EFFECT OF RESVERATROL SUPPLEMENTATION INTO *IN VITRO* CULTURE MEDIUM ON DEVELOPMENTAL COMPETENCE, CRYOTOLERANCE, AND GENE EXPRESSION OF *IN VITRO* PRODUCED BOVINE EMBRYOS

KAMOLCHANOK TONEKAM

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science Program in Biotechnology
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ผลของการเติมเรสเวอราทอลในน้ำยาเลี้ยงตัวอ่อนต่อความสามารถในการพัฒนา ความอยู่รอดหลังการแช่แข็ง และการแสดงออกของยีนในตัวอ่อนโคที่ผลิตในหลอดแก้ว

นางสาวกมลชนก โทนคำ

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

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กมลชนก โทนคำ: ผลของการเติมเรสเวอราทรอลในน้ำยาเลี้ยงตัวอ่อนต่อความสามารถในการพัฒนา ความอยู่รอดหลังการแช่แข็ง และการแสดงออกของยีนในตัวอ่อนโคที่ผลิตในหลอดแก้ว (EFFECT OF RESVERATROL SUPPLEMENTATION INTO *IN VITRO* CULTURE MEDIUM ON DEVELOPMENTAL COMPETENCE, CRYOTOLERANCE AND GENE EXPRESSION OF *IN VITRO* PRODUCED BOVINE EMBRYOS) อาจารย์ที่ปรึกษา: ศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 67 หน้า.

คำสำคัญ: โค/การพัฒนาของตัวอ่อน/เรสเวอราทรอล/ภาวะเครียดออกซิเดชัน/การแช่แข็งแบบ vitrification

ในเทคโนโลยีช่วยการสืบพันธุ์ (ART) การแช่แข็งตัวอ่อนโดยเฉพาะวิธี vitrification เป็นสิ่งจำเป็นในการรักษาคุณภาพตัวอ่อนของสัตว์เคี้ยวเอื้อง อย่างไรก็ตาม การแช่แข็งวิธี vitrification อาจส่งผลต่อความมีชีวิตหลังการละลายตัวอ่อน เนื่องจากเกิดภาวะเครียดออกซิเดชันจากอนุมูลอิสระ (ROS) การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบผลของเรสเวอราทรอลซึ่งเป็นสารต้านอนุมูลอิสระ ต่อการรอดชีวิตและความสามารถในการพัฒนาของตัวอ่อนโคระยะบลาสโตซิสต์ที่เลี้ยงในหลอดแก้ว ในการทดลองที่ 1 ตัวอ่อนที่ผลิตในหลอดทดลองถูกแบ่งออกเป็น 2 กลุ่มคือ 1) เติมเรสเวอราทรอล 0.5 µM ในน้ำยาเลี้ยงตัวอ่อน (+R) และ 2) กลุ่มควบคุม ซึ่งไม่เติมเรสเวอราทรอลในน้ำยาเลี้ยงตัวอ่อน (-R) จากผลการศึกษา พบว่าอัตราการแบ่งเซลล์และการพัฒนาสู่ระยะบลาสโตซิสต์ ในกลุ่ม +R (81.70% และ 37.75%) สูงกว่ากลุ่ม -R (75.13% และ 29.82%) อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) การทดลองที่ 2 นำตัวอ่อนระยะบลาสโตซิสต์ที่ผลิตในกลุ่ม +R และ -R ที่นำไปแช่แข็งโดยวิธี vitrification แล้วนำไปทำละลายในน้ำยาที่ไม่เติมเรสเวอราทรอลก่อนนำไปเลี้ยงต่อในหลอดทดลองที่เติมและไม่เติมเรสเวอราทรอล ซึ่งพบว่าการเติมเรสเวอราทรอลเฉพาะระหว่างการเลี้ยงตัวอ่อนแต่ไม่เติมในน้ำยาเลี้ยงตัวอ่อนหลังละลาย (+R/–R) ได้อัตราการฟักตัวของตัวอ่อน (71.50%) สูงกว่ากลุ่มเติมเฉพาะน้ำยาเลี้ยงตัวอ่อนหลังละลาย (–R/+R, 45.03%) อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) อย่างไรก็ตาม เรสเวอราทรอลไม่ส่งผลต่อจำนวนเซลล์โทรเฟคโทเดิร์ม (TE) เซลล์ไอซีเอ็ม (ICM) หรือจำนวนเซลล์ทั้งหมดในตัวอ่อนสดและตัวอ่อน vitrification การวิเคราะห์การแสดงออกของยีนแสดงให้เห็นว่า เรสเวอราทรอลช่วยเพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับการป้องกันอนุมูลอิสระ (*SOD1, CAT*) ความต้านทานต่อความเครียด (*SIRT1*) การทำงานของไมโตคอนเดรีย (*TFAM*) การต้านการตายของเซลล์ (*BCL2*) การควบคุมอีพีเจเนติกส์ (*DNMT1, DNMT3A*) การคงความเป็นเซลล์พลูโพเทนท์ (*OCT4*) และการส่งสัญญาณการตั้งท้อง (*IFN-tau*) ในตัวอ่อนสด ส่วนในตัวอ่อนที่ผ่านการ vitrification เรสเวอราทรอลสามารถคงระดับการแสดงออกของเอนไซม์ต้านอนุมูลอิสระ

GPX4 ไว้สูงโดยเฉพาะเมื่อได้รับเรสเวอราทรอลในช่วงเลี้ยงตัวอ่อน นอกจากนี้เรสเวอราทรอลยังลดการแสดงออกของยีนที่กระตุ้นการตายของเซลล์ (*BAX*) ในตัวอ่อนที่ได้รับการเติมสารระหว่างการเลี้ยงในหลอดทดลองอย่างมีนัยสำคัญทางสถิติซึ่งบ่งชี้ถึงการเพิ่มความมีชีวิตของเซลล์โดยสรุปผลลัพธ์ทั้งหมดชี้ให้เห็นถึงผลดีของการเติมเรสเวอราทรอลระหว่างเลี้ยงตัวอ่อนต่อการเพิ่มความทนทานต่อการแช่แข็ง การควบคุมการแสดงออกของยีน และความสามารถในการพัฒนาของตัวอ่อนโค

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา...........กมลชนก...........

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา..................................

KAMOLCHANOK TONEKAM: EFFECT OF RESVERATROL SUPPLEMENTATION INTO *IN VITRO* CULTURE MEDIUM ON DEVELOPMENTAL COMPETENCE, CRYOTOLERANCE AND GENE EXPRESSION OF *IN VITRO* PRODUCED BOVINE EMBRYOS. THESIS ADVISOR: RANGSUN PARNPAI, Ph.D., 67 PP.

Keyword: bovine/embryo development/resveratrol/oxidative stress/vitrification

In assisted reproductive technology (ART), embryo cryopreservation, particularly via vitrification, is essential for preserving the quality of ruminant embryos. However, vitrification can adversely affect embryo viability post-thawing due to oxidative stress induced by reactive oxygen species (ROS). The present study aimed to evaluate the effects of resveratrol, a known antioxidant, on the survival and developmental competence of bovine embryos cultured *in vitro*. In Experiment 1, *in vitro*-produced embryos were divided into two groups: (1) supplemented with 0.5 µM resveratrol in embryo culture medium (+R) and (2) without resveratrol in embryo culture medium (-R) which served as control group. Results indicated that cleavage and blastocyst developmental rates in the +R group (81.70% and 37.75%, respectively) were significantly higher ($P < 0.05$) than the -R group (75.13% and 29.82%, respectively). Experiment 2, investigated the developmental outcomes of blastocysts from the +R and -R groups subjected to vitrification, warmed in media without resveratrol, and then subsequently cultured *in vitro* with or without resveratrol. The results found that supplemented resveratrol only in culture medium (not in post-warming, +R/-R) exhibited significantly higher ($P < 0.05$) hatching rates (71.50%) compared to those supplemented only in post-warming culture medium (-R/+R, 45.03%). However, resveratrol supplementation did not affect the number of trophectoderm (TE), inner cell mass (ICM), or total cell numbers in both fresh and vitrified embryos. Gene expression analysis revealed that resveratrol enhanced expression of antioxidant-related genes (*SOD1*, *CAT*), stress resistance (*SIRT1*), mitochondrial function (*TFAM*), anti-apoptosis (*BCL2*), epigenetic regulation (*DNMT1*, *DNMT3A*), pluripotency (*OCT4*), and pregnancy signaling (*IFN-tau*) in fresh embryos. In vitrified embryos, resveratrol maintained high levels of *GPX4* expression, particularly when administered during embryo culture. Moreover, resveratrol significantly reduced the expression of the pro-apoptotic gene (*BAX*) in embryos cultured *in vitro*, suggesting improved cell viability. In conclusion, the findings demonstrate the beneficial effects of resveratrol supplementation during embryo culture, enhancing cryotolerance, regulating gene expression, and promoting developmental competence of bovine embryos.

School of Biotechnology
Academic Year 2024

Student's Signature....Kamolchanok....
Advisor's Signature..

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Kamolchanok Tonekam

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

Figure

Page

LIST OF ABBREVIATIONS

ART	=	Assisted Reproductive Technology
BAX	=	BCL2 Associated X Protein
BCL2	=	B-cell Lymphoma 2
BM	=	Base Medium
CAT	=	Catalase
cDNA	=	Complementary Deoxyribonucleic Acid
CO_2	=	Carbon Dioxide
COX2	=	Cyclooxygenase-2
COCs	=	Cumulus-Oocyte Complexes
CR1aa	=	Charles Rosenkrans 1 Amino Acid Medium
DMSO	=	Dimethyl Sulfoxide
DNMT1	=	DNA Methyltransferase 1
DNMT3A	=	DNA Methyltransferase 3 Alpha
EG	=	Ethylene Glycol
ES	=	Equilibration Solution
FBS	=	Fetal Bovine Serum
FSH	=	Follicle Stimulating Hormone
GAPDH	=	Glyceraldehyde 3-Phosphate Dehydrogenase
GPX4	=	Glutathione Peroxidase 4
HCG	=	Human Chorionic Gonadotropin
HSD	=	Honest Significant Difference
HSPA8	=	Heat Shock Protein Family A (Hsp70) Member 8
IFN-tau	=	Interferon Tau
ICM	=	Inner Cell Mass
IVC	=	In Vitro Culture
IVF	=	In Vitro Fertilization
IVM	=	In Vitro Maturation

LIST OF ABBREVIATIONS (Continued)

mDPBS	=	Modified Dulbecco's Phosphate Buffered Saline
OCT4	=	Octamer-binding Transcription Factor 4
OPU	=	Ovum Pick-Up
PBS	=	Phosphate Buffered Saline
PI	=	Propidium Iodide
PVP	=	Polyvinylpyrrolidone
qPCR	=	Quantitative Polymerase Chain Reaction
ROS	=	Reactive Oxygen Species
SCNT	=	Somatic Cell Nuclear Transfer
SIRT1	=	Sirtuin 1
SOD1	=	Superoxide Dismutase 1
TCM-199	=	Tissue Culture Medium 199
TE	=	Trophectoderm
TFAM	=	Transcription Factor A, Mitochondrial
VS	=	Vitrification Solution

CHAPTER I

INTRODUCTION

1.1 Background and significance

Cryopreservation is an important technique in assisted reproductive technologies (ARTs). In addition to preserving livestock animals and endangered genotypes, cryopreservation allows the store oocytes or embryos for later use (Woods et al., 2004). In livestock, one cryopreservation benefit is that it allows for oocyte and embryo transport over long distances, which can be more practical and cost-effective than live-animal transportation. Vitrification, a rapid method of cryopreservation associated with creating an amorphous, non-crystalline solid, successfully prevents the formation of intracellular and extracellular ice crystals (Rall & Fahy, 1985). In the fields of oocyte and embryo cryopreservation, the approach of vitrification methods has recently become dominant because the procedure is relatively simple and efficient, with higher post-thaw survival rates for oocytes and embryos compared to slow-freezing methods.

