EFFECTS OF RESVERATROL SUPPLEMENTATION IN *IN VITRO* EMBRYO CULTURE MEDIUM AND POST WARMING SOLUTION ON DEVELOPMENTAL RATES AND QUALITY OF VITRIFIED BOVINE BLASTOCYSTS

YANAPON ANTHAKAT

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ผลของการเสริมเรสเวอราทอลในน้ำยาเลี้ยงตัวอ่อนและน้ำยาเลี้ยงตัวอ่อนหลังละลายต่ออัตราการพัฒนาและคุณภาพของตัวอ่อนโคแช่แข็งระยะบลาสโตซิส

นางสาวญาณภร อันทะเกตุ

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มหาวิทยาลัยเทคโนโลยีสุรนารี

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

Thesis Examining Committee

(Asst. Prof. Dr. Pakpoom Boonchuen)
Chairperson

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

(Dr. Ampika Thongphakdee)
Member

(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)
Vice Rector for Academic Affairs
and Quality Assurance

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

ญาณภร อันทะเกตุ: ผลของการเสริมเรสเวอราทอลในน้ำยาเลี้ยงตัวอ่อนและน้ำยาเลี้ยงตัวอ่อนหลังละลายต่ออัตราการพัฒนาและคุณภาพของตัวอ่อนโคแช่แข็งระยะบลาสโตซิส (YANAPON ANTHAKAT: EFFECTS OF RESVERATROL SUPPLEMENTATION IN *IN VITRO* EMBRYO CULTURE MEDIUM AND POST WARMING SOLUTION ON DEVELOPMENTAL RATES AND QUALITY OF VITRIFIED BOVINE BLASTOCYSTS) อาจารย์ที่ปรึกษา: รศ.ดร. รังสรรค์ พาลพ่าย, 53 หน้า

คำสำคัญ: ตัวอ่อนวัว/เรสเวอราทรอล/ไวติฟิเคชั่น

ปัจจุบันเทคโนโลยีการแช่แข็งตัวอ่อนและการย้ายฝากตัวอ่อนโคมีการศึกษาและพัฒนากันอย่างต่อเนื่อง เพื่อใช้ในการขยายพันธุ์โคที่มีคุณสมบัติพันธุกรรมดีเยี่ยม เรสเวอราทอลเป็นสารต้านอนุมูลอิสระที่มีบทบาทสำคัญในการช่วยลดความเครียดในตัวอ่อน โดยงานวิจัยนี้มีวัตถุประสงค์ในการศึกษาประสิทธิภาพของน้ำยาเลี้ยงตัวอ่อน (IVC) โดยไม่เสริม (IVC-) และเสริม (IVC+) 0.5 μM เรสเวอราทอลต่อการพัฒนาของตัวอ่อนโคในหลอดแก้วและการอยู่รอดของตัวอ่อนโคหลังการแช่แข็งด้วยวิธีไวติฟิเคชั่น (vitrification) เมื่อเปรียบเทียบกับกลุ่มตัวอ่อนสด พบว่าการเพิ่ม 0.5 μM เรสเวอราทอล ในน้ำยาเลี้ยงตัวอ่อน (IVC) มีอัตราการเพิ่มขึ้นของตัวอ่อนระยะบลาสโตซิสเล็กน้อย (35.20%) ซึ่งไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเทียบกับกลุ่มควบคุม (32.40%) จากนั้นเมื่อศึกษาผลของตัวอ่อนระยะบลาสโตซิสที่ถูกแช่แข็งและละลายในน้ำยาเลี้ยงที่เสริม (VT+) และไม่เสริม (VT-) เรสเวอราทอลเป็นเวลา 2 ชั่วโมง ก่อนเลี้ยงในน้ำยาเลี้ยงตัวอ่อนเป็นเวลา 24 ชั่วโมง อัตราการอยู่รอดของตัวอ่อนระยะบลาสโตซิสในกลุ่ม IVC-VT- (87.04%), IVC+VT- (88.89%), IVC+VT+ (94.34%) และ IVC-VT+ (94.74%) ซึ่งไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ตัวอ่อนระยะบลาสโตซิสจากกลุ่ม IVC+VT+ มีอัตราการพัฒนาสูงที่สุด 81.25% ซึ่งไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเปรียบเทียบกับทุกกลุ่มแช่แข็ง การประเมินผลเรสเวอราทรอลที่มีผลต่อจำนวนเซลล์ inner cell mass (ICM), trophectoderm (TE) และจำนวนเซลล์ทั้งหมดของตัวอ่อนระยะ บลาสโตซิส (total cell number) พบว่าการเสริมด้วยเรสเวอราทอลทั้งก่อนและหลัง vitrification ไม่มีผลต่อจำนวนเซลล์ ICM, TE และจำนวนเซลล์ทั้งหมดในกลุ่มตัวอ่อนสดและแช่แข็ง

การวิจัยนี้ยังประเมินระดับการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการ apoptosis (*BAX* และ *BCL2*), การเผาผลาญไขมัน (*PNPLA2*) และ transcription factor (*SIRT1* และ *FOXO3A*) พบว่าในกลุ่มตัวอ่อนแช่แข็งที่เสริมด้วยเรสเวอราทอลมีการแสดงออกของยีน *BAX*, *PNPLA2* และ *FOXO3A* สูงขึ้นแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) มากกว่าในกลุ่มตัวอ่อนสด ในขณะเดียวกันกลุ่มตัวอ่อนแช่แข็งพบการแสดงออกของยีน *BCL2* ลดลงแตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มตัวอ่อนสด การแสดงของยีน *SIRT1* ระหว่างกลุ่มควบคุมและกลุ่มที่เสริมด้วยเรสเวอราทอลไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ

อย่างไรก็ตาม การศึกษาในอนาคตจำเป็นต้องหาความเข้มข้นที่เหมาะสมของ resveratrol เพื่อเพิ่มประสิทธิภาพของการแช่แข็งตัวอ่อนต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2566

ลายมือชื่อนักศึกษา..ณุกณภร ธันทะเกตุ.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....

YANAPON ANTHAKAT: EFFECTS OF RESVERATROL SUPPLEMENTATION IN *IN VITRO* EMBRYO CULTURE MEDIUM AND POST WARMING SOLUTION ON DEVELOPMENTAL RATES AND QUALITY OF VITRIFIED BOVINE BLASTOCYSTS. THESIS ADVISOR: ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 53 PP.

Keyword: Bovine embryo/Resveratrol/Vitrification

Currently, the technology for embryo cryopreservation and embryo transfer of cattle has been continuously studied and developed to propagate cattle with desirable genetic traits. Resveratrol is a crucial antioxidant that plays a significant role in alleviating stress in embryos. This research aimed to examine in vitro culture (IVC) medium without (IVC-) and with (IVC+) 0.5 μM resveratrol on the development of in vitro fertilized embryos and survival of vitrified-warmed embryos, compared with fresh embryos. Results showed that the addition of 0.5 μM resveratrol in IVC medium showed slightly increased blastocyst formation rate (35.20%), which was not significantly different ($p < 0.05$) when compared to the control group (32.40%). Then, the vitrified blastocysts were placed in warming solution with (VT+) and without (VT-) resveratrol and incubated for 2 h before cultured for another 24 h. Survival rates of blastocysts of IVC-VT- (87.04%), IVC+VT- (88.89%), IVC+VT+ (94.34%) and IVC-VT+ (94.74%) were not significantly different ($p < 0.05$). The blastocysts derived from IVC+VT+ showed the highest developmental rate at 81.25%, which was not significantly different ($p < 0.05$) among the vitrified groups. The effect of resveratrol on inner cell mass (ICM), trophectoderm (TE) and the total cell numbers of blastocysts was also evaluated. Supplementation of resveratrol at pre- and post-vitrification did not affect ICM, TE, and the total cell numbers in all fresh and vitrified groups.

This research also examined the expression level of genes related to apoptosis (*BAX* and *BCL2*), lipid metabolism (*PNPLA2*), and transcription factor (*SIRT1* and *FOXO3A*). The vitrified-warmed blastocysts in all resveratrol treated groups significantly higher ($p < 0.05$) expressions of *BAX, PNPLA2*, and *FOXO3A* genes than the fresh blastocysts. Meanwhile, the expression of the *BCL2* gene in all vitrified groups was significantly lower than fresh blastocysts. The expression of the *SIRT1* gene was not significantly different among all control and resveratrol treated groups.

However, further study is needed to determine the optimal concentration of resveratrol for enhancing the efficiency of embryo cryopreservation.

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Student's Signature Yanapon Anthakat
Advisor's Signature

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Yanapon Anthakat

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LIST OF ABBREVIATIONS

ARTs	=	Assisted reproductive technologies
ATP	=	Adenosine triphosphate
BM	=	Base medium
BSA	=	Bovine serum albumin
cDNA	=	Complementary DNA
CPAs	=	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
IU	=	International unit
RT	=	Room temperature
IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilization
IVM	=	*In vitro* maturation
IVP	=	*In vitro* embryo production
LN_2	=	Liquid nitrogen
mtDNA	=	Mitochondrial DNA
PBS	=	Phosphate-buffer saline
ROS	=	Reactive oxygen species
RT	=	Reverse transcription

LIST OF ABBREVIATIONS (Continued)

TCM-199	=	Tissue culture medium-199
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
˚C	=	Degree Celsius
µM	=	Micromolar
µl	=	Microliter

CHAPTER I
INTRODUCTION

1.1 Background

In the past, cattle production industry was highly popular due to the commercial value of cattle as a source of meat and milk. Assisted reproductive technologies (ART) have played a crucial role in enhancing the efficiency of bovine embryo production, particularly in processes of *in vitro* embryo production (IVP) and cryopreservation by vitrification technique. Several researches have effectively enhanced the efficiencies of ART, increasing the number of viability oocytes and transfer embryos (Milachich and Shterev, 2016). However, the efficiency of ART is influenced by the environmental conditions during *in vitro* embryo development, which could potentially impact the health of the next generation (Duranthon and Palmer, 2018). Especially in case of IVP, embryos are particularly affected by external factors from the culture conditions (Rizos et al., 2008; Rizos et al., 2002). Numerous studies have indicated that handling gametes and growing embryos in vitro can cause significant oxidative damage due to the excessive production of reactive oxygen species (ROS). This phenomenon can result in abnormal embryo development and may trigger apoptosis responses (Agarwal and Majzoub, 2017).

