	-	76 (72.4)[a]
				Vitrified –	109	109 (100)	84 (77.1)[a]
				Vitrified +	108	108 (100)	83 (76.9)[a]

Five independent replicates were performed.

Different superscripts within columns indicate significant differences ($P < 0.05$, ANOVA).

Vitrified – = vitrified group without resveratrol supplementation in vitrification solution; ES and VS.

Vitrified + = vitrified group with 0.5 μM resveratrol supplementation vitrification solution; in ES and VS.

4.4 Effects of resveratrol supplementation in vitrification solution and/or culture media on gene expression profiles of fresh and vitrified mouse embryos.

To study effects of resveratrol supplementation in KSOM medium and/or vitrification solution on gene expression profiles, expression levels of *Bax*, *Bcl-2* and *ErbB4* in embryos at the hatched blastocyst stage from experiment 4.3 were analyzed.

Relative expression level of *Bax* of vitrified was no significant difference when compared with fresh control embryos. No beneficial effect of resveratrol supplementation in culture media and/or vitrification solution on *Bax* expression. However, vitrified embryos using vitrification solution supplemented with resveratrol and cultured in KSOM media without resveratrol had significantly higher levels of *Bax* expression when compared with other groups (Fig. 4.1).

Vitrification had detrimental effect on *Bcl-2* expression of hatched blastocysts as the *Bcl-2* relative expression levels of vitrified embryos were significantly lower than those of fresh embryos (Fig. 4.2, $P < 0.05$). Supplementing KSOM culture media with 0.5 μM resveratrol significantly increased *Bcl-2* expression levels of fresh embryos ($P < 0.05$). However, no beneficial effect was found in vitrified embryos (Fig. 4.2).

For *ErbB4* gene, supplementing KSOM culture media with 0.5 μM resveratrol significantly increased relative expression level of *ErbB4* when compared with fresh embryos (Fig. 4.3, $P < 0.05$). Supplementing vitrification solution with 0.5 μM resveratrol had no beneficial effect on *ErbB4* expression of vitrified embryos. Moreover, the expression level of *ErbB4* of vitrified embryos using vitrification solution supplemented with 0.5 μM resveratrol and cultured in KSOM supplemented with 0.5 μM resveratrol was extremely low and significantly different when compared

with other groups ($P < 0.05$).

Taken together, supplementing KSOM with 0.5 μM resveratrol positively modulated expression levels of *Bcl-2* and *BrbB4* of fresh embryos. However, no beneficial effect was found in *Bax* gene or vitrified embryos.

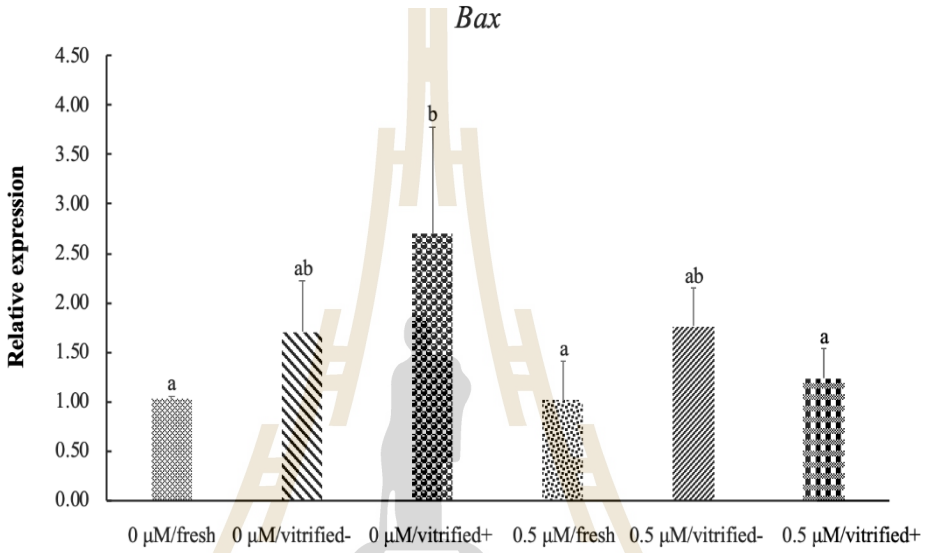

Figure 4.1 Changes (±S.E.M) in the relative expression of *Bax* gene in mouse hatched blastocyst in different group. a, b, c on error bar indicated values significantly different at P<0.05 using one-way ANOVA.

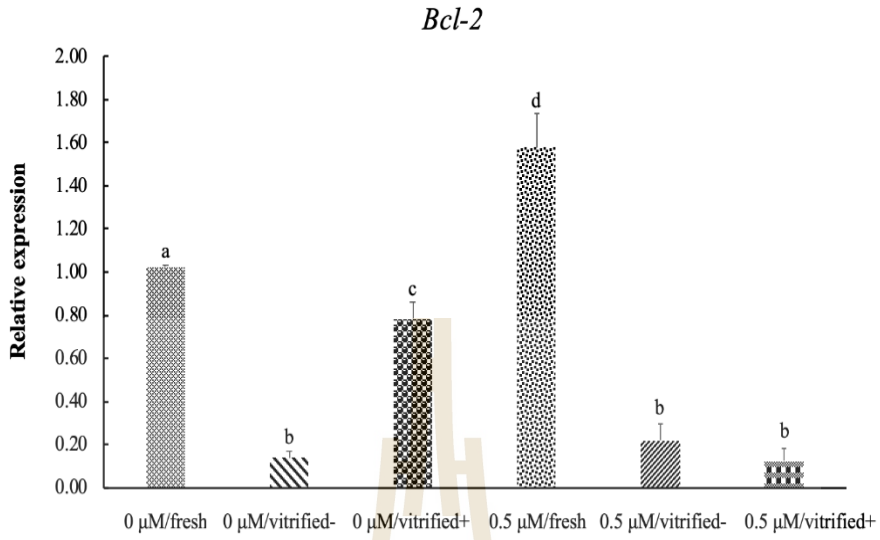

Figure 4.2 Changes (±S.E.M) in the relative expression of *Bcl-2* gene in mouse hatched blastocyst in different group. a, b, c, d on error bar indicated values significantly different at P<0.05 using one-way ANOVA.

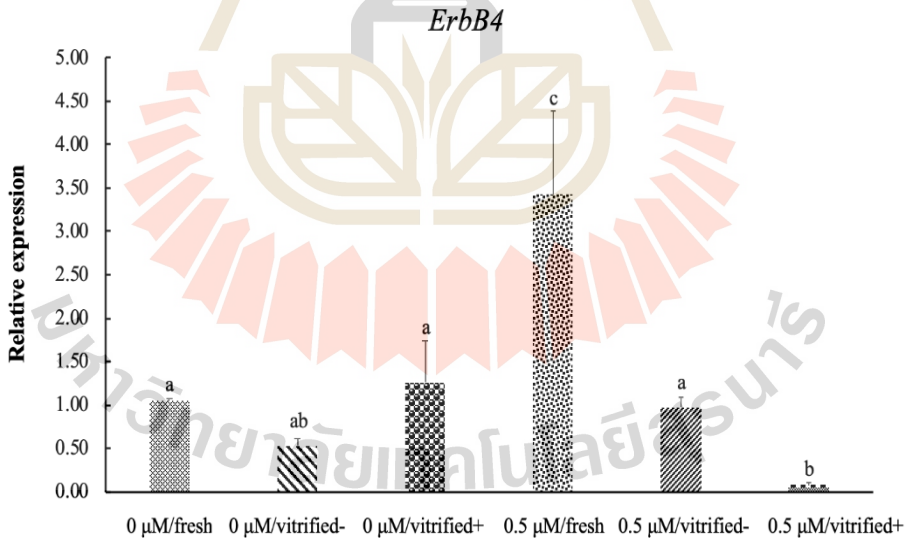

Figure 4.3 Changes (±S.E.M) in the relative expression of *ErbB4* gene in mouse hatched blastocyst in different group. a, b, c on error bar indicated values significantly different at P<0.05 using one-way ANOVA.