However, cryopreserved *in vitro* produced (IVP) embryos have lower survival rates than *in vivo*-derived embryos, as indicated by post-warming survival in culture or successful pregnancies after embryo transfer (Kaidi et al., 1998; Lonergan et al., 2003; Massip et al., 1995). This is related to the formation of damaging reactive oxygen species (ROS), which causes oxidative stress, mitochondrial damage, a shift in calcium oscillation during fertilization, apoptosis, and embryonic development failure. As a result, cells have evolved adaptations to utilize the reactive nature of ROS for beneficial purposes while avoiding their negative impacts (Morado et al., 2009). The female reproductive system has a variety of oxygen scavengers that shield oocytes and embryos from oxidative damage (Guerin et al., 2001). Nevertheless, *in vitro* systems, oocytes, and embryos lack these natural defenses and are constantly exposed to a variety of stressors (Guerin et al., 2001; Marques et al., 2007). To optimize embryo development *in vitro*, one option is to supplement the culture media with antioxidant molecules that can reduce ROS formation, neutralize it, and help cells repair ROS-induced damage.

In recent years, there have been reports focused on several substances, such as antioxidants, to prevent cell damage caused by free radicals, such as enzymes, thiol compounds, vitamins, flavonoids, amino acids, and amino acid derivatives. Resveratrol (3,4,5-trihydroxy-trans-stilbene), a well-known antioxidant, has been reported to reduce oocyte damage from vitrification injuries and improve blastocyst formation rate in various species, including mice, goats, pigs, and cattle (Piras et al., 2019; Itami et al., 2015; Chinen et al. 2020). Due to their physical and molecular features, resveratrol is a phytoalexin with intra- and extracellular antioxidant potentials (Gambini et al., 2015). In cattle, resveratrol-supplemented *in vitro* maturation medium (IVM) improved cumulus expansion, polar body formation, blastocyst rate, and the number of cells in blastocysts while reducing ROS levels and increasing glutathione (GSH) levels (Wang et al., 2014). In previous studies in our laboratory, supplementing resveratrol in the culture medium, and vitrification solution improved the cryotolerance of mouse embryos and altered the expression of apoptotic and implantation genes (Puangjit, 2019). Likewise, the addition of resveratrol in short-term recovery culture before *in vitro* fertilization (IVF) resulted in improved blastocyst formation rate and reduced oxidative stress (Chinen et al., 2020). Consistent with these findings, it has also been reported that adding 0.5 μM resveratrol to the embryo culture medium can improve the cryotolerance of embryos. (Salzano et al., 2014). This study aimed to investigate whether 0.5 μM resveratrol supplementation *in vitro* culture (IVC) and post-warming culture media affects the survivability and gene expression of post-warmed vitrified bovine embryos. Additionally, to evaluate the developmental competence (based on blastocyst yield) and quality of blastocysts by counting total cell numbers through IVF and IVC.

1.2 Research objective

1.2.1 To evaluate developmental competence and quality of IVF-derived bovine embryos treated with 0.5 μM resveratrol in IVC medium.

1.2.2 To investigate the effects of 0.5 μM resveratrol in IVC and post-warming culture media on the survivability of post-warmed vitrified bovine embryos.

1.2.3 To examine the effects of 0.5 μM resveratrol in IVC and post-warming culture media on gene expression of post-warmed vitrified bovine embryos.

1.3 Research hypothesis

1.3.1 Resveratrol in IVC medium could enhance developmental competence after IVF and improve the quality of bovine blastocysts.

1.3.2 Resveratrol in IVC and post-warming culture media could increase the survival rate of post-warmed vitrified blastocysts.

1.3.3 Resveratrol supplementation in both the IVC and post-warming culture media could enhance cell programming, pluripotency, embryonic quality, mitochondrial activity, and the expression of stress response-related genes.

1.4 Scope and limitations of the study

1.4.1 The effects of 0.5 μM resveratrol supplementation IVC medium on the developmental competence and quality of bovine embryos through IVF and IVC were examined until the blastocyst stage.

1.4.2 The effects of 0.5 μM resveratrol supplementation in IVC and post-warming culture media on the survivability of post-warmed vitrified bovine embryos were investigated, with survival, re-expansion, and hatching rates assessed at 24 and 48 h post-warming.

1.4.3 The effect of 0.5 μM resveratrol supplementation in IVC and post-warming culture media on the gene expression of post-warmed vitrified bovine embryos was examined using quantitative PCR (qPCR).

1.5 Research methodology

1.5.1 Instrumentation

The 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, supplied all their instruments and materials.

1.5.2 Location of research

The experiments were conducted at ESRC, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

CHAPTER II

RITERATURE REVIEWS

2.1 *In vitro* embryo production (IVP)

An alternate method for generating bovine embryos utilizes immature oocytes obtained from donor animals of diverse ages and physiological states. Current laboratory techniques enable the effective *in vitro* maturation and fertilization of these oocytes, followed by a culture period of roughly seven days to achieve the developmental stage appropriate for embryo transfer or cryopreservation. Originally conceived as a research technique, IVP was originally employed to recover follicular oocytes from ovaries obtained from slaughterhouses. Nonetheless, its utilization has since broadened considerably. In bovine, IVP is now frequently utilized to produce embryos from live donors, serving as either an adjunct to or replacement for multiple ovulation and embryo transfer (MOET), due to its adaptability and other practical benefits. The IVP method comprises three distinct and extensively utilized biological stages, including IVM of oocytes, IVF, and subsequent IVC of embryos (Galli et al., 2003).

2.1.1 *In vitro* maturation (IVM)

Assisted reproductive methods, especially IVF and somatic cell nuclear transfer (SCNT), depend on maturing oocytes from bovines in a lab. Using IVM, immature oocytes taken from the ovaries of an animal may develop in a lab setting under controlled conditions. In the 1960s, early studies showed that it was possible to mature bovine oocytes *in vitro*. But it was not until the 1980s that reliable protocols were set up that supported both nuclear and cytoplasmic maturation, which allowed for successful fertilization and embryo development (Lonergan et al., 1994; Zhang et al., 1992). Establishing successful IVM systems was a big step forward in bovine breeding because it made possible to collect oocytes from ovaries taken from animals that were slaughtered or from live donors using ovum pick-up (OPU). This made a lot more oocytes available to produce embryos. IVM is based on starting meiosis again after it has been stopped at the germinal vesicle (GV) stage in the mature oocytes inside follicles. *In vivo*, a rise in the luteinizing hormone (LH) before ovulation starts meiotic resumption. This causes the germinal vesicle to break down (GVBD), the shift from metaphase I to metaphase II, and the oocyte to become fertile (Mehlmann, 2005). These hormonal signals can be simulated in the laboratory by adding gonadotropins like follicle-stimulating hormone (FSH) and LH to the maturation medium along with estradiol. This helps the oocytes go through a similar nuclear maturation process (Lonergan et al., 1994). But it is still hard to get the cytoplasm to mature properly, which includes moving organelles around, conserving maternal mRNAs, and rearranging cortical granules. This is because oocytes that have been matured in the laboratory often don't develop as well as their *in vivo* counterparts (Watson, 2007; Palhares et al., 2022). A big part of the success of bovine IVM is the way of culture media is put together. A lot of people have used traditional media like TCM-199 with added FBS, FSH, LH, and estradiol to help oocytes mature (Ali & Sirard, 2002). Recently, people have been using chemically defined media to make the *in vitro* environment more stable and reduce batch variability (Ali & Sirard, 2002). Adding growth factors like epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) has also been shown to speed up the maturation of oocytes and improve their ability to develop by turning on signaling pathways that act like the microenvironments inside follicles (Lorenzo et al., 1995). Oxidative stress during IVM is another thing that can hurt the health of an oocyte. ROS and high oxygen stress can harm DNA, proteins, and lipids, which can affect embryo development less than ideal (Feuchard et al., 2025). Some ways to reduce oxidative stress are to bring the oxygen level in incubators down to a healthy level (5% O_2) and add antioxidants like cysteamine, melatonin, and vitamin E to the media (Tamura et al., 2013). Researchers have found that these actions lead to better mitochondrial function, fewer deaths, and more blastocysts after IVF. Getting nuclear and cytoplasmic maturation to happen at the same time is another area of active study. Many oocytes make it to the MII stage, but their cytoplasmic maturation may be slower than expected, which can hurt their growth. To keep oocytes temporarily at the GV stage (called "capacitation of oocytes"), scientists have investigated delaying nuclear maturation with certain inhibitors like recovering or colchicine. This lets cytoplasmic processes catch up, which improves the ability of the embryo to develop (Lorenzo et al., 1995). Even though they are better, embryos derived from IVM often have different gene expression patterns than embryos derived from oocytes that have matured *in vivo*. Studies that use transcriptomic studies have found that embryos made from *in vitro*-matured oocytes have problems with key genes that control metabolism, stress response, and epigenetic regulation (Watson, 2007). These differences at the molecular level probably play a part in why embryos made after IVM have lower cryotolerance and implantation rates. This shows how important it is to keep improving the culture conditions. In conclusion, the ability to grow bovine oocytes in the laboratory has opened a lot of pathways for investigation and commercial applications in reproductive biotechnology. The basic steps for nuclear maturation are well known, but work is still being done to improve cytoplasmic maturation, lower oxidative damage, and make the environment more like the follicular environment *in vivo*. Molecular biology, especially omics technologies, is making progress in finding key processes that can be targeted to improve the quality of oocytes. In the end, making IVM systems that are more biologically relevant and reliable will be necessary to get the most out of bovine IVF and other related technologies.

2.1.2 *In vitro* fertilization (IVF)

IVF in bovines has changed the field of reproductive biotechnology by conserving valuable genetics and enhancing reproductive efficiency. In the early 1980s, Brackett et al. recorded the first successful IVF with bovine, leading to a live calf. Technology has come a long way since then and is now an important part of both research and commercial production systems. IVF includes getting oocytes from donor cows, letting them mature, fertilizing them with sperm in a laboratory, and then developing the embryos until they are ready to be transferred. Traditional MOET cannot be equally successful as IVF because it cannot collect embryos as often and can use oocytes from both young cows and older cows (Mapletoft & Hasler, 2005). The number and quality of oocytes available are very important for the success of IVF. OPU from live donors or collected from slaughterhouse ovaries. After collecting, immature oocytes must undergo maturation *in vitro* to reach the metaphase II stage, which means they can be fertilized. For IVF to work, the sperm must also be properly prepared. In bovine IVF, sperm are often put through a process called "capacitation," which is usually caused by heparin. This helps the acrosome react and the oocytes penetrate (Parrish et al., 1988). The use of Percoll or discontinuous gradient centrifugation methods improved sperm selection by collecting spermatozoa that were highly motile and had normal morphology (Morrell & Rodriguez-Martinez, 2010). Higher rates of fertilization and cleavage have been achieved by improving the concentrations of insemination, the times that sperm and oocytes are co-incubated, and the activation methods. Understanding how the quality of the gametes, the conditions of fertilization, and the surroundings in which the embryos develop all affect each other is still important for improving IVF outcomes and making sure that it is successful for a lot of individuals in both commercial and conservation settings.

2.1.3 *In vitro* culture (IVC)

Current bovine reproductive technology relies heavily on IVP, which facilitates genetic enhancement and advanced assisted reproductive techniques (Lonergan et al., 1994). A critical component of IVP is the culture medium, designed to emulate the physiological conditions of the female reproductive tract, thereby supporting oocyte maturation, fertilization, and early embryonic development (Pinyopummintr & Bavister, 1994). Traditionally, undefined systems such as co-culturing with somatic cells or serum-based media have been utilized; however, there has been a shift towards chemically defined and serum-free formulations to enhance repeatability and reduce variability (Van der Valk et al., 2010).

Prominent culture media like synthetic oviduct fluid (SOF), CR1aa, and KSOM have demonstrated efficacy in promoting development to the blastocyst stage by replicating the ionic and nutritional composition of the oviductal environment (Takahashi & First, 1992; Rosenkrans et al., 1993; Biggers et al., 1997). Serum supplementation, particularly with fetal bovine serum, has historically enhanced embryo development by providing hormones, growth factors, and antioxidants that mitigate oxidative stress (Abdel-Wahab et al., 2018). Nonetheless, concerns regarding animal welfare, variability, ambiguous composition, and potential disease transmission have propelled the development of serum-free techniques (Van der Valk et al., 2010).

The addition of amino acids to culture media has been shown to improve embryo quality through mechanisms such as protein synthesis, reduction of ammonia accumulation, and maintenance of metabolic needs (Steeves & Gardner, 1999; Lane & Gardner, 2000). Currently, bicarbonate-buffered systems under 5% CO_2 are standard practice, as maintaining appropriate pH and osmolarity is essential for embryonic survival (Lane & Gardner, 2000). Oxidative stress remains a significant challenge in vitro cultivation, as reactive oxygen species can damage cellular components. Supplementation with antioxidants like melatonin and L-carnitine has been found to enhance embryo development and reduce oxidative damage (Tamura et al., 2013; Li et al., 2021). Apoptosis, or programmed cell death, also contributes to early embryo loss; thus, media formulations that reduce excessive apoptosis can improve blastocyst development and post-transfer viability (Brison & Schultz, 1997).