Vitrification has proven to be a method that significantly improves the survival rates of oocytes and embryos after thawing (Cao et al., 2009). Moreover, this could lead to an increased chance of pregnancy (Hayashi et al., 2019). Cryotop vitrification has been widely used to freeze oocytes and embryos in various species, particularly cattle (Punyawai et al., 2015). However, a challenge arises because bovine embryos are susceptible to ROS-induced stress, which can adversely affect their development (Prentice and Anzar, 2011). The imbalance between ROS production and cellular antioxidant defenses can damage cytoskeleton structure, membrane lipids, proteins, and DNA. These effects could lead to apoptotic responses and a decrease in embryo viability (Gaviria et al., 2019). The thawing of bovine embryos and the use of culture media for their recovery are crucial in embryo cryopreservation, as they impact the survival rate, quality, and long-term development of

the embryos. Appropriate culture media enhance pregnancy rates and result in healthier calves (Galli et al., 2003).

Resveratrol (3,5,40-trihydroxytrans-stilbene) is a natural antioxidant polyphenol found in various plants and foods, including peanuts, mulberries, cocoa, grapes, and red wine. Previous research has demonstrated that resveratrol has the potential to reduce ROS-induced stress in cat oocytes (Piras et al., 2020). When added to the *in vitro* culture (IVC) medium of pig embryos, resveratrol acts as an antioxidant, which help reduce ROS levels, and increase glutathione (GSH) levels (Wang et al., 2014). Furthermore, resveratrol supplementation in pig IVC medium has been shown to promote embryo development and enhance the expression of genes involved in antioxidant mechanism (Iwata, 2021). Notably, there are reports indicating that resveratrol supplementation in the IVC medium of bovine embryos before vitrification can enhance the expression levels of growth factor genes (Gaviria et al., 2019, 2019; Gaviria et al., 2019).

1.2 Research objectives

1.2.1 To examine the effect of resveratrol in IVC medium on developmental rate of the bovine embryos.

1.2.2 To examine the effect of resveratrol in IVC medium and warming solution on survival rate and gene expression of vitrified bovine blastocysts.

1.3 Hypothesis

1.3.1 The resveratrol in the IVC medium will positively impact the developmental rate of bovine embryos.

1.3.2 The resveratrol in both the IVC medium and warming solution will enhance the survival rate of bovine blastocysts after vitrification, and it will also influence the gene expression profile quality.

CHAPTER II

LITERATURE REVIEWS

2.1 Assisted reproductive technologies (ART).

In cattle, ART including artificial insemination (AI), ovum pick up (OPU), *in vitro* embryo production (IVP), cryopreservation and embryo transfer (ET) constitute a potential pipeline to produce entire genetics, sex determination and assessment of genomic estimated breeding values (GEBVs) of embryos. This technique can be used to improve the quality of oocytes and embryos. (Najafzadeh et al., 2021)

2.2 *In vitro* embryo production (IVP) of bovine is composed of 3 steps

2.2.1 *In vitro* maturation (IVM)

It is widely known that oocytes with cumulus cells are important in the development of oocytes during IVM. So, it is important to allow low-quality oocytes to mature by staying on the cumulus cells. It has been reported that oocytes with cumulus cells cultured in tissue culture medium-199 (TCM-199) supplemented with FSH and cysteamine, can increase the growth rate and cleavage of oocytes. There are reported that the optimal duration for this procedure is 16-24 hours after initiation of IVM. (Atef et al., 2005)

2.2.2 *In vitro* fertilization (IVF)

Fertilization would be considered the most critical step for IVP technology in any species. Many factors may affect the efficiency of IVF, such as sperm viability and capability, optimum *in vitro* environment for survival of gametes, time of insemination, duration of co-incubation, the presence of cumulus cells and the acquisition of the oocyte developmental competence during the complicated process of cytoplasmic maturation. Tyrode's albumin lactate pyruvate (TALP) solution is supplemented with many agents, such as bovine serum albumin (BSA), heparin, caffeine, penicillamine, hypotaurine, and calcium ionophore. There are several methods utilized for sperm preparation, such as swim up and percoll gradient centrifugation before being fertilized with oocytes. (Galli et al., 2003)

2.2.3 *In vitro* culture (IVC)

In recent years, the remarkable improvement of blastocyst formation rate (35-40%) is attained principally due to the optimization of the IVM and in part of the IVF systems rather than to environmental modifications applied to the IVC system. Despite high blastocyst formation rates, pregnancy rates following ET of cryopreserved bovine embryos are very poor. Bovine embryos develop in several media, such as synthetic oviductal fluid (SOF) (Carolan et al., 1995), TCM-199 and Chrerrles Rozenkrans 1aa medium (CR1aa) (Somfai et al., 2010) supplemented with various development influencing agents like essential and non-essential amino acids, fetal calf serum (FCS), insulin and insulin-like growth factor-1 (IGF-1). The presence of oviductal cells in the culture medium during IVC of the embryo is found to be very important for enhancing its developmental potential. Subsequently, zygotes/embryos have been successfully cultured in both SOF and another chemically defined medium, known as potassium simplex optimized medium (KSOM) (Sirisathien, 2002) with similar embryo development. In the IVC medium, the cleavage rate is improved up to 72% and the blastocyst rate per cleavage up to 52%. To prevent the oxidative damage of DNA, some antioxidants like ascorbic acid and alpha-tocopherol could play an active role in the growth and development of cattle embryos by neutralizing free radicals. Recently, much higher concentrations of glucose in both IVM and IVC have been proven beneficial for blastocyst development. It is also reported that the addition of leukemia inhibitory factor to the IVC medium improves blastocyst development and quality (Rizos et al., 2008). An improvement in embryo quality indicated by faster development and increased cryotolerance, was determined by a high concentration of hyaluronic acid during late IVC (Bousquet et al., 1999).

2.3 The environment of culture bovine embryo

In vitro embryo production serves as an essential tool for investigating pre-implantation embryonic development. Normally, embryos produced within the body develop in oviduct and uterus. This is unlike embryos produced *in vitro* where inappropriate environmental factors result in differences in biochemical signals that affect the genome (Rizos et al., 2008). Given the high sensitivity of pre-implantation embryos to environmental factors, inadequacies in culture conditions often result in suboptimal development (Ahmadi et al., 2023; Feuer and Rinaudo, 2012). The culture of mammalian embryos *in vitro* necessitates an appropriate environment wherein the early embryo can

undergo multiple cleavage divisions and eventually develop into a blastocyst (Campbell, 2013; Rizos et al., 2002; Thompson, 1997).

Embryos at the pre-implantation stage can be cultured in various media, ranging from simple balanced salt solutions like CR1aa, SOF, and KSOM, to more complex compositions such as TCM-199, often supplemented with serum and/or a feeder layer of somatic cells (Niemann and Wrenzycki, 2000; Summers and Biggers, 2003). However, the most substantial decline in *in vitro* development occurs during the latter phase of the process (post-fertilization culture), which involves in spanning from the two-cell to the blastocyst stages (Rizos et al., 2008). Moreover, increased oxidative stress is another factor that hinders *in vitro* embryo development. Therefore, there is a compelling need to enhance and refine the culture system aiming to potentially enhance the development of cultured embryos. This can be achieved by antioxidants supplementing such as cysteamine, cysteine, β-mercaptoethanol (Nedambale et al., 2006), and resveratrol (Gaviria et al., 2019; Wang et al., 2019) to the IVC media.

2.4 Evaluation and classification of bovine embryos

The IETS grading and classification system for embryos involves the assessment of embryos based on their stage of development and quality. Embryos are typically recovered 6-8 days after the onset of estrus. The classification includes a two-digit code: the first digit indicates the stage of development, and the second digit represents the quality grade (Stringfellow and Seidel, 1998).

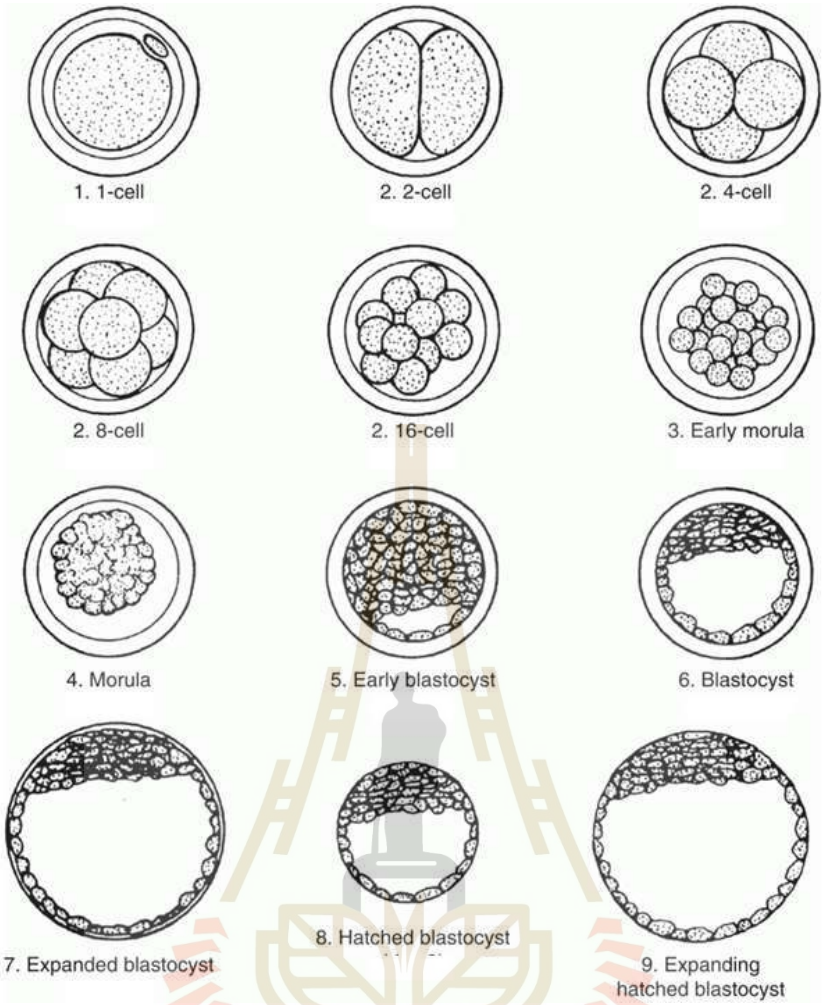

Figure 2.1 Normal embryonic development of bovine embryos (Stringfellow and Seidel, 1998).

