EFFECTS OF RESVERATROL SUPPLEMENTATION IN *IN VITRO* EMBRYO CULTURE MEDIUM ON PREGNANCY RATES AFTER TRANSFER VITRIFIED AND SLOW FREEZING BOVINE BLASTOCYSTS DERIVED FROM OPU OOCYTES

DARIKA PAEKIATCHALORN

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ผลของการเติมเรสเวอราทรอลในน้ำยาเลี้ยงตัวอ่อนในหลอดแก้วต่ออัตราการตั้งท้องของตัวรับหลังจากย้ายฝากตัวอ่อนโคที่ผ่านการแช่แข็งโดยวิธี vitrification และ slow freezing ซึ่งผลิตจากไข่ที่เจาะเก็บจากการทำ OPU

นางสาวดาริกา แพร่เกียรติเจริญ

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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ดาริกา แพร่เกียรติเจริญ: ผลของการเติมเรสเวอราทรอลในน้ำยาเลี้ยงตัวอ่อนในหลอดแก้วต่ออัตราการตั้งท้องของตัวรับหลังจากย้ายฝากตัวอ่อนโคที่ผ่านการแช่แข็งโดยวิธี vitrification และ slow freezing ซึ่งผลิตจากไข่ที่เจาะเก็บจากการทำ OPU (EFFECTS OF RESVERATROL SUPPLEMENTATION IN *IN VITRO* EMBRYO CULTURE MEDIUM ON PREGNANCY RATES AFTER TRANSFER VITRIFIED AND SLOW FREEZING BOVINE BLASTOCYSTS DERIVED FROM OPU OOCYTES) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 91 หน้า.

คำสำคัญ: ตัวอ่อนในหลอดแก้ว/ย้ายฝากตัวอ่อน/การแช่แข็งตัวอ่อน/การตั้งท้องในโค/เรสเวอราทอล

การทดลองนี้มีวัตถุประสงค์เพื่อทดสอบผลการเติมเรสเวอราทอลในน้ำยาเลี้ยงตัวอ่อนต่อการพัฒนาของตัวอ่อนและการตั้งท้องในโคหลังจากย้ายฝากตัวอ่อนที่ผ่านการแช่แข็งโดยวิธี vitrification และ slow freezing การทดลองนี้เก็บไข่จากแม่โคที่มีพันธุกรรมดีเยี่ยมทางช่องคลอดภายใต้การตรวจด้วยคลื่นเสียงความถี่สูง ก่อนนำไข่ไปเลี้ยงต่อในน้ำยาให้พร้อมปฏิสนธิ หลังจากทำปฏิสนธิในหลอดแก้วนำตัวอ่อนเลี้ยงในน้ำยาเลี้ยงตัวอ่อนในหลอดแก้วที่เติมและไม่เติมเรสเวอราทอลที่ความเข้มข้น 0.5 ไมโครโมลาร์ จนครบ 7 วัน หลังจากนั้นตัวอ่อนระยะบลาสโตซีสทั้งสองกลุ่มถูกแบ่งไปย้ายฝากตัวอ่อนสดและแช่แข็งด้วยวิธี vitrification และ slow freezing และตรวจการตั้งท้องด้วยคลื่นเสียงความถี่สูงหลังการย้ายฝาก 23-30 วัน ผลการศึกษาพบว่าโคพันธุ์วากิวให้ไข่น้อยกว่าโคพันธุ์บราห์มันและบีฟมาสเตอร์ อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ส่วนโคพันธุ์บีฟมาสเตอร์ให้ไข่สูงที่สุดแต่ไม่แตกต่างทางสถิติกับโคพันธุ์บราห์มัน ผลการเติมเรสเวอราทอลในน้ำยาเลี้ยงตัวอ่อนพบว่าช่วยเพิ่มอัตราการแบ่งเซลล์ของตัวอ่อนโคพันธุ์บราห์มัน (-Res 59.16%, and +Res 69.29%) และบีฟมาสเตอร์ (-Res 66.50%, and +Res 80.19%) อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) แต่เพิ่มอัตราการแบ่งเซลล์ของ ตัวอ่อนโคพันธุ์วากิวเพียงเล็กน้อยอย่างไม่มีนัยสำคัญทางสถิติ (-Res 60.39%, and +Res 67.73%, $P>0.05$) และสามารถเพิ่มอัตราการเจริญเติบโตของตัวอ่อนไปเป็นระยะบลาสโตซิสในโคพันธุ์บราห์มัน (-Res 33.99%, and +Res 40.32%) และบีฟมาสเตอร์ (-Res 37.14%, and +Res 44.43%) อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) แต่ไม่มีผลต่อการเจริญเติบโตของตัวอ่อนไปเป็นระยะบลาสโตซิสในโคพันธุ์วากิว (-Res 26.12%, and +Res 26.36%, $P>0.05$) อัตราการตั้งท้องในกลุ่มตัวอ่อนสดที่เติมเรสเวอราทอลเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (-Res Fresh 27.80% และ +Res Fresh 37.86%, $P<0.05$) ตัวอ่อนที่เติมเรสเวอราทอลและแช่แข็งด้วยวิธี vitrification (-Res Vit 26.87% และ +Res Vit 29.31%) และ slow freezing (-Res Slow 18.18% และ +Res Slow 21.56%) มีอัตราการตั้งท้องที่สูงขึ้นแต่ไม่มีความแตกต่างทางสถิติ ($P>0.05$) กับตัวอ่อนที่ไม่เติมเรสเวอราทอล การเติมเรสเวอราทอลในตัวอ่อนสดยังช่วยลดการสูญเสียการตั้งท้องอย่างมีนัยสำคัญทางสถิติ (-Res

Fresh 21.23% และ +Res Fresh 10.75%, P<0.05) แต่ในตัวอ่อนที่เติมเรสเวอราทอลและไม่เติมเรสเวอราทอลและแช่แข็งด้วยวิธี vitrification (-Res Vit 16.66% และ +Res Vit 11.76%) และ slow freezing (-Res Slow 20.00% และ +Res Slow 18.18%) มีอัตราการสูญเสียการตั้งท้องไม่แตกต่างกันทางสถิติ (P>0.05) การแช่แข็งวิธี slow freezing ให้ผลทางลบต่ออัตราการตั้งท้อง เมื่อเปรียบเทียบกับกลุ่มอื่นๆ อิทธิพลจากความแตกต่างของสายพันธุ์แม่โคตัวให้และแม่โคตัวรับไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (P>0.05) ต่ออัตราการตั้งท้องและการสูญเสียการตั้งท้อง การศึกษาครั้งนี้สรุปได้ว่าการเติมเรสเวอราทอลในน้ำยาเลี้ยงตัวอ่อนช่วยเพิ่มอัตราการแบ่งเซลล์ของตัวอ่อนและอัตราการเจริญเติบโตของตัวอ่อนไปเป็นระยะบลาสโตซิส และช่วยเพิ่มอัตราการตั้งท้องและลดการสูญเสียการตั้งท้อง อีกทั้งยังช่วยปรับปรุงคุณภาพของตัวอ่อนรวมถึงเพิ่มอัตราการรอดชีวิตของตัวอ่อนหลังการแช่แข็ง นอกจากนี้ยังพบว่าการแช่แข็งด้วยวิธี vitrification เป็นวิธีที่มีประสิทธิภาพมากกว่าการแช่แข็งแบบ slow-freezing ในการเก็บรักษาตัวอ่อนให้มีชีวิต

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2566 ลายมือชื่ออาจารย์ที่ปรึกษา _____

DARIKA PAEKIATCHALORN : EFFECTS OF RESVERATROL SUPPLEMENTATION IN *IN VITRO* EMBRYO CULTURE MEDIUM ON PREGNANCY RATES AFTER TRANSFER VITRIFIED AND SLOW FREEZING BOVINE BLASTOCYSTS DERIVED FROM OPU OOCYTES. THESIS ADVISOR: ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 91 PP.

Keyword: Bovine/*In vitro*/Slow freezing/Vitrification/Embryo transfer/ Pregnancy

This study aimed to examine the effects of resveratrol supplemented in culture medium on embryo development and pregnancy rate of vitrified and slow frozen bovine embryos. The oocytes were collected from elite genetics cattle using a transvaginal ultrasound-guided ovum pick-up (OPU) technique and cultured in *in vitro* maturation (IVM) medium. After *in vitro* fertilization (IVF), the embryos were cultured in *in vitro* culture (IVC) medium with (+Res) or without (-Res) 0.5 µM resveratrol supplement for 7 days. Grade 1 blastocysts from both treatments were selected for fresh transfer and freezing using vitrification and slow freezing methods. The embryo transfer (ET) was conducted in recipients using vitrified blastocysts in comparison with fresh and slow frozen blastocysts. Then, pregnancy was examined at 23 to 30 days post-ET using a transrectal ultrasonography. Results showed that Brahman and Beefmaster cattle had a significantly higher ($P<0.05$) numbers of collected oocytes than Wagyu cattle. Moreover, Beefmaster cattle yielded the highest number of collected oocytes but not significantly different with Brahman cattle. Resveratrol supplementation in IVC medium resulted in a significant ($P<0.05$) increase of cleavage rate in Brahman (-Res 59.16%, and +Res 69.29%) and Beefmaster (-Res 66.50%, and +Res 80.19%) in comparison with those of untreated group. However, there was slightly higher effect on cleavage rate of Wagyu (-Res 60.39%, and +Res 67.73%, $P>0.05$). Similar to cleavage rate, resveratrol also significantly ($P<0.05$) increased blastocyst rate of Brahman (-Res 33.99%, and +Res 40.32%) and Beefmaster (-Res 37.14%, and +Res 44.43%) compared to the untreated group. However, there was no beneficial effect on blastocyst rate of Wagyu (-Res 26.12%, and +Res 26.36%, $P>0.05$). Pregnancy rates of fresh blastocysts with resveratrol supplement were significantly increased (-Res Fresh 27.80% and +Res Fresh 37.86%, $P<0.05$) over the untreated fresh blastocysts. Pregnancy rates of slow freezing (-Res Slow 18.18%, and +Res Slow 21.57%), and

vitrified blastocysts (-Res Vit 26.87%, and +Res Vit 29.31%) with resveratrol were slightly higher than those of untreated oocytes, but there was no significant difference (P>0.05). Pregnancy losses significantly decreased in recipients receiving fresh embryos from +Res in comparison with -Res (-Res Fresh 21.23%, and +Res Fresh 10.75%, P<0.05). However, pregnancy losses obtained from slow freezing embryos (-Res Slow 20.00%, and +Res Slow 18.18%, P>0.05) and vitrified embryos (-Res Vit 16.66%, and +Res Vit 11.76%, P>0.05) did not significantly differ. Furthermore, slow freezing had a more negative effect on pregnancy rate showed by a significantly lower pregnancy rate compared to other groups. The impact of different recipient breeds and donor breeds did not significantly effect on pregnancy rates and pregnancy loss. In conclusion, resveratrol supplementation in IVC medium was found to significantly enhance cleavage and blastocyst formation rates. Embryos treated with resveratrol showed higher pregnancy rates and reduced pregnancy loss. These results suggest that resveratrol is a beneficial supplement for improving the quality and viability of embryos. In addition, vitrification is more effective than slow-freezing in preserving embryo viability.

School of Biotechnology
Academic Year 2023

Student's Signature P. Darika
Advisor's Signature

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Darika Paekiatchalorn

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LIST OF ABBREVIATION

°C	=	Degree Celsius
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
•OH	=	Hydroxyl radical
1^{st} PB	=	First polar body
8-oxo-dG	=	8-oxodeoxyguanosine
ACACA	=	Acetyl-CoA carboxylase 1
ARTs	=	Assisted reproductive technologies
ATP	=	Adenosine triphosphate
AU	=	Arbitrary units
BAX	=	Bcl-2-associated protein x
BCL2	=	B-cell CLL/lymphoma 2
BCL2L1	=	BCL2-like protein 1
bFGF	=	Basic fibroblast growth factor
BM	=	Basal medium
BMP15	=	*Bos taurus* bone morphogenetic protein 15
BO	=	Bracket and Oliphant
BSA	=	Bovine serum albumin
CAT	=	Catalase
CDC2	=	Cell division cycle protein 2 homolog
CGs	=	Cortical granules
CH_3	=	Methyl
Cl–	=	Chlorine ions
CL	=	Corpus luteum
c-MOS	=	mRNA is necessary for oocyte maturation
CO_2	=	Carbon dioxide

LIST OF ABBREVIATION (Continued)

COCs	=	Cumulus-oocyte complex
COX	=	Cyclooxygenase
CPAs	=	Cryoprotectant agents
CTZ	=	Critical temperature zone
Cu	=	Copper
CyCB2	=	Cyclin-B2
CYP51A1	=	Cytochrome P450, family 51, subfamily A, polypeptide 1
DGC	=	Density gradient centrifugation
DG-SU	=	Density gradient-swim up
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DNMT3A	=	DNA (cytosine-5-) methyltransferase 3 alpha
E2	=	Estrogen
EAAs	=	Essential amino acids
EC	=	Electrical Conductivity
ECS	=	Estrus bovine serum
EDTA	=	Ethylenediaminetetraacetic acid
EG	=	Ethylene glycol
EGF	=	Epidermal growth factor
ERK2	=	Extracellular signal-regulated kinases
ES	=	Equilibration solution
ET	=	Embryo transfer
FACS	=	Fluorescence activated cell sorting
FBS	=	Fetal bovine serum
FCS	=	Fetal calf serum
Fe	=	Iron
Fe_3O_4	=	Iron oxide
FoxO3A	=	Forkhead box protein O3
FSH	=	Follicle-stimulating hormone

LIST OF ABBREVIATION (Continued)

GDF-9	=	Growth differentiation factor-9
GLY	=	Glycerol
GnRH	=	Gonadotropin-releasing hormone
GPx	=	Glutathione peroxidase
GPX1	=	Glutathione peroxidase 1
GPx4	=	Glutathione peroxidase 4
GSH	=	Glutathione
GSSG	=	Glutathione disulfide
GV	=	Germinal vesicle
GVBD	=	Germinal vesicle breakdown
GWF	=	Glass wool filtration
h	=	Hour
H_2O_2	=	Hydrogen peroxide
hCG	=	Human chorionic gonadotropin
HGF	=	Hepatocyte growth factor
HOO–	=	Hydroperoxide
HTU	=	Hypotaurine
ICM	=	Inner cell mass
IETS	=	International Embryo Transfer Society
IU	=	International units
IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilization
IVM	=	*In vitro* maturation
IVP	=	*In vitro* embryo production
Kg	=	Kilogram
KGF	=	Keratinocyte growth factor
KL	=	Kit ligand
LH	=	Luteinizing hormone
LIPE	=	Lipase, hormone-sensitive

LIST OF ABBREVIATION (Continued)

M	=	Molarity
MACS	=	Magnetic activated cell sorting
MAPK1	=	Mitogen activated protein kinase
mDPBS	=	Dulbecco's phosphate buffered saline modified
MF	=	Microfluidics sorting
MI	=	Metaphase I
MII	=	Metaphase II
min	=	Minute
ml	=	Milliliter
MNP	=	Magnetic nanoparticles
MnSOD	=	Superoxide dismutase 2
mOsm	=	Milliosmole
MPF	=	Maturation-promoting factor
MPGF	=	Masculine pronucleus growth factor
mPT	=	Mitochondrial permeability transition
MS	=	Migration sedimentation
MSC	=	Migration sedimentation chamber
mtDNA	=	Mitochondrial DNA
mTOR	=	Mammalian target of rapamycin
MTORC1	=	Mechanistic target of rapamycin
N_2	=	Nitrogen
NAC	=	N-acetyl cysteine
NAD	=	Nicotinamide adenosine dinucleotide
NEAAs	=	Non-essential amino acids
NF-κB	=	Nuclear factor kappa B
nm	=	Nanometer
O_2	=	Oxygen
O_2•–	=	Superoxide anion
OECs	=	Oviductal epithelial cells