CHAPTER V

DISCUSSION AND CONCLUSION

This study demonstrated that resveratrol had beneficial effects on preimplantation development of mouse embryos. Supplementation of the culture medium with 0.5 μM resveratrol increased developmental rates to the hatched blastocyst stage in both fresh and vitrified embryos, and positively upregulated expression levels of genes involved in apoptosis and implantation of fresh embryos.

In vitro culture of mammalian embryos requires suitable environment in which the early stage embryos can undergo number of cleavage divisions and ultimately form blastocysts (Rizos et al., 2008). Resveratrol is a natural polyphenolic product found in food. Resveratrol may play a role as an antioxidant and anti-apoptosis (Fremont et al., 2000). In cell culture studies, resveratrol had a dose-dependent effect, at high concentration resveratrol exerted an anti-proliferation effect and induced apoptosis whereas at low concentration resveratrol promoted cell division in various human cell lines (Szende et al., 2000; Kuwajerwala et al., 2002). There are a number of studies about the effects of resveratrol on embryonic development. Supplementing culture medium with 0.5 μM resveratrol improved pig and cattle embryonic development (Lee et al., 2010; Abe et al., 2017). Also, supplementation of 1 μM resveratrol in culture medium enhanced cattle embryonic development (Hayashi et al., 2018). To my best knowledge, there was no study on the effect of resveratrol in mouse embryos. Only a study mentioned that 25 μM resveratrol supplementation in vitrification and warming

solution, and IVC media had a beneficial effect on MII mouse oocyte vitrification (Wang et al., 2018). In this study, beneficial effect of resveratrol on mouse 4-cell embryos was observed. The preliminary dose-response trial highlighted a distinct dose-dependent effect of resveratrol, with signification decrease of blastocyst rate at the concentration of 5 μM. The high concentration (5 μM) of resveratrol in the culture medium had toxic side effect and were detrimental to the developmental competency. However, low concentration (0.5-1 μM) of resveratrol in culture medium tended to be advantage as blastocyst and hatched blastocyst rates were increased. These results are consistent with the pro-proliferation characteristic and developmental competency of resveratrol reported as mentioned earlier (Lee et al., 2010; Abe et al., 2017; Hayashi et al., 2018).

Under IVC conditions, embryos are subjected to a variety of homeostatic pressures including physicochemical (temperature and pH) and oxidative (prooxidant and antioxidant balance) stresses, all of which can compromise further development (Summers and Biggers 2003). Especially, oxidative stress can be lethal to embryos developmental competency (Betts and Madan 2008). Low concentration of ROS can activate cellular pathways that determine differentiation or proliferation (Rajendran et al., 2014), while high concentration appears to affect the integrity of cellular constituents such as lipids, proteins, amino acids and nucleic acids, which may induce apoptotic responses and reduce embryo viability (Castillo-Martín et al. 2014; Dehghani-Mohammadabadi et al. 2014; Zhao et al. 2016). However, several analyses have shown that one of the ways in which culture conditions are detrimental to embryo developmental competence is by increasing oxidative stress. In this study, low concentration of resveratrol (0.5-1 μM) supplementation in IVC medium significantly increased hatched blastocyst rates of mouse embryos. Thus, one strategy to reduce the

damage caused by oxidative stress is supplementation of culture media with antioxidant molecules, such as resveratrol.

The unique advantage of vitrification over conventional freezing is the elimination of mechanical injury caused by intra- or extra-cellular ice crystallization (Ling et al., 2009). The survivability of oocytes and embryos following cryopreservation procedures depends on different mechanisms of cell injury such as the chemical toxicity of the cryoprotectants, osmotic shock in the dehydration and rehydration procedures, and vitrification effects on the ultrastructure of oocytes and embryos (Shi et al., 2007). The main factors of vitrification/warming technique involved with 1) vitrification and warming solution, need to be suitable with stage of embryo, time, temperature; 2) cryo-device, minimize sample volume and rapid cooling/warming; 3) post-warmed treatment, would reduce the high sensitivity of embryos (Yavin and Arav, 2007). Moreover, another hypothesis could explain to support the result of improving survival rate after vitrification, known as cryo-treatment. During freezing/warming process is a way to activate the endogenous survival and repair response in preimplantation embryos. The term for favorable biological response to low exposures to stress is called hormesis. The mechanism involved hormesis response consists of two processes; reducing ROS levels and mutated mtDNA by removing out of cells during vitrification process; rapid recovery (jumping effect) of mitochondrial activity during warming process (Vladimirov et al., 2016). This study, mouse blastocysts had high survivability following vitrification (100% all group; within and without resveratrol supplementation) by using Cryotop as a device and freezing condition; equilibration solution (ES) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulphoxide (DMSO) for 10 minute, followed with vitrification solution (VS) containing 15% (v/v) EG, 15% (v/v) DMSO

and 0.5 mol/l sucrose for 60 second at room temperature. After cryo-storage, warming condition by using a four-step dilution procedure with sucrose (1.0, 0.5, 0 and 0 mol/l sucrose for 1, 3, 5 and 5 minutes respectively) at 37°C. Similarly, it is reported that low level of resveratrol in culture medium (0.5 μM) improved the resistance to cryopreservation of IVP bovine embryo (Abdel-Wahab et al., 2012; Salzano et al., 2014; Abe et al., 2017). Recent studies have reported that the addition of 0.5 μM resveratrol during IVC media reduce ROS production and increases ATP generation, leading to increasing of survival rates and developmental competency after vitrification (Abe et al., 2017). However, the post-warmed culture conditions have a major influence on survival and development of embryos following vitrification (Hosseini et al., 2009; Hwang and Hoshi, 2014). The results from this study indicated that the post-warmed culture conditions of mouse blastocysts with resveratrol (0.5 μM) improving developmental competency after warming, as indicated by hatched blastocyst rate.

Resveratrol has been reported to act as antioxidant because of its ability to decrease mitochondria ROS production, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes (Pervaizand and Holme, 2009). Supplementing the vitrification solution with an antioxidant reduces oxidative stress and increases the survival rates of embryos. An antioxidant treatment can be useful for development of embryos and cells differentiation (Lane et al., 2002). This study investigated the combined effect of resveratrol supplementation in culture medium and vitrification solution. Supplementation of 0.5 μM resveratrol to the culture medium and/or vitrification solution increased hatched blastocyst rates after warming. Similarly, the use of 0.5 μM resveratrol increased developmental and hatching rates following vitrification and warming (Salzano et al. 2014). Moreover, the addition of resveratrol in various phases

of IVM, vitrification/warming and IVC media can modulate the apoptotic process, improving the resistance of porcine oocytes to cryopreservation-induced damage (Giaretta et al., 2013).

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues (Pizzino et al., 2017). One of the effects of resveratrol is to maintain the redox state of cells and protect them against the harmful effects of oxidative injuries. During IVC condition, increased oxidative stress can be induced apoptosis in preimplantation embryos and its incidence in the developing embryos can be correlated to embryo quality (Kitagawaa et al., 2004; Fabian et al., 2005). Following vitrification, embryos become more sensitive to the oxidative stress (Somfai et al., 2007a). Glutathione (GSH) content, a major non-enzymatic antioxidant in oocyte and embryo to defense ROS, sharply decreases after cryopreservation in bovine (George et al., 2008) and porcine IVF embryos (Somfai et al., 2007b), confirming that oxidative stress occurs during the freezing-thawing process.