Table 2.1 Stages of Embryonic Development (Stringfellow and Seidel, 1998).

Stage code	Name Stage code	Description
1	Unfertilized	A single cell, typically a UFO (unfertilized ovum).
2	2-cell to 12-cell	Contains 2-12 blastomeres, usually dead or degenerate.
3	Early morula	Group of at least 16 cells, not well-suited for cryopreservation.
4	Compact morula	Tightly compacted ball of cells, difficult to discern individual blastomeres.
5	Early blastocyst	Presence of a small fluid-filled cavity (blastocoele).
6	Blastocyst	Defined trophoblast layer, blastocoele cavity, and ICM cells.
7	Expanded blastocyst	Increased diameter and thinning of the zona pellucida.
8	Hatched blastocyst	Embryo undergoing or having completed hatching from the zona pellucida.
9	Expanded hatched blastocyst	Larger diameter than stage 8, uncommon to recover unless flushing is done more than 8 days after estrus.

Table 2.2 Quality grades of embryo (Stringfellow and Seidel, 1998).

Quality grade code	Name	Description
1	Excellent or good	Symmetrical, spherical embryo mass with uniform blastomeres; at least 85% viable cells.
2	Fair	Moderate irregularities in shape, size, color, and density of blastomeres; at least 50% viable cells.
3	Poor	Major irregularities in embryonic mass or blastomere characteristics; at least 25% viable cells.
4	Dead or degenerating	Nonviable entities, not suitable for transfer or cryopreservation.

2.5 Cryopreservation

Cryopreservation of embryos is widely used and plays an important role in the breeding and conservation of animal species. Therefore, technology must be developed that can effectively preserve embryos at low temperatures and maintain embryo survival. There are two methods of cryopreservation that are popular currently (Najafzadeh et al., 2021).

2.5.1 Cryoprotectant (CPAs)

Cryoprotectants (CPAs) are essential for the cryopreservation of bovine blastocysts to protect them from damage during the freezing and thawing processes. The use of CPAs enables the preservation of embryo viability and function post-thawing, which is crucial for successful embryo transfer and subsequent development.

Types of cryoprotectants: Cryoprotectants can be broadly classified into two categories: permeating and non-permeating.

Permeating cryoprotectants are essential for the successful cryopreservation of bovine blastocysts. These CPAs can penetrate the cell membrane and include substances such as Dimethyl Sulfoxide (DMSO), Glycerol, Ethylene Glycol (EG), and Propylene Glycol. DMSO is one of the most used CPAs, offering effective protection by

replacing water within the cells and preventing ice crystal formation. Glycerol is often used for its ability to permeate cells and reduce ice formation, although it can sometimes be toxic to embryos. Ethylene Glycol is widely used due to its relatively low toxicity and high permeability, making it effective for cryopreserving bovine embryos. Propylene Glycol is another permeating CPA, known for its compatibility with various embryo stages and relatively low toxicity. (Leibo and Pool, 2011)

Non-permeating cryoprotectants (CPAs) remain outside the cell and primarily work by dehydrating cells and forming a protective glass-like matrix during freezing. Examples include sucrose, which is often used in combination with permeating CPAs to enhance dehydration and reduce ice formation, and trehalose, which similarly helps to stabilize cell membranes and proteins during freezing. Polyvinylpyrrolidone (PVP) is another non-permeating CPA that creates a viscous environment, reducing ice formation and mechanical stress on cells. (Leibo and Pool, 2011)

High concentrations of cryoprotectants (CPAs), especially during vitrification, can be toxic to embryos, necessitating a careful balance of CPA concentration and exposure time. The permeability of CPAs varies, influencing the selection and combination of these agents. Different stages of embryonic development may require specific CPA protocols to achieve optimal cryopreservation outcomes. The effectiveness of cryopreservation protocols is ultimately measured by the post-thaw survival and developmental competence of the embryos. (Mazur, 1984; Rall and Fahy, 1985)

2.5.2 Slow freezing

It is a method for slowly freezing embryos. It will cause cold from the outside into the inside. It is an easy method that does not require much expertise, but it takes a longer time, after slow freezing and decreases the survival rate when compared with fresh embryos (Saragusty and Arav, 2011).

The success of slow freezing has been reported with the first calves born after embryo freezing (Fuku et al., 1992). Since then, it has progressed and developed a simpler and more stable protocol (Ferré et al., 2020).

2.5.3 Vitrification

Vitrification is a method of rapid freezing of embryos. It directly cools the embryo with a high concentration of cryoprotective agents (CPA) in the medium. It is a simple method and requires a lot of expertise but takes less time. Embryos can be frozen in large numbers; however, the embryos after being frozen in this way still have a low survival rate when compared with fresh embryos (Nedambale et al., 2006), but the survival rate is higher than slowly freezing technique (Najafzadeh et al., 2021). Previously report found that vitrified MII-stage oocytes have higher survival rates than GV-stage oocytes (Chaves et al., 2017).

Vitrification is a better alternative than slow freezing because of its cost-effectiveness, speed and simplicity (Mochida and Ogura, 2010). So, vitrification become to the routine method for freezing oocytes and embryos of human (Azawi et al., 2013; Dhali et al., 2019) along with freezing embryos in animals such as bovine (Mohr and Trounson, 1981), rabbits (Silvestre et al., 2002), horses (Massip, 2001) and pigs (Dobrinsky, 2002).

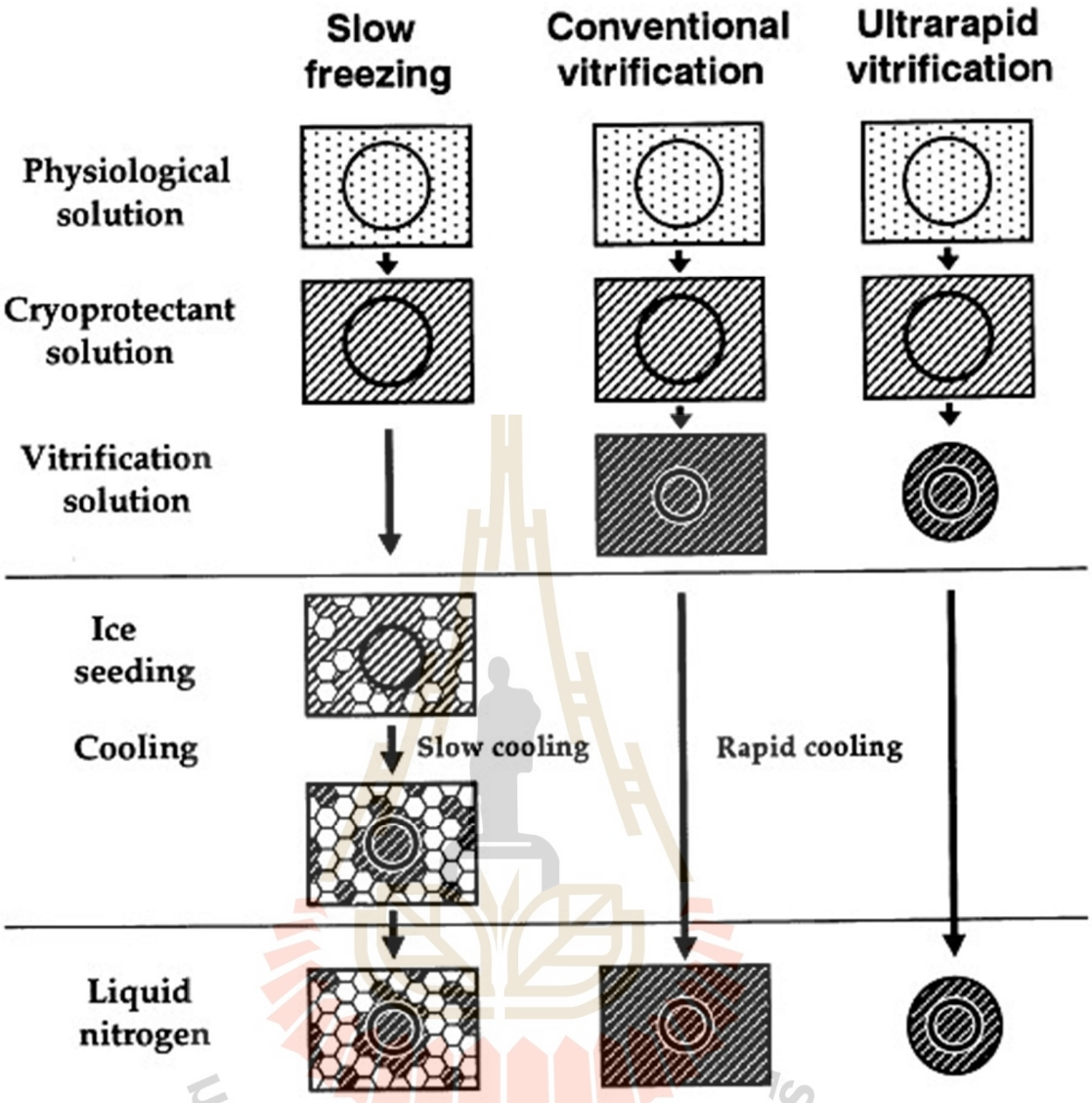

Figure 2.2 Schematic representation of an embryo during slow freezing and vitrification steps. Darker shading represents a higher osmolality. Embryo size changes represent shrinking and re-expansion in response to solution osmolality and cryoprotectant concentrations. Hexagons show ice crystal formation (Ferré et al., 2020).

Cryotop is the device for vitrification. The design facilitates for loading of the oocytes and makes it easy for vitrification (Kitasato, Japan). The Cryotop method represents a significant advancement in cryopreservation techniques, offering improved outcomes for individuals seeking fertility preservation and enhancing the success rates of assisted reproductive technologies. Its ability to rapidly vitrify and subsequently thaw cells with minimal damage makes it a cornerstone technique in modern reproductive medicine. So, cryotop become the gold standard for vitrification of animals such as rabbits (Hochi et al., 2004), bovine (Sprícigo et al., 2017), pigs (Galeati et al., 2011) and humans (Kuwayama et al., 2005).