LIST OF ABBREVIATION (Continued)

OPS	=	Open-pulled straws
OPU	=	Ovum pick-up
p21	=	Cyclin-dependent kinase inhibitor 1
P4	=	Progesterone
P450aro	=	Aromatase in cumulus cells
P450scc	=	Cholesterol side-chain cleavage enzyme in cumulus cells
p53	=	Tumor protein P53
pAMPK	=	phosphorylated AMP-activated protein kinase
PBS	=	Phosphate-buffered saline
PG	=	Prostaglandin F2α
PGC-1α	=	PPARG coactivator 1 alpha
pH	=	Potential of hydrogen ion
PNA	=	Peanut agglutinin
PNPLA2	=	Adipose triglyceride lipase
PROH	=	Propanediol
PTX3	=	Pentraxin 3 in cumulus cells
PVP	=	Polyvinylpyrrolidone
RER	=	Rough endoplasmic reticulum
Res	=	Resveratrol
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species
rpm	=	Revolutions per minute
SER	=	Smooth endoplasmic reticulum
SIRT1	=	Sirtuin1
SLC2A1	=	Solute carrier family 2 facilitated glucose transporter member 1
SOD	=	Superoxide dismutase
SOD1	=	Superoxide dismutase 1
SOF	=	Synthetic oviduct fluid medium
SSV	=	Solid surface vitrification

LIST OF ABBREVIATION (Continued)

SU	=	Swim-up
TALP	=	Tyrode's albumin lactate Pyruvate
TCM 199	=	Tissue culture medium 199
TE	=	Trophoblasts
TFAM	=	Transcription factor A, mitochondrial
TNF-α	=	Tumor necrosis factor-α
TOMM20	=	Outer mitochondrial membrane protein
VS	=	Vitrification medium
WS	=	Warming solution
Zn	=	Zinc
ZP	=	Zona pellucida

CHAPTER I
INTRODUCTION

1.1 Background and significance

The cattle industry's efforts to develop animal performance, including growth rate, reproductive rate, carcass quality, adaptation, and animal yield. These are influenced by the genetics of the herd. However, there is a limitation related to unreliable breeders with excellent traits used for calf production to allow sustained genetic improvement. The application of assisted reproductive technology (ART) is a popular alternative to enhance reproductive performance and efficiency (Bousquet et al., 1999). When compared to superovulation (SOV), the combination of *in vitro* embryo production (IVP) and ultrasound-guided ovum pick-up (OPU) successfully produces a considerable number of calves (Ferré et al., 2019). Additionally, cryopreservation is crucial for maintaining female genetics and storing embryos. However, cryopreservation is detrimental to embryo quality and is the main cause of concern (Hwang & Hochi, 2014).

Previous researchs demonstrate that cryopreservation causes structural, biochemical, and molecular alterations that negatively affect quality of the embryo, such as weakened mitochondrial functions caused by increase reactive oxygen species (ROS) levels (Akiyama et al., 2006; Chen et al., 2019; Gupta et al., 2010; Gutnisky et al., 2013; Iwata, 2021; Piras et al., 2020; Wang et al., 2017). Antioxidants are substances that protect cells from free radicals during metabolism, including enzymes, thiol compounds, vitamins, flavonoids, amino acids, and amino acid derivatives. Moreover, it is also reported that resveratrol, α-tocopherol, melatonin, and L-proline protect oocytes from vitrification damage (Chinen et al., 2020).

Resveratrol (Res: 3,4,5-trihydroxy-trans-stilbene) is an antioxidant that belongs to a group of compounds called polyphenols, mostly found in red grapes, red wines, and some berries. In embryo development, previous researches demonstrate that resveratrol enhances the *in vitro* development of embryos, modulates redox homeostasis (Pasquariello et al., 2020), and control turnover of ROS production in

mitochondria as well as enhance the quality and subsequent embryo development in cryopreserved embryos. At the electron transport chain level, ROS levels are decreased along with abnormal mitochondrial distribution and apoptotic rates (Giaretta et al., 2013; Pervaiz & Holme, 2009; Piras et al., 2020; Truong et al., 2018; Wang et al., 2018; Xia et al., 2017). Furthermore, addition of resveratrol in *in vitro* culture (IVC) medium may enhance development and revivability of cryopreserved embryos using vitrification and slow freezing methods.

1.2 Research objective

To investigate the effects of resveratrol supplemented in IVC medium on quality and quantity of bovine embryos, the pregnancy rate after cryopreservation using vitrification and slow freezing protocols.

1.3 Research hypothesis

1.3.1 Resveratrol supplemented in IVC medium enhances embryo development.

1.3.2 Resveratrol supplemented in IVC medium could increase the pregnancy rate after cryopreservation

1.4 Scope and limitations of the study

The effect of resveratrol supplemented in IVC medium on the developmental potential of cryopreserved embryo and transfer to the recipient was investigated in terms of the pregnancy rate.

1.5 Research methodology

1.5.1 Instrumentation

The provider of all the supplies and instruments was Siam Vet Tech IVF Center, Khon Kaen, Thailand.

1.5.2 Location of research

The experiments were conducted at Siam Vet Tech IVF Center, Khon Kaen, Thailand.

CHAPTER II

LITERATURE REVIEW

2.1 *In vitro* embryo production (IVP)

In vitro embryo production (IVP) is the primary assisted reproductive technology, encompassing oocyte retrieval, *in vitro* fertilization, and embryo transfer. Key steps include controlled ovarian stimulation, oocyte retrieval, fertilization, embryo culture, and transfer. Additional techniques such as preimplantation genetic testing and intracytoplasmic sperm injection may be incorporated. Cryopreservation via vitrification is utilized for surplus embryo storage or fertility preservation of oocytes or embryos (Jain & Singh, 2022).

IVP consists of *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* culture (IVC). IVP is a method of improving reproductive performance that is used for commercial purposes. This technology become popular when compared with conventional embryo production (Bousquet et al., 1999). The main advantages of this technology include a higher number of embryos, a higher pregnancy rate per year, potential oocytes from female donors, high-index genomic calves, and a reduced number of sperm required to produce embryos and give birth to the desired sex of calves. However, there are still many problems with IVP embryos that are the limitations of this technology, including potentially reduced fertility from sexing semen, reduced oocyte quality after IVM, and lower embryo cryotolerance, which reduces pregnancy rates compared to *in vivo* produce embryos (Ferré et al., 2019).

In a decade, there had been an increase in the number of transfers of frozen-thawed IVP embryos. The reason might be that the culture media with specific supplements promotes embryo quality. Another reason might improve the efficiency of the ultrasound-guided oocyte retrieval technique (Pieterse et al., 1988), usually called ultrasound-guided ovum pick-up (OPU) (Ward et al., 2000). OPU-IVP is the primary tool to produce superior genetic cattle. Currently, IVP has been researching to improve reproductive performance at all stages such as ovarian stimulation, oocyte recovery,

maturation, fertilization, embryo development, freezing, transfer, and pregnancy establishment (Ferré et al., 2019).

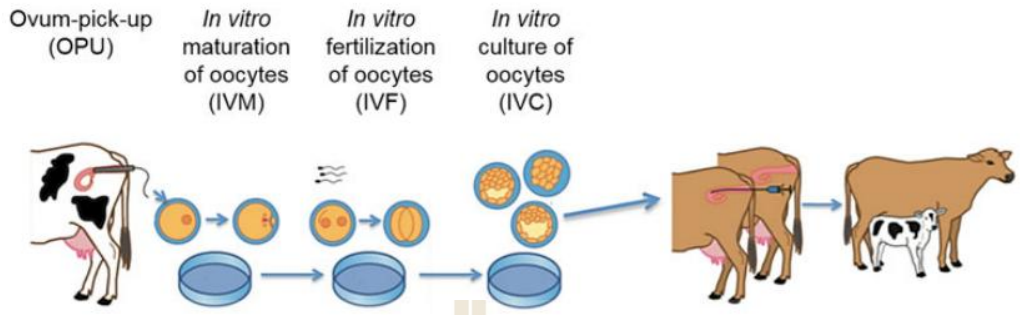


Figure 2.1 Flowchart of the process for IVP consists of ovum OPU, IVM, IVF, IVC, and embryo transfer (ET) (Ferré et al., 2019)

2.1.1 Ovulation

The mechanism of ovulation starts with the hypothalamus producing and releasing gonadotropin-releasing hormone (GnRH) to stimulate the anterior pituitary to produce and release follicle-stimulating hormone (FSH). FSH then binds to receptors on the granulosa cells of the follicle, stimulating the development of a small follicle into an antrum follicle. Additionally, granulosa cells produce estrogen (E2) and activin, which further stimulate follicle growth until it reaches maturity, leading to an increase in E2 levels. Elevated E2 levels exert negative feedback, inhibiting FSH production and triggering the release of luteinizing hormone (LH) from the anterior pituitary, which induces ovulation (Mihm et al., 2002).

Ovulation occurs from the preovulatory follicle during the dominant phase, where only one oocyte mature. The stages of follicle growth in the ovaries can be divided into three stages: primordial follicle, recruited follicle, and preovulatory follicle, respectively (Hafez, 2000). Following ovulation, the site of follicle rupture is stimulated by LH to undergo further growth into luteal cells and subsequently develop into the corpus luteum (CL), which is responsible for producing progesterone (P4). After fertilization occurs in the fallopian tube, the embryo implants in the uterus, leading to pregnancy (Ginther et al., 2001; Ginther et al., 2003). The thicker endometrium that is created and maintained by P4, which is produced by the CL and later by the placenta, provides a nurturing environment for implantation and growth.

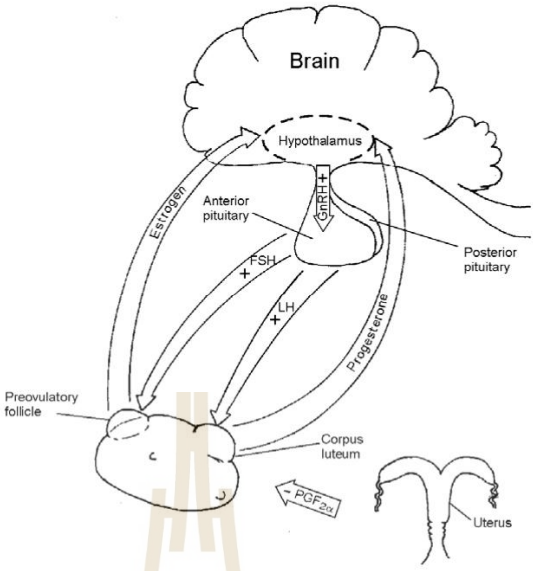


Figure 2.2 Impacts of ovarian, uterine, anterior pituitary, and hypothalamus hormones on the regulation of reproduction (Larson & Randle, 2008).

2.1.2 The estrous cycle in bovine

For mature cows, the estrous cycle lasts 21 days on average. There are four phases in a cycle: estrus, metestrus, diestrus, and proestrus (Larson & Randle, 2008). The time when a heifer shows signs of sexual desire and accepts a bull by standing to be mounted is known as estrus. The duration of this phase ranges from 6 to 30 hours, with an average of 20 hours. Vaginal and cervical mucus production increases significantly during estrus. Before this time, CL has been lysed, which results in very little P4 being produced and subsequently in the blood. During estrus, LH increases to a high level, triggering ovulation. Additionally, E2 levels are falling from their peak right before estrus.

Metestrus lasts 3 to 5 days, 10–15 hours following the end of estrus, during metestrus, ovulation takes place. The CL is still in its early stages of development. A slightly bloody vulvar discharge from certain heifers may be noticed by watchful producers as a result of endometrial tiny vessel rupture. Although still low, P4 levels are modestly rising during the menstrual cycle. This is a result of the CL's tiny size and its limited ability to release significant levels of P4 at this developmental stage. Large dosages of E2 are efficient luteolytic drugs during metestrus, while prostaglandin F2**α** (PG) is ineffective at lysing the immature, growing CL, and causing a return to estrus.

Diestrus, the CL's period, lasts almost a month. During the early stages of diestrus, the CL is making more P4 since it has a greater potential to produce steroids as it matures. Blood progesterone concentrations plateau during diestrus. While high amounts of E2 do not cause the CL to lyse and trigger a return to estrus, PG does during diestrus.

Proestrus, which lasts for 2-3 days, is marked by the ovulatory follicle's final development phase and the regression of the CL. LH and E2 positively feedback on each other as P4 levels in the blood drop, causing the ovulatory follicle to produce more E2 quickly and raising blood concentrations. Since the animal is currently experiencing regression due to the CL, administering PG would not have an impact on its typical return to estrus (Ball & Peters, 2008; Jainudeen & Hafez, 2000; Yizengaw, 2017)

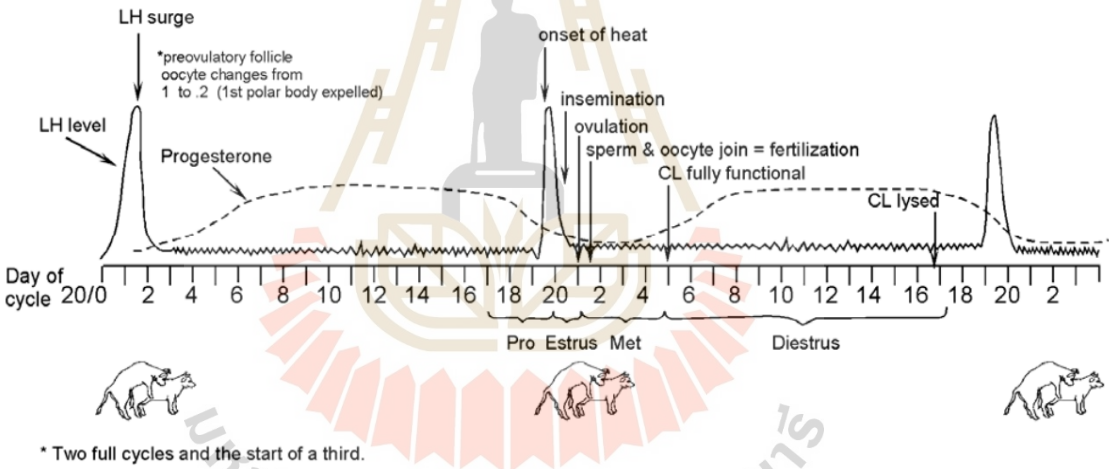


Figure 2.3 Diagram showing the phases of the estrous cycle, serum P4 levels, and serum levels of the hormone LH (Larson & Randle, 2008).

2.1.3 Estrus synchronization

It is necessary to regulate the luteal and follicular phases of the estrous cycle in order to successfully synchronize estrus. Protocols for synchronizing estrous cycles can be divided into four major categories (Perry & Salverson, 2016).