Developing effective bovine embryo culture media necessitates consideration of various biochemical and physiological elements, including nutritional content and redox balance, to optimize embryo development and ensure consistent outcomes in reproductive biotechnology (Pinyopummintr & Bavister, 1994; Van der Valk et al., 2010).

2.1.4 Embryo

Researchers have been studying bovine embryos as an important part of developmental biology and reproductive engineering for many years. For artificial reproductive technologies like IVF, somatic cell nuclear transfer (SCNT), and gene editing to get more effectively, scientists must have complete knowledge of the early stages of bovine embryo development. The bovine zygote goes through a number of mitotic divisions after fertilization, including the 2-cell, 4-cell, 8-cell, and morula stages before it forms a blastocyst, which usually happens 7 or 8 days after fertilization (Farin et al., 2001). It is important for the morula stage to successfully compact and for the blastocyst stage to differentiate into the inner cell mass (ICM) and trophectoderm (TE). These are the events that affect viability and implantation potential after development (Thompson et al., 1995). As a bovine embryo changes from an oocyte to a blastocyst, its metabolism undergoes a lot of changes. Embryos get most of their energy from pyruvate and lactate in the early stages of cleavage. After compaction, glucose uptake becomes important (Gardner & Lane, 1993). The "quiet embryo" hypothesis says that embryos with lower metabolic activity, which means they use nutrients more slowly, are better able to develop (Leese, 2002). These insights into embryonic metabolism have shaped *in vitro* culture media formulations to better mimic the physiological conditions of the female reproductive tract, aiming to support optimal development while avoiding metabolic stress. *In vitro* culture systems for bovine embryos have evolved significantly. In early methods, co-culture with somatic cells was used to provide support and lower oxidative stress (Eyestone & First, 1989). But these kinds of methods led to variation and limited standardization. Creating defined media like SOF and CR1aa and adding amino acids and antioxidants to them made the conditions more controllable and increased the number and quality of blastocysts (Takahashi & First, 1992; Rosenkrans et al., 1993). Reducing the oxygen tension in culture to normal levels (5% O_2) instead of atmospheric levels (20% O_2) has also been shown to improve embryo development by lowering oxidative damage (Goto et al., 1993). Evaluating embryo quality remains a challenge. Traditionally, morphological assessment has been the main method for grading embryos, but this approach is subjective and limited in predictive power (Lindner & Wright, 1983). As genetic markers have improved, they have become more objective ways to measure quality. It is now known that the amounts of expression of genes related to pluripotency (*OCT4*, *SOX2*), trophectoderm specification (*CDX2*), and metabolism (*GLUT1*) are good ways to tell if an embryo is still alive (Kues et al., 2008; Berg et al., 2011). Epigenetic profiling, which includes DNA methylation patterns of imprinted genes, is also being used more and more to evaluate the development of embryos, especially those made in laboratories (Reik et al., 2001). Cryopreservation of bovine embryos has become a critical component of commercial embryo transfer programs. Conventional slow freezing was initially the method of choice but often resulted in suboptimal post-thaw survival rates, especially for *in vitro*-produced embryos that typically have higher cytoplasmic lipid content (Massip et al., 1995). The use of vitrification methods, which involve very fast cooling and high levels of cryoprotectants, has made it much more likely for bovine embryos to survive and implant after being warmed up (Vajta & Kuwayama, 2006). Still, getting the best cryopreservation methods is important, especially for embryos that come from specific, serum-free culture systems and may have different membrane and cytoplasmic features. Molecular methods have helped us learn a lot more about how cow embryos grow and develop. Different gene expression patterns have been found between embryos that were grown *in vivo* and those that were grown *in vitro*. This shows how culture factors can change how embryos develop (Wrenzycki et al., 2001). Proteomic and metabolomic profiling add to these results by giving a more complete, holistic view of embryo physiology and creating new signals for choosing high-quality embryos (Gutierrez-Adan et al., 2004). Also, recent improvements in genome editing tools like CRISPR/Cas9 have made it easier to change bovine embryos to add features that are wanted or to study how genes work during early development (Tan et al., 2016). Finally, the study of bovine embryos includes many different fields, ranging from traditional embryology to cutting-edge molecular biology. To make *in vitro* culture systems better, cryopreservation methods better, and eventually assisted reproductive technologies more successful, we need to know how embryos grow, what their metabolic needs are, and their molecular signatures. Suppose omics technologies are kept together with conventional methods of evaluating embryos. In that case, it might be possible to make bovine embryo production higher-quality, reliable, and efficient in both scientific and commercial settings.

2.1.5 Embryo quality

Embryo quality is also assessed using a numerical grading system, which is determined by evaluating the morphological integrity of the embryos. The quality grades range from 1 to 4, with each number reflecting a specific level of structural quality (Bo & Mapletoft, 2013).

Grade 1 – Excellent or Good: Embryos in this category display a well-rounded, symmetrical structure with blastomeres that are consistent in size, color, and texture. Their appearance aligns with the expected developmental stage, and any imperfections should be minimal. At least 85% of the cellular content must be intact and viable, based on the amount of cell material displaced into the perivitelline space. The zona pellucida should be smooth, without flat or indented areas that could lead to adhesion in culture dishes or transfer straws. These embryos are highly tolerant of cryopreservation and are often referred to as "freezable" or suitable for international shipment.

Grade 2 – Fair: These embryos exhibit some moderate deviations in shape or variability among blastomeres in terms of size, color, or density. At least half of the embryonic mass must remain viable. Although these embryos have lower survival rates after freezing and thawing compared to Grade 1, they are still considered suitable for fresh embryo transfer, which can yield acceptable pregnancy outcomes. They are generally labeled as "transferable" but not ideal for freezing.

Grade 3 – Poor: Embryos classified in this group show significant abnormalities in shape or inconsistencies among individual cells. A minimum of 25% of the embryonic structure must still be intact. Their ability to survive cryopreservation is minimal, and when used for fresh transfer, their potential to result in pregnancy is notably reduced compared to embryos of higher quality.

Grade 4 – Non-viable or Degenerating: This category includes embryos that are dead or showing signs of degeneration, which may also encompass unfertilized oocytes or single-cell embryos. These are considered non-viable and are not recommended for use, they should be discarded.

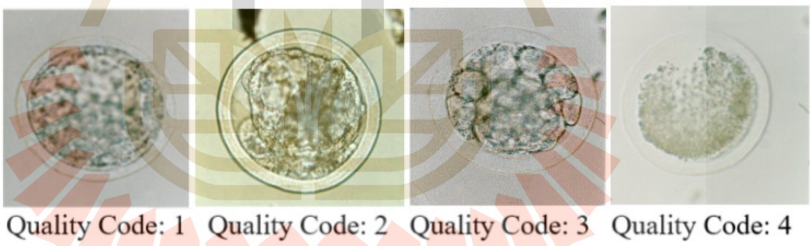

Figure 1. Numerical grading system used to assess embryo morphology, with quality classifications from 1 to 4 based on structural characteristics (adapted from Bo & Mapletoft, 2013).

2.2 Cryopreservation

In the control of bovine reproduction, cryopreservation has emerged as a crucial technology that allows genetic material to be stored and transported over extended periods of time and space. When mammalian spermatozoa were frozen using cryoprotective chemicals like glycerol in the middle of the 19^{th} century, the idea of cryopreserving living cells was first proved (Polge et al., 1949). Based on these groundbreaking findings, scientists started using cryopreservation methods on cow embryos, and in 1973, the first viable calf was born from a frozen-thawed embryo (Wilmut & Rowson, 1973). Since then, cryopreservation has transformed cow breeding and genetic improvement techniques by becoming a standard component of bovine embryo transfer (ET) programs across the globe.

Slow freezing and vitrification have historically been the two main techniques used in the cryopreservation of bovine embryos. Developed in the 1970s, the slow freezing approach includes cooling embryos gradually while including cryoprotectants such as ethylene glycol or glycerol to prevent ice formation (Leibo & Mazur, 1971). Embryos are placed in straws, chilled gradually, and then submerged in liquid nitrogen at -196°C during the slow freezing process. By allowing for some embryonic dehydration, slow freezing reduces the production of intracellular ice, which could otherwise harm cellular structures. To control cooling rates, precise equipment is needed, and the process is still time-consuming.

A more recent development, vitrification, uses extremely high concentrations of cryoprotectants and extremely fast cooling rates to completely avoid ice crystal formation (Vajta & Kuwayama, 2006). The embryos are submerged straight into liquid nitrogen, which causes the solution to solidify into a glass-like state without generating harmful ice crystals, as opposed to freezing gradually. For bovine embryos created *in vitro*, which are more prone to cryoinjury due to their higher lipid content than *in vivo*-derived embryos, vitrification has shown promise (Massip et al., 1995). Methods like Cryotop vitrification and open pulled straw (OPS) vitrification have significantly improved pregnancy outcomes and post-thaw survival rates.

Bovine embryo physiology presents cryopreservation difficulties. Compared to their *in vivo* counterparts, embryos created *in vitro* typically have a higher concentration of cytoplasmic lipid droplets, which makes them more susceptible to freezing and thawing stress (Seidel, 2006). Higher rates of intracellular ice production and freezing-induced membrane damage are linked to lipid accumulation. Using dilapidation techniques, altering culture conditions to lower lipid content, or adding metabolic regulators like L-carnitine to culture media are some ways to increase cryosurvival (Sudano et al., 2011). Furthermore, the developmental stage of the embryo also affects cryotolerance; early blastocysts and compact morulae have a higher survival rate than extended blastocysts because of their smaller size and smaller blastocoelic cavity capacity (Dochi et al., 1998).

The selection and concentration of cryoprotectants are essential to the success of cryopreservation. Conventional cryoprotectants like glycerol and ethylene glycol work by entering cells and preventing the production of ice, but prolonged exposure can potentially have harmful consequences (Pedro et al., 2005). Because of its greater membrane permeability and decreased toxicity, ethylene glycol has become more popular than glycerol for direct transfer techniques. This allows embryos to be transferred into recipients without the cryoprotectant having to be diluted or removed after thawing. To help with osmotic dehydration during freezing and reduce the production of ice crystals, non-permeating substances such as sucrose are frequently added to freezing media (Leibo, 2004).

Assessing post-thaw embryo viability is crucial to determining how effective cryopreservation procedures are. Initial markers of embryo quality are provided by morphological evaluation as soon as the embryo is thawed, with an emphasis on membrane integrity, blastocoel re-expansion, and cellular fragmentation (Lindner & Wright, 1983). But to more accurately predict embryo competence after thawing, more sensitive methods such as detecting mitochondrial activity, membrane permeability assays, and apoptotic markers have been used. Pregnancy rates after embryo transfer are still the gold standard for determining the success of cryopreservation.

Additionally, the global movement of bovine genetics is made possible by cryopreservation, which makes it easier to export valuable embryos across continents without running the same biosecurity risks as live animal transportation. The World Organization for Animal Health (OIE) and the International Embryo Transfer Society (IETS) have established regulatory systems that guarantee exported frozen embryos adhere to strict quality and health requirements (Stringfellow & Givens, 2010). This has facilitated the quick spread of superior genetics, which has increased genetic variety and production in cow populations all over the world.

Cryopreservation of bovine embryos is, in summary, a fundamental component of contemporary genetic improvement and cattle reproductive initiatives. Post-thaw survival and pregnancy rates have continuously increased due to ongoing developments in cryoprotectant formulations, vitrification processes, embryo culture systems, and evaluation methodologies. There are still difficulties, nevertheless, especially in improving the cryotolerance of embryos created *in vitro* and expanding the efficacy of cryopreservation to include bovine oocytes. To further improve cryopreservation techniques and optimize the reproductive capacity of cryopreserved bovine germplasm, future initiatives will incorporate molecular insights, metabolic regulation, and innovative biotechnological treatments

2.2.1 Slow freezing

In bovine reproduction, slow freezing has long been the mainstay of cryopreservation, allowing for the long-term transfer and storage of valuable genetic material. This technique, which was created in the 1970s, made it possible to preserve bovine embryos at extremely low temperatures without sacrificing their ability to survive after thawing. A significant advancement in reproductive biotechnology was made when Wilmut and Rowson (1973) reported the first calf born from a cryopreserved cow embryo. Since then, slow freezing has been used extensively in commercial ET operations as well as research, especially when combined with *in vivo*-derived embryos. The slow freezing method reduces intracellular ice formation, which is believed to be the main source of cellular damage during freezing, by gradually cooling embryos in a cryoprotectant solution. Usually, embryos are loaded into plastic straws, suspended in a medium that contains permeating cryoprotectants like ethylene glycol or glycerol, and then put through a programmable freezing curve that gradually lowers the temperature from room temperature to -35°C before submerging them in liquid nitrogen (Leibo & Mazur, 1971). By allowing for controlled cell dehydration, this slow temperature drop lowers the possibility of ice crystals forming in the cytoplasm.