Figure 2.3 Cryotop used for vitrification.

Vitrification is the process of solidifying a highly viscous liquid (Shaw and Jones, 2003). This method, known as a physical process, involves rapidly transforming high-concentration CPAs (cryoprotective agents) into a glass-like state at low temperatures (-196°C) (Hunt, 2019). Therefore, transforming a viscous liquid into a glass-like state helps reduce damage caused by ice crystal formation during the cooling process (Mahato et al., 2019). Vitrification technique can be separated into 2 parts including vitrified and warming/thawing parts. In vitrified step, there are 2 main factors: water and CPAs influencing cell survival rate (Leonard et al., 2003). Role of CPAs is to help decrease the freezing point and reduce ice crystal formation (Liu et al., 2021). However, addition of CPAs in solvent has been significantly influenced by increased osmolarity (Raju et al., 2021), which makes unstable in membrane polar absorption of oocytes. Consequently, These affect change of water in cells (Nolan and Hammerstedt, 1997). In the following step, warming/thawing is the process causing a rapid temperature to change and decrease formation of ice crystals. This process is involved in water influx into the cells of oocytes (Mphaphathi et al., 2023). Transfer of oocyte and embryo from high concentration of CPAs to isotonic solution causes a reverse osmotic shock or over-swelling. This phenomenon can be prevented by thawing oocyte and embryo in hypotonic solution (Fahy and Wowk, 2015). Monosaccharides and disaccharides such as sucrose are the most common CPAs

used in a thawing vitrification medium (Rajan and Matsumura, 2018; Sánchez et al., 2011) due to their ability to reduce osmotic stress, and prevent cell damage (Jin and Mazur, 2015; Pedro et al., 1997).

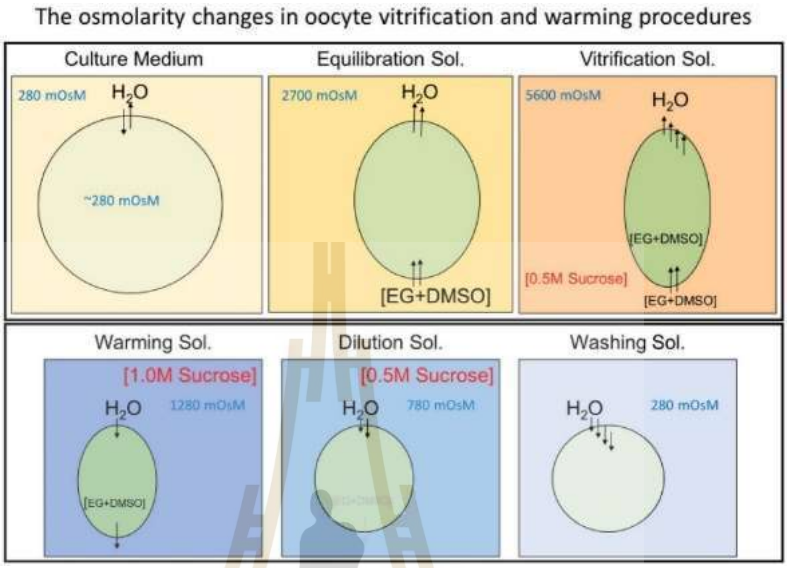

Figure 2.4 The process of vitrification and warming (Chang et al., 2022)**.**

2.6 Bovine blastocyst vitrification

Embryo cryopreservation through vitrification has been safely practiced since 1990. In 1990, vitrification was first successfully employed to cryopreserve cleavage-stage blastocyst of humans, leading to a viable birth (Gordts et al., 1990). As techniques of *in vitro* advance and the demand for single embryo transfer grows, there is a greater need for an efficient and dependable cryopreservation technique for surplus embryos. Cryopreservation was performed on zygotes immediately at the pronuclear stage after fertilization, embryos due to cleavage stages (2–8 cells), or at the expanded blastocyst stage. Vitrification of early-stage blastocysts is considered a preferable and superior alternative to a slow freezing rate due to its higher viability rate and improved pregnancy rates. Previous research has indicated that vitrified blastocysts using the open-pulled straw method exhibited developmental competence like that of fresh embryos. This suggests that the early blastocyst stage is the most optimal stage for the vitrification of bovine embryos (Martinez & Moreno, 2000). However, according to Michal et al. (2016), cryopreserving cleaved embryos are not as efficient as vitrifying blastocysts (Michal et al., 2016). Embryos, the highest quality have surpassed the developmental barrier linked to

genomic activation, and they exhibit relatively strong developmental potential. The blastocyst stage represents the last developmental phase before implantation, which offers the prime opportunity to screen and select the healthiest blastocysts for transfer (Ling et al., 2009). Additionally, the presence of numerous small cells provides an advantage, as the potential cell loss during the freezing and thawing process is likely to be less detrimental to the embryo's future development (Hunt, 2019). Moreover, during prolonged cultivation, embryos with poor survival are ceased in their result of development in no embryo cryopreservation needed (Wong et al., 2014). Hence, the vitrification of blastocyst-stage embryos plays a crucial role in assisted reproduction.

The optimal procedure for vitrifying mammalian embryos varies across different stages of development, even within the same species (Kasai and Edashige, 2010; Ling et al., 2009). The observed specificity may arise from variations in the cryobiological characteristics of embryos across different stages of development and species. These properties include sensitivity to cold temperatures, susceptibility to the potentially harmful effects of cryoprotectant chemicals, the ability of the cell membrane to allow water to pass through and cryoprotectants, and the ability to withstand changes in osmotic pressure causing swelling or shrinkage (Mazur, 2004). Likewise, The ability of embryos' plasma membrane to allow water and cryoprotectants to pass through remains crucial as a crucial property, as it is intimately linked to primary reasons for cell damage during vitrification (Edashige, 2017). It is essential to prevent such damage to ensure the viability of embryos after vitrification. As a result, the ability of the cell membrane to allow substances to pass through greatly influences the suitability of situations for vitrification.

Identifying the mechanism is essential for determining the optimal conditions for blastocyst vitrification. Determining the primary pathway for water movement and cryopreserved oocytes and embryos at various stages would aid in selecting appropriate cryoprotectants and optimal vitrification conditions. This includes various factors such as the duration, temperature, and steps involved in treating oocytes and embryos with cryoprotectant solutions before cryopreserved, as well as the process of removing cryoprotectants after warming (Prentice and Anzar, 2011). To develop protocols for vitrifying mammalian embryos, it's crucial to assess how water and cryoprotectants move at each developmental stage. During vitrification, the duration and temperature of exposure to the vitrification solution are critical factors due to its high cryoprotectant

concentration, which can be highly toxic to oocytes and embryos. The temperature and duration of exposure to vitrification solution are critical factors when cryoprotectants and water permeate embryos mainly via basic diffusion. This is because temperature directly influences the ability of water and cryoprotectants to pass through. When water and cryoprotectants primarily move through blastocysts via promote diffusion channels, the duration of showing to vitrification solution becomes more critical, since the permeability is less influenced by temperature. Nevertheless, subjecting blastocysts to vitrification solution at elevated temperatures could be modified, as cryoprotectants exhibit increased toxicity under such conditions

2.6.1 Vitrification and Warming solution

Today, many researchers are exploring various ways to enhance the success of cryopreservation by adjusting key factors such as cryoprotectants, freezing and warming rates, and adding chemical supplements like antioxidants. In a study by Chian et al. (2004), bovine embryos were first equilibrated in a solution containing 7.5% EG and 7.5% DMSO for 10 minutes. They were then transferred to a vitrification solution containing 15% EG, 15% DMSO, and 0.5 M sucrose for 30-60 seconds at room temperature before being loaded onto cryotop and plunged into liquid nitrogen. The warming procedure involved a stepwise dilution with sucrose solutions at 37°C, resulting in high survival and developmental rates post-thaw (Dujíčková et al., 2021). A similar study on bovine embryos utilized a vitrification protocol with a first solution containing 7.5% EG and 7.5% DMSO for equilibration, followed by a vitrification solution containing 16.5% EG, 16.5% DMSO, and 0.5 M sucrose. The embryos were placed on a cryotop and quickly plunged into liquid nitrogen. The warming procedure involved stepwise dilution with sucrose solutions of decreasing concentration at 37°C. This method resulted in improved survival and developmental rates for bovine embryos (Gómez et al., 2022). Interestingly, Hosseini and colleagues (2010) demonstrated a beneficial impact of antioxidants in the cultivation medium following embryo thawing. Likewise, the addition of **α-**tocopherol to the recovery culture medium significantly increased the blastocyst yield from post-warm bovine oocytes compared to control oocytes (Hosseini and Hosseini, 2017). Therefore, refining vitrification procedures with post-vitrification treatments could mitigate embryo sensitivity to cryopreservation, offering insights into advanced thawing procedures. Additionally, strategic selection of embryonic culture media is crucial for optimizing the freezing and warming processes successfully.

2.7 Oxidative stress by ROS and apoptosis

Oxidative stress is a monstrosity resulting from an imbalance between the generation and buildup of ROS in tissue and cells. ROS are small molecules. It acts as an oxidizing agent and is highly reactive. ROS is generated during the oxygen reduction step. These are called free radicals derived from oxygen, such as superoxide anions (O_2^-), hydroxyl radicals (OH) or non-oxidative molecules and hydrogen peroxide (H_2O_2) (Pizzino et al., 2017). In the IVP step, oocytes and embryos can be declarative to high levels of ROS because the antioxidant defense mechanism is lost and ultimately causing oxidative stress conditions (Agarwal et al., 2006). However, ROS can be produced within mitochondria as well as from external sources when cells are exposed to changes in environment culture factors, such as oxygen levels, light exposure, pH, and temperature (Caparros et al., 2021).