1) Prostaglandin F2α (PG)-based protocols

If a pregnancy does not materialize, the naturally occurring hormone PG signals the CL to degenerate (regress), allowing the cow to resume standing estrus. PG controls the luteal phase of the estrous cycle since it causes a CL to regress before

it would ordinarily regress on its own when an injection of PG is provided. The CL is not responsive to PG during the first five days of luteal development or during natural CL regression, which occurs after day 17 of the estrous cycle. As a result, PG will only attempt to regress the CL during the estrous cycle's days 5 through 17. If PG is injected within the responsive period (days 5–17), the animal exhibits standing estrus 48–120 hours following the administration due to CL regress. An animal will not react to a PG injection if there is no CL present (a cow in the postpartum anestrus stage, or a heifer that has not reached puberty). An animal must be in the estrous cycle and be between days 5 and 17 of the cycle.

2) Gonadotropin releasing hormone (GnRH)-based protocols

GnRH regulates the estrous cycle's follicular phase. Each estrous cycle consists of two or three follicular waves, which are the product of the wave-like growth patterns of follicles. Each of these waves has a dominant follicle that is fertile and able to ovulate or release an oocyte. On the other hand, P4 prevents ovulation in a dominant follicle. Naturally occurring gonadotropin-releasing hormone triggers a spike in LH, which leads to the dominant follicle ovulating even when progesterone is present. A dominant follicle can be stimulated to ovulate with a GnRH injection at three different times during an estrous cycle with three follicular waves. An injection of GnRH will not work when a follicular wave is forming, and a dominant follicle is absent. A CL will form, and a new follicular wave will begin after an injection of GnRH induces ovulation in a dominant follicle.

3) Progestin-based protocols

Standing estrus and ovulation are suppressed during the estrous cycle while a CL is present and P4 levels are high; nevertheless, the animal reverts to standing estrus when the CL gets smaller and P4 levels fall. By prolonging the luteal phase of the cycle, progestins regulate the estrous cycle by inhibiting ovulation and acting as a mimic of the P4 generated by the CL. When the CL naturally recedes, the animal will not display standing estrus or ovulation; instead, the progestin will cause the follicle to grow larger. It will inhibit ovulation. P4 levels will be low when the progestin is stopped, and standing estrus and ovulation will happen. On the other hand, the dominant follicle will become a persistent follicle if a CL regresses and cows are

exposed to progestin to prevent ovulation of the follicle. Animals exposed to progestin for longer than seven days will have lower fertility when breeding at the initial estrus; nevertheless, future ovulations will have normal fertility.

4) GnRH-PG combination protocols

As was previously indicated, P4 can still promote ovulation of a dominant follicle, but GnRH is a naturally occurring hormone that, when injected, causes an LH surge. CL will form, and a new follicular wave will begin after the dominant follicle undergoes induced ovulation. 7 days after this forced ovulation, a PG injection can stop (regress) the CL that occurs. After the CL by PG recedes, the new follicular wave that was triggered by the forced ovulation by GnRH will develop naturally, and on day 7, a new dominant follicle will be present and prepared to ovulate. Nevertheless, ovulation cannot be triggered in animals if there isn't a dominant follicle present at the moment of the GnRH injection. These animals may demonstrate standing estrus prior to the time of the PG injection, depending on the stage of the estrous cycle.

5) Progestin-PG combination protocols

Exposure for 7 days. Progestins prevent ovulation by mimicking the P4 produced by the CL, as was previously described. All animals will have a CL that is at least 7 days old at the time of PG injection when ovulation is suppressed for 7 days. As a result, the PG will cause all animals with a CL to react. After the progestin is removed, animals whose CL had regressed during the 7-day period will exhibit standing estrus.

Exposure for 14 days. Progestins prevent ovulation by mimicking the P4 produced by the CL, as was previously described. Thus, when a CL regresses, follicular growth will continue and ovulation will be suppressed, rather than the animal demonstrating standing estrus and ovulating. Within seven days of the progestin being removed, animals typically exhibit standing estrus. The follicle that ovulates after this standing estrus is elderly (referred to as a persistent follicle) and has decreased fertility because ovulation was suppressed for up to 14 days. Consequently, PG is often administered 19 days after the elimination of progestin. An estrus with good fertility will result from this PG injection, which will regress the CL that occurs following the ovulation of a persistent follicle.

6) GnRH-Progestin-PG combination protocols

The addition of a GnRH injection at the beginning of progestin exposure will synchronize follicular waves, as progestin administration will not synchronize follicular waves, and animals that regress their CL during a 7-day period of progestin treatment can create a persistent follicle. Since a follicular wave typically lasts seven to ten days, starting a fresh wave at the beginning of progestin exposure will decrease the likelihood of a persistent follicle forming. Moreover, the progestin that is administered in between the GnRH and PG injections will reduce the possibility that animals will show symptoms of standing estrus prior to the PG injection.

2.1.4 Importance of media for *in vitro* production

The commercial bovine embryo transfer by IVP focuses on increasing blastocysts quality and yield. One of the most popular is the addition of supplements such as fetal bovine serum (FBS), serum replacements or bovine serum albumin (BSA). FBS may improve embryo development (Holm et al., 1999; Thompson et al., 1998), but they reduce cryotolerance (Rizos et al., 2003). BSA gives similar or even higher blastocyst rates and quality as compared to FBS (Stroebech et al., 2018). BSA can bind many low molecular weight compounds including heavy metal ions, free radicals, citrate, and steroids. It can protect cellular constituents against the effect of toxins, and regulate redox potential, potential of hydrogen ion (pH) and osmolarity (George et al., 2008). Although serum contains substances beneficial for embryonic development, such as antioxidants, growth factors, and heavy metal chelators, it can adversely affect the quality of the embryo by inducing excessive lipid droplet accumulation (Abe et al., 2002; Ferré et al., 2019).

Moreover, they are concerned about the import-export of embryos cultured in media animal-based serum because of the risk of spreading pathogens and want to exclude serum from IVP media and developed IVP media with synthetic serum replacement in all steps from IVM, IVF and IVC until the blastocyst stage in media without serum. Normally bovine commercial and research laboratories prepared all their own culture media, such as Tissue culture medium 199 (TCM 199), Tyrode's albumin lactate Pyruvate (TALP) (Parrish et al., 1986), and Synthetic oviduct fluid medium (SOF) (Holm et al., 1999) with additives required balanced salt solution, sodium

bicarbonate, non-essential and essential amino acids, pyruvate, phosphatase, L-glutamine, vitamins, antibiotics, sugar (glucose or fructose) and other protector factors (antioxidants) (Ferré et al., 2019; Gardner, 2007).

2.1.5 *In vitro* maturation (IVM)

Oocytes for IVP can be obtained from OPU, ovariectomy, and postmortem. Oocytes are aspirated from follicles, then selecting cumulus-oocyte complex (COCs) retrieval is highly correlated with embryo production and the number of pregnancies (Ferré et al., 2019; Watanabe et al., 2018)In the maturation process, oocytes in the germinal vesicle (GV) stage are raised to develop until the Meiosis-II stage. The first change found in nuclear membrane breakdown is called germinal vesicle breakdown (GVBD). Afterward, the oocyte will enter the cell division process called meiosis. At the time during metaphase I (MI) meiotic division to metaphase II (MII), nuclear maturation, organelles changes, proteins and transcripts take place in the oocyte, constituting the cytoplasmic oocyte maturation (Hyttel et al., 1997). In this stage of Meiosis-II, the first polar body (1^{st} PB) results from the pushing of chromosomes out of the nucleus, creating haploid pronuclei that are prepared for fertilization.

Rose and Bavister (1992) and Gliedt et al. (1996) found that TCM199 had the best results when comparing several types of medium. In the IVM medium, serum is added, including fetal calf serum (FCS), FBS, estrus bovine serum (ECS), and BSA (Blanco & Simonetti, 2002; Lee et al., 1996). Serum supplementation in IVM solution influenced embryo development during the cleavage to blastocytes. The hormone FSH plays a role in stimulating the expansion of COCs *in vitro*, which is similar to the LH surge that occurs before ovulation in animals. In addition, the hormones FSH and LH are important for maturation and cytoplasmic maturation (McGee & Hsueh, 2000).

COCs secretes substances or cytokines that optimize the environment for ovulation, support fertilization, and stimulate many factors to be activated such as tumor necrosis factor-α (TNF-α), neurotrophins, inhibins, and growth differentiation factor-9 (GDF-9)(Morrison & Marcinkiewicz, 2002; Wang & Roy, 2006). At the same time, oocyte responds to the stimulation of kit ligand (KL) and produces various substances that are factors in oocyte development, such as basic fibroblast growth factor (bFGF), GDF-9, and bone morphometric factor-15 (BMP-15) (Skinner, 2005). Also, theca cells

produce and secrete keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) (Skinner, 2005).

When follicles get to 2-3 mm in size, bovine oocytes can finish the process of GVBD during miosis. Additionally, oocytes with a diameter of 110 μm can develop nuclei until the MII stage. Cytoplasmic maturation also occurs in the oocyte, bringing about changes in vital organelles such as the cortical granules (CGs), which stop other sperm from accessing the oocyte after fertilization, and the mitochondria, which provide energy. The production of ribosomal proteins is carried out by the rough endoplasmic reticulum (RER). and moves other materials, including proteins that can be made. Within cells, the smooth endoplasmic reticulum (SER) transfers a number of materials, including ribonucleic acid (RNA), lipoproteins, fatty acid production, and steroid hormones (Hyttel et al., 1997). The completion of cell development depends on each of these modifications. masculine pronucleus growth factor (MPGF) is a protein that regulates the activity of several proteins and organelles and acts to promote cytoplasmic maturation. Furthermore, a protein called maturation-promoting factor (MPF) encourages cells to enter the cell cycle and grow until they are ready to fertilize (Hyttel et al., 1997).

There are also many important substances, including sodium pyruvate (Arlotto et al., 1996), sodium lactate, glutamine (Fukui, 1990), and glucose, which help accelerate the process of nuclear maturation. Also, sodium bicarbonate (Younis et al., 1989) epidermal growth factor (EGF)(Blanco & Simonetti, 2002) has a significant impact on oocyte growth.

2.1.6 Fertilization

A zygote is created when a female ovum and a male sperm combine. As soon as the sperm is deposited in the vagina, the oocyte and sperm must go through important alterations for fertilization to take place. To facilitate its migration to the fallopian tube, sperm undergo capacitation, which increases their motility and metabolism. The vagina's acidic environment is the cause of capacitation. It causes the sperm's cytoplasm to become active with adenosine triphosphate (ATP) enzymes. One of the two modifications the sperm undergoes during capacitation is an alteration in

the lipid and glycoprotein content of the plasma membrane, which makes the process significant (De Jonge, 2017).

The second modification facilitates matrix penetration. It is challenging to pierce the extracellular matrix of an oocyte. Important lysosomal enzymes are found in the sperm acrosome. These enzymes are thought to be produced during exocytosis and are necessary for the oocyte to penetrate (Okabe, 2014). The cells contained in the hyaluronic acid surrounding the oocyte are broken down by the acrosome's hyaluronidase enzyme. The sperm's inner membrane contains acrosin, which is exposed by this process. It takes acrosin to break down the zona pellucida (ZP). It is essential that no further sperm pass through the ZP once the acrosome reaction occurs to ensure that the proper number of chromosomes is available and that a trisomy zygote does not form. An elevated level of calcium is caused by the sperm's acrosome fusing with the ZP. An increase in calcium causes CGs, which are secretory vesicles, to release their contents, changing the extracellular matrix. By breaking down the sperm receptor glycoproteins ZP2 and ZP3, which prevent spermatozoon binding, the cortical granules release enzymes that render them impermeable to sperm entry (Georgadaki et al., 2016).

2.1.7 *In vitro* fertilization (IVF)

An essential step in the IVF process is capacitation. Consequently, glycosaminoglycan serves as an energy source that stimulates the motility of sperm and conditions to enable fertilization, such as caffeine and heparin. TALP and bracket and oliphant (BO) are two often-used IVF formulas. The optimal time is 9–18 hours (Ward et al., 2003).

2.1.8 Sperm preparation

Potentially fertile spermatozoa actively migrate through the cervical mucus in the female genital tract under *in vivo* conditions to differentiate from immotile spermatozoa, detritus, and seminal plasma (Mortimer, 1989). Male germ cells go through physiological changes known as capacitation during this process, which is necessary for the sperm to be functionally competent in terms of the acrosome reaction in addition to the selection of progressively motile spermatozoa (Bedford, 1983). Andrology to comprehend the physiology of male germ cells and provide new

methods for distinguishing viable spermatozoa from immotile, abnormally morphological, or incapable of fertilizing oocytes. Beginning with the basic step of washing spermatozoa, separation techniques based on various concepts.

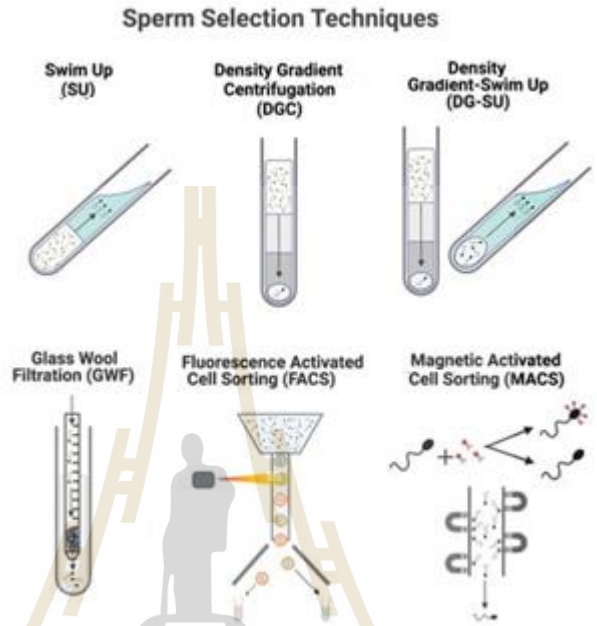


Figure 2.4 Selection techniques used to select quality sperm include swim-up (SU), density gradient centrifugation (DGC), density gradient-swim up (DG-SU), glass wool filtration (GWF), fluorescence activated cell sorting (FACS), and magnetic activated cell sorting (MACS) (Orsolini et al., 2021).

1) Swim-up (SU)

Sperm separation via SU from a washed pellet is the most traditional and widely utilized technique. This technique, which was first detailed by Mahadevan and Baker (1984), is still widely employed in IVF facilities worldwide. Spermatozoa actively migrate into an overlying medium from the pre-washed cell pellet according to the standard SU technique. The incubation period is normally 60 minutes. The technique is characterized by a high proportion of motile sperm (>90%).

Potentially motile spermatozoa in the lower levels of the pellet may never reach contact with the medium layer due to the numerous cell layers in the pellet. Furthermore, following the SU process, a noteworthy reduction in the proportion of spermatozoa with typically compacted chromatin has been documented (Henkel et

al., 1994). The fact that spermatozoa are pelleted during its use, putting them in close contact with leukocytes, cell debris, and each other all of which are known to create extremely high amounts of ROS (Ford, 1990). The sperm's plasma membranes have an exceptionally high concentration of polyunsaturated fatty acids (Aitken & Clarkson, 1987), which results in lipid peroxidation and a sharp decline in sperm functions, such as motility (Mortimer, 1991).

The advantages of SU include its ease of use, cost-effectiveness, and restriction to ejaculates with high sperm count and motility. Nevertheless, ROS can severely harm spermatozoa, low yield, and a significant decrease in the percentage of normally chromatin-condensed spermatozoa.