An essential part of the slow freezing process is cryoprotectants. The first cryoprotectant to be effectively employed in the cryopreservation of bovine embryos was glycerol (Willadsen et al., 1976). By lowering the freezing point of intracellular water and penetrating the cell membrane, it reduces the generation of ice. After thawing, glycerol must be diluted gradually since it causes osmotic stress during addition and removal. A substitute that is less toxic and has a higher membrane permeability is ethylene glycol, which makes field application easier by enabling direct transfer without dilution (Dochi et al., 1998). To improve osmotic dehydration during freezing, both cryoprotectants are frequently mixed with non-permeating substances like sucrose. The effectiveness of slow freezing depends on the source and stage of development of the embryo. Compared to expanded blastocysts or embryos created *in vitro*, *in vivo*-derived embryos, especially those at the morula and early blastocyst stages, have shown better post-thaw survival (Massip et al., 1995). Later-stage and *in vitro*-produced embryos are more vulnerable to cryodamage due to their larger blastocoel cavity and higher cytoplasmic lipid content. Selection of embryos before freezing is therefore essential. The morphological grading system created by Lindner and Wright (1983) is still commonly used to evaluate the quality of embryos before freezing; Grade 1 (excellent) embryos have the highest survival rates.

Lipid content has emerged as a significant barrier in the cryopreservation of *in vitro*-produced embryos. Because of the changed metabolic conditions *in vitro*, these embryos tend to collect more cytoplasmic lipids, which lower their cryotolerance (Seidel, 2006). Modifying culture media, dilapidation methods, and supplementing with metabolic regulators such as L-carnitine, which encourages fatty acid oxidation and decreases lipid buildup, have all been used to increase the success of slow freezing in these embryos (Sudano et al., 2011). Slow freezing has a few drawbacks despite its proven benefits. The procedure takes a long time, needs certain tools, and is subject to change depending on operator skill and protocol accuracy. Furthermore, the mechanical stress brought on by ice crystal formation and the toxicity of cryoprotectants still affect embryo viability. Slow freezing is still the preferred method for routine cryopreservation in many field settings because of its standardization and track record of success with *in vivo* embryos. To increase the post-warming survival of bovine embryos, ongoing attempts have been made to improve slow freezing procedures. To reduce intracellular ice formation, which is still a leading cause of cryoinjury, these strategies include maximizing cooling and seeding rates (Leibo &

Mazur, 1971). Osmotic stress and cytotoxicity during the freezing process have also been demonstrated to be reduced by shortening the exposure duration to cryoprotectants such as glycerol and ethylene glycol (Vajta & Kuwayama, 2006). Furthermore, incorporating post-thaw embryo cleaning and rehydration procedures enhances blastocyst viability and re-expansion (Massip et al., 1995). To reduce oxidative stress, some research has investigated adding antioxidants such as L-carnitine and cysteine to freezing or post-thaw culture conditions (Silva et al., 2015; Tamura et al., 2008). Other studies have investigated the function of mitochondrial activity and gene expression, including stress response (*HSPA1A*) and apoptotic markers (*BAX, BCL2*), as possible indicators of cryotolerance and post-thaw developmental competence (Sudano et al., 2011; Rizos et al., 2002).

Finally, ET programs still heavily utilize slow freezing, which was pivotal in the development of bovine embryo cryopreservation. The quality, origin, and developmental stage of the embryos, together with the cautious use of cryoprotectants and chilling procedures, all have a significant impact on their success. Even though newer techniques like vitrification present encouraging substitutes, slow freezing is still a dependable and practical procedure, especially for high-quality *in vivo*-derived embryos. Molecular and metabolic therapies to improve embryo cryotolerance and guarantee high post-thaw viability are probably going to be the focus of future developments in slow freezing.

2.2.2 Vitrification

In the field of bovine embryo cryopreservation, vitrification has become a game-changing method that provides a productive substitute for conventional slow freezing. Vitrification is a rapid-freezing method that turns cellular water into a glass-like solid without generating ice crystals, in contrast to slow freezing, which uses controlled dehydration and gradual chilling to avoid ice formation (Vajta & Kuwayama, 2006; Rall & Fahy, 1985). High concentrations of cryoprotectants and instantaneous immersion in liquid nitrogen are used to accomplish this ultrarapid chilling, which reduces cryoinjury and maintains cell structure (Vajta & Kuwayama, 2006). Interest in vitrification has increased because of slow freezing limitations, especially its decreased effectiveness with IVP bovine embryos. IVP embryos are particularly susceptible to ice crystal injury because of their high lipid content and altered membrane permeability (Massip et al., 1995; Seidel, 2006). In these embryos, vitrification has shown enhanced post-thaw survival and developmental competency by completely avoiding crystallization (Vajta et al., 1998). Various methods have been developed to increase handling and cooling rates during vitrification, including the open pulled straw (OPS) method, Cryoloop, and Cryotop (Vajta et al., 1998; Kuwayama, 2007). The composition of the cryoprotectant solution, which typically combines non-penetrating compounds like sucrose or trehalose with permeating agents like ethylene glycol and dimethyl sulfoxide (DMSO), is a crucial component of efficient vitrification. These substances prevent ice nucleation during cooling by reducing the freezing point and increasing the viscosity of intracellular and extracellular fluids (Rall & Fahy, 1985). For this method to swiftly move across the critical temperature range of roughly 15°C to -5°C, an extremely rapid cooling rate typically surpassing 10,000°C per minute is needed.

Temperature and exposure duration are crucial factors since large doses of cryoprotectants can be harmful. Stepwise loading of cryoprotectants and shorter exposure intervals are two examples of methods that researchers have modified to reduce toxicity while preserving vitrification efficiency (Dinnyes et al., 2000). There are still issues, even if vitrification has definite benefits in terms of embryo survival and pregnancy rates. Open systems like Cryotop and OPS expose embryos to liquid nitrogen directly, which raises questions about biosafety, particularly when transporting embryos internationally. As a result, closed vitrification systems have been developed, which seek to maintain high cooling rates while lowering the possibility of contamination, albeit at the possible expense of some efficiency (Kuwayama et al., 2005). The goal of recent developments in vitrification has been to enhance the quality of embryos after warming. Antioxidants like melatonin, cysteine, and L-carnitine have been demonstrated to lower oxidative stress and enhance mitochondrial activity and developmental competence when added to post-thaw culture medium (Tamura et al., 2013). Furthermore, apoptosis (e.g., *BAX, BCL2*), oxidative stress (e.g., *SOD1*, *GPX*), and stress response (e.g., *HSP70*) genes have been identified by molecular investigations as possible biomarkers for evaluating vitrification success (Sudano et al., 2011).

In the context of oocyte preservation, vitrification has also grown in significance, especially for commercial IVP programs and genetic conservation. However, the sensitivity of the meiotic spindle and the high lipid content make oocyte vitrification more difficult. However, improvements in oocyte vitrification have also been attributed to developments in spindle imaging, cytoskeletal stabilization, and antioxidant inclusion (Arav et al., 1996). In conclusion, vitrification significantly advances bovine embryo cryopreservation, particularly for IVP embryos that exhibit low cryotolerance. Pregnancy outcomes and embryo viability have been greatly improved by the introduction of ultrarapid cooling methods, better cryoprotectant formulations, and encouraging post-warming treatments. Vitrification is still a very flexible and successful method in both commercial cattle breeding and reproductive biotechnology research, despite persistent worries about biosafety and cytotoxicity.

2.3 Oxidative stress

Oxidative stress is defined as a physiological imbalance between the production of ROS and the capacity of antioxidant defense systems to neutralize them, leading to potential cellular damage (Agarwal et al., 2005). This redox imbalance is a major factor in determining the quality and developmental potential of both oocytes and embryos during bovine reproduction. Normal reproductive activities, including oocyte maturation, folliculogenesis, ovulation, and early embryo development, depend on ROS, which are normally created in trace amounts during cellular metabolism (Gupta et al., 2010; Dumollard et al., 2007). However, when produced in excess, ROS can damage gametes and embryos' lipids, proteins, and DNA, therefore compromising reproductive competence (Guérin et al., 2001). With the development of ARTs, such as IVM, IVF, and IVC, the importance of oxidative stress in bovine reproduction has become even more apparent. Because of increased oxygen tension, light exposure, and a lack of maternal antioxidant factors, *in vitro* conditions frequently lack the dynamic antioxidant environment of the reproductive canal, leading to elevated ROS levels (Rizos et al., 2002; Lonergan & Fair, 2008). Reduced cleavage rates, poor blastocyst formation, and increased apoptosis in developing embryos have all been related to these increased ROS levels (Goud et al., 2008; Tamura et al., 2008).

Furthermore, mitochondria are particularly important in oocyte physiology since they are the main source of ROS during oxidative phosphorylation. Oxidative stress-induced mitochondrial dysfunction decreases meiotic competence and developmental potential by decreasing ATP synthesis and increasing the generation of ROS (Van Blerkom, 2004). Because of their high lipid content, which makes membrane structures more susceptible to lipid peroxidation, bovine oocytes and embryos are much more sensitive to oxidative damage (Sudano et al., 2011). Bovine reproductive cells use both enzymatic antioxidants (such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) and non-enzymatic antioxidants (such as reduced glutathione (GSH), vitamins C and E, and melatonin) to counteract the harmful effects of oxidative stress (Takahashi, 2012; Ali et al., 2003). The natural antioxidant defense, however, can be insufficient in *in vitro* conditions, requiring supplementation with culture media improvements (Nabenishi et al., 2012).

2.3.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are naturally occurring by products of cellular metabolism, specifically mitochondrial oxidative phosphorylation. These include hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (OH•) (Agarwal et al., 2005). Under normal circumstances, ROS function as signaling molecules in ovulation, embryo implantation, and folliculogenesis (Guerin et al., 2001). However, oxidative stress occurs when ROS production is excessive or antioxidant defenses are inadequate, which impairs gamete activity and embryo development (Ruder et al., 2009). When energy is produced in bovine oocytes, ROS is mostly produced by mitochondria. Increased ROS levels due to mitochondrial dysfunction can harm mitochondrial DNA (mtDNA), cause lipid peroxidation of oolemma membranes, and interfere with spindle formation, all of which can affect meiotic competence and developmental potential (Van Blerkom, 2004; Nabenishi et al., 2012). Environmental factors, including light exposure, temperature or pH changes, and increased oxygen tension (20% vs. 5% in vivo) can greatly increase ROS generation in oocytes and embryos during IVP (Rizos et al., 2002; Lonergan & Fair, 2008). According to Goud et al. (2008), the absence of maternal antioxidants in the oviductal and uterine environments in vivo increases this oxidative burden. According to Takahashi (2012) and Sudano et al. (2011), high ROS levels in embryos can cause cytochrome c release, caspase activation, and DNA fragmentation, which can set off apoptotic pathways and lower blastocyst formation rates and developmental competence. The peroxidation of membrane phospholipids by ROS also impairs ionic transport and membrane fluidity, both of which are necessary for healthy embryonic physiology (Gupta et al., 2010). Bovine reproductive cells use both enzymatic (SOD, CAT, and GPx, GSH, vitamins C and E, melatonin) antioxidant mechanisms to reduce ROS-induced damage (Abedelahi et al., 2010; Ali et al., 2003). However, it is frequently necessary to supplement culture medium due to the absence of adequate antioxidant support under *in vitro* settings (Silva et al., 2023). According to recent research, increased ROS levels also affect the expression of genes that can be used as molecular indicators of oxidative damage in oocytes and embryos, including stress-response genes like *HSP70*, pro-apoptotic genes like *BAX*, and antioxidant genes like *SOD1* and *GPX1* (Rizos et al., 2002; Sudano et al., 2011).