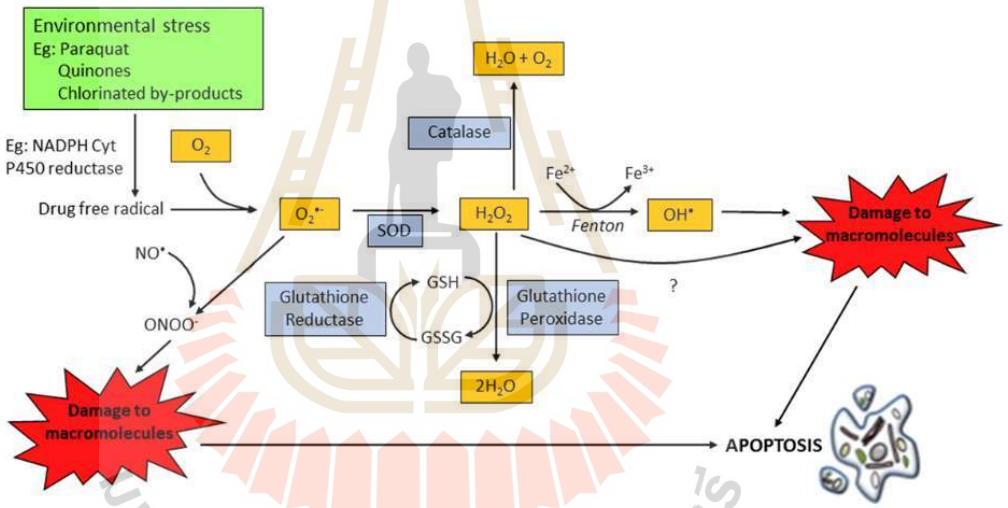

Figure 2.5 The production of ROS causes cell damage and apoptosis. Xenobiotics can contribute to the generation of xenobiotic-derived free radicals through one-electron reduction catalyzed by cytochrome P450 reductases (Kehrer and Klotz, 2015). The xenobiotic-derived free radicals rapidly react with oxygen to produce superoxide (O_2^-), which is reacted with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), or undergoes transformation to form H_2O_2, which is catalyzed by SOD. H_2O_2 can be detoxified by antioxidants such as catalase and GSH (Dutordoir and Bates, 2016). Alternatively, OH can be generated by metal-accelerated fenton reactions OH and $ONOO^-$ can damage intracellular proteins, lipids and nucleic acids. This causes to cell death by apoptosis (Vadgama, 2021).

Apoptosis, also known as the program of cell death, is a meticulously formulated and evolutionarily preserved process in which cells undergo self-destruction (Naessens, 2012). In multicellular organisms, it serves as a vital process for eliminating undesired or surplus cells due to the development or neutralizing of potential cells with DNA damage (Dutordoir and Bates, 2016). The maintenance of normal cellular balance depends greatly on the regulation of apoptosis. Apoptosis can be initiated by a range of external and internal signals, including various stressors like heat shock, viral infection, serum deprivation, hypoxia, DNA-damaging agents (such as radiation), and ROS (Pallepati, 2012). Multiple studies have affirmed that apoptosis plays a role in the reduction of oocyte and embryo development following vitrification (Sudano et al., 2011) and showed that cryopreserving could lead to apoptosis in bovine blastocysts. They discovered that the incidence of apoptotic cells in bovine embryos rose following undesired. Inaba et al. (2016) also discovered that vitrification of cattle embryos could lead to increased apoptosis after thawing.

2.8 Resveratrol

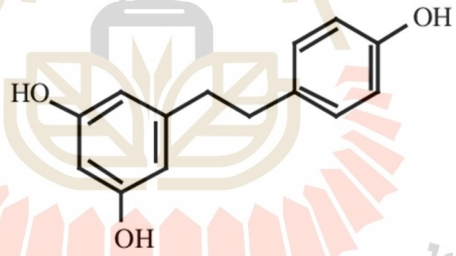

Figure 2.6 The chemical structure of resveratrol (3,5,4′-trihydroxy-trans-stilbene).

Resveratrol (3,5,40 -trihydroxytrans-stilbene) is a natural polyphenol antioxidant. It is found in many plants and foods such as peanuts, cocoa, grapes, and red wine. Its biological function is to shield the plant from parasitic attacks or environmental stresses, such as UV radiation and exposure to ozone (Dobrzynska, 2013). Resveratrol, a plant polyphenol, is synthesized when plants interact with microorganisms during pathogen attacks and is a protective agent against fungal and bacterial infections (Lattanzio et al., 2009). Resveratrol is present in cis- and trans-isomeric forms (Fig. 2.5). The trans-resveratrol form is steric and remains relatively stable when protected from high pH and light exposure (Lastra and Villegas, 2007).

Resveratrol exhibits various health benefits, including anti-inflammatory (Conte et al., 2015), antimicrobial (Pale et al., 2015), cancer prevention (Gusman et al., 2001), antioxidant (Zheng et al., 2012), heart protection (Tang et al., 2013), anti-cancer (Kucinska et al., 2014), and anti-aging (Smoliga et al., 2011) properties. For example, resveratrol facilitated bovine oocyte maturation and subsequent embryo development post-*in vitro* fertilization by stimulating progesterone secretion and exerting an antioxidant effect, likely through a mechanism dependent on sirtuin-1 (Wang et al., 2014).

Moreover, resveratrol has strong anti-apoptotic and antioxidant, serving as an antioxidative agent for IVP. The antioxidant potential of polyphenolic compounds depends on the redox properties of their phenolic hydroxyl groups and their ability to delocalize electrons throughout their chemical structure (Lastra and Villegas, 2007). Resveratrol has been found to operate through three distinct antioxidant mechanisms: (1) competing with coenzyme Q to reduce activity in the oxidative chain complex, where ROS is generated, (2) scavenging $O_2^{\bullet-}$ radicals produced in the mitochondria, and (3) inhibiting lipid peroxidation triggered by Fenton reaction products. Indeed, multiple studies have shown that resveratrol can scavenge both $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals (Leonard et al., 2003; Martinez and Moreno, 2000). Many studies have demonstrated that resveratrol has a positive effect on the culture medium environment. The beneficial effects of resveratrol on reproductive system function and vitrification have been widely recognized in various species, including cats (Comizzoli et al., 2009), pigs (Lee et al., 2010), goats (Mukherjee et al., 2014) and cattle (Abe et al., 2017; Gaviria et al., 2019; Hayashi et al., 2019; Salzano et al., 2014; Sprícigo et al., 2017). The supplemental resveratrol can be categorized into phases: IVM medium, IVC medium, and vitrification or warming media.

Resveratrol treatment has been shown to create a beneficial microenvironment within the embryo increasing mitochondrial DNA copy number, GSH level and ATP content while decreasing ROS levels and downregulating apoptosis-related genes such as *Bax* and *Bcl-2* expressions. These effects can significantly enhance the blastocyst formation rate and quality after parthenogenetic activation or IVF (Abe et al., 2017; Hayashi et al., 2018; Wang et al., 2014; Wang et al., 2019). During IVC, treating cattle embryos with 0.5 μM resveratrol in the culture medium improves embryo quality, especially by increasing the total cell number in blastocysts (Gaviria et al., 2019). However, after cryopreservation, treating cattle embryos with 0.5 μM resveratrol resulted in similar numbers of inner cell

mass (ICM), trophectoderm (TE), and total cells (Salzano et al., 2014). Moreover, supplementing with the addition of resveratrol in the IVC medium before vitrification enhances embryo survival rates and cryotolerance. This is evidenced by higher development and hatching rates after post-warming culture, increased ATP generation in mitochondrial function through elevated Surtuin family member 1 (*SIRT1*) expression levels, and reduced lipid content (Abe et al., 2017; Salzano et al., 2014; Sprícigo et al., 2017). Interestingly, research has shown that the addition of resveratrol during the vitrification/warming procedure and in the IVC medium can have positive effects, which help embryos to partially restore the initial quality, compared to that of pre-cryopreserved embryos (Gaviria et al., 2019; Gaviria et al., 2019). The diverse impacts of resveratrol result from its ability to interact with numerous components of intracellular signaling pathways, influencing a broad range of enzymes such as kinases, lipo- and cyclooxygenases, sirtuins, and transcription factors (Frojdo et al., 2008)

CHAPTER III

RESERCH METHODOLOGY

3.1 Chemicals:

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

3.2 *In vitro* maturation (IVM)

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

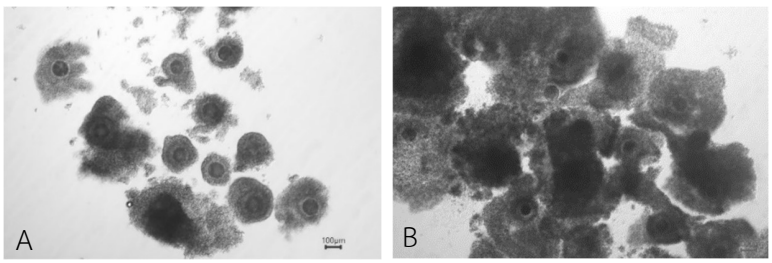

Figure 3.1 A, Oocytes before culture in IVM medium.

B, Oocytes after culture 23 hr in IVM medium.