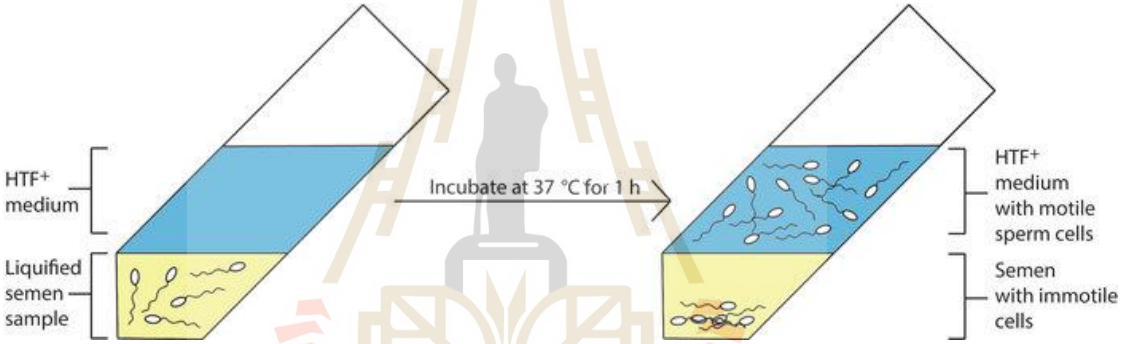


Figure 2.5 SU purification of motile sperm cells (Rehfeld et al., 2019).

2) Migration-sedimentation (MS)

Tea et al. (1984) developed MS, a more advanced sperm-separating technology. This approach consists mostly of a SU technique with a sedimentation step. There are specific glass or plastic tubes that have an interior cone. Unlike in the traditional SU process, within 1 hour, spermatozoa swim up directly from the liquid semen into the supernatant medium and then sediment in that inner cone. As a result, this approach is very gentle, especially when compared to other approaches like the traditional SU that call for centrifugation stages prior to sperm separation. A portion of extremely motile and functionally competent spermatozoa can be acquired in the original version. Unfortunately, because of the extremely low yield, the original IVF technique was not widely accepted.

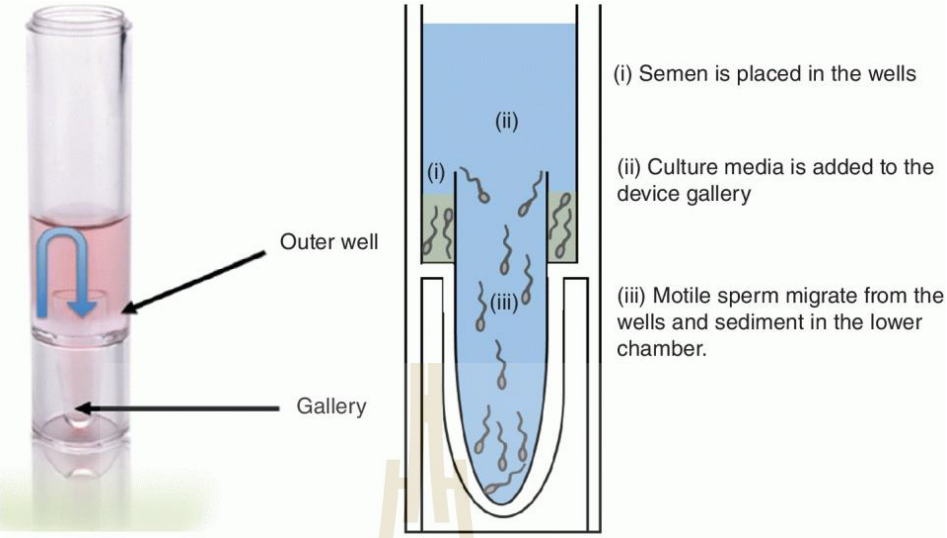


Figure 2.6 The migration sedimentation chamber (MSC)(Ramos et al., 2015).

3) Density gradient centrifugation (DGC)

DGC is a commonly used sperm separation method in assisted reproduction (Bolton & Braude, 1984; Pousette et al., 1986). It employs either discontinuous or continuous gradients, with highly motile sperm accumulating at the bottom due to their aggressive migration through the gradient (Harrison, 1976). While Ficoll® was initially used, Percoll® is made of polyvinylpyrrolidone (PVP)-coated silica particles became more prevalent despite its drawbacks such as endotoxin contamination and membrane alteration (Andersen & Grinsted, 1997; Arcidiacono et al., 1983; De Vos et al., 1997; Scott & Smith, 1997; Strehler et al., 1998)

An alternative to Percoll® is Nycodenz, which has shown promise in producing highly motile spermatozoa, particularly from low-quality semen samples (Gellert-Mortimer et al., 1988). Substitutes like Isolate®, PureSperm®, IxaPrep®, and SilSelect® are made of silane-coated silica particles and offer improved biocompatibility compared to Percoll® (Strehler et al., 1998). Nitric oxide levels significantly decrease after IxaPrep® preparation, possibly due to guanylyl cyclase activation (Chan et al., 1990; Rosselli et al., 1995). However, results from different studies vary due to diverse sperm separation variables, underscoring the importance of standardization (Chen & Bongso, 1999; Claassens et al., 1998; Ding et al., 2002; Makkar et al., 1999; McCann & Chantler, 2000; Söderlund & Lundin, 2000; Yang et al., 1998).

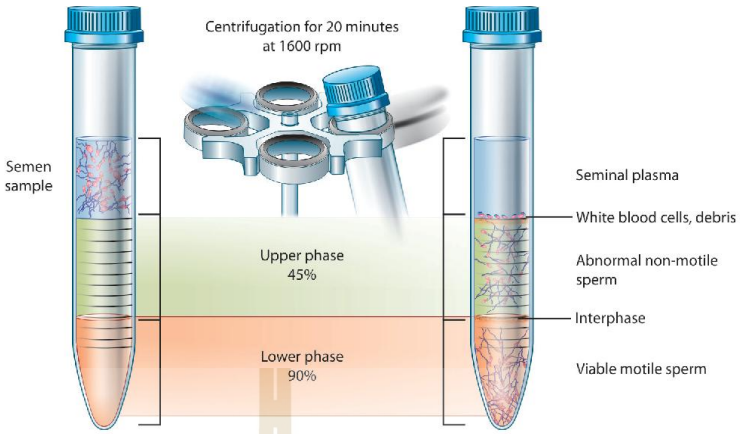


Figure 2.7 DGC depending on their motility and density, the immature, aberrant sperm are visible along the gradient. Highly mobile normal sperm actively descends the gradient and is collected as a pellet (Beydola et al., 2013).

4) Combination Density Gradient-Swim Up (DG-SU)

Additionally, sperm have been pelleted using DGC and allowed to swim upward through an overlaying medium in a technique known as DG-SU. It has been demonstrated that DG-SU selects for a population with greater overall DNA integrity than DGC alone, enriches for motility and morphology more effectively than SU alone, and lowers the proportion of ultrastructural defects in the selected sample (Ng et al., 1992; Yamanaka et al., 2016). This technique has also been demonstrated to be a successful means of separating healthy spermatozoa from contaminated semen and eliminating pathogens such the cattle viral diarrhea virus and equine arteritis virus (Galuppo et al., 2013; Morrell & Geraghty, 2006). As a result, this approach may be very beneficial for other illnesses that can be spread via ejaculate.

5) Glass wool filtration (GWF)

Densely packed GWF are used to differentiate motile spermatozoa from immotile sperm cells (Van der Ven et al., 1988). The self-propelled mobility of the spermatozoa and the filtering effect of the glass wool are the fundamental components of this sperm separation technology. The type of glass wool used has a direct bearing on how well this technique works (Sánchez et al., 1996). Therefore, it is necessary to take into account elements such as the chemical makeup of the glass (such as borate, silicate, or quartz glass), the surface structure and charge of the glass wool, the thickness

of the fibers in the glass wool, and the filter's pore size. Glass wool (code number 112) from Manville Fiber Glass Corp. (Denver, CO, USA) and SpermFertil® columns (code number Mello; Holzhausen, Germany) have undergone thorough testing in clinical settings. The type of glass wool used and the degree of cleaning before filtration are the main factors that determine potential dangers associated with the procedure, such as injury to the spermatozoa or the presence of glass wool pieces in the filtrate.

GWF, like DGC, uses the entire volume of the ejaculate and produces a much larger total number of motile spermatozoa when compared to SU or migration-sedimentation methods (Berger et al., 1985; Henkel et al., 1994). The benefit of GWF, similar to DGC, is that sperm separation can be done straight from the ejaculate. Centrifugation will not be required to extract the seminal plasma until the motile spermatozoa have been separated from the leukocytes and detritus. This is a crucial step because it lessens the harm that ROS does to cells. GWF has been demonstrated to remove leukocytes to a degree of up to 90% in addition to spermatozoa separation (Henkel et al., 1997). Free radicals in the ejaculate are greatly reduced by this impact since leukocytes are abundant even in normal ejaculates (Wallach & Wolff, 1995) and create 100 times more ROS than spermatozoa (Ford, 1990). This is critical to the functioning of spermatozoa because the very high concentration of polyunsaturated fatty acids in the plasma membrane of male germ cells makes them especially vulnerable to oxidation by ROS (Aitken & Clarkson, 1987; Iwasaki & Gagnon, 1992; John Aitken et al., 1989).

6) Fluorescent Activated Cell Sorting (FACS)

Numerous quality parameters in ejaculate, including membrane integrity, ROS generation, capacitation, acrosome reaction, mitochondrial state, apoptotic markers, and deoxyribonucleic acid (DNA) integrity, are commonly examined using flow cytometry (Martínez-Pastor et al., 2010). FACS has emerged to recover living cells, overcoming the need for fixatives in flow cytometry. FACS operates by selectively removing damaged cells from a sample using fluorescent stains and dyes that bind to sperm based on specific sorting criteria (negative selection). Various indicators such as membrane permeability, apoptotic markers, mitochondrial membrane potential, and

sex chromosomes have been utilized for flow-sorting live sperm (Chaveiro et al., 2007; Funaro et al., 2013; Hoogendijk et al., 2009; Johnson, 2000; Sousa et al., 2011).

FACS finds widespread use in sperm selection, notably in sex selection across diverse animal species. However, it has been noted that FACS sorting can induce oxidative and DNA damage in spermatozoa despite its advantages (Balao da Silva et al., 2016). Moreover, the high-pressure throughput of FACS may subject sperm to mechanical stress, while exposure to lasers and dyes can impair mitochondrial activity and motility in bovine sperm (Carvalho et al., 2010; Garner, 2006). Consequently, varying combinations of dyes, laser wavelengths, and flow pressures may lead to mechanical and functional stress induction. Additionally, flow cytometry's expense and time-consuming individual cell characterization make it less favorable for routine selection (Aurich & Schneider, 2014; Rath et al., 2013; Rath & Johnson, 2008).

7) Magnetic Activated Cell Sorting (MACS)

Using magnetic nanoparticles (MNP) to select viable spermatozoa based on various quality parameters, known as MACS, is a relatively recent method (Grunewald et al., 2001; Said et al., 2008). These nanoparticles, typically less than 100 nm in diameter, can be coated with different magnetic compounds and linked to various biomarkers indicative of sperm's physicochemical qualities (Durfey et al., 2019; Falchi et al., 2018). Iron oxide (Fe_3O_4) MNP, due to its versatile applications, is a popular choice for magnetic conjugation (Falchi et al., 2018; Huang & Juang, 2011). By subjecting spermatozoa-incubated magnetized conjugates to a magnetic field, physiologically relevant selection is achievable (Grunewald et al., 2001; Said et al., 2008). MACS has been utilized for high-quality sperm selection in humans, pigs, cows, and donkeys, identifying characteristics associated with apoptotic and prematurely acrosome-reacted sperm, leading to improved fertilization and embryo development (Durfey et al., 2019; Faezah et al., 2014; Grunewald et al., 2001; Grunewald et al., 2006; Said et al., 2008; Yousef et al., 2020).

Specifically, paramagnetic microbeads conjugated to Annexin-V have been employed to selectively remove human spermatozoa undergoing apoptosis, resulting in improved sperm quality without adverse effects (Grunewald et al., 2001; Paasch et al., 2003). For pigs, MACS utilizing MNP conjugated with Lectin and Annexin-V

has enhanced motility, and the use of negatively selected sperm for artificial insemination has shown no harmful effects (Durfey et al., 2019; Feugang et al., 2015). Furthermore, Peanut agglutinin (PNA)-lectin conjugated nanoparticles have improved donkeys' sperm motility and membrane viability by removing damaged spermatozoa (Yousef et al., 2020). MACS has also been effective in enhancing the population of X spermatozoa with high accuracy and is suggested as an alternative to flow cytometry sex sorting (Domínguez et al., 2018). Notably, MACS sex-sorted semen has shown high motility and viability without DNA damage or premature capacitation (Domínguez et al., 2018). Despite its effectiveness, MACS application in the industry remains limited.

2.1.9 *In vitro* culture (IVC)

Typically, between 20% and 40% of the cultured embryos will progress to the blastocyst stage (Rizos et al., 2008). Ideally, the culture medium utilized during IVC exerts significant effects on embryo quality and cryotolerance (Rizos et al., 2002). However, IVC-supplemented medium is designed to support embryo development, enhance blastocyst formation rates, and improve both embryo quality and cryopreservation outcomes (Orsi & Reischl, 2007).

In comparison to the absence of FCS supplementation in IVC medium at a concentration of 10–20%, it was observed that more embryos progressed to the blastocyst stage (Duque et al., 2003; Hasler, 2000). However, this led to a decline in embryo quality, affecting factors such as compaction, post-freezing survival rate, and large offspring. The abnormal DNA methylation, characterized by methyl (CH_3) attachment to DNA-controlling growth genes, was identified as a contributing factor. This methylation inhibits the function of the synthesized genes, resulting in abnormal acceleration or enlargement of growth, which may manifest during preimplantation or fetal stages (Niemann & Wrenzycki, 2000; Young et al., 2001). Moreover, compared to the absence of FCS supplementation, an increase in lipid droplets sized 2-6 μm was noted, accompanied by a higher number of immature mitochondria, consequently leading to a reduced survival rate after freezing (Abe et al., 2002).

Lactate and pyruvate serve as vital energy sources within the IVC medium (Takahashi & First, 1992). Across the developmental stages spanning from cleavage to blastocyst, pyruvate and lactate are indispensable for the progression of

the bovine embryo (Biggers et al., 1967; Rosenkrans Jr et al., 1993; Takahashi & First, 1992). As reported, supplements containing growth hormone and EGF have been identified. These proteins possess the capacity to stimulate cell development or regulate the proliferation and differentiation of cells(Teruel et al., 2000).

Supplementation with essential amino acids (EAAs) and non-essential amino acids (NEAAs) has been found to enable embryos to develop to the blastocyst stage closely resembling those cultured with oviductal epithelial cells (OECs)(Seshagiri & Bavister, 1991; Takahashi & First, 1992). Co-culture with OECs aids in reducing oxygen toxicity (Nagao et al., 1994), generating growth factors (Edwards & Beard, 1997), and modulating the environment within the culture medium to mimic the conditions in the uterus. Consequently, embryos are able to reach the blastocyst stage up to 42% when compared to co-culture with fibroblasts, which only achieved 4.5% development (Gliedt et al., 1996).