2.3.2 Antioxidant

Oocytes include a number of defensive mechanisms against oxidative stress under normal physiological settings, including both non-enzymatic and enzymatic antioxidants (such as peroxidase, catalase, and superoxide dismutase) (Combelles et al., 2009). Through the regulation of ROS levels, these systems aid in the maintenance of redox equilibrium. A variety of internal and external factors, however, have the potential to upset this equilibrium, leading to elevated ROS production and changed intracellular antioxidant capacity (Li et al., 2016; Somfai et al., 2007; Succu et al., 2018). Numerous antioxidant compounds, such as enzymatic agents, thiol-based molecules like β-mercaptoethanol and cysteamine, vitamins like α-tocopherol and vitamin C (ascorbic acid), flavonoids like resveratrol, and other bioactive molecules like L-proline and melatonin, are used to combat oxidative damage. Notably, substances including resveratrol, melatonin, L-proline, and α-tocopherol have shown protective benefits in vitrified oocytes, reducing cryoinjury and preserving developmental potential (Chinen et al., 2020; Sovernigo et al., 2017).

2.3.3 Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a naturally occurring polyphenolic compound classified as a stilbenoid, consisting of two aromatic rings linked by a double bond (Salehi et al., 2018). Japanese researcher Michio Takaoka isolated it for the first time in 1939 from the roots of the medicinal plant Veratrum grandiflorum (Pezzuto, 2018). According to Baur and Sinclair (2006), resveratrol is a phytoalexin, which means that plants produce it in reaction to environmental stressors, including pathogen invasion, UV radiation, or mechanical damage. According to Burns et al. (2002), dietary sources of resveratrol include peanuts, red wine, grape skins, and a variety of berries, including mulberries and blueberries. The trans isomeric form is more physiologically active than the cis isomeric form. Nevertheless, trans-resveratrol can change into the less potent cis form when exposed to UV light (Almeida et al., 2009). Due to its suggested link to the "French Paradox," which is the finding that French populations have low rates of coronary heart disease despite eating a diet high in saturated fats, a phenomenon partly ascribed to red wine consumption, the compound attracted international attention in the early 1990s (Renaud & de Lorgeril, 1992). Since then, numerous studies have shown that resveratrol has a variety of biological properties, such as anti-inflammatory, cardioprotective, neuroprotective, antioxidant, and anti-cancer benefits (Baur & Sinclair, 2006; Salehi et al., 2018). It is believed that these health advantages are mediated by processes such as sirtuin 1 (SIRT1) activation, mitochondrial function regulation, and oxidative stress signaling pathway modulation (Baur & Sinclair, 2006; Das & Das, 2007). However, because of its rapid metabolism and excretion, resveratrol has a limited oral bioavailability, which is a significant barrier to its therapeutic usage. As a result, new formulations and analogs have been created to enhance their pharmacokinetic characteristics (Walle, 2011).

About bovine reproductive technology, resveratrol, a naturally occurring polyphenolic molecule mostly found in grapes and red wine, has attracted considerable attention due to its antioxidant properties. It is a viable option for improving the results of IVP due to its capacity to reduce oxidative stress (Wang et al., 2014). Resveratrol supplementation has been demonstrated to enhance developmental competence in bovine oocyte maturation. According to Wang et al.

(2014), 1.0 μM resveratrol during IVM improved oocyte cytoplasmic maturation, activated the Mos/MEK/p42 MAPK signaling pathway, and boosted cumulus expansion, all of which led to a greater cell count and blastocyst formation. Because oxidative stress damages oocyte quality and embryonic development, resveratrol also lowers intracellular ROS and raise GSH levels in oocytes (Wang et al., 2014). According to Gaviria et al. (2019), adding resveratrol to vitrified embryos during post-warming culture increased both the number of cells overall and the rates of re-expansion and hatching. These advantages are dose-dependent, though; larger resveratrol concentrations during IVM may have detrimental effects on embryonic development, according to Sovernigo et al. (2017), underscoring the necessity for cautious optimization. According to Wang et al. (2014), the protective benefits of resveratrol are partly mediated by the activation of SIRT1, a NAD^{+}-dependent deacetylase linked to enhanced mitochondrial function and cellular stress responses. Its anti-apoptotic properties also supplement its antioxidant and mitochondrial advantages. For example, Silva et al. (2021) showed that resveratrol improved survival and developmental potential by lowering apoptotic cells in fresh and vitrified embryos.

2.3.4 Mechanisms of Resveratrol on SIRT1

One of the central mechanisms of resveratrol action is the activation of sirtuin 1 (SIRT1), a NAD^{+}-dependent deacetylase that regulates mitochondrial biogenesis and cellular energy metabolism. By activating SIRT1, resveratrol enhances the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), promoting mitochondrial function, a key factor for cytoplasmic maturation in bovine oocytes and energy production during early embryo development (Takeo et al., 2014).

Resveratrol supplementation during IVM has been shown to upregulate SIRT1 expression in bovine oocytes, leading to improved mitochondrial function, increased ATP production, and enhanced oocyte quality (Takeo et al., 2014). This upregulation of SIRT1 also contributes to the reduction of ROS levels and the increase of GSH concentrations, thereby mitigating oxidative stress and improving embryonic development (Wang et al., 2014). Furthermore, the activation of SIRT1 by resveratrol influences apoptotic pathways by modulating the expression of apoptosis-related genes, decreasing pro-apoptotic markers such as BAX and increasing anti-apoptotic markers like BCL2L1 in bovine embryos, leading to reduced apoptosis and improved embryo quality (Gaviria et al., 2019). In addition, resveratrol has been found to enhance the cryotolerance of bovine embryos by reducing ROS levels, restoring GSH content, and improving re-expansion and hatching rates after warming, effects that are associated with SIRT1 activation (Gaviria et al., 2019).

The mitogen-activated protein kinase (MAPK) signaling pathway, which is involved in oocyte maturation and embryo development, is also modulated by resveratrol through SIRT1 activation. Resveratrol has been reported to activate the Mos/MEK/p42 MAPK cascade in bovine oocytes, leading to enhanced cumulus expansion, polar body extrusion, and improved blastocyst formation rates (Wang et al., 2014). *In vivo* studies have demonstrated that direct injection of resveratrol into the uterus of cows during artificial insemination increases plasma SIRT1 levels and improves conception rates, indicating the potential of resveratrol as a therapeutic agent in bovine reproduction (Kim et al., 2020).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

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

3.2 Ethic statement

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

3.3 Experimental design

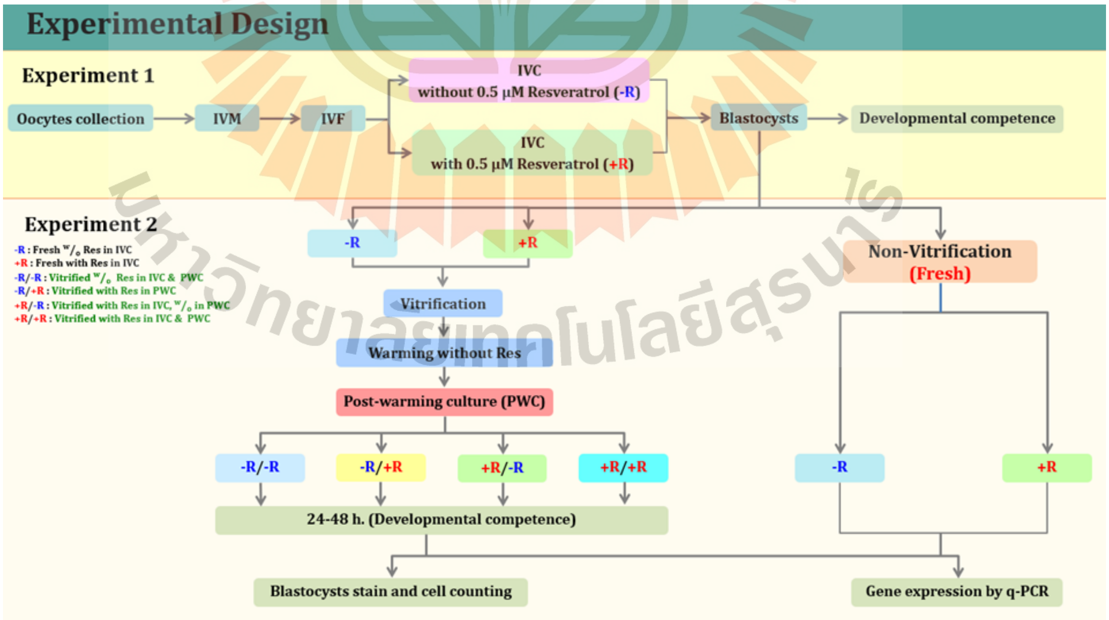

Figure 2. Diagrammatic representation of the experimental design.

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

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

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

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

3.3.1.2 *In vitro* fertilization (IVF)

Frozen fertile semen of Wagyu bull (Pornchai Intertrade Ltd., Ratchaburi, Thailand) in 0.25 ml straws was thawed in the air for 10 seconds, followed by immersion in a water bath at 37.5°C for 1 min. The semen was then placed at the bottom of a 5 ml snap tube (Corning, Glendale, AZ, USA) containing 2 ml of TALP medium (Lu et al., 1987). The snap tube was incubated under a humidified atmosphere of 5% CO_2 in air at 38°C for 30 min. After incubation, the top 1.8 ml layer of the medium was gently collected and transferred to a 15 ml conical tube (SPL Life Sciences) containing 5 ml of TALP medium. The sperm suspension was centrifuged at 400 g for 5 min, and the supernatant was removed. The sperm pellet was diluted and adjusted to a concentration of 2×10^6 sperm/ml using a TALP medium. Then, 50 µl droplets of the sperm suspension were transferred to a 35 mm culture dish covered with mineral oil. At 23 h of IVM, COCs were washed three times with TALP medium and placed in droplets of sperm suspension (10 COCs/drop) and incubated under a humidified atmosphere of 5% CO_2 in the air at 38°C for 10 h.

3.3.1.3 *In vitro* embryo culture (IVC)

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

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

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

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

3.3.2.1 Vitrification and warming of blastocysts

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

3.3.2.2 Evaluation of TE and ICM cell numbers

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

3.3.2.3 Quantitative real-time PCR (q-PCR)

Twenty blastocysts from each group were washed three times with PBS (-) and stored at -80°C until further use. The manufacturer extracted Total mRNA using the FavorPrep™ Tissue Total RNA Mini Kit (Favorgen Biotech Crop., Pingtung, Taiwan). cDNA synthesis was performed using biotechrabbit™ cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany), and the expression of specific genes was assessed using the KAPA SYBR FAST qPCR Master Mix (Applied Biosystems) on the CFX Opus 96 real-time PCR system (Biorad, Hercules, California, USA). Melting curve analysis was performed for all primers, which were optimized. The primer sequences are provided in Table 1. GAPDH was used as a housekeeping gene to normalize the expression of target genes. qPCR was performed in triplicate, and statistical analysis was conducted using the $2^{-\Delta\Delta Ct}$ method.

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

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

3.4 Statistical analysis

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

CHAPTER IV

RESULTS

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

Embryo development was evaluated based on cleavage and blastocyst rates in embryos cultured with 0.5 μM resveratrol (Table 4.1). Cleavage rate was significantly higher in the resveratrol group (81.70% ± 3.20) vs control (75.13% ± 4.04), ($P < 0.05$). Similarly, the blastocyst development rate in the resveratrol-treated group (37.75% ± 2.31) was also significantly higher ($P < 0.05$) than that in the control group (29.82% ± 1.95). The present results show that supplementation with 0.5 μM resveratrol during IVC improved cleavage and blastocyst rates, with enhanced early embryo development achieved.

Table 2. Effects of resveratrol during *in vitro* culture (IVC) of bovine embryos on cleavage rates and blastocyst formation rates.