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

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

3.4 *In vitro* fertilization (IVF)

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

3.5 *In vitro* embryo culture (IVC)

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

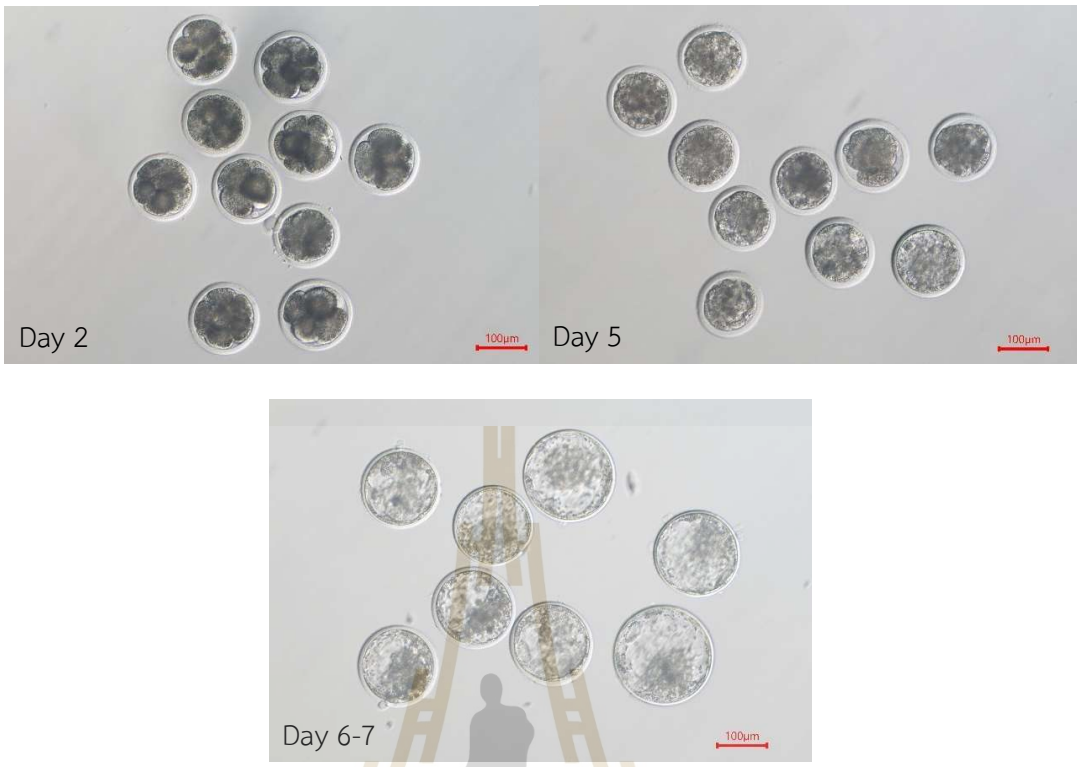

Figure 3.2 The development of the embryos at day 2, 5 and 6-7 after culture in IVC medium.

3.6 Cryopreservation of embryos using vitrification technique

3.6.1 Vitrification procedure

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

3.6.2 Warming technique

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

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

3.7 Blastocyst staining

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

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

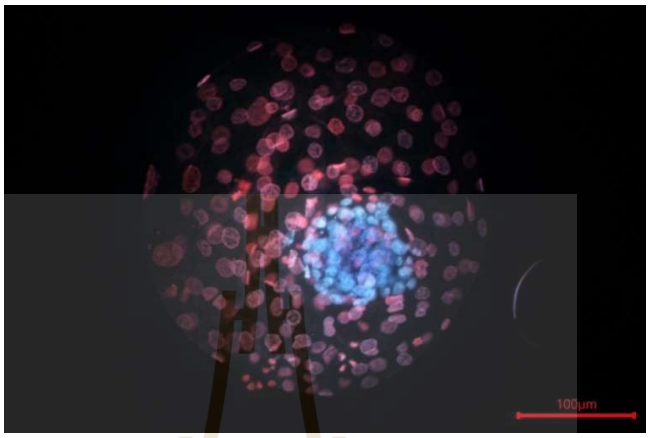

Figure 3.4 TE and ICM cells of blastocyst after PI and Hoechst 33342 staining.

3.8 Evaluation of gene expression in blastocysts

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

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

Table 3.1 Primer for qPCR

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

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

3.8 Statistical analysis

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

CHAPTER IV
RESULTS

4.1 Effects of supplemented resveratrol in IVC medium on embryo development

The development of bovine embryos with and without resveratrol supplemented is shown in Table 4.1. This finding found that the blastocyst formation rate in the control group (0 µM resveratrol) was 32.40%, while in the 0.5 µM resveratrol group slightly increased to 35.20%. Although resveratrol influenced the embryo development of blastocysts, there was no statistical difference.

Table 4.1 Effects of resveratrol supplementation in IVC media on bovine embryo development.

Groups	No. *in vitro* cultured	No. (%) cleaved	No. (%) embryos developed to		
			8 cells	Morulae	Blastocysts
0 µM Resveratrol (control)	500	397 (79.40)	272 (54.40)	170 (34.00)	162 (32.40)
0.5 µM Resveratrol	500	402 (80.40)	275 (55.00)	179 (35.80)	176 (35.20)

No significant difference ($p < 0.05$, t-test)

4.2 Resveratrol supplemented in post-warming medium on survival of vitrified blastocysts

The development of vitrified blastocysts after thawing and culturing in warming solution supplemented with or without resveratrol is summarized in Table 4.2. There was no significant difference in survival and development to expanded, hatching and hatched blastocysts rates of fresh blastocysts without and with resveratrol supplemented (IVC- vs IVC+), which were 100% vs 100% and 91.67% vs 100%, respectively. Blastocysts without

resveratrol supplemented in IVC medium and warming solution (IVC-/VT-) and blastocysts with resveratrol supplemented in IVC medium and without resveratrol in warming solution (IVC+/VT-) showed no significant difference in survival and development to expanded, hatching and hatched blastocysts rates, which were 87.04% vs 88.89% and 74.47% vs 79.17%, respectively. Likewise, no significant difference of survival and development to expanded, hatching and hatched blastocysts rates was noticeable between vitrified blastocysts without resveratrol in IVC medium and with resveratrol in warming solution (IVC-/VT+) and vitrified blastocysts with resveratrol in IVC medium and with resveratrol in warming solution (IVC+/VT+), which were 90.74% vs 94.34% and 77.08% vs 81.25%, respectively.

Survival rates of fresh blastocysts with and without resveratrol added into IVC medium were similar (100 and 100%, respectively), but were not significantly different with those of all vitrified blastocysts (87.04 – 94.34%). Likewise, development rates of fresh blastocysts with and without resveratrol in IVC medium were not significantly different (100% and 91.67%, respectively), which were significantly higher than those of all vitrified blastocysts (74.47-81.25%).

Blastocysts in IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ showed no significant differences of survival rate (87.04, 90.74, 88.89 and 94.34%), respectively. Development rates of blastocysts in IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ were not significantly different (74.47, 77.08, 79.17 and 81.25%), respectively. Among vitrified groups, blastocysts in IVC+/VT+ had the highest survival and developmental rates (94.34%, 81.25%), however, no significant difference was observed.

Table 4.2 Effects of resveratrol supplementation in post-warming solution on developmental competency of embryos after post-warming

Conc. Resveratrol in IVC medium	Groups	No. vitrified blastocysts	No. warmed blastocysts	No. (%) blastocyst survival	No. blastocysts cultured in post warming medium	No. (%) expanded, hatching and hatched blastocysts
0 µM (IVC-)	Fresh	-	-	48/48 (100)	48	44/48 (91.67)[a]
	VT-	54	54	47/54 (87.04)	47	35/47 (74.47)[b]
	VT+	54	54	49/54 (90.74)	48	37/48 (77.08)[b]
0.5 µM (IVC+)	Fresh	-	-	48/48 (100)	48	48/48 (100)[a]
	VT-	54	54	48/54 (88.89)	48	38/48 (79.17)[b]
	VT+	54	53	50/53 (94.34)	48	39/48 (81.25)[b]

a, b in the same column indicates significant differences ($p < 0.05$, one-way ANOVA)

VT-: vitrification without resveratrol in post-warming medium; VT+: vitrification with 1 µM resveratrol in post-warming medium

4.3 Effect of resveratrol supplemented in post-warming solution on ICM, TE and total cell number of blastocysts

The cell numbers of fresh and vitrified blastocysts after thawing in warming solution supplemented with or without resveratrol are summarized in Table 4.3. The ICM, TE, and total cell numbers of blastocysts in the fresh control groups and IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ groups were not significantly different. The ICM, TE, and total cell numbers of fresh blastocysts with or without resveratrol were higher than those of IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ blastocysts, but no significant difference was observed. Particularly, fresh blastocysts derived from IVC+ had the highest TE, and total cell numbers (162.0±15.9 and 208.4±19.1), which were significantly higher than those in IVC-/VT- group, but not significantly different with other groups. In addition, among vitrified groups, the ICM, TE, and total cell numbers of IVC+/VT+ blastocysts were slightly higher than those of other groups, but no significant difference was noted.

Table 4.3 Effects of resveratrol supplementation in post-warming solution on ICM, TE, and total cell numbers of blastocysts.

Conc. resveratrol in IVC medium	Groups	No. blastocyst examined	ICM	TE	Total cell numbers
0 µM (IVC-)	Fresh	25	48.8±3.8^{ab}	158.0±15.3^{ab}	202.8±17.6^{ab}
	VT-	26	40.0±3.3^{b}	137.0±13.5^{b}	177.0±16.5^{b}
	VT+	25	43.0±2.2^{ab}	152.0±9.3^{ab}	195.0±11.0^{ab}
0.5 µM (IVC+)	Fresh	25	46.4±4.1^{a}	162.0±15.9^{a}	208.4±19.1^{a}
	VT-	26	43.6±3.0^{ab}	146.8±8.8^{ab}	190.4±11.1^{ab}
	VT+	25	43.8±1.6^{ab}	156.4±5.4^{ab}	200.2±5.8^{ab}

The analysis was performed in 4 replicates. a, b in the same column indicate significant difference ($p < 0.05$, one-way ANOVA) VT; vitrification group, -/+; without or with 1 µM resveratrol in warming solution

4.4 Effects of resveratrol supplementation in IVC medium and/or post-warming solution on gene expression of fresh and vitrified bovine blastocysts

From Fig. 4.1, the expression of *BAX* gene in IVC- fresh embryos was slightly higher than IVC+ fresh embryos but was not significantly different. However, *BAX* gene expressions of fresh IVC- and IVC+ embryos were significantly lower than those of the IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ embryos. Among vitrified groups, IVC+/VT- and IVC+/VT+ embryos showed slightly lower *BAX* gene expression than those of IVC-/VT- and IVC-/VT+ embryos, but there were no significant differences.

Effect of resveratrol on *BCL2* expression in bovine embryos is illustrated in Fig. 4.1. In contrast to *BAX* gene expression, *BCL2* gene expressions of IVC- and IVC+ fresh embryos were significantly higher than those of the IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ embryos. The IVC+ fresh embryos had the highest *BCL2* gene expression and were significantly higher than IVC- fresh embryos. Among vitrified groups, *BCL2* gene expression of embryos in the IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ were not significantly different.

From Fig. 4.2, the expression of *SIRT1* gene in all fresh and vitrified groups showed no significant difference. The *SIRT1* gene expressions of embryos in IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ were slightly higher than those of fresh embryos, but no significant difference was observed.