The successful of vitrification/warming technique not only can lead to high survival rates (high cryo-survival rates) but also enable maintenance of biological functions, leading to higher success rates of development competency after warming. Thus, we evaluated the possible impact of the resveratrol supplementation association with vitrification process on expression of apoptosis and implanting gene. Apoptosis is a regulated program that initiate cell death. Members of *Bcl-2* genes family play an important role in apoptosis regulation. *Bcl-2* is anti-apoptotic and is responsible for cell survival, whereas *Bax* is pro-apoptotic and induced cell death (Yang and Rajamahendran, 2002). Implanting gene is the interaction between *ErbB4* and HB-EGF mediated the initial attachment of blastocyst to uterine luminal epithelium (Davidson

and Coward, 2016; Paria et al., 1993). The signalling of HB-EGF simultaneous function to embryo and activation the program of trophoblast differentiation required for adhesive functions during subsequent attachment and invasion (Davidson and Coward, 2016). This present study found clearly that the addition of resveratrol to the culture medium did not alter the expression of *Bax* in fresh hatched blastocysts, similar results have been reported by Torres et al., 2018 and Gaviria et al., 2018, but up regulated in *Bcl-2* and *ErbB4,* compared with fresh group without addition of resveratrol. Not only the combined effect of adding resveratrol to the culture medium and/or vitrification solutions but the vitrification/warming process also altered gene expression.

In conclusion, 0.5 μM resveratrol supplementation during *in vitro* culture improved developmental competency of mouse embryo, indicated by their effective for development of mouse hatched blastocyst with enhanced cryotolerance. The association of resveratrol supplementation and vitrification process can alter the expression levels of genes related to apoptosis and implantation (*Bax*, *Bcl-2* and *ErbB4*) in mouse embryos at the hatched blastocyst stage.

REFERENCES

Abdel-Wahab, A., Zullo, G., Boccia, L., De Blasi, M., Longobardi, V., Albero, G., and Gasparrini, B. (2012). Resveratrol during in vitro culture improves cryotolerance of in vitro produced bovine embryos. **Reprod. Fertil. Dev.** 25: 213-214.

Abe, T., Kawahara-miki, R., HARA, T., Noguchi, T., Hayashi, T., Shirasuna, K., Kuwayama, T., and Iwata, H. (2017). Modification of mitochondrial function, cytoplasmic lipid content and cryosensitivity of bovine embryos. **J. Reprod. Dev.** 63: 455-461.

Agarwal, A., and Majzoub, A. (2017). Role of Antioxidants in Assisted Reproductive Techniques. **World J. Mens. Health.** 35: 77-93.

Agarwal, A., Said, T.M., Bedaiwy, M.A., Banerjee, J., and Alvarez, J.G. (2006). Oxidative stress in an assisted reproductive techniques setting. **Fertil. Steril.** 86: 503-512.

Alarcón de la, C., and Villegas, I. (2007). Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. **Biochem. Soc. Trans.** 35: 1156-1160.

Almasi-turk, S., and Roozbehi, A. (2013). Mouse Oocytes and Embryos Cryotop-vitrification Using Low Concentrated Solutions: Effects on Meiotic Spindle, Genetic Material Array and Developmental Ability. **Iran. J. Basic. Med. Sci.** 16: 590-601.

Anchamparuthy, V., Dhali, A., Butler, S., Pearson, R., Jiang, H., and Gwazdauskas, F. (2007). Effect of Vitrification of Zygotes in Nylon Mesh on the Differential

Expression of Apoptotic Genes in Mouse Embryos. **World. J. Dairy. Food. Sci.** 2: 54-61.

Arav, A. (2014). Cryopreservation of oocytes and embryos. **Theriogenology** 81: 96-102.

Baur, J.A., and Sinclair, D.A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. **Nat. Rev. Drug. Discov.** 5: 493-506.

Betts, D.H. and Madan, P. (2008). Permanent embryo arrest: molecular and cellular concepts.**Mol. Hum. Reprod.** 14: 445-453.

Castillo-Mart´ın, M., Bonet, S., Morato´, R., and Yeste, M. (2014). Supplementing culture and vitrification-warming media with L-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of GPX1 and SOD1 expression. **Cryobiology** 68: 451-458.

Chen, H., Zhang, L., Wang, Z., Chang, H., Xie, X., Fu, L., Zhang, Y., and Quan, F. (2019). Resveratrol improved the developmental potential of oocytes after vitrification by modifying the epigenetics. **Mol. Reprod. Dev.** 86: 862-870.

Chen, S.U., Lien, Y.R., Cheng, Y.Y., Chen, H.F., Ho, H.N., and Yang, Y.S. (2001). Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. **Hum. Reprod.** 16: 2350-2356.

Chian, C.R., Wang, Y., and Li, R.Y. (2014). Oocyte vitrification: advances, progress and future goals. **J. Assist. Reprod. Genet.** 31: 411-420.

Chian, R.C., and Quinn, P. (2010). **Fertility cryopreservation.** Cambridge: Cambridge University Press.

Chian, R.C., Son, W.Y., Huang, J.Y., Cui, S.J., Buckett, W.M., and Tan, S.L. (2005). High survival rates and pregnancies of human oocytes following vitrifi- cation: preliminary report. **Fertil. Steril**. 84: 36.

Chung, J.H., Manganiello, V., and Dyck, J.R. (2012). Resveratrol as a calorie restriction mimetic: therapeutic implications. **Trends Cell Biol.** 22: 546-554.

Circu, M.L., and Aw,T.Y. (2010). Reactive Oxygen Species, Cellular Redox Systems And Apoptosis. **Free Radic. Biol. Med.** 48: 749-762.

Cobo, A., and Diaz, C. (2011). Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials. **Fertil. Steril.** 96: 277-285.

Comizzoli, P., Wildt, D.E., and Pukazhenthi, B.S. (2009). In Vitro Compaction of Germinal

Vesicle Chromatin is Beneficial to Survival of Vitrified Cat Oocytes. **Reprod. Dom. Anim.** 44: 269-274.

Conte, E., Fagone, E., Fruciano, M., Gili, E., Iemmolo, M., and Vancheri, C. (2015). Anti-inflammatory and antifibrotic effects of resveratrol in the lung. **Histol. Histopathol.** 30: 523-529.

Coutinho, A.R., Mendes, C.M., Caetano, H.V., Nascimento, A.B., Oliveira, V.P., Hernadez-Blazquez, F.J., Sinhorini, I.L., Visintin, J A., and Assumpção, M.E. (2007). Morphological changes in mouse embryos cryopreserved by different techniques. **Microsc. Re. Tech.** 70: 296-301.

Cuello, C., Gil, M.A., Parrilla, I. Tornel, J., Vázquez, J.M., Roca, J., Berthelot, F., Martinat-Botté, F., and Martínez, E.A. (2004). Vitrification of porcine embryos at various developmental stages using different ultra- rapid cooling procedures.

Theriogenology 62: 353-361.

Dalcin, L., Silva, R.C., Paulini, F., Silva, B.D., Neves, J.P., and Lucci, C.M. (2013). Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. **Cryobiology** 67: 137-145.

Davidson, L.M., and Coward, K. (2016). Molecular mechanisms of membrane interaction at implantation. **Birth Defects Res. Part C Embryo Today** 108: 19-32.

Dehghani-Mohammadabadi, M., Salehi, M., Farifteh, F., Nematollahi, S., Arefian, E., Hajjarizadeh, A., Parivar, K., and Nourmohammadi, Z. (2014). Melatonin modulates the expression of BCL-xl and improve the development of vitrified embryos obtained by IVF in mice. **J. Assist. Reprod. Genet.** 31: 453-461.

Dhali, A., Anchamparuthy, V., Butler, S., Pearson, R., Mullarky, I., and Gwazdauskas, F. (2007). Gene expression and development of mouse zygotes following droplet vitrification. **Theriogenology** 68: 1292-1298.

Dickinson, B.C., and Chang, C.J. (2011). Chemistry and biology of reactive oxygen species
in signaling or stress responses. **Nat. Chem. Biol.** 7: 504-511.