Groups	No. Oocytes	No. (%) Cleavage (Mean ± SEM)	No. (%) Blastocyst (Mean ± SEM)
Control	546	404 (75.13 % ± 4.04)[a]	153 (29.82 % ± 1.95)[a]
0.5 μM Resveratrol	546	441 (81.70 % ± 3.20)[b]	196 (37.75 % ± 2.31)[b]

10 replicate; %: Mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA)

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

4.2.1 Effects of supplemented resveratrol in IVC and post-warming culture (PWC) media on the developmental potential of vitrified blastocysts

The effect of resveratrol addition in post-warming culture medium on embryonic developmental competency following post-warming is shown in Table 3. The post-warming culture medium supplementation of 0.5 µM resveratrol could not significantly improve the survival rate of blastocysts or developmental competency. The survival rates at 24 h post-warming were not significantly different among all groups, ranging from 88.96 ± 3.55% to 93.85 ± 2.88% (P > 0.05). Likewise, there were no significant differences in the re-expanded blastocyst percentage between treatments, ranging from 51.01 ± 7.82% to 64.02 ± 5.97% (P > 0.05). At 48 h following post-warming culture, all groups had similar survival rates (81.80 ± 4.56% to 90.67 ± 2.87%, P > 0.05). In contrast to the –R/+R group (45.03 ± 5.48%, P < 0.05), the group that was cultured with resveratrol during IVC but not in post-warming culture (+R/–R) exhibited the highest hatching and hatched rate at 71.50 ± 5.35%. There was no appreciable variance in the fully expanded blastocyst rates, which ranged from 19.18 ± 3.41% to 36.77 ± 5.42% (P > 0.05). These findings suggest that whereas resveratrol supplementation had no discernible effect on developmental competency or overall survival, the use of resveratrol during IVC combined with its absence in the post-warming culture medium may enhance blastocyst hatching and hatched competency.

Table 3. Effect of resveratrol supplementation in post-warming culture on developmental competency of embryos after post-warming.

Resveratrol in IVC	Resveratrol in post-warming culture medium	No. Blastocyst	Post-warming culture					
			24 h.			48 h.		
			No. (%) Survival	No. (%) Re-expanded blastocysts	No. (%) Hatching and hatched blastocysts	No. (%) Survival	No. (%) Fully expanded blastocysts	No. (%) Hatching and hatched blastocysts
-	-	91	82	57	25	77	24	53
			(90.16 ± 3.72)	(64.02 ± 5.97)	(26.14 ± 5.39)	(83.22 ± 4.71)	(27.22 ± 4.13)	(56.00 ± 4.82)[ab]
-	+	91	81	58	22	74	34	40
			(88.96 ± 3.55)	(63.73 ± 4.22)	(22.73 ± 4.48)	(81.80 ± 4.56)	(36.77 ± 5.42)	(45.03 ± 5.48)[b]
+	-	95	89	54	34	86	19	67
			(93.85 ± 2.88)	(54.81 ± 6.64)	(36.58 ± 8.02)	(90.67 ± 2.87)	(19.18 ± 3.41)	(71.50 ± 5.35)[a]
+	+	95	87	51	35	81	22	59
			(90.83 ± 3.54)	(51.01 ± 7.82)	(37.32 ± 6.50)	(84.06 ± 5.05)	(22.67 ± 5.98)	(61.39 ± 6.45)[ab]

10 replicates; %: Mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA)

4.2.2 Effect of resveratrol supplemented in IVC and post-warming culture media on ICM, TE, and total cell numbers of fresh and vitrified bovine embryo

Figure 3 presents the cell counts of both fresh and vitrified blastocysts following post-warming culture. The ICM, TE, and total cell counts were not significantly different between the vitrified and fresh control groups, regardless of resveratrol treatment. Fresh blastocysts with and without resveratrol had higher ICM, TE, and total cell counts than vitrified groups, but these differences were not statistically significant. These results imply that resveratrol supplementation does not affect the development of cells in both fresh and cultured bovine embryos.

Table 4. Effect of resveratrol supplemented in IVC and post-warming culture on ICM, TE, and total cell numbers of fresh and vitrified bovine embryos

Groups	No. blastocyst	No. ICM Mean ± SEM	No. TE Mean ± SEM	Total cell numbers Mean ± SEM
-R	10	54.50 ± 3.24	155.7 ± 5.34	210.2 ± 7.242
+R	10	53.70 ± 1.606	156.7 ± 4.95	210.4 ± 5.82
-R/-R	10	51.50 ± 4.11	144.7 ± 9.66	196.2 ± 12.65
-R/+R	10	50.90 ± 1.87	152.2 ± 8.30	203.1 ± 9.01
+R/-R	10	54.00 ± 0.83	150.9 ± 13.07	204.9 ± 13.39
+R/+R	10	53.80 ±1 .988	150.1 ± 13.50	203.9 ± 13.72

A total of 10 embryos per group were analyzed. Data are presented as mean ± SEM. No significant difference at P > 0.05 (ANOVA).

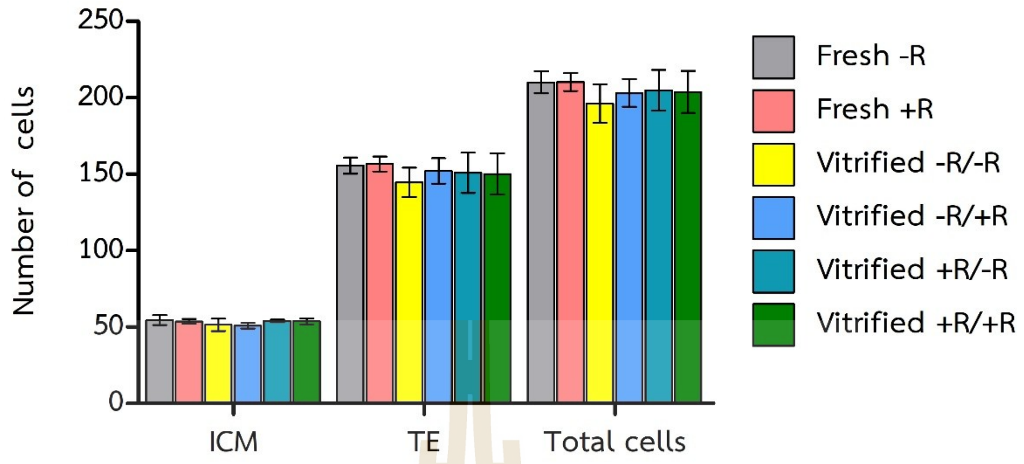

Figure 3. Analysis of the TE, ICM, and total cell numbers of fresh and vitrified embryos cultured in IVC with or without 0.5 μM resveratrol. A total of 10 embryos per group were analyzed. Data are presented as mean ± SEM. No significant difference at P > 0.05 (ANOVA).

4.2.3 Effect of resveratrol supplementation on fresh and vitrified bovine blastocyst gene expression in IVC and/or post-warming culture media

Gene expression related to stress response and antioxidant defense

HSP70, a heat shock protein involved in the cellular stress response, displayed a unique expression pattern with the largest increase in the -R/-R group (P < 0.05), whereas the +R groups displayed similar low expressions. *HSP70* expression differed between the control and the -R/-R group (P < 0.05) in the other vitrification groups (-R/+R, +R/-R, and +R/+R). All resveratrol-treated groups showed a significant increase in the antioxidant enzyme *SOD1* (P < 0.05) when compared to the control. The +R and -R/-R groups had the highest *SOD1* expression (Figure 4), whereas the vitrification groups, including resveratrol, maintained high expression throughout certain treatment stages. *CAT* expression was much higher in the +R group than in any other group (P < 0.05). The vitrification treatments resulted in significantly decreased *CAT* expression (P < 0.05) than both the -R and +R groups. *GPX4* showed a distinct expression pattern with significant overexpression in the -R/-R, +R/-R, and +R/+R groups compared to the -R and +R groups (P < 0.05). Unless resveratrol is added only post-warming culture (-R/+R), this suggests that vitrification may result in overexpression of *GPX4* (Figure 5).

Gene expression related to stress-resistance and mitochondrial function

SIRT1 exhibited significantly higher expression in the +R group compared to the control ($P < 0.05$). Conversely, all vitrified groups had markedly reduced *SIRT1* levels, with no statistically significant variations among them ($P > 0.05$). Similarly, *TFAM* expressions significantly increased in the +R group compared to the other groups ($P < 0.05$). The +R/+R group had the lowest *TFAM* expression ($P < 0.05$) following vitrification treatments (Figure 6).

Gene expression related to apoptosis

The fresh control group without resveratrol supplementation had the highest relative expression of the pro-apoptotic gene *BAX* ($p < 0.05$). When compared to the -R group, resveratrol supplementation during IVC significantly decreased *BAX* expression ($p < 0.05$). The *BAX* expression levels in vitrified embryos were lowest in the -R/+R group and significantly lower than in the -R/-R and +R/-R groups ($p < 0.05$). On the other hand, the +R group had the highest expression of the anti-apoptotic gene *BCL2*, which was significantly higher than that of any other group ($p < 0.05$). Regardless of when resveratrol was administered, all vitrification groups showed a significant decrease in *BCL2* expression ($p < 0.05$), and there were no significant differences among the vitrified groups ($P > 0.05$) (Figure 7).

Gene expression related to cell-programming, pluripotency and recognition of pregnancy

The +R group had significantly higher *DNMT1* expression than any other group ($p < 0.05$). The -R/-R and +R/-R groups showed intermediate levels of expression, which were significantly higher than both the -R/+R and +R/+R groups ($p < 0.05$) but significantly lower than the +R group. The -R group had the lowest *DNMT1* expression ($p < 0.05$). The +R group once again showed the highest expression of *DNMT3A* ($p < 0.05$), whereas all vitrified groups (-R/-R, -R/+R, +R/-R, and +R/+R) showed significantly lower expression levels that did not differ significantly from one another ($p > 0.05$). The expression level of the -R group was intermediate; it was lower than that of the

+R group but significantly higher than all vitrified groups ($p < 0.05$) (Figure 8). The +R group showed significantly higher levels of *OCT4/POU5F1* expression than all other groups, according to the analysis ($p < 0.05$). Although the expression of the -R group was significantly lower than that of the +R group, it was higher than that of other vitrified groups ($p < 0.05$). The -R/+R group had the lowest expression ($p < 0.05$) among vitrified embryos, while the -R/-R, +R/-R, and +R/+R groups all had comparably low levels with no significant differences ($p > 0.05$). The +R group had the highest expression of *IFN-tau*, higher than all other groups by a significant amount ($p < 0.05$). While the -R/+R and +R/-R groups had moderate expression levels that were not significantly different from the other groups ($p > 0.05$), they were significantly lower than the +R group ($p < 0.05$). The -R/-R group also showed higher expression than the -R group ($p < 0.05$). Comparable to the -R control, the +R/+R group had the lowest expression of all vitrified groups ($p > 0.05$) (Figure 9).

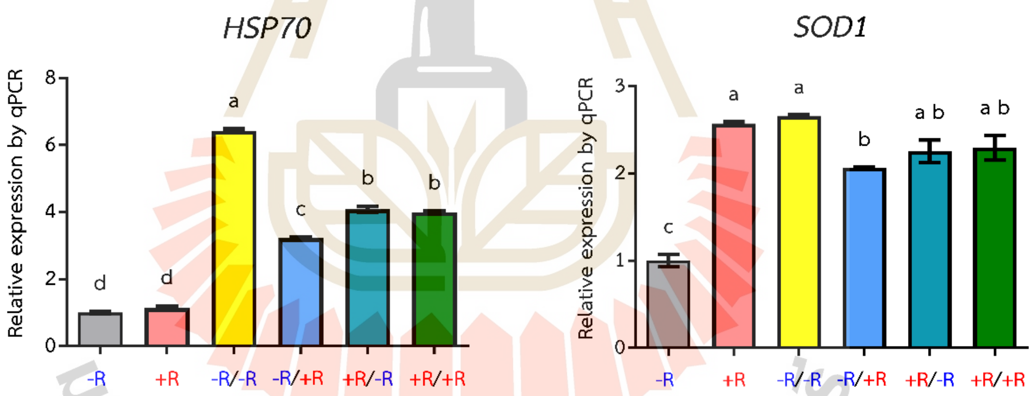

Figure 4 Expression levels of *HSP70* and *SOD1* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA).

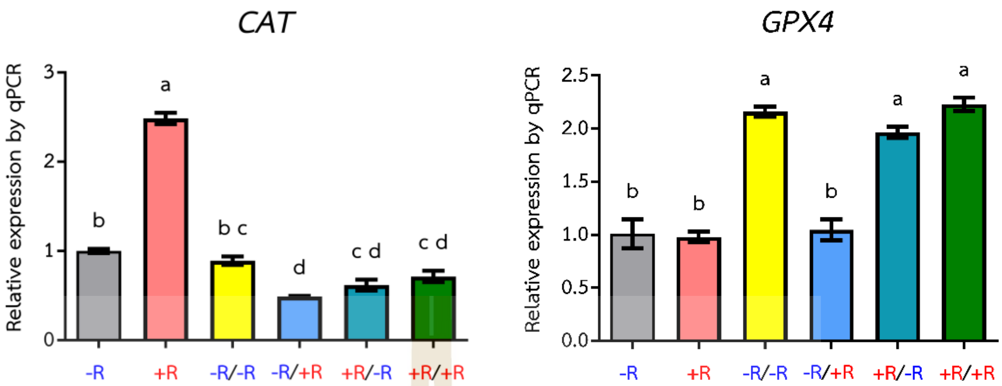

Figure 5 Expression levels of *CAT* and *GPX4* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA).