From Fig. 4.2, the expressions of *FOXO3A* and *PNPLA2* genes in IVC+ fresh embryos were higher than IVC- fresh embryos but were not significantly different. However, *FOXO3A* and *PNPLA2* gene expressions of IVC- and IVC+ fresh embryos were significantly lower than those of the IVC-/VT-, IVC-/VT+, IVC+/VT-, and IVC+/VT+ embryos. Among vitrified groups, there were no significant differences of the expression of *FOXO3A* and *PNPLA2* genes in IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ groups.

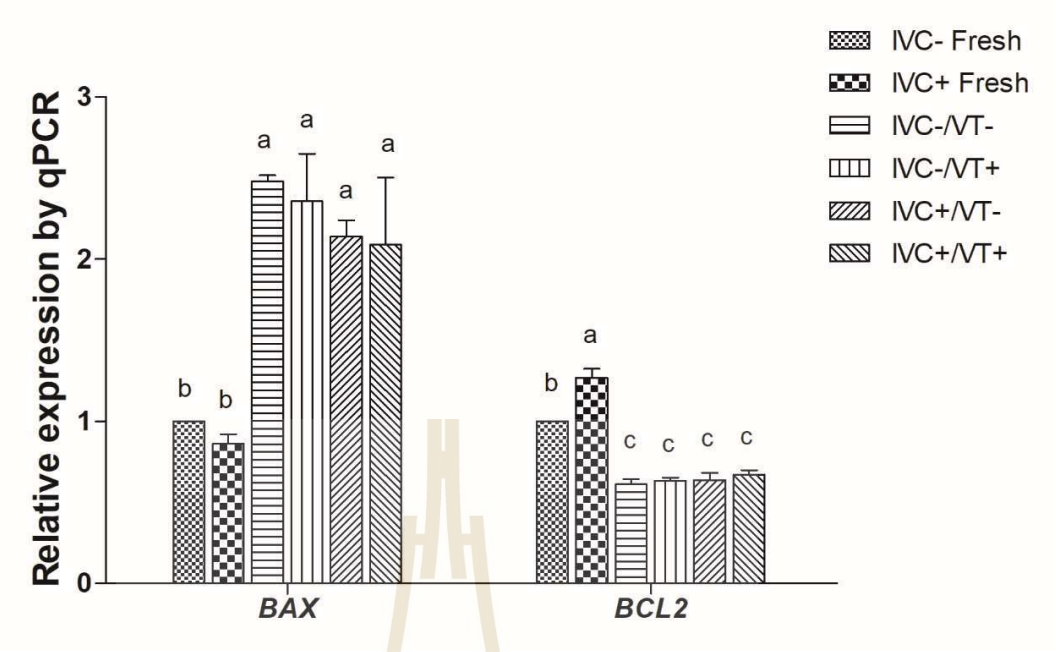

Figure 4.1 Changes (±S.E.M.) in the relative expression of *Bax* and *Bcl2* genes in bovine blastocysts across different groups. Error bars marked with a, b, c indicate values that are significantly different at $P<0.05$ according to one-way ANOVA. IVC-: cultured embryos in IVC medium without resveratrol; IVC+: cultured embryos in IVC medium with 0.5 µM resveratrol; VT-: vitrification without resveratrol in post-warming solution; VT+: vitrification with 1 µM resveratrol in post-warming solution

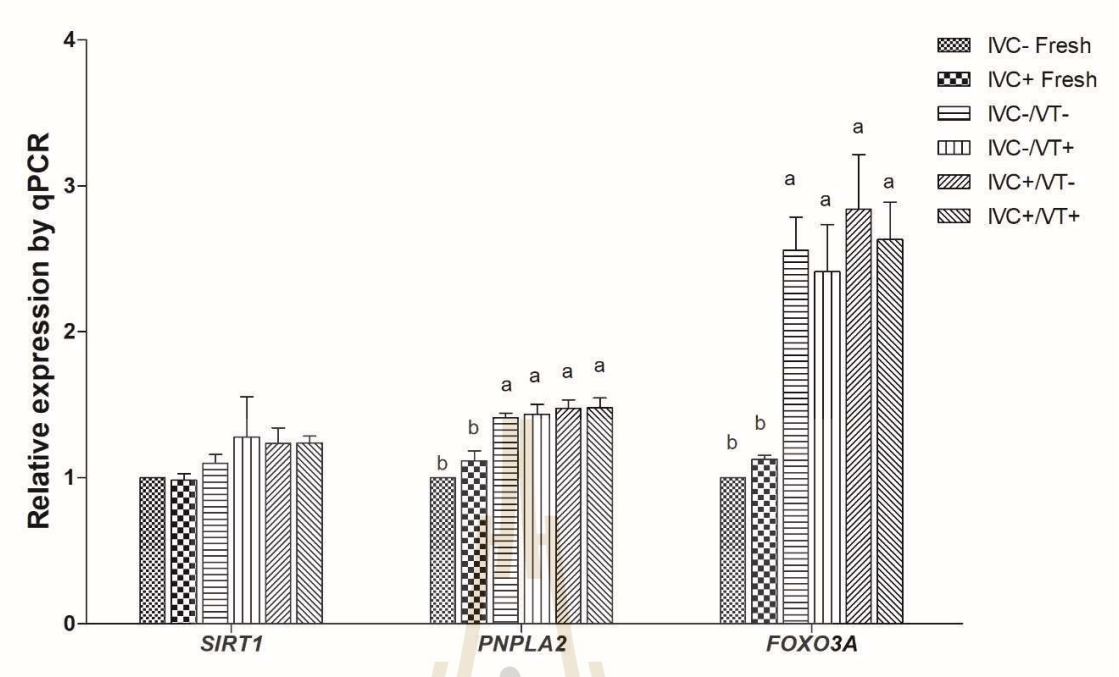

Figure 4.2 Changes (±S.E.M.) in the relative expression of *SIRT1, PNPLA2* and *FOXO3A* genes in bovine blastocysts across different groups. Error bars marked with a; b indicate values that are significantly different at P<0.05 according to one-way ANOVA. IVC-: cultured embryos in IVC medium without resveratrol; IVC+: cultured embryos in IVC medium with 0.5 μM resveratrol; VT-: vitrification without resveratrol in post-warming solution; VT+: vitrification with 1 μM resveratrol in post-warming solution

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

In the present study, the blastocyst formation rate of IVC- fresh embryos was 32.40%, whereas it increased to 35.20% in IVC+ fresh embryos. However, there was no statistical difference between both groups. These results indicated no positive effects of resveratrol on bovine embryo development. Similarly, several reports focused on resveratrol supplementation have shown a neutral benefit to bovine embryo development. For example, Salzano et al. (2014) investigated the effect of supplementing the IVC medium with varied concentrations of resveratrol at 0, 0.25, 0.5, and 1 μM. They found that resveratrol did not significantly increase blastocyst yields, which were 57.1%, 57.7%, 59.2%, and 46.6%, respectively. Afterward, Gaviria et al. (2019) reported that supplementing the culture medium with 0.5 μM resveratrol resulted in no significant difference in blastocyst rates compared to the control group. It is generally accepted that resveratrol might contribute to fostering embryo development, potentially due to its antioxidant properties and its ability to influence cellular processes (Hayashi et al., 2018). Resveratrol can also reduce mitochondrial ROS production. Anti-superoxide radicals inhibit fat oxidation and modulate the expression of cofactors and antioxidant enzymes (Abbasi et al., 2021; Abe et al., 2017; Agarwal and Majzoub, 2017). Additionally, it has been reported that supplementing the culture medium with 0.5 μM resveratrol can enhance the hatching rate of blastocysts (Iwata, 2021). Therefore, a potential strategy to alleviate oxidative stress-induced damage involves enriching culture media with antioxidant molecules, such as resveratrol. However, in the present study, a slight improvement of embryo development was noticed in IVC+ fresh group. This phenomenon may be a consequence of different culture environments. Since supplementation of resveratrol in IVC medium was not necessary in the study, our IVC condition may be optimal for the embryos by producing less ROS.

Post-warmed vitrified blastocysts in IVC-/VT- showed the significantly lowest survival rate (87.04%), compared to other groups. Despite no significant difference observed, the

addition of resveratrol yielded higher survival rate of blastocysts in the ranges of 90.74 – 94.34%. In accordance with Gaviria et al. (2019), resveratrol added into the IVC medium caused an increased survival rates after 4 hours of post-warming culture. Moreover, in this study, vitrified blastocysts derived from IVC+/VT+ exhibited the highest developmental rates (81.25%), indicated by the highest expanded and hatching blastocyst stages after culturing in warming solution. However, no significant difference was found in the developmental rate after 24 hours of culture. These results were in contrast with the findings performed by Salzano et al. (2014), who reported an increase in developmental and hatching rates of post-warmed embryos with 0.5 μM resveratrol supplement in IVC medium. Other studies have also shown that resveratrol supplementation during embryo culture or as a pretreatment before cryopreservation could improve viability and hatching rates after thawing (Abe et al., 2017; Hayashi et al., 2019). Despite most of the research indicating that low concentrations of resveratrol positively affect embryo competence, the results of this study did not show a substantial improvement. This suggests that resveratrol effects may not be sufficiently robust to create statistically significant differences in this context. These results were consistent with the report by Gaviria et al. (2019), who found no difference in the hatching rate after 24 hours of culture with resveratrol added to both the IVC medium and the vitrification solution. It is possible that resveratrol alters some metabolic characteristics of the embryo, potentially generating detrimental effects following vitrification e.g. increase in the proportion of cell death (Gadacha et al., 2009; Gueguen et al., 2015). This may be related to induction of cell cycle arrest by resveratrol through the upregulation of p53 expression leading to efflux of cytochrome c into cytosol and the activation of the caspase apoptotic pathway (Brill et al., 1999; Yuan et al., 2015). Some features, such as concentration, effective cell uptake, species, cell type, cellular redox state, and stressor factor might interact and form a negative effect by the added supplement (Lastra and Villegas, 2007; Gadacha et al., 2009)). In addition, similar survival rate and developmental competency of post-warmed embryos between the control and resveratrol treated groups may be associated with gene expression as observed in the present study. Apoptotic inducing *BAX* gene was increased simultaneously with a decrease in anti-apoptotic *BCL2* gene in post-warmed embryos despite resveratrol addition. This may be explained why no significant enhancement of survival in post-warmed embryos in resveratrol treated solution. The present study indicated that resveratrol supplemented had no beneficial effects on stress resistance and

survival of bovine embryos following vitrification. Comparatively, IVC+ fresh and IVC- fresh embryos showed significantly higher developmental rates to expanded and hatching blastocyst stages than all vitrified groups. This outcome underscores the inherent advantage of fresh embryos over vitrified ones, likely due to the absence of stressors associated with the freezing and thawing processes. This finding suggested that while resveratrol supplementation in IVC media can improve certain developmental outcomes for bovine embryos, particularly in fresh conditions, its benefits for vitrified embryos are limited. These insights highlight the complexity of optimizing embryo culture and cryopreservation protocols and the need for further research to enhance the efficacy of these techniques.