Dinnyes, A., Dai, Y., Jiang, S., and Yang, X. (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. **Biol. Reprod.** 63: 513-518.

Duranthon, V., Watson, A.J., and Lonergan, P. (2008). Preimplantation embryo programming: transcription, epigenetics, and culture environment. **Reprod. Fertil.** 135: 141-150.

Edashige, K. (2016). The movement of water and cryoprotectants across the plasma

membrane of mammalian oocytes and embryos and its relevance to vitrification. **J. Reprod. Dev.** 62: 317-321.

Edashige, K. (2017). Permeability of the plasma membrane to water and cryoprotectants in mammalian oocytes and embryos: Its relevance to vitrification. **Reprod. Med. Biol.** 16: 36-39.

Edashige, K., Ohta, S., Tanaka, M., Kuwano, T., Valdez, D.M.Jr., Hara, T., Jin, B., Takahashi, S., Seki, S., Koshimoto, C., and Kasai, M. (2007). The role of aquaporin 3 in the movement of water and cryoprotectants in mouse morulae. **Biol Reprod.** 77: 365-375.

Edashige, K., Tanaka, M., Ichimaru, N., Ota, S., Yazawa, K., Higashino, Y., Sakamoto, M., Yamaji, Y., Kuwano, T., Valdez, D.M. Jr., Kleinhans, F.W., and Kasai, M. (2006). Channel-dependent permeation of water and glycerol in mouse morulae. **Biol. Reprod.** 74: 625-632.

Fabian, D., Koppel, J., and Maddox-Hyttel, P. (2005). Apoptotic process during mammalian preimplantation development. **Theriogenology** 64: 213-221.

Fadini, R., Brambillasca, F., Renzini, M., Merola, M., Comi, R., De Ponti, E., and Dal Canto, M. (2009). Human oocyte cryopreservation: comparison between slow and ultrarapid methods. **Reprod. BioMed Online.** 19: 171-180.

Fahy, G.M., MacFarlane, D.R., Angell, C.A., and Meryman, H.T. (1984). vitrification as an approach to cryopreservation. **Cryobiology** 21: 407-426.

Flurkey, K., Currer, J.M., Leiter, E.H., and Witham, B. (2009). **The Jackson Laboratory Handbook on Genetically Standardized Mice.** Sixth Edition. Bar Harbor: ME 04609.

Fremont, L. (2000). Biological Effects of Resveratrol. **Life Sci.** 66: 663-673.

Fulda, S., Gorman, A.M., Hori, O., and Samali, A. (2010). Cellular stress responses: cell survival and cell death. **J. Cell Biol.** 10: 1-23.

Fuller, B.J., and Bernard, A. (1984). Successful in vitro fertilization of mouse oocytes after cryopreservation using glycerol. **Cryolett.** 5: 307-312.

Gambini, J., Ingle´s, M., Olaso, G., Lopez-Grueso, R., Bonet-Costa, V., Gimeno-Mallench, L., Mas-Bargues, C., Abdelaziz, K. M., Gomez- Cabrera, M. C., Vina, J., and Borras, C. (2015). Properties of resveratrol: in vitro and in vivo studies about metabolism, bioavailability, and biological effects in animal models and humans. **Oxid. Med. Cell. Longev.** 2015, 837042.

Gaviria, S.M., Herrera, A.L., Urrego, R., Betancur, G.R., and Zuluaga, J.J.E. (2019). Effect of resveratrol on vitrified in vitro produced bovine embryos: Recovering the initial quality. **Cryobiology** 89: 42-50.

Gaviria, S.M., Morado, S.A., Lo´pez Herrera, A., Betancur, G.R., Urrego A´ lvarez, R.A., Zuluaga, J.J.E., and Ce´tica, P.D. (2018). Resveratrol supplementation promotes recovery of lower oxidative metabolism after vitrification and warming of in vitro-produced bovine embryos. **Reprod. Fertil. Dev.** 31: 521-528.

George, F., Daniaux, C., Genicot, G., Verhaeghe, B., Lambert, P., and Donnay, I. (2008). Set up of a serum-free culture system for bovine embryos:embryo development and quality before and after transient transfer. **Theriogenology** 69: 612-623.

Ghandy, N., and Malekshah, A.A.K. (2017). Which Stage of Mouse Embryos Is More Appropriate for Vitrification?. **Int. J. Fertil. Steril.** 10: 357-362.

Gharenaz, N.M., Movahedin, M., and Mazaheri, Z. (2018). Effects of re-vitrification of mouse morula and early blastocyst stages on apoptotic gene expression and

developmental potential. **Cell Journal (Yakhteh)** 19: 614-619.

Gharenaz, N.M., Movahedin, M., Mazaheri, Z., and Beiranvand, S.P. (2016). Alternation of apoptotic and implanting genes expression of mouse embryos after re-vitrification. **Int. J. Reprod. Biomed. (Yazd)** 14: 511-518.

Giaretta, E., Spinaci, M., Bucci, D., Tamanini, C., and Galeati, G. (2013). Effects of Resveratrol on Vitrified Porcine Oocytes. **Oxidative med. cell. longev. (Online).** e920257.

Gordts, S., Rozires, P., Campo, R., and Noto, V. (1990). Survival and pregnancy outcome after ultrarapid freezing of human embryos. **Fertil. Steril.** 53: 469-472.

Gusman, J., Malonne, H., and Atassi, G. (2001). A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. **Carcinogenesis** 22: 1111-1117.

Hafez, Y.M. (2015). Assisted Reproductive Technologies in Farm Animals. **ICMALPS**: 91-118.

Halliwell, B. (2011). Free radicals and antioxidants - quo vadis?. **Trends Pharmacol. Sci.** 32: 125-130.

Halliwell, B. (2012). Free radicals and antioxidants: updating a personal view. **Nutr. Rev.** 70: 257-265.

Hamawaki, A., Kuwayama, M., and Hamano, S. (1999). Minimum volume cooling method for bovine blastocyst vitrification. **Theriogenology** 51:165.

Hara, T., Kin, A., Aoki, S., Nakamura, S., Shirasuna, K., Kuwayama, T., and Iwata H. (2018). Resveratrol enhances the clearance of mitochondrial damage by vitrification and improves the development of vitrified-warmed bovine embryos. **PLoS ONE** 13: e0204571.

Hayashi, T., Ueda, S., Mori, M., Baba, T., Abe, T., and Iwata, H. (2018). Influence of resveratrol pretreatment on thawed bovine embryo quality and mitochondrial DNA copy number. **Theriogenology** 106: 271-278.

Herrero, L., Martínez, M., and Garcia-Velasco, J.A. (2011). Current status of human oocyte and embryo cryopreservation. **Curr. Opin. Obstet. Gynecol.** 23: 245-250.

Hosseini, S.M., Forouzanfar, M., Hajian, M., Asgari, V., Abedi, P., Hosseini, L., Ostadhosseini, S., Moulavi, F., Safahani Langrroodi, M., Sadeghi, H., Bahramian, H., Eghbalsaied, Sh., and Nasr-Esfahani, M.H. (2009). Antioxidant supplementation of culture medium during embryo development and/or after vitrification-warming; which is the most important?. **J. Assist. Reprod. Genet.** 26: 355-364.

Houghton, F.D. (2006). Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst. **Differentiation** 74: 11-18.

Huang, F.J., Chin, T.Y., and Chan, W.H. (2013). Resveratrol protects against methylglyoxalinduced apoptosis and disruption of embryonic development in mouse blastocysts**. Environ Toxicol.** 28: 431-441.

Hunter, J.E. (1995). Cryopreservation of mammalian embryos. Slow cooling. **Methods. Mol. Biol.** 38: 199-209.