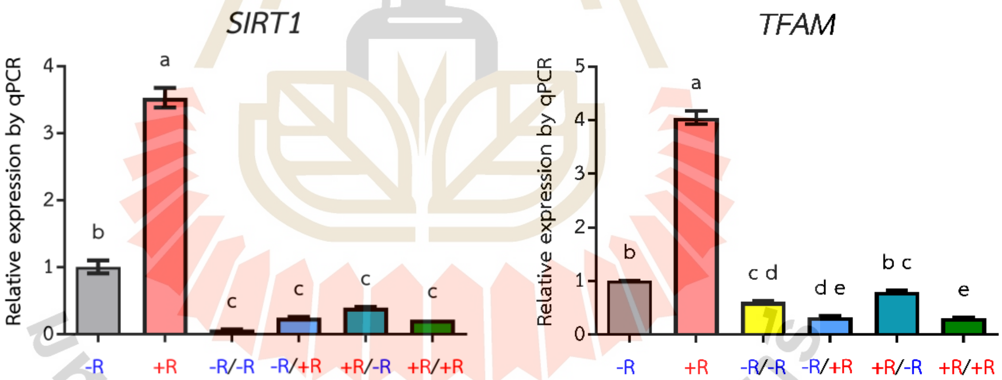

Figure 6 Expression levels of *SIRT1* and *TFAM* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA).

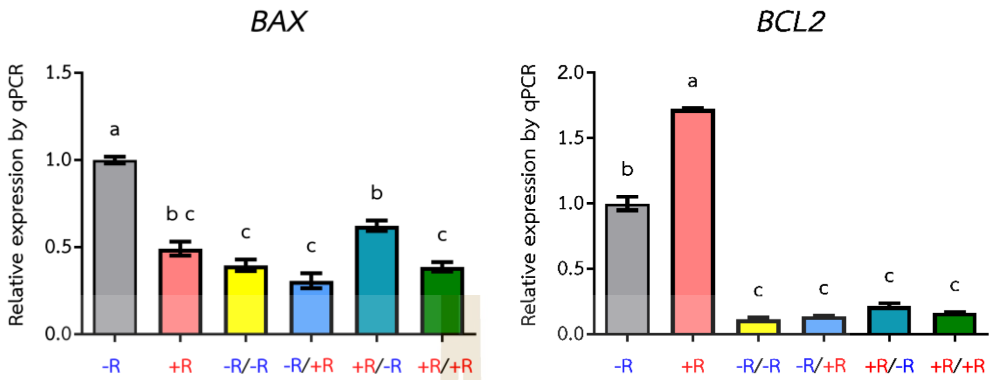

Figure 7 Expression levels of *BAX* and *BCL2* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at P < 0.05 (ANOVA test).

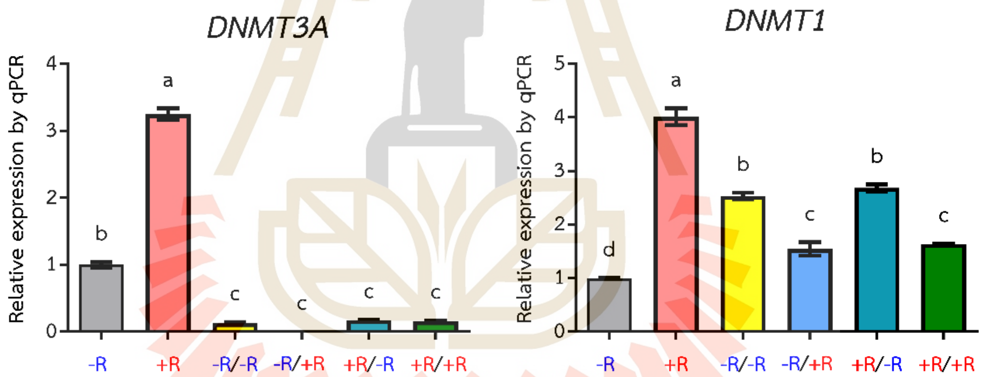

Figure 8 Expression levels of *DNMT1* and *DNMT3A genes* in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at P < 0.05 (ANOVA).

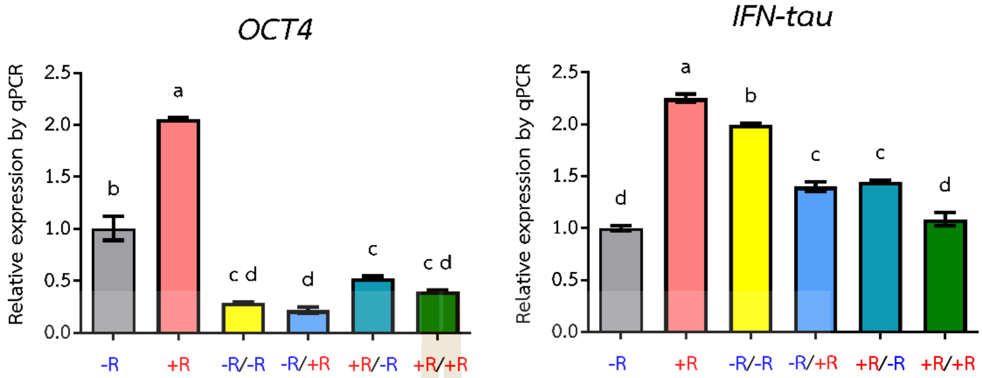

Figure 9 Expression levels of *OCT4* and *IFN-tau genes* in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA).

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

Recent years have seen a lot of interest in how resveratrol influences the development of bovine embryos *in vitro*, which has prompted studies on its mechanisms of action and impact on embryo quality. This study showed that 0.5 µM resveratrol added to the IVC medium affects the different morphology of embryos at both the cleavage and blastocyst phases (Figure 1). The findings revealed that embryos at these stages exhibit different characteristics under different conditions. Consistent cleavage stage development in the control group embryos reflected normal early embryogenesis, as indicated by symmetric blastomeres with clear boundaries. Similar morphology of embryos treated with 0.5 µM resveratrol also suggested that resveratrol does not influence the early cleavage process. These findings were consistent with studies indicating that low resveratrol levels had little impact on early developmental stage cell division (Piras et al., 2020). Control group embryos showed suitable expansion and compaction at the blastocyst stage, suggesting normal blastocyst development. Embryos treated with 0.5 µM resveratrol showed increasing structural integrity, despite this, showing trophectoderm layers and well-formed inner cell masses. This result supports earlier research indicating that resveratrol may increase mitochondrial activity and lower oxidative stress, raising blastocyst quality (Piras et al., 2019). Resveratrol-treated embryos also showed no morphological abnormalities or developmental delays, hence verifying its probable role in improving embryonic viability (Gaviria et al., 2019). Consistent with its stated antioxidant qualities, the findings mostly suggested that resveratrol treatment at a dose of 0.5 µM had no impact on early cleavage and might enhance blastocyst quality. On the other hand, the results of resveratrol supplementation in a post-warming culture medium. The addition of 0.5 µM resveratrol to the post-warming culture medium at 24 and 48 h did not affect blastocyst survival rates or developmental competency.

Though after 48 h, the +R/-R group outperformed the -R/+R group in hatching rates, indicating a context-dependent effectiveness of resveratrol supplementation. These findings support recent studies indicating that the impact of resveratrol can vary depending on concentration, duration, and cellular environment, maybe because it modulates mitochondrial activity and oxidative stress (Hara et al., 2018). Surprisingly, resveratrol has been demonstrated to have both pro-oxidant and antioxidant actions, hence affecting the destinies of embryos through mitochondrial homeostasis and apoptosis. Higher hatching rates in the +R/-R group (Abe et al., 2017) suggest that resveratrol exposure before cryopreservation may have enhanced the balance of ROS, hence enhancing mitochondrial efficiency and developmental ability. Exposure to resveratrol after cryopreservation, such as in the -R/+R group, may, however, alter the redox balance in favor of pro-oxidative stress, hence reducing its antioxidant properties and showing the intricate function of resveratrol in embryonic systems (De La Lastra & Villegas, 2007). These findings help us to know more about the important link between mitochondrial function, antioxidant supplements, and embryonic survival. Future studies on the temporal modulation of resveratrol in culture systems should help to maximize its advantages and avoid any undesirable cytotoxic consequences. This will aid in strengthening techniques for improving embryo cryotolerance and developmental results in assisted reproductive technologies.

Moreover, the findings of this study revealed that including 0.5 μM resveratrol to bovine embryos, whether fresh or vitrified, has no noticeable impact on the total counts of ICM, TE. Not having any notable changes in cell counts suggested that resveratrol does not interfere with vital developmental pathways since ICM cells are involved in embryonic development and TE cells express interferon-tau (IFN-tau), which is required for implantation. Though the findings indicated that ICM or TE numbers were unaffected by 0.5 μM resveratrol, this does not negate potential molecular effects on embryonic physiology. Earlier research has suggested that, rather than directly affecting cell proliferation, resveratrol may work via pathways connected to mitochondrial bioenergetics, stress response modulation, and gene expression control (Yuan et al., 2015).

In this study, resveratrol-supplemented and vitrified bovine embryos showed notable variations in the expression of genes linked to antioxidant defense and cellular stress response. Interestingly, different expression patterns of *GPX4*, *HSP70*, *SOD1*, and *CAT*) were found. The group without resveratrol supplementation showed the highest expression of *HSP70* both before and after vitrification (-R/-R), indicating more cellular stress when antioxidant assistance is lacking. On the other hand, embryos supplemented with resveratrol consistently had low expression of *HSP70*, suggesting that the antioxidant qualities of resveratrol effectively reduce cellular stress (Arredondo et al., 2021) Every group that received resveratrol showed a notable rise in *SOD1*, a vital enzyme that shields embryos from oxidative stress by transforming superoxide radicals into less toxic forms (Zarbakhsh, 2021). Resveratrol supplementation can improve embryo survival under oxidative stress, as evidenced by the continued high expression of *SOD1* in vitrified embryos (Keane and Ealy, 2024). Additionally, embryos regularly treated with resveratrol showed much increased *CAT* gene expression, whereas all vitrification groups showed a dramatic drop in this gene expression. This implies that embryos are more susceptible to oxidative damage during vitrification. Such findings are consistent with earlier research showing that vitrification can increase oxidative stress to a level that may surpass the inherent antioxidant capability of embryos (Amin et al., 2014). An interesting pattern was shown by the significantly higher *GPX4* expression in vitrified groups, specifically -R/-R, +R/-R, and +R/+R, as compared to the control. This upregulated expression may be a compensatory reaction to elevated lipid peroxidation brought on by vitrification, which is in line with earlier findings in oxidative stress scenarios and highlights the critical function of *GPX4* in reducing the effects of lipid peroxidation during embryonic development (Saber et al., 2024). The results point to resveratrol as a useful additive for oxidative stress management in bovine embryo culture, especially during vitrification procedures. According to earlier studies, resveratrol effectively lowers the formation of ROS, raises intracellular glutathione levels, and has anti-apoptotic properties, all of which improve embryo survival after vitrification (Silva et al., 2021).