The findings presented in the impact of resveratrol supplementation on the cell numbers of vitrified blastocysts following thawing and culturing in warming solution. Specifically, the inner cell mass (ICM), trophectoderm (TE), and total cell numbers did not show significant differences between vitrified blastocysts supplemented with resveratrol (VT+) and those without resveratrol (VT-). Moreover, IVC+ fresh blastocysts exhibited significantly higher ICM, TE, and total cell numbers compared to vitrified-warmed blastocysts incubated in warming solution without resveratrol (VT-). The results of Salzano et al. (2014) were consistent with the present finding. They reported that the adding 0.5 μM resveratrol to the culture media did not affect the cell allocation between the ICM and TE or the total cell number within the embryos. Similarly, Abe et al. (2017) found no difference in total cell number between the control group and the 0.5 μM resveratrol group (65.5 and 65.4, respectively). This observation underscores the potential vulnerability of blastocysts to cryopreservation-induced cellular damage in the absence of resveratrol (Nashtaei et al., 2018). Interestingly, no significant differences were noted between the control group and the group subjected to vitrification with resveratrol supplementation regarding the counts of ICM cells, TE cells, or total cell numbers. These findings suggested that the absence of resveratrol supplemented during both culture and post-warming stages resulted in a significant reduction in cell numbers in vitrified blastocysts. However, when comparing these blastocysts to those from groups with resveratrol supplementation, the difference in cell numbers is less pronounced. Further investigations focused on the mechanisms underlying the protective effect and the optimal supplementation protocols are warranted to improve the success rates of cryopreservation in assisted reproductive technologies.

Vitrification/warming technique not only ensures high cryo-survival rates but also maintains biological functions, leading to higher developmental competency after warming. Thus, we evaluated the potential impact of resveratrol supplementation in conjunction with the vitrification process on the expression of apoptosis-related and transcription factor genes. Apoptosis is a regulated program that initiates cell death, with members of the *BCL2* gene family playing a crucial role in its regulation. *BCL2* is anti-apoptotic and promotes cell survival, whereas *BAX* is pro-apoptotic and induces cell death (Yang and Rajamahendran, 2002). In this study, the aim was to evaluate whether resveratrol addition during IVC or post-warming caused change in the relative expression of genes related to mitochondrial function, antioxidant defense and embryo quality. The results of this study indicated a significant impact of vitrification on the relative expression of *BAX* and *BCL2* genes in bovine blastocysts. As expected, vitrified embryos exhibited significantly higher *BAX* and lower *BCL2* expression, compared to both IVC- and IVC+ fresh embryos. Similar results have been reported by Torres et al., 2018 and Gaviria et al., 2018. This suggests that the vitrification process alters the expression of pro-apoptotic genes, potentially affecting the viability and quality of the embryos. Despite the known antioxidant properties of resveratrol, the supplementation in the IVC medium and/or warming solution did not confer any beneficial effect on *BAX* expression in vitrified embryos. Interestingly, the IVC+/VT+ embryos showed slightly lower *BAX* expression compared to other vitrified groups. This marginal reduction, while not indicative of a substantial protective effect, suggests a minor influence of resveratrol on *BAX* regulation under vitrification stress conditions. In contrast, vitrification had a pronounced detrimental effect on *BCL2* expression, a gene associated with anti-apoptotic functions. The relative *BCL2* expression in all vitrified embryos (IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+) were significantly lower than those observed in IVC- and IVC+ fresh embryos. The ratio of *BAX* and *BCL2* can be used to evaluate the vulnerability of cells to a mortality signal (Gross et al., 1999). Fresh embryos represented low *BAX/BCL2* ratio while IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ vitrified embryos presented a higher ratio, indicating a major tendency to apoptosis. Obviously, in the present study, the supplementation of the IVC medium with resveratrol (IVC+) significantly increased *BCL2* expression levels in fresh embryos, highlighting the potential of resveratrol to enhance embryo resilience under non-vitrified conditions (Gross et al., 1999). However, this beneficial effect was not observed in IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ vitrified embryos, indicating that

the protective mechanisms of resveratrol may be insufficient to counteract the severe stress induced by the vitrification process.

The findings of this study highlight the differential impact of resveratrol supplementation on gene expression in vitrified bovine blastocysts. *SIRT1* is known for its role in promoting cell survival and longevity by deacetylating key transcription factors and enzymes involved in stress resistance, DNA repair, and metabolism (Brooks and Gu, 2009). There was no significant difference of the expression of *SIRT1* gene between fresh and vitrified groups in this study. Similarly, Gaviria et al. (2019) reported that culturing bovine blastocysts in the IVC medium and/or the vitrification/warming process did not alter *SIRT1* mRNA expression. On the contrary, recent studies have reported that the addition of 0.5 or 1 μM resveratrol into the IVC medium increased the expression of *SIRT1* protein (Abe et al., 2017; Hayashi et al., 2018, 2019). However, Torres et al. (2018) found that bovine blastocysts cultured with 1 μM resveratrol in complex with cyclodextrin (RV-CD) showed a significant reduction in *SIRT1* mRNA expression compared to the control.

This study also found that the addition of resveratrol to IVC+ fresh embryos resulted in a slight increase in *FOXO3A* and *PNPLA2* gene expressions. *FOXO3A*, a transcription factor regulated by *SIRT1*, is critical for the expression of genes involved in apoptosis, oxidative stress resistance, and metabolism (Salminen et al., 2013). The upregulation of *FOXO3A* suggests enhanced cellular defense mechanisms and stress response pathways in resveratrol-treated groups (Qin et al., 2021; Yun et al., 2012). *PNPLA2*, an enzyme involved in lipid metabolism and energy homeostasis through triglyceride hydrolysis resulting to the formation of diglyceride and fatty acid (Taxiarchis et al., 2019; Zhang et al., 2019). The slight increase in *FOXO3A* and *PNPLA2* expression in the present study may imply partial maintaining cellular energy balance and membrane integrity of resveratrol during and after vitrification. Resveratrol supplemented in the IVC medium or warm solution may help restore the initial quieter, or lower metabolic state of bovine blastocysts (Baumann et al., 2007; Gaviria et al., 2019; Krisher and Prather, 2012). Furthermore, the supplementation of resveratrol in the IVC medium and/or warming solution (IVC-/VT+, IVC+/VT- and IVC+/VT+) in this study did not change the expression of *FOXO3A* and *PNPLA2* in post-warmed embryos compared to IVC- and IVC+ fresh embryos, but vitrification significantly increased both gene expression. Similar result was reported by Gaviria et al. (2019). They observed no alteration in *FOXO3A* and *PNPLA2* gene expression

following resveratrol administration, but vitrification-waring cycle was the main cause of the increased relative abundance of *FOXO3A* and *PNPLA2* genes. A previous study demonstrated that the vitrification-warming cycle increases active mitochondria *in vitro* produced bovine blastocysts, leading to an increase in mitochondrial O_2^- production. The significant increase in expression of *FOXO3A* and *PNPLA2* reflects the elevated embryo metabolism in response to cryopreservation (Gaviria et al., 2019). When embryos are exposed to stressors, they activate energy-consuming repair mechanisms, such as apoptosis, which increases their ATP consumption (Baumann et al., 2007; Marsico et al., 2019). In this context, the increased expression of *PNPLA2* may indicate the hydrolysis of triacyl glycerides stored in cytosolic lipid droplets and subsequent production of fatty acid to support energy demand of IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ embryos in the present study (Gruber et al., 2010; Schweiger et al., 2008).

5.2 Conclusion

Our study reveals the potential benefits of resveratrol supplementation in the development and cryopreservation of bovine embryos. Supplementing the culture medium with resveratrol slightly increased blastocyst formation rates, suggesting a positive impact on embryo development, although this increase was not statistically significant. Resveratrol had no desired effects on cryotolerance improvement of vitrified embryos evidenced by similar survival rate and development of blastocysts in post-warming solution. Fresh embryos cultured with resveratrol had the highest ICM, TE and total cell mass, while similar ICM, TE and total cell mass were produced in IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ embryos. However, these numbers were not statistically significant. In addition, resveratrol did not play a role in any changes of expression of genes related to apoptosis and stress response indicated by no significant difference of the gene expression in the IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ embryos. However, vitrification played an important role in the alteration of targeted genes. Vitrification caused significant increases in *BAX*, *PNPLA2* and *FOXO3A* expression while significant decrease in *BCL2* expression. No changes were observed in the expression of *SIRT1* gene following vitrification and/or resveratrol supplementation.

Further research is necessary to optimize the concentration and timing of resveratrol supplementation to maximize its benefits. Understanding the precise molecular mechanisms through which resveratrol exerts its effects will be crucial in refining

cryopreservation techniques and improving embryo quality. By advancing our knowledge and application of resveratrol in embryo culture, we can enhance reproductive success rates and the overall efficiency of breeding programs.

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BIOGRAPHY

Miss. Yanapon Anthakat was born on August 12th, 1998, in Nakhon Phatom, Thailand. She graduated with a Bachelor's degree in Biological Science, Faculty of Liberal Arts and Science, Kasetsart University, Nakhon pathom, Thailand in 2021. Then she studied Master's Degree under the supervision of Assoc. Prof. Dr. Rangsun Parnpai at School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima, Thailand in 2021-2024, and received Oner Research One Graduate (OROG) scholarship from Suranaree University of Technology. The research topic is "Effects of Resveratrol supplementation in *in vitro* embryo culture medium and warming solution on developmental competence after vitrification". Some parts of the results from this study have been presented as a poster presentation at the 35th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2023) during 26-29 November, 2023.