Hunter, J.E., Fuller, B.J., Bernard, A., Jackson, A., and Shaw, R.W. (1995). Vitrification of human oocytes following minimal exposure to cryoprotectants; initial studies on fertilization and embryonic development. **Hum. Reprod.** 10: 1184-1188.

Hwang, I.S. and Hochi, S. (2014). Recent progress in cryopreservation of bovine

oocytes. **Bio. Med. Res. Int.** e570647.

Ignatowicz, E., and Baer-Dubowska, W. (2001). Resveratrol, a natural chemopreventive agent against degenerative diseases. **Pol. J. Pharmacol.** 53: 557-569.

Inaba, Y., Miyashita, S., Somfai, T., Geshi, M., Matoba, S., Dochi, O., and Naqai, T. (2016). Cryopreservation method affects DNA fragmentation introphectoderm and the speed

of re-expansion in bovine blastocysts. **Cryobiology** 72: 86-92.

Isachenko, V., Folch, J., Isachenko, E., Nawroth, F., Krivokharchenko, A., Vajta, G., Dattena, M., and Alabarta, J.L. (2003). Double vitrification of rat embryos at different developmental stages using an identical protocol. **Theriogenology** 60: 445-452.

Ito, J., Kuramochi, M., Inoue, A., Yabe, K., Fujiwara, K., and Sonoki, S. (2010). Cryotop facilitates high developmental ability of re-vitrified mouse embryos. **Reprod. Eng.** 13: 21-26.

Jo, J.W., Jee, B.C., Lee, J.R., and Suh, C.S. (2011). Effect of antifreeze protein supplementation in vitrification medium on mouse oocyte developmental competence. **Fertil. Steril.** 96: 1239-1245.

Jo, J.W., Jee, B.C., Suh, C.S., and Kim, S.H. (2012). The Beneficial Effects of Antifreeze Proteins in the Vitrification of Immature Mouse Oocytes. **PLoS ONE** 7: e37043.

Johnson, M. (2012). Laboratory Mice and Rats. **Mater. Methods** 2:113doi:10.13070/ mm.en.2.113.

Kaidi, S., Bernard, S., Lambert, P., Massip, A., Dessy, F., and Donnay, I. (2001). Effect

of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced in vitro. **Biol. Reprod.** 65: 1127-1134.

Kannan, K., and Jain, S.K. (2000). Oxidative stress and apoptosis. **Pathophysiology** 7: 153-163.

Kasai, M., and Edashige, K. (2010). Movement of water and cryoprotectants in mouse oocytes and embryos at different stages: Relevance to cryopreservation. In: Chian, R., Quinn, P., editors. **Fertility Cryopreservation.** Cambridge: Cambridge University Press 16-23.

Kehrer, J.P., and Klotz, L.O. (2015). Free radicals and related reactive species as mediators of tissue injury and disease: implications for health. **Crit. Rev. Toxicol.** 45: 765-798.

Kitagawaa, Y., Suzukib, K., Yonedaa, A., and Watanabea, T. (2004). Effects of oxygen

concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. **Theriogenology** 62, 1186-1197.

Koutlaki, N., Schoepper, B., Maroulis, G., Diedrich, K., and Al-Hasani, S. (2006). Human oocyte cryopreservation: past, present and future. **Reprod. Biomed. Online.** 13: 427-436.

Krisher, R.L., Petters, R.M., Johnson, B.H., Bavister, B.D., and Archi- bong, A.E. (1989). Development of porcine embryos from the one-cell stage to blastocyst in mouse oviducts maintained in organ culture. **J. Exp. Zool.** 249: 235-239.

Kuc, P., Kuczynska, A., Stankiewicz, B., Sieczynski, P., Matysiak, J., and Kuczynski, W. (2010). Vitrification vs. slow cooling protocol using embryos cryopreserved in the 5th or 6th day after oocyte retrieval and IVF outcomes. **Folia. Histochem. Cytobiol.** 48: 84-88.

Kuwajerwala, N., Cifuentes, E., Gautam, S., Menon, M., Barrack, and E.R., Reddy, G.P.V. (2002). Resveratrol induces prostate cancer cell entry into S phase and inhibits DNA synthesis. **Cancer. Res.** 62: 2488-2492.

Kuwayama, M. (2007). Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. **Theriogenology** 67: 73-80.

Kuwayama, M., Vajta, G., Ieda, S., and Kato, O. (2005a). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. **Reprod. Biomed. Online.** 11: 608-614.

Kuwayama, M., Vajta, G., Kato, O., and Leibo, S.P. (2005b). Highly efficient vitrification method for cryopreservation of human oocytes. **Reprod. Biomed. Online.** 11: 300-308.

Kwak, S.S., Cheong, S.A., Jeon, Y. and Lee, E. (2012). The effects of resveratrol on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic

activation and in vitro fertilization. **Theriogenology.** 78: 86-101.

Lane, M., Maybach, J.M., and Gardner, D.K. (2002). Addition of ascorbate during cryopreservation stimulates subsequent embryo development. **Hum. Reprod.** 17: 2686-2693.

Lane, M., Schoolcraft, W.B., Gardner, D.K., and Phil, D. (1999). Vitrification of mouse and human blastocysts using a novel cryoloop containerless technique. **Fertil.**

Steril. 72: 1073-1078.

Larman, M.G., and Gardner, D.K. (2010). Vitrifying mouse oocytes and embryos with super-cooled air. **Hum. Reprod.** 25: 265.

Lawitts, J.A. and Biggers, J.D. (1993) Culture of preimplantation embryos. **Methods Enzymol.** 225: 153-164.

Lee, H.H., Lee H.J., Kim H.J., Lee J.H., Ko, Y., Kim, S.M., Lee, J.R., Suh, C.S., and Kim S.H. (2015). Effects of antifreeze proteins on the vitrification of mouse oocytes: comparison of three different antifreeze proteins. **Hum. Reprod.** 30: 2110-2119.

Lee, K., Wang, C., Chaille J.M., and Machaty, Z. (2010). Effect of Resveratrol on the Development of Porcine Embryos Produced In Vitro. **J. Reprod. Dev.** 56: 330-335.

Leibo, S.P., and Mazur, P. (1978). Methods for the cryopreservation of mammalian embryos by freezing. In: Daniel, Jr. K.C., editor. **Methods in Mammalian Reproduction.** New York: Academic Press 179-201.

Leme, L.O., Dufort, I., Sprícigo, J.F.W., Braga, T.F., Sirard, M.A., Franco, M.M., and Dode, M.A.N. (2016). Effect of vitrification using the Cryotop method on the gene expression profile of in vitro-produced bovine embryos. **Theriogenology** 85: 724-733.

Leonard, S., Xia, C., Jiang, B.H., Stinefelt, B., Klandorf, H., Harris, G.K. and Shi, X. (2003). Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. **Biochem. Biophys. Res.** 309: 1017-1026.

Levi Setti, P.E., Porcu, E., Patrizio, P., Vigiliano, V., de Luca, R., d'Aloja, P., Spoletini, R., and Scaravelli, G. (2014). Human oocyte cryopreservation with slow freezing

versus vitrification**. Fertil. Steril.** 102: 90-95.

Li, H., Horke, S., and Forstermann, U. (2013). Oxidative stress in vascular disease and its pharmacological prevention. **Trends Pharmacol. Sci.** 34: 313-319.

Li, Z., Wang, Y.A., Ledger, W., Edgar, D.H., and Sullivan, E.A. (2014). Clinical outcomes following cryopreservation of blastocysts by vitrification or slow freezing: a population-based cohort study. **Hum. Reprod.** 29: 2794-2801.

Liang, Q.X., Lin, Y.H., Zhang, C.H., Sun, H.M., Zhou, L., Schatten, H., Sun, Q.Y., and Qian, W.P. (2018). Resveratrol increases resistance of mouse oocytes to postovulatory aging in vivo. **Aging.** 10: 1586-1596.