This study found that bovine embryos exposed to resveratrol supplementation and vitrification treatments showed notable changes in gene expression linked to stress resistance and mitochondrial function, namely the expression of *SIRT1* and *TFAM*. The main conclusions showed that whereas all vitrified groups showed a significant decrease in the expression of *SIRT1* and *TFAM*, embryos constantly supplied with resveratrol +R showed significantly greater expression levels of these genes. Resveratrol is a strong activator of *SIRT1*, which improves mitochondrial activity and may improve embryo developmental competence, as seen by the greater expression of *SIRT1* in the (+)R group when compared to the control. Prior research by Abe et al. (2017) showed that resveratrol-mediated activation of *SIRT1* promoted mitochondrial biogenesis and turnover through pathways involving phosphorylated AMP-activated protein kinase (AMPK), which in turn increased ATP generation and improved mitochondrial function. This is consistent with our research and implies that resveratrol supplementation may be a useful tactic for improving embryo viability, especially when mitochondrial quality is essential for later developmental success. On the other hand, *SIRT1* expression levels were markedly reduced following vitrification treatments, indicating mitochondrial malfunction and lower fetal viability post-warming culture. Vitrification-induced mitochondrial damage, a frequent side effect of cryopreservation techniques, may account for this phenomenon. According to earlier research, vitrification significantly impairs the integrity of the mitochondrial membrane, lowers ATP synthesis, and causes oxidative stress in embryos (Hara et al., 2018). *SIRT1* expression suppression in vitrified embryos may result from cryoinjury-induced mitochondrial malfunction and oxidative stress. Comparable patterns were noted for *TFAM*, a crucial modulator of transcription and replication of mitochondrial DNA. The positive impact of resveratrol in preserving the integrity and function of the mitochondrial genome is further supported by the notable increase in *TFAM* expression in the +R group supplemented with resveratrol. Especially, the +R/+R group showed the least *TFAM* expression after vitrification. This pattern could imply that cryopreservation does not provide benefits from extended or consecutive exposure to resveratrol before and after, and in some situations, may cause dysregulation of mitochondrial gene expression. One reasonable theory is that resveratrol supplementation during both IVC and post-warming culture media phases might excessively shift the redox balance, possibly cause mild cytotoxic effects or interfere with mitochondrial signaling pathways under post-thaw conditions (De la Lastra & Villegas, 2007; Hara et al., 2018). Particularly through altering mitochondrial dynamics and oxidative stress, resveratrol has been said to have both antioxidant and pro-oxidant effects depending on quantity, time, and cellular environment (Abe et al., 2017; Mesalam et al., 2018). This finding highlights the requirement of exact management of treatment duration and timing to prevent unintentional consequences on mitochondrial function and embryo viability, as well as the of the interaction between resveratrol and developmental physiology. These findings have significant ramifications for techniques involving IVP. A crucial part of embryo transfer programs in bovine breeding is that improved mitochondrial activity via resveratrol supplementation may significantly increase embryo survival rates following cryopreservation. According to Alarcón de la Lastra & Villegas (2007), resveratrol has strong antioxidant qualities that can potentially lessen cryo-induced mitochondrial damage by scavenging ROS and lowering oxidative stress. Since oxidative stress and mitochondrial dysfunction are the main factors limiting embryo quality after thawing, the judicious use of antioxidants like resveratrol could significantly enhance research and commercial results in IVP systems. However, there are certain restrictions on this study. First, actual protein activities or mitochondrial functional outcomes cannot be accurately represented by gene expression data alone. Consequently, assays for mitochondrial functional parameters such as ATP content, membrane potential, and mitochondrial turnover, as well as protein expression analysis, should be a part of future studies. According to Ren et al. (2019), additional research on the molecular signaling pathways that resveratrol activates, specifically SIRT1-mediated mechanisms involving PGC-1α and downstream transcription factors like NRFs and ERRs, may also shed light on mitochondrial biogenesis and function under stressful circumstances. Our findings highlight the potential benefits of resveratrol in enhancing mitochondrial activity and mitigating the negative effects of vitrification on bovine embryos. To fully utilize the positive effects of resveratrol on embryo quality and mitochondrial integrity, it is highly advised that future research refine resveratrol supplementation strategies, especially optimizing treatment timing about vitrification protocols, and conduct thorough functional studies.

The current study found that resveratrol supplementation had a significant impact on the expression of genes linked to apoptosis in bovine embryos, including *BAX* and *BCL2*, which have conflicting functions in controlling cell survival and death. The fresh control group, from -R group had the highest expression of the pro-apoptotic gene *BAX*, indicating that increased apoptotic signaling during *in vitro* embryo development may result from a lack of antioxidant support. On the other hand, resveratrol supplementation during *in vitro* culture markedly decreased *BAX* expression, which is in line with the preventive function of resveratrol against oxidative stress-induced apoptosis (Huang et al., 2007). The -R/+R group had the lowest *BAX* expression among the vitrified groups, suggesting that resveratrol supplementation in post-warming culture may be a more effective way to reduce cryoinjury-induced apoptosis than pre-warming treatment. This is consistent with research by Gaviria et al. (2019), who found that vitrification dramatically raises the expression of apoptotic genes and that antioxidant supplements, especially resveratrol, can reduce this effect, though the degree of protection may vary depending on the time of treatment and other factors. Conversely, the +R group had the highest and considerably higher expression of the anti-apoptotic gene *BCL2* compared to the other treatment groups. This finding supports the anti-apoptotic effects of resveratrol during culture, possibly through modulating mitochondrial integrity or activating *SIRT1* (Hayashi et al., 2018). Regardless of the degree of resveratrol treatment, *BCL2* expression was considerably lower in all vitrified groups. This implies that cellular stress caused by vitrification weakens anti-apoptotic defenses, which resveratrol by itself might not be able to fully repair. These trends suggest that whereas resveratrol effectively lowers the expression of apoptotic genes in non-cryopreserved circumstances, its protective effects are somewhat lessened in vitrified embryos. According to earlier research, vitrification-related injuries can overwhelm cellular defense systems, resulting in decreased anti-apoptotic responses, and the positive effects of antioxidants are more noticeable in fresh embryos (Lee et al., 2010; Gaviria et al., 2019). These findings significantly impact the improvement of embryo cryotolerance. To improve post-thaw embryo survival, *BAX* expression should be decreased and *BCL2* expression should be increased. The potential of resveratrol as a culture supplement is demonstrated by its capacity to change the *BAX/BCL2* ratio in fresh embryos toward a more survival-favorable condition. Its limited post-vitrification impact, however, highlights the necessity of combining strategies, perhaps incorporating synergistic antioxidant combinations or enhanced vitrification protocols to protect embryo quality better.

The expression of *DNMT1* and *DNMT3A* in +R group suggests better epigenetic programming, most likely due to the regulatory action of resveratrol on chromatin remodeling enzymes. According to Giroto et al. (2019), DNA methyltransferases like *DNMT1* preserve methylation patterns, while *DNMT3A* participates in de novo methylation throughout early embryogenesis. Both processes are critical for lineage commitment and genome stability. The decreased expression of these genes in every vitrified group supports research showing cryopreservation might affect epigenetic markers, resulting in problems with development (Yodruk et al., 2021). Importantly, it has been demonstrated that resveratrol activates *SIRT1* and promotes histone deacetylation and methylation, which indirectly supports DNMT activity and preserves the epigenetic integrity of the embryo (Adamkova et al., 2017). Similarly, the function of resveratrol in maintaining pluripotency is supported by the markedly increased *OCT4* expression in the +R group. One important indicator of inner cell mass and a major self-renewal regulator is *OCT4*. Cryopreservation lowers *OCT4* expression in bovine blastocysts, perhaps because of stress-induced cellular damage, according to Mori et al. (2015). Our findings build on existing findings by showing that resveratrol-pretreated embryos retain higher *OCT4* levels, suggesting improved resistance to stress brought on by vitrification. The findings of Sabre et al. (2024), who also documented an increase in *OCT4* expression after low-dose resveratrol intake, corroborate this observation. In bovine, a similar pattern was seen for *IFN-tau*, a cytokine generated from trophoblasts that is essential for the mother to recognize pregnancy. The expression of *IFN-tau* was lowest in the vitrified groups, particularly +R/+R, and highest in the +R group. This result illustrates the detrimental impact of vitrification on trophectoderm function as well as the restricted ability of resveratrol to reinstate *IFN-tau* expression when administered solely post-warming culture. Previous studies have also shown that vitrification impairs the expression of *IFN-tau*, which may lessen embryo-endometrial signaling (Mori et al., 2015).

5.2 Conclusions

The present study demonstrated that supplementing bovine embryo culture with 0.5 µM resveratrol during IVC or PWC modulates gene expression related to stress response, mitochondrial function, apoptosis regulation, epigenetic programming, and developmental competence in both fresh and vitrified embryos. In fresh embryos, resveratrol supplementation during IVC promoted favorable gene expression, including lower pro-apoptotic *BAX*, higher anti-apoptotic *BCL2*, enhanced antioxidant defense (*SOD1*, *CAT*), improved mitochondrial-related genes (*SIRT1*, *TFAM*), and increased expression of pluripotency (*OCT4*) and maternal recognition of pregnancy (*IFN-tau*) markers. These results indicate improved embryo quality and developmental potential under non-stressed conditions. For vitrified embryos, resveratrol treatment before vitrification (+R/-R) proved superior to supplementation in post-warming culture medium (-R/+R) in preserving embryo quality. The +R/-R group exhibited higher expression of mitochondrial function genes (*TFAM*), epigenetic regulator (*DNMT1*), and pluripotency marker (*OCT4*), along with improved antioxidant defense (*CAT*, *SOD1*, *GPX4*) and a reduced *BAX* level, contributing to better resistance to oxidative stress and higher hatching rates. In contrast, embryos supplemented only during post-warming culture (-R/+R) showed limited benefits, with persistent downregulation of *IFN-tau* expression and no significant improvement in other critical gene markers. Notably, embryos receiving continuous resveratrol treatment both before and after vitrification (+R/+R) displayed the lowest *IFN-tau* expression, suggesting that prolonged or excessive resveratrol exposure may impair embryo recovery or disrupt redox homeostasis following cryopreservation. Despite these positive effects, vitrification remained associated with downregulation of implantation (*IFN-tau*), epigenetic (*DNMT1*, *DNMT3A*), and mitochondrial (*TFAM*) genes across groups, highlighting the molecular stress induced by cryopreservation, which resveratrol alone could not fully overcome. These findings emphasize the importance of optimizing resveratrol treatment strategies, particularly in terms of timing and duration, to enhance cryotolerance and developmental potential in bovine embryos. Further research should explore protein-level changes, oxidative stress indicators (GSH, ROS), mitochondrial membrane potential, and apoptotic markers to refine supplementation protocols. Overall, this study supports the potential application of resveratrol as a culture supplement to improve embryo quality and developmental outcomes, particularly when administered prior to vitrification. (Figure 10)

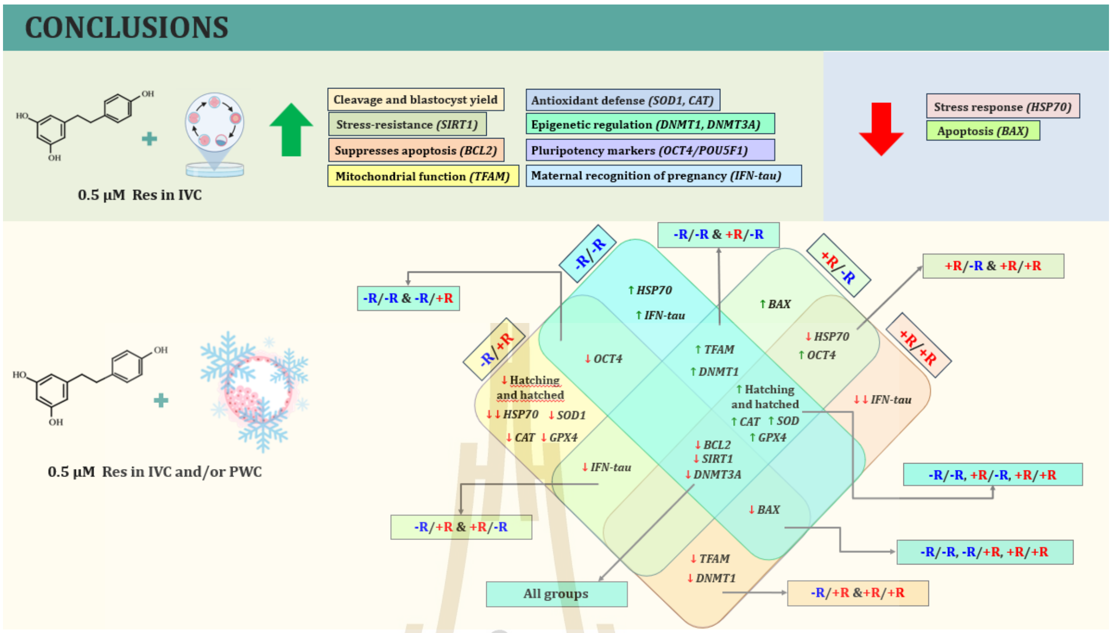

Figure 10 Graphical conclusions of resveratrol supplementation in IVC and post-warming culture media on bovine embryo development and gene expression.

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