The natural carbon dioxide (CO_2) concentrations in the uterus and fallopian tubes are low, blastocysts tend to develop more effectively in environments with CO_2 levels ranging between 5% and 7% as opposed to 20% (Van Soom, Tanghe, et al., 2002; Yuan et al., 2003). High concentrations of CO_2 lead to increased apoptosis (Van Soom, Tanghe, et al., 2002), DNA damage (Takahashi et al., 1996), and oxidative stress due to the production of hydrogen peroxide (H_2O_2), which inhibits embryo development (Legge & Sellens, 1991). This issue can be addressed by supplementing antioxidants such as thioredoxin, superoxide dismutase (Nonogaki et al., 1991), ethylenediaminetetraacetic acid (EDTA), catalase (Vermeiden & Bast, 1995), vitamin C, and vitamin E (Olson & Seidel Jr, 2000) into the culture medium.

2.1.10 Embryo

Substantial developments occur at the zygote's cellular level following fertilization. The zygote is a single cell that divides into numerous cells via mitosis. The zygote transforms into a morula when it reaches the 32-cell stage. On the fourth day, blastulation starts, and hollow balls first form before cavities start to form. According to some research, implantation may be impacted by the time of this procedure (Franasiak et al., 2018).

The inner and outer cell types are now distinct from one another. The outside cells are referred to as trophoblasts (TE), which subsequently aid in the formation of the placenta, while the inner cells are known as the inner cell mass (ICM), which gives rise to the embryo. The inner cell mass will undergo further differentiation into hypoblasts and epiblasts. The amniotic sac will develop from the epiblast, while the primitive yolk sac will arise from the hypoblast. The entity is a blastula during this phase, and the ZP has disappeared to allow for growth and differentiation.

The third week, called the gastrulation phase, is when tubes start to form. Planar polarity, chemokinesis, chemotaxis, and differential cell adhesion all influence movement during gastrulation (Solnica-Krezel & Sepich, 2012). Three layers of cells will eventually become the various organ systems, such as endoderm, mesoderm, and ectoderm. The brain, spinal cord, peripheral nervous system, nails, hair, and epidermis are all made of ectoderm. The notochord, kidney, gonads, circulatory system, muscle, bone, and connective tissue are all formed by mesoderm. The endoderm creates the epithelial lining of the pancreas, liver, stomach, colon, and digestive system (Oliver & Basit, 2023).

2.1.11 Evaluation and classification of bovine embryos

Bo and Mapletoft (2013) in Chapter 9 of the International Embryo Transfer Society (IETS) Manual outline standardized coding systems for describing embryo quality and developmental stage. Appendix D provides illustrations for reference. The developmental stages are indicated by numbers as follows:

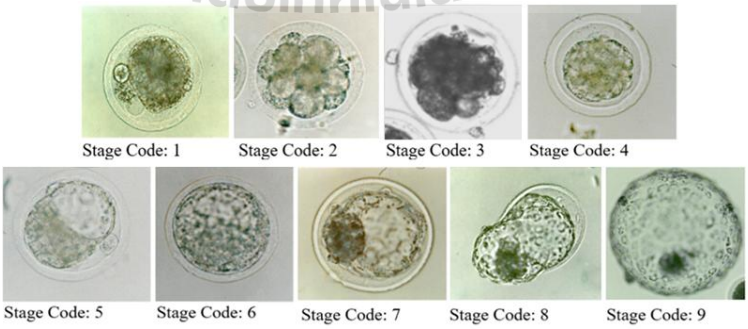


Figure 2.8 Standardized coding systems that can be used to describe the developmental stage of the embryo (Stages Code: 1–9)(Bo & Mapletoft, 2013)

Table 2.1 Standardized coding systems that can be used to describe the developmental stage of the embryo (Stages Code: 1–9) (Bo & Mapletoft, 2013)

Code	Name Stage	Developmental stage
1	1-cell	Unfertilized oocyte or 1-cell embryo
2	Cleavage	Embryo unable to develop
3	Morula	Minimum of 16 cell mass, challenging to distinguish individual blastomeres
4	Compact morula	Coalescence of blastomeres, 60–70% perivitelline space occupied
5	Early blastocyst	Resembles signet ring, blastocele present, 70–80% perivitelline space occupied
6	Blastocyst	Clear distinction between ICM and TE layers, blastocele noticeable
7	Expanded blastocyst	Sharp increase in diameter, thinning ZP
8	Hatching blastocyst	One-third reduction in ZP thickness, may be hatching or collapsed
9	Hatched blastocyst	Without ZP, identification may be challenging without re-expansion

The quality codes assigned to embryos are based on their morphological integrity. Here are the codes indicating embryo quality, ranging from code 1 to 4:

Excellent or Good (Quality Code 1): Embryos are spherical, symmetrical masses with uniformly sized, colored, and dense blastomeres. At least 85% of the cellular material should be intact, with minor irregularities. Smooth ZP is essential. Grade 1 embryos are recommended for freezing, thawing, and international trade.

Fair (Quality Code 2): Embryos show some variation in cell size, color, and density, with around 50% intact embryonic bulk. While less suitable for freezing/thawing than Grade 1, they are still transferable for fresh transfer.

Poor (Quality Code 3): Significant abnormalities in cell size, color, and density, with at least 25% of the mass not surviving intact. Lower pregnancy rates are expected compared to fair-quality embryos.

Dead or degenerating (Quality Code 4): Not viable and should be discarded, regardless of their form (1-cell, oocyte, or embryonic).

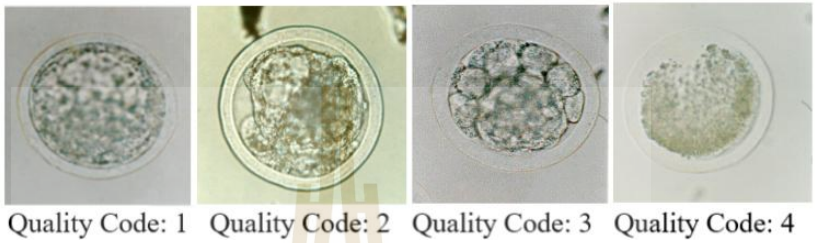


Figure 2.9 Standardized coding systems that can be used to describe the morphological integrity of embryos (Quality Code: 1–4) (Bo & Mapletoft, 2013)

2.2 Cryopreservation

Embryo cryopreservation employs two primary techniques including slow freezing and vitrification, both utilizing similar cryoprotectant agents (CPAs) (Martínez-Burgos et al., 2011). CPAs, predominantly non-electrolyte substances, include sugar-based compounds (Koster et al., 2000). However, sugars, unable to permeate cell membranes, elevate extracellular salt concentrations, posing osmotic stress risks pre-freezing (Meryman, 1968, 1971). CPAs like ethylene glycol (EG), dimethyl sulfoxide (DMSO), and glycerol (GLY), capable of membrane permeation, are thus preferred over sugar-based CPAs.

CPAs function by extracting water from cells in a hypotonic solution, maintaining pressure levels upon entering cells, thereby averting osmotic stress and ice crystal formation (Songsasen et al., 2002). Controlling CPA concentration during freezing and thawing is crucial to prevent excessive cell shrinkage and re-swelling-induced damage (Chaveiro et al., 2006). CPA toxicity manifests in specific protein disruptions. Concentrations exceeding 30% can compromise gap junction integrity, leading to oocyte developmental arrest and demise, while concentrations of certain CPAs like Propanediol (PROH) above 0.5M induce intermediate filament disintegration (Hurtt et al., 2000). DMSO disrupts cytoskeleton fiber and glycolytic pathway function, especially

above 10% concentration (Aman & Parks, 1994; George et al., 1996; Parks & Ruffing, 1992; Vincent et al., 1990). Combining CPAs like EG and DMSO can mitigate their toxicity.

The damages observed from freezing include structural damage to cell membranes, cell functionality impairment, cell shock from sudden osmotic pressure changes (osmotic-shock), toxicity from CPAs, and the formation of intracellular and extracellular ice crystals. Reports on oocyte culturing in phosphate-buffered saline (PBS) with varying osmotic pressures show that at 290 milliosmoles (mOsm) for 20 minutes, development to the blastocyst stage is higher compared to other osmotic pressure levels (Agca et al., 2000). Supplementation with CPAs in sugar-based solutions like sucrose or trehalose can prevent osmotic shock and reduce intracellular water volume to prevent intracellular ice crystal formation. However, excessive water removal can damage cell membranes and lead to cell death (Orief et al., 2005).

Cryopreservation induced damage from freezing and cold shock when cells are stored below body temperature (Smith, 1950), affecting the function of lipids and proteins of the cell membrane, that lipid phase transitions from liquid to solid state, impairing membrane function (Gao & Critser, 2000; Lin et al., 2013). Additionally, damage from ROS generated during cryo-capacitation reaction at reduced temperatures may occur (Gupta et al., 2010). Moreover, the cooling process can disrupt cellular homeostasis, affecting microtubules, tubulin, and spindle fibers, consequently impairing cell division function and resulting in decreased developmental rates post-thaw (Roser Morató et al., 2008).

2.2.1 Slow freezing

Slow freezing embryo cryopreservation involves immersion in a cryoprotective solution and gradual reduction of temperature below the freezing point. During certain temperature reduction stages, ice crystal formation may occur, along with the preservation of cells or solution components in an unfrozen state (Bianchi et al., 2012). The concentration of solutes such as sugars, salts, and CPAs increase in the unfrozen fraction, leading to osmotic pressure buildup and water efflux from cells during continuous temperature reduction. The unfrozen fraction experiences increased viscosity and transitions into an amorphous solid state without internal ice crystal formation (Chen & Yang, 2009; Chen & Cui, 2006; Martínez-Burgos et al., 2011). However,

increased salt concentration within cells leads to decreased structural integrity of cell membranes, and protein denaturation occurs (Crowe & Crowe, 1984; Hvidt & Westh, 1992; Lovelock, 1953; Tanford, 1980).

An unstable liquid state, known as supercooling, occurs during temperature reduction below the freezing point (Seki & Mazur, 2011). Still, ice nucleation does not occur at the freezing point due to lower ice nucleation temperatures compared to ice, attributed to higher surface tension (Gao & Critser, 2000). With continuous temperature reduction to -5°C to -15°C, ice nucleation occurs. Moreover, the likelihood of intracellular ice formation increases with increased supercooling (Jin et al., 2016; Mazur et al., 2005). To minimize supercooling, nucleation induction (seeding) can be initiated (Liebermann et al., 2003). Additionally, ice nucleating agents supplementation with freezing media facilitates water molecule orientation into an ice-like structure, forming active germ crystals (Morris & Acton, 2013; Petersen et al., 2006).

Temperature reduction in slow freezing affects survival rates because the movement of water through cell membranes occurs slowly due to the membrane's resistance to permeation (Mazur et al., 1972). As the temperature decreases, changes within the cell occur more slowly compared to the external environment, leading to intracellular ice formation if the temperature decreases too rapidly. Conversely, if the temperature decreases too slowly, excessive water extraction can damage cell membranes (Smith et al., 2011). The temperature reduction rate depends on the surface area-to-volume ratio, cell membranes' permeability to water influx and efflux, and the permeability of CPAs into cells (Mazur, 1963).

2.2.2 Vitrification

Vitrification is the rapid transformation of a liquid into a glass-like solid, free from ice crystal formation (Wowk, 2010), using concentrated CPAs. Achieved by exceeding a temperature reduction rate of -10,000°C/min, swiftly traversing the temperature range of 15°C to -5°C or the critical temperature zone (CTZ). This process minimizes ice crystal formation inside and outside cells, ensuring non-toxicity of CPAs. Additionally, it prevents lipid aggregation and extrusion from cell membranes, preserving zona pellucida and cytoskeleton fiber integrity (Vajta, 2000). Simultaneously,

the use of highly concentrated CPAs may result in osmotic shock (Steif et al., 2007), water loss (Wolfe & Bryant, 1999), or CPA toxicity (Wusteman et al., 2002). Equilibration can mitigate the impact of osmotic shock and CPA toxicity (Taniguchi et al., 2011), including the use of multiple types of CPAs or additives such as BSA (Van Wagtendonk-de Leeuw et al., 1997) to mitigate toxicity effects. Vitrification results in higher post-thaw survival and developmental rates (Massip, 2003).

Currently, there are advancements in equipment and methods for temperature reduction to enhance the cooling rate and reduce the volume of solution. Examples include open-pulled straws (OPS)(Vajta et al., 1998), electron microscope grids (Martino et al., 1996), hemi-straws (Liebermann & Tucker, 2002), cryoloops (Succu et al., 2007), solid surface vitrification (SSV)(Roser Morató et al., 2008), and polypropylene strips (cryotop) (Kuwayama, 2007). Among these, cryotop is capable of reducing the volume of solution to as little as 0.1µl before cryopreservation and has shown superior performance compared to OPS and SSV (Liu et al., 2008; R. Morató et al., 2008). Additionally, rates of cryotop vitrification, cooling, and warming were 69,000 and 118,000°C/min, respectively (Mazur & Seki, 2011).

2.3 Reactive oxygen species (ROS)

Oxygen (O_2) and nitrogen molecules with one or more unpaired electrons are referred to as "reactive species." When a molecule is called a "free radical," it means that one or more unbound electrons are trying to restore electron stability by oxidizing with other molecules, which makes the molecule extremely reactive (Phaniendra et al., 2015). Free radicals can react with almost any type of biomolecule, although the macromolecules that are most frequently oxidized by them are proteins, lipids, and nucleic acids (Nandi et al., 2019).

O_2 free radical species that are known to cause cell damage include alkoxyl, peroxyl, hydroperoxyl, superoxide anion (O_2•–), hydroxyl radical (•OH), and hydroperoxyl radicals (Halliwell & Whiteman, 2004; Phaniendra et al., 2015). Although all of these ROS can be produced in different parts of the cell, mitochondria are the primary source. A steady, low rate of electron escape from the proton gradient produced by electron transport occurs during the generation of ATP (He et al., 2017). These electrons will quickly react with O_2 to produce O_2•–. This ROS will be converted

to H_2O_2 by an enzyme known as superoxide dismutase (SOD), which is mostly found in the inner membrane or matrix of the mitochondria (Rasouli et al., 2024). This nonradical can pass via peroxiporins, which are particular aquaporin channels, and cross the mitochondrial membrane. The damage induced by •OH radical garners significant attention due to its swift degradation of nucleic acids, proteins, lipids, and carbohydrates. This peril is heightened by the absence of known enzymatic processes identified to inactivate •OH. Moreover, •OH is generated through reactions involving O_2•– with metal ions and iron–sulfur clusters in the presence of H_2O_2, as well as via the reaction of H_2O_2 with chlorine ions (Cl–) or transition metals like copper (Cu) or iron (Fe) in Fenton and Haber–Weiss reactions, amplifying its reactivity (Fenton, 1894; Haber et al., 1934; Liemburg-Apers et al., 2015).

2.3.1 Effect of ROS

Exposure to ROS is a contributing factor in diminishing the efficacy of *in vitro* embryo production, such as reduced post-transfer pregnancy rates, increased pregnancy loss, chromosomal abnormalities, and large offspring syndrome (Ealy et al., 2019; Hansen, 2020; Kępka et al., 2023; Tšuiko et al., 2017). Although minute quantities of these highly reactive molecules are naturally generated through regular cellular processes, their levels escalate during embryo culture. This escalation is primarily attributed to various environmental stressors encountered by oocytes and embryos, such as fluctuations in temperature, exposure to light, pH variations, atmospheric oxygen levels, suboptimal compositions of culture media, and the process of cryopreservation (Ramos-Ibeas et al., 2019).