Liebermann, J., Tucker, M., Graham, J., Han, T., Davis, A., and Levy, M. (2002). Blasto- cyst development after vitrification of multi pronuclear zygotes using the flexipet denuding pipette. **Reprod. Biomed. Online.** 4: 146-150.

Lin, T.K., Su, J.T., Lee, F.K., Lin, Y.R., and Lo, H.C. (2010). Cryotop vitrification as compared to conventional slow freezing for human embryos at the cleavage stage: survival and outcomes. **Taiwan J. Obstet. Gynecol.** 49: 272-278.

Ling, X.F., Zhang, J.Q., Cao, S.R., Chen, J., Peng, Y., Guo, X., Heng, B.C., Tong, G.Q., and Wang, X. (2009). Effect of cryotop vitrification on preimplantation developmental competence of murine morula and blastocyst stage embryos. **Reprod. Biomed. Online.** 19: 708-713.

Liu, Y., He, X.Q., Huang, X., Ding, L., Xu, L., Shen, Y.T., Zhang, F., Zhu, M.B., Xu, B.H., Qi, Z.Q., and Wang, H.L. (2013). Resveratrol Protects Mouse Oocytes from Methylglyoxal-Induced Oxidative Damage. **PLoS ONE** 8: e77960.

Liu, Y., Wang, Y.L., He, S.W., Chen, M. H., Zhang, Z., Fu, X.P., Fu, B.B., Liao, B.Q., Lin, Y.H., Qi, Z. Q., and Wang, H.L. (2017). Protective effects of resveratrol

against mancozeb induced apoptosis damage in mouse oocytes. **Oncotarget** 8: 6233-6245.

Lonergan, P. (2007). State-of-the-art embryo technologies in cattle. **Soc. Reprod. Fertil.** 64: 315-325.

Losa, G.A. (2003). Resveratrol modulates apoptosis and oxidation in human blood mononuclear cells. **Eur. J. Clin. Invest.** 33: 818-823.

Mandawala, A. A., Harvey, S. C., Roy, T. K., and Fowler, K. E. (2016). Cryopreservation of animal oocytes and embryos: current progress and future prospects. **Theriogenology** 86: 1637-1644.

Martino, A., Songsasen, N., and Leibo, S.P. (1996). Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 54: 1059-1069.

Martı́nez, J., and Moreno, J.J. (2000). Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. **Biochem. Pharmacol.** 59: 865-870.

Matsumoto, H., Jiang, J.Y., Tanaka, T., Sasada, H., and Sato, E. (2001). Vitrification of large quantities of immature bovine oocytes using nylon mesh. **Cryobiology** 42: 139-144.

Mattson, M.P. (2008). Hormesis Defined. **Ageing. Res. Rev.** 7: 1-7.

Mazur, P. (1990). Equilibrium quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. **Cell. Biophys.** 17: 53-92.

Michal, J., Jana, Z., Pavel, V., Kempisty, B., and Igor, C. (2016). Cryopreservation of Human Gametes and Embryos: Current State and Future Perspectives. In: Marco-Jiménez, F., and Akdemir, H., editors. **Cryopreservation in Eukaryotes.**

IntechOpen. doi: 10.5772/64950.

Mikula-Pietrasik, J., Sosinska, P., and Ksiazek, K. (2014). Resveratrol inhibits ovarian cancer cell adhesion to peritoneal mesothelium in vitro by modulating the production of alpha5beta1 integrins and hyaluronic acid. **Gynecol. Oncol.** 134: 624-630.

Milachich, T. and Shterev, A. (2016). Are there optimal numbers of oocytes, spermatozoa and embryos in assisted reproduction?. **JBRA Assist. Reprod**. 20: 142-149.

Minoru, S. H. (2006). Expression profiling of the mouse early embryo: Reflections and Perspectives. **Dev. Dyn.** 235: 2437-2448.

Momozawa, K., Matsuzawa, A., Tokunaga, Y., Abe, S., Koyanagi, Y., Kurita,M., Nakano, M., and Miyake, T. (2017). Efficient vitrification of mouse embryos using the Kitasato Vitrification System as a novel vitrification device. **Reprod. Biol. Endocrinol.** 15: 1-9.

Mora-Pale, M., Bhan, N., Masuko, S., James, P., Wood, J., McCallum, S., Linhardt, R.J., Dordick, J.S., and Koffas, M.A. (2015). Antimicrobial mechanism of resveratrol-trans-dihydrodimer produced from peroxidase-catalyzed oxidation of resveratrol. **Biotechnol. Bioeng.** 112: 2417-2428.

Morató, R., Romaguera, R., Izquierdo, D., Paramio, M.T., and Mogas, T. (2011). Vitrification of in vitro produced goat blastocysts: effects of oocyte donor age and development stage. **Cryobiology**. 63: 240-244.

Moussa, M., Shu, J., Zhang, X.H., and Zeng, F.Y. (2014). Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. **Sci. China Life Sci.** 57: 903-914.

Mukaida, T., Wada, S., Takahashi, K., Pedro. P.B., An, T.Z., and Kasai, M. (1998). Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. **Hum. Reprod.** 13: 2874-2879.

Mukherjee, A., Malik, H., Saha, A.P., Dubey, A., Singhal, D.K., Boateng,S., Saugandhika,S., Kumar, S., De, S., Guha, S.K., and Malakar, D. (2014). Resveratrol treatment during goat oocytes maturation enhances developmental competence of parthenogenetic and hand-made cloned blastocysts by modulating intracellular glutathione level and embryonic gene expression. **J. Assist. Reprod. Genet.** 31: 229-239.

Nakagata, N. (1989). High survival rate of unfertilized mouse oocytes after vitrification**. J. Reprod. Fertil.** 87: 479-483.

Niemann, H., and Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. **Theriogenology** 53: 21-34.

Niu, Y., Dai, J., Wu, C., Chen, Y., Zhang, S., and Zhang, D. (2016). The application of apoptotic inhibitor in apoptotic pathways of MII stage porcine oocytes after vitrification. **Reprod. Domest. Anim.** 51: 953-959.

Nowshari, M.A., Nayudu, P.L., and Hodges, J.K. (1994). Effect of cryo- protectant concentration, equilibration time and thawing procedure on survival and development of rapid-frozen–thawed mature mouse oocytes. **Theriogenology.** 42: 1193-1204.

Padhee, M., Zhang, S., Lie, S., Wang, K.C., Botting, K.J., Caroline McMillen, I., MacLaughlin, S.M., and Morrison, J.L. (2015). The Periconceptional

Environment and Cardiovascular Disease: Does In Vitro Embryo Culture and Transfer Influence Cardiovascular Development and Health?. **Nutrients** 7: 1378-1425.

Pallepati, P. and Averill-Bates, D.A. (2012). Reactive oxygen species, cell death signaling and apoptosis. In: Pantopoulos, K., Schipper H., editors. **Principles of Free Radical Biomedicine.** New York: Nova Science Publishers 513-546.

Paria, B., Das, S., Andrews, G., and Dey, S. (1993). Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. **Proc. Nat. Acad. Sci.** 90: 55-59.

Pegg, D.E. (2005). The role of vitrification techniques of cryopreservation in reproductive medicine. **Hum. Fert. 8**: 231-239.

Pervaiz, S. (2003). Resveratrol: from grapevines to mammalian biology. **FASEB J.** 17: 1975-1985.

Pervaizand, S., and Holme, A.L. (2009). Resveratrol: its biologic targets and functional activity. **Antioxid. Redox. Sign.** 11: 2851-2897.

Pfaff, R.T., Liu, J., Gao, D., Peter, A.T., Li, T.K., Critser, J.K. (1998). Water and DMSO membrane permeability characteristics of in-vivo and in-vitro de- rived and cultured murine oocytes and eandmbryos**. Mol. Hum. Reprod.** 4: 51-59.

Pirola, L., and Frojdo, S. (2008). Resveratrol: one molecule, many targets. **IUBMB Life** 60: 323-332.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., and Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. **Oxidative med. cell. longev. (Online).** e8416763.

Plessis, S.S., Makker, K., Desai, N.R., and Agarwal, A. (2008). Impact of oxidative

stress on IVF. **Expet. Rev. Obstet. Gynecol.** 3: 539-554.

Polge, C., and Willadsen, S.M. (1978). Freezing eggs and embryos of farm animals. **Cryobiology** 15: 370-373

Portmann, M., Nagy, Z.P., and Behr, B. (2010). Evaluation of blastocyst survival following vitrification/warming using two different closed carrier systems. **Hum. Reprod.** 25: 261.

Punyawai, K., Anakkul, N., Srirattana, K., Aikawa, Y., Sangsritavong, S., Nagai, T., Imai, K. and Parnpai, R. (2015). Comparison of Cryotop and micro volume air cooling methods for cryopreservation of bovine matured oocytes and blastocysts. **J. Reprod. Dev.** 61: 431-437.

Quinn, P., Moinipanah, R., Steinberg, J.M. and Weathersbee, P. (1995) Successful human in vitro fertilization using a modified human tubal fluid medium lacking glucose and phosphate ions. **Fertil. Steril.** 63: 922-924.

Rajendran, P., Nandakumar, N., Rengarajan, T., Palaniswami, R., Gnanadhas, N.E., and Lakshminarasaiah, U. (2014). Antioxidants and human diseases. **Clinica. Chimica. Acta.** 436: 332-347.

Rall, W.F., and Fahy, G.M. (1985). Ice-free cryopreservation of mouse embryos at 196 °C by vitrification. **Nature.** 313: 573-575.

Redza-Dutordoir, M., and Averill-Bates, D.A. (2016). Activation of apoptosis signalling pathways by reactive oxygen species. **Biochim. Biophys. Acta** 1863: 2977-2992.

Rizos, D., Clemente, M., Bermejo-Alvarez, P., de La Fuente, J., Lonergan, P., and Gutie´rrez-Ada´, A. (2008). Consequences of In Vitro Culture Conditions on Embryo Development and Quality. **Reprod. Dom. Anim.** 43: 44-50.

Rizos, D., Ward, F., Duffy, P., Boland, M.P., and Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. **Mol. Reprod. Dev.** 61: 234-248.

Roy, T.K., Brandi, S., Tappe, N.M., Bradley, C.K., Vom, E., Henderson, C., Lewis, C., Battista, K., Hobbs, B., Hobbs, S., Syer, J., Lanyon, S.R., Dopheide, S.M., Peura, T.T., McArthur, S.J., Bowman, M.C., and Stojanov, T. (2014). Embryo vitrification using a novel semi-automated closed system yields in vitro outcomes equivalent to the manual Cryotop method. **Hum. Reprod.** 29: 2431-2438.

Rubiolo, J. A., Mithieux, G., and Vega, F. V. (2008). Resveratrol protects primary rat hepatocytes against oxidative stress damage. Activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes. **Eur. J. Pharmacol.** 591: 66-72.

Sakurai, K. (2017). Vitrification - A front-runner of human oocyte and embryo cryopreservation technology. **FUJIFILM Irvine Scientific.** Retrieved from http://www.irvinesci.com/blog/vitrification-front-runner-of-human-oocyte-embryo-cryopreservation-tech.

Salzanoa, A., Alberoa, G., Zulloa, G., Negliaa, G., Abdel-Wahabb, A., Bifulcoa, G., Zicarelli, L., and Gasparrini, B. (2014). Effect of resveratrol supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos. **Anim. Reprod. Sci.** 151: 91-96.

Santos, E.C.S., Appeltant, R., Dang-Nguyen, T.Q., Noguchi, J., Kaneko, H., Kikuchi, K., and Somfai, T. (2018). The effect of resveratrol on the developmental competence of porcine oocytes vitrified at germinal vesicle stage. **Repord. Dom.**

Anim. 53: 304-312.

Saragusty, J., and Arav, A. (2011). Current progress in oocyte and embryo cryo-preservation by slow freezing and vitrification. **Reproduction** 141: 1-19.

Scaravelli, G., and Spoletini, R. (2015). The Application of Reproductive Techniques (ART): Worldwide Epidemiology Phenomenon and Treatment Outcomes. In: Watson, R. R., editors. **Handbook of Fertility Nutrition, Diet, Lifestyle and Reproductive Health.** first Edition. Cambridge: Academic Press 75-87.

Seidel, G. E. (2015). Lessons from Reproductive Technology Research. **Annu. Rev. Anim. Biosci.** 3: 467-487.

Shaw, P.W., Bernard, A.G., Fuller, B.J., Hunter, J.H., and Shaw, R.W. (1992). Vitrification of mouse oocytes using short cryoprotectant exposure: effects of varying exposure times on survival. **Mol. Reprod.** 33: 210-214.

Shi, L.Y., Jin, H.F., Kim, J.G., Mohana Kumar, B., Balasubramanian, S., Choe, S.Y., and Rho, G.J. (2007). Ultra-structural changes and developmental potential of porcine oocytes following vitrification. **Anim. Reprod. Sci.** 100: 128-140.

Shoraki, F.F., and Akhondi, M.M. (2016). Examining laboratory mouse oocytes and vitrification. **Adv. Biores.** 7: 16-21.

Smoliga, J.M., Baur, J.A., and Hausenblas, H.A. Resveratrol and health–a comprehensive review of human clinical trials. **Mol. Nutr. Food. Res.** 55: 1129-1141.

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Kuriani Karja, N.W., Farhudin, M., Dinnyés, A., Nagai, T., and Kikuchi, K. (2007a). Developmental competence of in vitro- fertilized porcine oocytes after in vitro maturation and solid surface vitrification: effect of cryopreservation on oocyte antioxidative system and cell

cycle stage. **Cryobiology** 55: 115-126.

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Ohnuma, K., Karja, N.W.K., Farhudin, M., Maedomari, N., Dinnyes, A., Nagai, T., and Kikuchi, K. (2007b). Vitrification causes damage of the antioxidant defense system in IVM porcine oocytes. **Reprod. Fertil. Dev.** 19: 184.

Spikings, E.C., Alderson, J., and St John, J.C. (2007). Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development. **Biol. Reprod.** 76: 327-335.

Sprícigo, J.F., Morató, R., Arcarons, N., Yeste, M., Dode, M.A., López-Bejar,M., and Mogas, T. (2017). Assessment of the effect of adding L-carnitine and/or resveratrol to maturation medium before vitrification on in vitro-matured calf oocytes. **Theriogenology** 89: 47-57.

Srirattana, K., Sripunya, N., Sangmalee, A., Imsoonthornruksa, S., Liang, Y.Y., Ketudat-Cairns, M., and Parnpai, R. (2013). Developmental potential of vitrified goat oocytes following somatic cell nuclear transfer and parthenogenetic activation. **Small Rumin. Res.** 112: 141-146.

St John, J.C., Facucho-Oliveira, J., Jiang, Y., Kelly, R., and Salah, R. (2010). Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. **Hum. Reprod. Update.** 16: 488-509.

Summers, M.C., and Biggers, J.D. (2003). Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. **Hum. Reprod. Update** 9: 557-582.

Sun, X., Li, Z., Yi, Y., Chen, J., Leno, G.H., and Engelhardt, J.F. (2008). Efficient term

development of vitrified ferret embryos using a novel pipette chamber technique. **Biol. Reprod.** 79: 832-840.

Szende, B., Tyihak, E., and Kiraly-Veghely, Z. (2000). Dose-dependent effect of resveratrol

on pro- liferation and apoptosis in endothelial and tumor cell cultures. **Exp. Mol. Med.** 32: 88-92.