In unregulated conditions, ROS inflict damage on lipid membranes, induce alterations in DNA methylation patterns, interfere with protein functionality, and adversely affect the integrity and functionality of both nuclear and mitochondrial DNA (mtDNA) (Keane & Ealy, 2024; Sturmey et al., 2008). The DNA damage from ROS leads to base structure alterations (Sturmey et al., 2008), resulting in single-strand breaks or more severe double-strand breaks. Specifically, oxidative damage primarily yields 8-oxodeoxyguanosine (8-oxo-dG) via •OH reaction with deoxyguanosine at the C8 position. Hydroxyl radicals also target imidazole ring-opened products, specifically the C5-C6 double bond of pyrimidines and C8-C5 bonds of purines (Gros et al., 2002; Haghdoost

et al., 2005). mtDNA is highly vulnerable due to its proximity to ROS production and the absence of protective histones (Ames et al., 1993). Oocytes, with their abundance of mitochondria exceeding 100,000 compared to 1000–2500 in other cells, are particularly prone to oxidative damage (Malott et al., 2022).

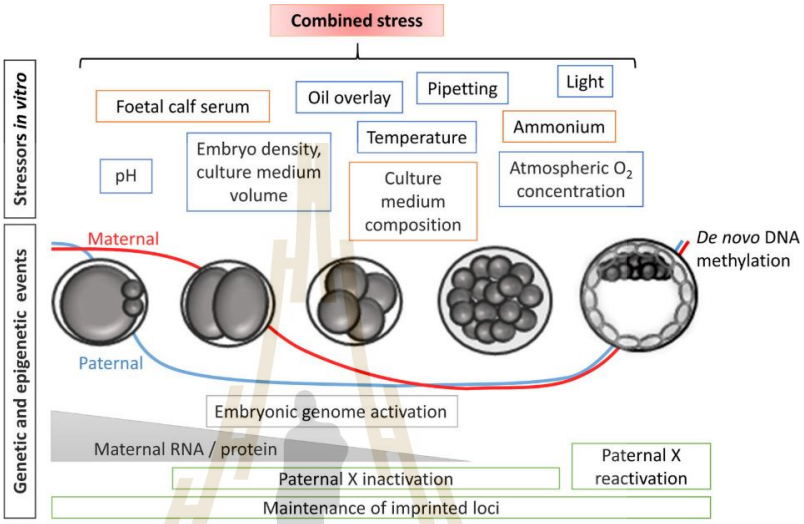


Figure 2.10 Overview of the important chemical (orange) and physical (blue) stresses affecting the embryo *in vitro*, as well as the sensitive genetic and epigenetic processes taking place during preimplantation embryo development (Ramos-Ibeas et al., 2019).

ROS primarily targets lipid bilayers within cells, including those of the mitochondria, nucleus, and other organelles. The main ROS implicated in this damage is •OH, initiating peroxidation reactions within lipid methylene groups (Pratt et al., 2011). Consequently, lipid peroxidation compromises membrane fluidity, disrupts functional integrity, and impairs the activity of membrane-bound proteins such as receptors and ion channels. Another significant source of ROS-induced lipid damage occurs through the reaction of various ROS with O_2, yielding peroxyl radicals that react with polyunsaturated fatty acids to form hydroperoxide (HOO–) and alkyl radicals (Juan et al., 2021). This process triggers a concerning feed-forward reaction, as HOO– and alkyl radicals further propagate radical formation by reacting with nearby fatty acids (Marnett, 1999). Excessive ROS production further compromises mitochondrial integrity by affecting mitochondrial permeability transition (mPT) pores. While low conductance is

reversible, high conductance leads to irreversible transmembrane potential alterations, triggering apoptosis via cytochrome-c release and subsequent caspase pathway activation (Wang et al., 2009).

Protein damage induced by ROS occurs through multiple pathways. Initially, •OH reacts to produce an alkyl radical, which in turn reacts with O_2 to form a peroxide radical. Subsequent reaction with adjacent proteins yields HOO– and alkyl radicals, capable of generating alkoxy radicals (Juan et al., 2021). Oxidative stress, mediated by •OH, leads to protein modification, conformational loss, and amino acid side chain oxidation, as well as protein cross-linking and fragmentation (Butterfield et al., 1998; Dean et al., 1997; Juan et al., 2021). Such modifications induce conformational alterations, potentially resulting in protein aggregation, distortion of secondary and tertiary structures, and impairment of normal function (Butterfield et al., 1998). Enzymatic activity may be compromised due to conformational changes, exacerbating the impact of oxidative stress (Juan et al., 2021). The levels of ROS damage and abundance are tightly controlled by intrinsic enzymatic pathways, and antioxidants play a crucial role in mitigating the reactivity of ROS by interacting with them to diminish their detrimental effects.

2.3.2 Systems for Mitigating Intrinsic ROS

Three key enzymes play a crucial role in reducing ROS accumulation within cells. Firstly, SOD catalyzes the conversion of O_2•– to O_2 and H_2O_2. There are three isoforms: SOD1 (Cu/Zn-SOD) located in the cytoplasm and nucleus, SOD2 (Mn-SOD) localized in mitochondria, and SOD3 (EC-Cu/Zn-SOD) found extracellularly (Sies & Jones, 2020). SOD1 and SOD3 utilize copper (Cu^{2+}) as a cofactor in a two-step process, where Cu^{2+} is reduced to Cu^{1+} to oxidize an O_2•– molecule to O_2, and then Cu^{1+} is oxidized back to Cu^{2+} while reducing a second O_2•– molecule into H_2O_2 (Eleutherio et al., 2021). SOD2 employs iron as a cofactor to achieve similar results (Ganini et al., 2018).

The second enzyme system involved in ROS mitigation is catalase (CAT), which further reduces H_2O_2 generated by SOD into H_2O and O_2 (Nandi et al., 2019).

The third group of ROS-mitigating enzymes utilizes glutathione (GSH), a tripeptide thiol antioxidant synthesized by most cells through two ATP-dependent

reactions: First, involving glutamate cysteine ligase and second adding glycine and γ-glutamylcysteine. The availability of cysteine usually governs the rate-limiting step in GSH synthesis (De Matos et al., 2002; Franklin et al., 2009). GSH renewal occurs via the γ-glutamyl cycle, where glutathione peroxidase (GPx) enzymatically oxidizes GSH to remove H_2O_2, generating water molecules. The oxidized form of GSH, GSH disulfide (GSSG), is then enzymatically reduced back to GSH by GSH reductase (Couto et al., 2016).

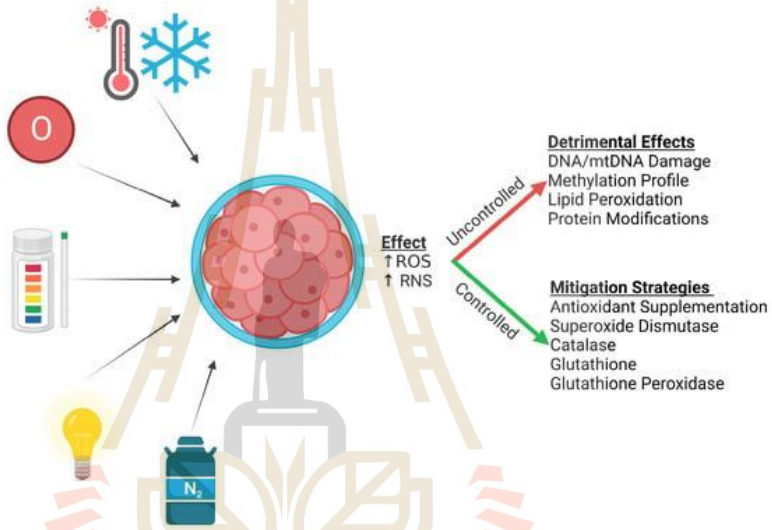


Figure 2.11 Overview of several ROS factors that assault IVP bovine oocytes and embryos; damage may occur when uncontrolled and controlled by intrinsic mitigation pathways or antioxidant supplementation (Keane & Ealy, 2024).

2.3.3 Antioxidant

Under physiological conditions, oocytes are equipped with antioxidant defense mechanisms, both enzymatic and non-enzymatic, which regulate excessive free radical generation (Combelles et al., 2009). However, various internal and external factors disrupt the redox balance of oocytes, leading to fluctuations in ROS production and intracellular antioxidant levels (Li et al., 2016; Somfai et al., 2007; Succu et al., 2018).

Antioxidants encompass enzymes such as superoxide dismutase, catalase, and peroxidase, as well as thiol compounds like β-mercaptoethanol and cysteamine, vitamins such as ascorbic acid and α-tocopherol, flavonoids like resveratrol, and amino acids and derivatives such as L-proline and melatonin. Among these, α-tocopherol, melatonin, L-proline, and resveratrol have been identified to shield oocytes from injuries induced by vitrification (Chinen et al., 2020; Sovernigo et al., 2017).

1) Cysteine

Cysteine serves as the pivotal amino acid in GSH biosynthesis. Externally, cysteine is unstable and prone to auto-oxidation, forming cystine (Van Soom, Yuan, et al., 2002). Nonetheless, within the cell, cystine can be regenerated to cysteine through reactions with β-mercaptoethanol or cysteamine (Caamano et al., 1996; De Matos et al., 2002). N-acetyl cysteine (NAC) is commonly used to supplement cysteine levels, as it is deacetylated within the cell to form cysteine. Additionally, NAC directly acts as an electron donor due to its thiol group, facilitating reactions with hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), and other ROS (Barrozo et al., 2021).

2) Cysteamine

Cysteamine, like cysteine, plays a role in GSH function and has been investigated as an antioxidant in bovine oocytes and embryos. Its primary functions include reducing cystine to cysteine within cells and enhancing cysteine uptake by cells, thus promoting cysteine availability for GSH synthesis (Deleuze & Goudet, 2010; Merton et al., 2013).

3) Hypotaurine

Hypotaurine (HTU) operates as an antioxidant beyond the functions of SOD, CAT, and GSH. It is a nonessential amino acid produced through the degradation of cysteine and the synthesis of pantothenate (Aruoma et al., 1988; Grove & Karpowicz, 2017). HTU serves as a precursor for taurine synthesis, wherein its dehydrogenase activity relies on H_2O_2 (Grove & Karpowicz, 2017). The conversion of HTU to taurine involves utilizing O_2•– and hydrogen ions as substrates, with the peroxytaurine intermediate swiftly transforming into taurine and H_2O_2. Additionally, HTU directly neutralizes •OH through a nonenzymatic pathway (Guerin et al., 2001).

2.3.4 Resveratrol

Resveratrol (Res; 3,4,5-trihydroxy-trans-stilbene) is a phytoalexin with antioxidant activity that modulates redox homeostasis in oocytes and improves *in vitro* embryo production. Many research that Res positively affects after oocyte maturation and subsequent embryonic development and supplementation of culture medium with Res be able to improve blastocysts development rates, hatching blastocyst rates, and the number of cells in blastocysts after IVM, IVF (Abe et al., 2017; Hayashi et al., 2018; Piras et al., 2020).

Mitochondria were most modified during development; these changes are about increased metabolism (Plante & King, 1994). However, vitrification induces mitochondrial damage, and Res may enhance the development of post-warming embryos and activate the degeneration of damaged mitochondria (Chen et al., 2019; Hara et al., 2018). After oocyte vitrification by mitochondrial turnover regulated ROS production at the electron transport chain level, resulting in decreasing ROS levels, abnormal mitochondrial distribution, and apoptotic rates (Giaretta et al., 2013; Pervaiz & Holme, 2009; Piras et al., 2020; Truong et al., 2018; Wang et al., 2018; Xia et al., 2017) improving metabolism, and promoting cell survival (Hwang et al., 2008).

1) Mechanisms of resveratrol

Res has demonstrated reversible inhibition of prostaglandin-endoperoxide synthase, commonly known as cyclooxygenase 1 (*COX-1*), and exhibits mild inhibition of *COX-2* (Szewczuk et al., 2004). Studies have revealed that mice lacking *COX-2* exhibited infertility, characterized by abnormalities in ovulation, fertilization, and implantation (Lim et al., 1997; Reese et al., 2001). *COX-2* deficient mice displayed irregularities in gonadotropin-induced oocyte maturation and cumulus expansion, resulting in disrupted ovulation patterns (Takahashi et al., 2006).

Res also indirectly targets tumor protein P53 (p53), a gene encoding a protein pivotal in regulating various cellular processes such as the cell cycle, apoptosis, and DNA repair (Soussi, 2000). In response to cellular stresses, p53 can arrest the cell cycle to facilitate DNA damage repair. However, in cases of severe DNA damage, p53 can trigger apoptosis to prevent the proliferation of genetically unstable cells and tumorigenesis (Soussi, 2000). p53 plays a crucial role in reproduction, contributing to

the regulation of embryo implantation; loss of p53 in female mice resulted in decreased implantation rates, pregnancy rates, and litter sizes (Hu et al., 2007). In humans, genetic variations in p53 have been linked to reproductive outcomes, particularly regarding implantation and pregnancy success (Fraga et al., 2014; Palomares et al., 2021). The activity of p53 is modulated by nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase Sirtuin1 (*SIRT1*) (Vaziri et al., 2001), which is implicated in metabolism, responses to environmental stresses, and cellular senescence (Duntas, 2011; Rahman & Islam, 2011).

Res acts as an activator of *SIRT1*, a protein known to regulate various molecular factors. This includes the inhibition of p53, Nuclear factor kappa B (NF-κB), and mammalian target of rapamycin (mTOR), as well as the promotion of PPARG coactivator 1 alpha (PGC-1α) expression, thereby enhancing mitochondrial biogenesis and glucose metabolism (Mc Auley et al., 2015; Olmos et al., 2011). *SIRT1* plays a crucial role in reproduction, as evidenced by infertility observed in mice with two null alleles for *SIRT1* (McBurney et al., 2003). Additionally, *SIRT1* has been implicated in oocyte maturation; a study on mouse oocytes highlighted the protective role of *SIRT1* signaling against oxidative stress (Di Emidio et al., 2014). However, mice engineered to overexpress *SIRT1* exhibited subfertility and implantation failure (Kim et al., 2022). In porcine ovarian cells, Res was found to induce *SIRT1* accumulation and apoptosis (Sirotkin et al., 2019). Furthermore, Res's treatment resulted in inhibited cellular proliferation and reduced P4 release, while increasing E2 and testosterone release. These findings underscore the direct impact of Res on ovarian cellular functions.

Res's activation of *SIRT1* prompts the deacetylation of p53, reducing its activity and promoting cell survival, particularly under cellular stress (Howitz et al., 2003; Suvorova et al., 2018). Res's dual impact on p53 and *SIRT1* highlights its mechanism of action. In colorectal cancer cell treatment, high concentrations of resveratrol (>10 μM) induce p53 acetylation, resulting in the upregulation of cyclin-dependent kinase inhibitor 1 (p21), caspase-3, and Bcl-2-associated protein x (*BAX*), ultimately leading to apoptosis. Conversely, lower concentrations of resveratrol (<5 μM) upregulate *SIRT1*, leading to a concentration-dependent downregulation of p53 and enhanced cell survival (Brockmueller et al., 2023). These findings underscore a

concentration-dependent effect of resveratrol, where higher concentrations induce apoptosis while lower concentrations exhibit anti-apoptotic effects (Podgrajsek et al., 2024).