The Jackson Laboratory. (2018). **B6D2 F1 hybrid mice.** Retrieved from https://www.jax.org/strain/100006.

The Jackson Laboratory. (2018). **C57BL/6NJc1 (B6) female mice.** Retrieved from https://www.jax.org/strain/000664.

The Jackson Laboratory. (2018). **DBA/2NJc1 (D2) male mice.** Retrieved from https://www.jax.org/strain/000671.

Thompson, J.G. (1997). Comparison between in vivo-derived and in vitro-produced pre- elongation embryos from domestic ruminants. **Reprod. Fertil. Dev.** 9: 341-354.

Thundathil, J., Filion, F., and Smith, L.C. (2005). Molecular control of mitochondrial function in preimplantation mouse embryos. **Mol. Reprod.** 71: 405-413.

Torres, V., Hamdi, M., Millán de la Blanca, M., Urrego, R., Echeverri, J. López-Herrera, A., Rizos, D., Gutiérrez-Adán, A., and Sánchez-Calabuig, M. (2018) Resveratrol-cyclodextrin complex affects the expression of genes associated with lipid metabolism in bovine in vitro produced embryos. **Reprod. Domest. Anim.** 1-9.

Turrens, J.F. (2003). Mitochondrial formation of reactive oxygen species. **J. Physiol** 552: 335-344.

Vajta, G. (2006). Are programmable freezers still needed in the embryo laboratory? Review on vitrification. **Reprod. BioMed. Online.** 6: 779-796.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T., and Callesen, H. (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. **Mol. Reprod.** 51: 53-58.

Vallorani, C., Spinaci, M., Bucci, D., Porcu, E., Tamanini, C., and Galeati, G. (2012). Pig oocyte vitrification by cryotop method and the activation of the apoptotic cascade. **Anim. Reprod. Sci.** 135: 68-74.

Vanderzwalmen, P., Bertin, G., Debauche, C., Standaart, V., and Schoysman, E. (2000). In vitro survival of metaphase II oocytes (MII) and blastocysts after vitrification in a hemi-straw (HS) system. **Fertil. Steril.** 74: 215-216.

Verkman, A.S., van Hoek, A.N., Ma, T., Frigeri, A., Skach, W. R., Mitra, A., Tamarappoo, B. K., and Farinas, J. (1996). Water transport across mammalian cell membranes. **Am. J. Physiol.** 270: 12-30.

Vladimirov, I.K., Tacheva, D., and Diez, A. (2017). Theory about the Embryo Cryo-Treatment. **Reprod. Med. Biol.** 16: 118-125.

Wakayama, S., Mizutani, E. and Wakayama, T. (2010). **Guide to Techniques in Mouse Development, Part A: Mice, Embryos, and Cells.** 2nd Edition. New York: Academic Press.

Wang, X.L., Zhang, X., Qin, Y.Q., Hao, D.Y., and Shi, H.R. (2012). Outcomes of day 3 embryo transfer with vitrification using Cryoleaf: a 3-year follow-up study. **J. Assist. Reprod. Genet.** 29: 883-889.

Wang, Y., Zhang, M., Chen, J.Z., and Du, Y. (2018). Resveratrol promotes the embryonic development of vitrified mouse oocytes after in vitro fertilization. **In**

Vitro Cell Dev.-An. 54: 430-438.

West, J.D., and Marnett, L.J. (2006). Endogenous reactive intermediates as modulators of cell signaling and cell death. **Chem. Res. Toxicol.** 19: 173-194.

Wilding, M., Dale, B., Marino, M., Matteo, L., Alviggi, C., Pisaturo, M.L., Lombardi, L., and De Placido, G. (2001). Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos**. Hum. Reprod.** 16: 909-917.

Willis-Owen, S.A., and Flint., J. (2006). The genetic basis of emotional behaviour in mice. **Eur. J. Hum. Genet.** 14: 721-728.

Winterbourn, C.C. (2015). Are free radicals involved in thiol-based redox signaling?. Free Radic. **Biol. Med.** 80: 164-170.

Wu, G.Q., Quan, G.B., Shao, Q.Y., Lv, C.R., Jiang, Y.T., Zhao, Z.Y., and Hong, Q.H. (2016). Cryotop vitrification of porcine parthenogenetic embryos at the early developmental stages. **Theriogenology**. 85: 434-440.

Yang, M.Y., and Rajamahendran, R. (2002). Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. **Anim. Reprod. Sci.** 70: 159-169.

Yavin, S., and Arav, A. (2007). Measurement of essential physical properties of vitrification solution. **Theriogenology** 67: 81-89.

Yavin, S., Aroyo, A., Roth, Z., and Arav, A. (2009). Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. **Hum. Reprod.** 24: 797-804.

Yoisungnern, T., Choi, Y.J., Han, J.W., Kang, M.H., Das, J., Gurunathan, S., Kwon, D.N., Cho, S.G., Park, C., Chang, W.K., Chang, B.S., Parnpai, R., and Kim, J.H. (2015). Internalization of silver nanoparticles into mouse spermatozoa results in

poor fertilization and compromised embryo development. **Sci. Rep.** 5: e11170.

Youm, J., Kim, S. K., Jee, B.C., and Kim, S.H. (2017). Embryonic survival, development and cryoinjury of repeatedly vitrified mouse preimplantation embryos. **Eur. J. Obstet. Gynecol. Reprod. Biol.** 217: 66-70.

Zhang, J., Cui, J., Ling, X., Li, X., Peng, Y., Guo, X., Heng, B.C., and Tong, G.Q. (2009). Vitrification of mouse embryos at 2-cell, 4-cell and 8-cell stages by cryotop method. **J. Assist. Reprod. Genet.** 26: 621-628.

Zhang, L., Xue, X., Yan, J., Yan, L. Y., Jin, X. H., Zhu, X. H., He, Z. Z., Liu, J., Li, R., and Qiao, J. (2016). L-proline: a highly effective cryoprotectant for mouse oocyte vitrification. **Sci. Rep.** 6: e26326.

Zhao, X.M., Hao, H.S., Du, W.H., Zhao, S.J., Wang, H.Y., Wang, N., Wang, D., Liu, Y., Qin, T., and Zhu, H.B. (2016). Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes. **J. Pineal. Res.** 60: 132–141.

Zhou, C. J., Wang, D.H., Niu, X.X., Kong, X.W., Li, Y.J., Ren, J., Zhou H. X., Lu, A., Zhao, Y.F., and Liang, C.G. (2016). High survival of mouse oocytes using an optimized vitrification protocol. **Sci. Rep.** 6: e19465

Zhou, G.B., Hou, Y.P., Jin, F. Yang, Q.E., Yang, Z.Q., Quan, G.B., Tan, H.M., and Zhu, S.E. (2005). Vitrification of mouse embryos at various stages by open-pulled straw (OPS) method. **Anim. Biotechnol.** 16: 153-163.

BIOGRAPHY

Miss Natchanok Puangjit was born on May 16th, 1993 in Phang-Nga Province, Thailand. She graduated with a Bachelor of Science degree from the Department of Biology, Faculty of Science, Khon Kaen University in 2014. After graduation, she spent eight months to following her soul-dreams. In the same time, she tried to improve her English skills.In 2016, she decided to study master's degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology with Assoc. Prof. Dr. Rangsun Parnpai. During her study, she practiced the skills in the field of animal biotechnology including oocyte and embryo freezing, *in vitro* fertilization (IVF), cell culture and molecular biology techniques. Her research topic was "The effect of resveratrol supplementation in culture and vitrification medium for mouse blastocyst vitrification on developmental competence and gene expression". The results from some part of this study have been published in The Thai Journal of Veterinary Medicine (Thai J. Vet. Med. 49(2): 162-165, 2019) and presented as a poster presentation at the 14th Asian Reproductive Biotechnology congress (ARBC) in Nakhon Ratchasima, Thailand during August 19-22, 2019.