2) Previous studies of resveratrol

Table 2.2 Previous studies of resveratrol in bovine

Medium	Conc.	Result	References
IVM	2 mM	(+) Normal Fertilization (-) Abnormal Fertilization	(S. Takeo et al., 2013)
IVM	2 μM 20μM	(+) Normal fertilization (+) Total cell number (+) ATP content (+) Mt number and mitochondrial membrane potential (+) The distribution and exocytosis of cortical granules (+) *SIRT1*	(Shun Takeo et al., 2013)
IVM	0.1 μM 1 μM 10 μM	(+) P4 (-) E2 (-) P450aro: Aromatase in cumulus cells (+) P450scc: Cholesterol side-chain cleavage enzyme in cumulus cells (+) Polar body rate (+) PTX3: pentraxin 3 in cumulus cells (=) Cleavage rate (=) Survival rates (+) Blastocyst rate (+) Hatched rate (+) The mean number of cells/blastocysts (-) ROS content (+) GSH (+) CAT (+) GPx4: Glutathione peroxidase 4 (+) SOD1: Superoxide dismutase 1 (+) *c-MOS: mRNA is necessary for oocyte maturation* (+) ERK2: Extracellular signal-regulated kinases (+) MAPK1: Mitogen activated protein kinase (+) CDC2: Cell division cycle protein 2 homolog (-) CyCB2: Cyclin-B2 (+) *SIRT1*	(Wang et al., 2013)

Table 2.2 (Continued)

Medium	Conc.	Result	References
IVC	0.25 μM 0.5 μM	(=) Cleavage rate (+) Morulae/blastocysts rates (+) Fast developing blastocysts (=) Survival rates (+) Development rates (+) Hatching rates (=) ICM, TE and total cells	(Salzano et al., 2014)
IVC	0.5 μM	(+) Blastocysts rates (=) Total number of cells (+) *SIRT1* (+) pAMPK: phosphorylated AMP-activated protein kinase (+) ATP content (-) ROS content (-) Lipid content (=) mtDNA copy numbers	(Abe et al., 2017)
IVM	2 μM	(-) ROS levels (=) GSH levels (=) Intermediate maturation stages (=) Metaphase II stage (=) Cleavage rates (+) Blastocysts rates (=) Hatched rates (+) The total cell numbers	(Sovernigo et al., 2017)
IVM	1 μM	(+) MII stage (-) Abnormal spindles (-) Chromosome configurations (-) DNA fragmentation (=) Cleavage rates (=) Blastocyst rates (+) Hatching rates (=) Early apoptosis (-) *ACACA: Acetyl-CoA carboxylase 1* (+) *SLC2A1: Solute carrier family 2 facilitated glucose transporter member 1* (+) SOD*1* (=) *GPX1: Glutathione peroxidase 1*	(Spricigo et al., 2017)

Table 2.2 (Continued)

Medium	Conc.	Result	References
IVC	0.5 μM	(+) Blastocyst rate (=) Mt-number (-) ROS content (-) TOMM20: Outer mitochondrial membrane protein (-) Levels of cell-free mtDNA in medium	(Hara et al., 2018)
IVM	1 μM	(-) *BAX* (-) *BCL2: B-cell CLL/lymphoma 2* (+) BMP15: *Bos taurus* bone morphogenetic protein 15	(Torres et al., 2018)
IVC	0.5 μM	(=) SOD2 (+) CYP51A1: Cytochrome P450, family 51, subfamily A, polypeptide 1 (=) TFAM: Transcription factor A, mitochondrial (=) Cleavage rate (=) Blastocyst yield (=) *SLC2A1* (+) *LIPE: Lipase, hormone-sensitive* (+) *PNPLA2: Adipose triglyceride lipase* (+) *DNMT3A: DNA (cytosine-5-) methyltransferase 3 alpha* (=) *SIRT1* (-) *MTORC1: Mechanistic target of rapamycin*	
IVC	1 μM	(=) Blastocyst stage (=) Survival rate (+) Hatching rate (+) *SIRT1* (+) The copy number of mtDNA (+) Pregnancy rate	(Hayashi et al., 2018)
IVC	0.5 μM	(+) Survival rate (=) The total cell numbers (+) *SIRT1* (+) Mt-cDNA in medium (=) Mt-DNA copy number (-) ATP content	(Hayashi et al., 2019)
IVC	0.2 μM 0.5 μM 1 μM	(=) Cleavage rates (+) Blastocyst rates (+) The total cell numbers	(Madrid Gaviria et al., 2019)

Table 2.2 (Continued)

Medium	Conc.	Result	References
IVC VS	0.5 µM	(=) Blastocyst rates (=) Survival rates (+) Active mitochondria (+) Mitochondrial superoxide (=) Lipid peroxidation levels	(Madrid Gaviria, et al., 2019)
IVC VS	0.5 µM	(=) Blastocyst rates (=) Hatching rates (+) Survival rates (=) ROS content (+) GSH content (=) Total cell number (+) live non-apoptotic cells (-) Early apoptotic cells (-) Dead cells (+) *SIRT1* (-) FoxO3A: Forkhead box protein O3 (-) MnSOD: Superoxide dismutase 2 (+) *PNPLA2* (-) *BCL2L1: BCL2-like protein 1* (-) *BAX*	(Madrid Gaviria, et al., 2019)

Data are presented as (+) significant increase when supplemented with resveratrol, (-) significant decrease when supplemented with resveratrol, and (=) No significant differences.

CHAPTER III

MATERIALS AND METHODS

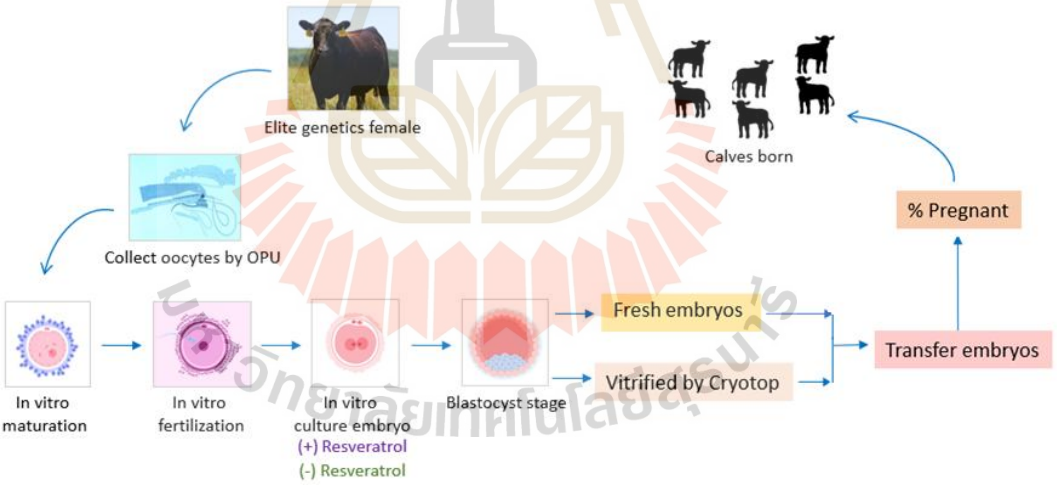
3.1 Chemicals

Unless otherwise noted, all chemicals utilized in this study were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

3.2 Ethic statement

Ethical approval for using cattle in this study was obtained from the Animal Ethics Committee of Suranaree University of Technology, Thailand.

3.3 Experimental design



The duration of this research started from December 2022 to April 2024. Oocytes were collected from each elite genetics' donors including Wagyu, Brahman, and Beefmaster breeds (n = 20) using an OPU technique every 3 weeks. Oocytes were matured in IVM medium prior to IVF. Embryos were cultured in IVC medium with or without resveratrol supplement. At day 7 of culture, blastocysts were divided into the following groups:

Group 1: The blastocysts derived from IVC medium with no resveratrol supplementation were freshly transferred to recipients.

Group 2: The blastocysts derived from IVC medium with no resveratrol supplementation were frozen using a slow freezing method prior to warming and directly transferring to recipients.

Group 3: The blastocysts derived from IVC medium with no resveratrol supplementation were vitrified by Cryotop, warmed and then transferred to recipients.

Group 4: The blastocysts derived from IVC medium with resveratrol supplementation were freshly transferred to recipients.

Group 5: The blastocysts derived from IVC medium with resveratrol supplementation were frozen using a slow freezing method, warmed and then transferred to recipients.

Group 6: The blastocysts derived from IVC medium with resveratrol supplementation were vitrified by Cryotop, warmed and then transferred to recipients.

3.4 Selection and preparation of recipients

Total of 1,612 heifers and cows of Holstein Friesian, Brahman, Charolais, Angus, and Beefmaster crossbred were prepared 6–8 weeks prior embryo transfer. The procedures were conducted at the Saim Vet Tech IVF Center in Phra Bu Subdistrict, Phra Yuen District, Khon Kaen Province, Thailand. The reproductive organs were examined and interpreted by a veterinarian. Recipients were raised under good physical conditions, with an average body score of 2.5–3.5 and a weight of 250–400 kg. They were free of underlying conditions, at least 50-60 days post-calving, and had a regular cycle. Cows used in this study were not older than 8 years and had no history of reproductive infections. The 2-3 years-old heifers with having a minimum of three normal cycles and a CL on at least one ovary were used in this study. Recipients were also evaluated through careful examination of the cervix, uterus and ovaries via rectal palpation to ensure that they lacked adhesions to neighboring organ structures and other palpable lesions. They were also fully dewormed and vaccinated before estrous synchronization.

Estrous synchronization of the recipients was started by insertion with a progesterone-releasing intravaginal device (CIDR®, Animal12 Healthcare Professionals, U.S.). After 7 days, CIDR was removed, and 2 ml of PG (ESTRUMATE®, USA) was injected. At 9 days post-injection, CL was checked using ultrasound scanning (Farmscan L60, BMV,

China). Only recipients with CL sizes greater than 14 mm were selected and injected with 2% lidocaine between the first and second coccygeal vertebrae before ET. Any form of stress to the recipient was avoided. Additionally, feeding regime was not changed during 3–4 weeks before and after the embryo transfer (Bozkurt et al., 2022).

3.5 Selection and preparation of donors

Donors are pure breed Wagyu, Brahman, and Beefmaster cattle. Prior to the beginning of experiment, the selected donors were evaluated in terms of the genetic value of the potential embryo donor based on performance, and reproduction criteria by a veterinarian. Two main factors were used for choosing the oocytes donation candidate in this study. Firstly, the donors with 2-10 years of age were to be physically firm, with an average 3.0 body score. Secondly, they had a regular cycle at least 50 to 60 days post-calving and be free of reproductive issues in the past. In order to obtain excellent donors, detailed examination of the cervix, uterus, and ovaries per rectum was performed to check palpable abnormalities and adhesions to surrounding organ structures. Any form of stress to the donors was avoided. Additionally, similar feeding regime was conducted during 3–4 weeks before and after the embryo transfer (Bozkurt et al., 2022).

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

Oocytes were collected from each donor (n = 20) using an ultrasound-guided ovum pick-up (OPU) procedure every 3 weeks. Oocytes were aspirated from follicles with a diameter of 3–8 mm using ultrasound scanning (ECO3 expert, Chison, China) connected to an aspiration needle and vacuum pump in Ringer solution containing 10 IU/ml heparin and 0.1% PVP. After that, oocytes were filtrated using Emcon filter (Agtech, Inc., 8801, Kansas, USA), and then selected under stereomicroscope (Olympus, Tokyo, Japan). Maturation was performed in IVM medium consisting of TCM199 supplemented with 10% FBS (Invitrogen, Bangalore, India), 50 IU/ml hCG (MSD Animal Health, Milton Keynes, UK), 0.02 AU/ml FSH (Kyoritsu Seiyaku Co., Tokyo, Japan), and 1 µg/ml 17β-estradiol. Oocytes were cultured in 80 µl IVM medium (20 oocytes/drop)

under mineral oil (tissue culture grade) on a culture dish (Thermo Fisher Scientific, Waltham, USA) at 38.5ºC under humidified atmosphere of 5% CO_2 for 23 h.

3.7 *In vitro* fertilization (IVF)

After maturation, oocytes were IVF with frozen-thawed semen from the same breed of donor. Semen was washed with a discontinuous 45% to 90% Percoll gradient solution by centrifugation (3,000 rpm) for 10 min. The final concentration of sperm was adjusted to $2x10^6$ sperm/ml in 50 µl of TALP medium (10 oocytes/drop). Oocytes and spermatozoa were co-incubated at 38.5ºC under humidified atmosphere of 5% CO_2 for 18 h.

3.8 *In vitro* embryo culture (IVC)

After fertilization, cumulus cells surrounding presumptive embryos were removed by pipetting. Embryos were cultured in 80-µl CR1aa medium (≤16 oocytes/drop) (Rosenkrans Jr et al., 1993) supplemented with or without 0.5 µM resveratrol at 38.5ºC under humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 days. The development of embryos was recorded in terms of cleavage and blastocyst rates at day 2 and 7 post-culture. Grade 1 blastocysts (Bo & Mapletoft, 2013) were selected for fresh transfer, slow freezing and vitrification.

3.9 Embryo transfer

Transfer of fresh embryos, an embryo was loaded into 0.25 ml of French straw in transfer medium (IVF Bioscience, United Kingdom) and maintained at 38.5 °C between transport. The straw was loaded into the ET gun, and the embryo was directly transferred to a uterine horn ipsilateral to CL. In order to produce a successful pregnancy, the embryonic stage must correspond to the uterine age of the recipients.

3.10 Vitrification

The basal medium (BM) used for vitrification and warming was TCM199-HEPES supplements with 20% FBS. The embryos were immersed in equilibration solution (ES: BM supplemented with 7.5% DMSO and 7.5% EG) for 3 minutes at 23-24 ºC. The

embryos were then immersed in vitrification solution (VS: BM supplemented with 16.5% DMSO, 16.5% EG, and 0.5 M sucrose), loaded on the Cryotop® device (Kitazato BioPharma, Fujinomiya, Japan), and plunged into liquid nitrogen within 1 minute. After vitrification, the vitrified blastocysts were warmed in serial warming solution (WS) consisting of BM supplemented with 0.5, 0.25, and 0 M sucrose for 5 minutes at each concentration and maintained at 38.5 °C. Finally, an embryo was loaded into 0.25 ml of French straw in transfer medium prior to transferring to each recipient.

3.11 Slow freezing

The embryo was immersed in slow freezing solution consisting of mDPBS supplemented with 20% FBS, 4 mg/ml BSA, 1.5 M EG, and 0.1 M sucrose. The embryo was loaded into 0.25 ml of French straw at room temperature. Straws were placed into slow freezing machine. The slow freezing machine (Cryologic, Victoria, Australia) cooled the embryos from 20°C to -6°C (3°C/min). At -6°C, seeding was induced by touching the straw with cold forceps. After 10 minutes, the straws were then cooled from -6°C to -33°C (0.5°C/min). At -33°C, the straws were plunged into liquid nitrogen. The slow freezing blastocyst was warmed in air for 10 seconds and in water at 30°C for 20 seconds before direct transfer to each recipient.

3.12 Diagnosis of pregnancy

After 14±3 days of ET, the recipients were carefully observed, non-pregnancy confirmed by the naturally returned to estrus. Between 23 to 30 days after ET, first diagnosis of pregnancy was performed when the embryo was at a gestational age of 30 to 37 days. This was done via transrectal ultrasonography, where an amniotic vesicle, a very turgid, fluid-filled sac surrounding and protecting the embryo during early gestation, is observed. The size of the amniotic vesicle was 0.6–0.8 cm in diameter. Additionally, the presence of a CL with an expected size of approximately 1 cm in diameter. was monitored. In the present study, a diagnosis of non-pregnancy was monitored by transrectal ultrasonography, was no presence of an amniotic vesicle in uterine horns (Christiansen, 2021; Jaśkowski et al., 2019; Szelenyi et al., 2023; Szenci, 2021).

Second diagnosis of pregnancy was carried out at the embryo stage of a gestational age of 60 to 70 days. The following criteria were used including the amniotic vesicle at about 8–8.5 cm, and the fetal body with approximately 5.5–10 cm in length. The fetal heartbeat was also measured using transrectal ultrasonography to confirm the viability of the fetus. The fetus with the absence of heartbeat was diagnosed as a pregnancy loss (Christiansen, 2021; JaśKowski et al., 2019; Szelenyi et al., 2023; Szenci, 2021).

Pregnancy loss in this study included; late embryo mortality, and fetal mortality. Late embryo mortality was referred to losses occurring between days 25 and 45 of gestation. Fetal mortality was defined as losses occurring from day 45 of gestation until the onset of parturition.

3.13 Statistical analysis

Data are expressed as mean ± SEM. All data were tested for normal distribution prior to statistical analyzes. The differences in the data pertaining to oocyte recovery, cleavage rate, blastocyst rate, pregnancy rate, and pregnancy loss were compared using a One-way Analysis of Variance ANOVA and followed by a Tukey's post hoc test using the GraphPad Prism 5 (GraphPad Software, Boston, MA, USA). Differences in $p < 0.05$ were considered significant.

CHAPTER IV
RESULTS

4.1 Oocytes recovery

Effect of different donor breeds between Wagyu, Brahman, and Beefmaster on number of collected oocyte is presented in Table 4.1. Wagyu cattle exhibited the lowest average number of oocytes per donor among the breeds studied, with a mean of 11.85 oocytes per donor. Brahman cattle had a significantly higher ($P<0.05$) average number of oocytes per donor compared to Wagyu, with a mean of 21.62. This indicates a more efficient oocyte production capability in Brahman cattle. Moreover, Beefmaster cattle produced the highest average number of oocytes per donor at 24.33. This breed demonstrated the most efficient oocyte production among the breeds.

Table 4.1 Oocyte collection efficiency in different cattle breeds

Donor breeds	No. Donors	No. Oocytes	No. Oocytes /donor
Wagyu	81	960	11.85±0.83 [b]
Brahman	303	6553	21.62±0.96 [a]
Beefmaster	80	1946	24.33±1.78 [a]

Superscript letters represent significant differences between treatments ($P<0.05$). Data are presented as mean ± SEM.

4.2 Embryo development

Effect of resveratrol supplemented in IVC medium on cleavage and blastocyst formation rates of three cattle breeds including Wagyu, Brahman, and Beefmaster under two conditions, untreated (-Res) and treated (+Res) is shown in Table 4.2. Resveratrol increased the cleavage rate in Wagyu but not significantly different (-Res 60.39%, and +Res 67.73%, $P>0.05$). In Brahman, cleavage rate of resveratrol treated group was significantly higher than that of untreated group (-Res 59.16%, and +Res 69.29%, $P<0.05$). Similar results were also observed in Beefmaster, cleavage rate of resveratrol treated group was significantly higher than that of untreated group (-Res 66.50%, and +Res

80.19%, P<0.05). Beefmaster showed the highest percentage of cleavage rates with resveratrol supplementation.

Table 4.2 Embryo development of untreated and treated resveratrol of oocytes collected from each bovine donor breed.

Res in IVC	Donor breed	No. Oocytes in IVC	Cleavage		Blastocyst	
			No.	Percentage	No.	Percentage
- Res	Wagyu	712	430	60.39±0.02 ^{cd}	186	26.12±0.02 ^{e}
	Brahman	4136	2447	59.16±0.01 ^{d}	1406	33.99±0.01 ^{cd}
	Beefmaster	824	548	66.50±0.02 ^{b}	306	37.14±0.02 ^{bc}
+Res	Wagyu	220	149	67.73±0.03 ^{bc}	58	26.36±0.03 ^{de}
	Brahman	1957	1356	69.29±0.01 ^{b}	789	40.32±0.01 ^{ab}
	Beefmaster	1060	850	80.19±0.01 ^{a}	471	44.43±0.02 ^{a}

Superscript letters represent significant differences between treatments (P<0.05). Data are presented as mean ± SEM.

Furthermore, in Wagyu, the blastocyst formation rates in supplemented resveratrol group were not significantly difference with untreated group (-Res 26.12%, and +Res 26.36%, P>0.05). In Brahman, resveratrol supplemented was significantly higher blastocyst formation rates than those in untreated group (-Res 33.99%, and +Res 40.32%, P<0.05). In Beefmaster, similar to cleavage rate, the blastocyst formation rate in resveratrol supplemented group were significantly higher than those in untreated group (-Res 37.14%, and +Res 44.43%, P<0.05). Beefmaster showed the highest blastocyst formation rates in both treated and untreated groups.

4.3 Pregnancy rate

Effect of resveratrol supplementation in IVC medium on pregnancy rate is presented in Table 4.3. Pregnancy rates were calculated on a basis of the number of pregnant recipients examined by ultrasound scanning at a gestational age of 30 to 37 days. Significantly higher pregnancy rates were noted in recipients receiving fresh embryos derived from resveratrol supplementation in comparison with no supplementation (-Res Fresh 27.80%, and +Res Fresh 37.86%, P<0.05). However,

pregnancy rates of recipients receiving slow freezing embryos (-Res Slow 18.18%, and +Res Slow 21.57%) and vitrified embryos (-Res Vit 26.87%, and +Res Vit 29.31%) did not significantly differ (P>0.05) between treatments. Slow freezing showed significantly lower pregnancy rate when compared with other groups. Although the improvement was modest, it indicates that resveratrol has a positive effect even on cryopreserved embryos.

Table 4.3 Pregnancy and pregnancy loss recorded in recipients who obtained embryos supplemented with (+) or without (-) 0.5μM resveratrol (Res). Blastocysts were allocated to fresh, slow freezing, and vitrified embryo transfer.

Res in IVC	Type embryo	No. Recipients	No. 1st Pregnancy Check (%)	No. Pregnancy loss (%)	No. 2nd Pregnancy Check	No. Ongoing pregnancy	No. Birth
- Res	(-) Fresh	644	179 [b] (27.80±0.02)	38/179 [a] (21.23±0.03)	141	40	101
	(-) Slow	55	10 [b] (18.18±0.05)	2/10 [ab] (20.00±0.13)	8	2	6
	(-) Vit	67	18 [ab] (26.87±0.05)	3/18 [ab] (16.66±0.09)	15	11	4
+ Res	(+) Fresh	737	279 [a] (37.86±0.02)	30/279 [b] (10.75±0.02)	249	73	176
	(+) Slow	51	11 [ab] (21.57±0.06)	2/11 [ab] (18.18±0.12)	9	6	3
	(+) Vit	58	17 [ab] (29.31±0.06)	2/17 [ab] (11.76±0.08)	15	11	4

Superscript letters represent significant differences between treatments (P<0.05). Data are presented as mean ± SEM.

Effect of resveratrol supplementation in IVC medium on pregnancy loss is presented in Table 4.3. Pregnancy rates were calculated on the basis of the pregnant number of recipients monitored by ultrasound scanning at a gestational age of 60 to 70 days. Significantly decreased pregnancy loss was noted in recipients those received fresh embryos derived from supplemented with resveratrol group in comparison with no supplementation (-Res Fresh 21.23%, and +Res Fresh 10.75%, P<0.05). However,

pregnancy loss of recipients received slow freezing embryos (-Res Slow 20.00%, and +Res Slow 18.18%) and vitrified embryos (-Res Vit 16.66%, and +Res Vit 11.76%) comparatively did not significantly differ (P>0.05) between treatments.

4.4 Effect of donor breeds

The effect of different donor breeds on pregnancy rates and pregnancy loss rates only from fresh embryos are presented in Table 4.4. Wagyu donor breed showed the pregnancy rate of 35.94%, with a pregnancy loss rate of 8.7%. This breed showed a relatively higher pregnancy rate and lower pregnancy loss rate compared to those of Brahman donors, but there were no significant differences (P>0.05). Brahman donor breed had the pregnancy rate of 31.85%, but the pregnancy loss rate was obviously higher at 15.71%. Brahman donors exhibited a lower pregnancy rate and a higher pregnancy loss rate compared to those of Wagyu and Beefmaster donors, but there were no significant differences (P>0.05). For Beefmaster donor breed, the pregnancy rate was 35.39%, and the pregnancy loss rate was 14.19%. Beefmaster donors demonstrated pregnancy rates similar to Wagyu but slightly higher pregnancy loss rates.

Table 4.4 Impact of donor breeds on pregnancy rates and pregnancy loss

Donor breeds	No. Recipients	No. Pregnancy (%)	No. Pregnancy loss (%)
Wagyu	64	23/64 (35.94±0.06)	2/23 (8.7±0.06)
Brahman	879	280/879 (31.85±0.02)	44/280 (15.71±0.02)
Beefmaster	438	155/438 (35.39±0.02)	22/155 (14.19±0.03)

Data are presented as mean ± SEM. No significant differences (P>0.05) among donor breeds.

4.5 Effect of recipient breeds

The effect of different recipient breeds on pregnancy rates and pregnancy loss rates only from fresh embryos are presented in Table 4.5. For Holstein Friesian crossbreed recipients, the pregnancy rate was 33.44% with a pregnancy loss rate of

12.75%. Holstein Friesian crossbreed recipients demonstrated a moderate pregnancy rate and a relatively lower pregnancy loss rate compared to other breeds. Brahman crossbreed recipients had the pregnancy rate of 29.89%, but the pregnancy loss rate was notably higher at 21.15%. Brahman crossbreed recipients exhibited a lower pregnancy rate and a higher pregnancy loss rate compared to Holstein Friesian crossbreed recipients, but there were no significant differences (P>0.05). Charolais crossbreed recipients represented the pregnancy rate of 34.48%, and the pregnancy loss rate of 15.26%. Charolais crossbreed recipients had a slightly higher pregnancy rate and lower pregnancy loss rate compared to Holstein Friesian crossbreed recipients, but there were no significant differences (P>0.05).

Table 4.5 Impact of different recipient breeds on pregnancy rates and pregnancy loss

Recipient breeds	No. Recipients	No. Pregnancy (%)	No. Pregnancy loss (%)
Holstein Friesian crossbreed	305	102/305 (33.44±0.03)	13/102 (12.75±0.03)
Brahman crossbreed	174	52/174 (29.89±0.03)	11/52 (21.15±0.06)
Charolais crossbreed	551	190/551 (34.48±0.02)	29/190 (15.26±0.03)
Angus crossbreed	192	67/192 (34.90±0.03)	6/67 (8.96±0.04)
Brangus crossbreed	159	47/159 (29.56±0.04)	9/47 (19.15±0.06)

Data are presented as mean ± SEM. No significant differences (P > 0.05) among donor breeds.

For Angus crossbreed recipients, the pregnancy rate was 34.90%, and the pregnancy loss rate was relatively low at 8.96%. Angus crossbreed recipients showed a higher pregnancy rate and a notably lower pregnancy loss rate compared to other breeds. In case of Brangus crossbreed recipients, the pregnancy rate was 29.56%, and the pregnancy loss rate was 19.15%. Brangus crossbreed recipients demonstrated a

lower pregnancy rate and a higher pregnancy loss rate compared to Holstein Friesian crossbreed recipients, but there were no significant differences (P>0.05). The results of this study showed variable pregnancy rates among different recipient breeds by which Angus crossbreed recipients had the highest pregnancy rate while Brahman crossbreed recipients showed the lowest pregnancy rate. Also, there is a pregnancy loss rate among recipient breeds, with Brahman crossbreed recipients having the highest loss rate and Angus crossbreed recipients having the lowest loss rate.

4.6 Cost-effectiveness

In this study, depreciation was calculated using the Straight-Line Method (SLN). This method ensures that the cost of the equipment is allocated evenly over its useful life, with a consistent expense per use based on the frequency of usage. Since the equipment is used around 64 times a year, depreciation expense per use was estimated by dividing the annual depreciation expense by 64. Table 4.6 shows the cost information for 20 donors per time. Therefore, the average cost per donor was ca. 2,445.20 Baht (48,904.08/20=2,445.20 Baht). As of 2024, resveratrol price is 8,300 Baht per 100 grams. Supplementation of 0.5 M resveratrol generates the additional expense at approximately 0.095 Baht per time. Therefore, the use of resveratrol in *in vitro* production of bovine embryos is highly cost-effective, with minimal cost per donor.

Table 4.6 Cost estimation for *in vitro* production of bovine embryos with resveratrol supplementation

Type of cost	**Expense/20 Donor (Bath)**
Culture Media	14,320
Equipment	1,940
Gas	967
Equipment depreciation	1,677.08
Wage	30,000
Total	48,904.08

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

The study of oocyte collection from different cattle breeds, specifically Wagyu, Brahman, and Beefmaster, reveals the significance of how breed variations can influence embryo development, pregnancy rates, and pregnancy loss. The study found that among the breeds studied, Wagyu cattle exhibited the lowest average number of oocytes per donor with average value of 11.85 oocytes per donor. The result was consistent with the findings of Du et al. (2024), who reported the number of oocytes collected from Wagyu in heifers and cows of 5.3 and 13.6–19.1 oocytes per donor, respectively. Additionally, the present results indicated that Wagyu breeds had significantly lower embryo development rates. Despite producing fewer oocytes and having lower embryo development rates, Wagyu cattle demonstrated a higher pregnancy rate and lower pregnancy loss compared to Brahman donors, although these differences were not significant different. This implied even though Wagyu cattle produced low number of oocytes and had lower embryo development rate, the embryos had high pregnancy potential with low abortion. Goto et al. (2021) studied the factors that play an important role in the fertility rate of Wagyu breed. They reported that environmental factors (season of transfer) and recipient factors (age, number of transfers, and clinical status of the ovaries) did not significantly differ between successful and unsuccessful fertility groups. Meanwhile, embryo quality, state, and breed were identified to be main factors responsible for the fertility. Their results were highly relevant to the present study and pointed out the importance of improving embryo quality in order to enhance overall reproductive efficiency of Wagyu cattle.

Brahman cattle exhibited a significantly higher average number of oocytes per donor compared to Wagyu cattle, indicating a more efficient capability for oocyte production. This breed also demonstrated significantly higher blastocyte rates compared to Wagyu. However, Brahman donors showed a lower pregnancy rate and a

higher pregnancy loss rate compared to both Wagyu and Beefmaster donors, although these differences were not significant different. Similarly, previous studies conducted by Holroyd et al. (1993) and Donaldson (1971) shown that Brahman crossbreeds had relatively high pregnancy loss rates. Specifically, the present study found that Brahman crossbreed recipients exhibited a lower pregnancy rate and the highest pregnancy loss rate among the breeds studied, though these differences were not statistically significant. These may be attributed to genetic factors. The overall results suggested that while Brahman cattle were more efficient in producing oocytes and developing embryos, there were potential genetic or physiological challenges in sustaining pregnancies. Further study is needed to explore the underlying causes of the pregnancy losses and to identify strategies to improve reproductive success in Brahman cattle.

The study found that Beefmaster cattle produced the highest average number of oocytes per donor, which were significantly higher than that of Wagyu cattle, demonstrating the most efficient oocyte production among the breeds. This breed also showed the highest blastocyst rates, which were significantly higher than those of Wagyu and Brahman. These results were consistent with the report by Donaldson (1984), who found that Brangus, Simbrah, and Beefmaster breeds could produce the highest number of embryos, compared to across 13 different cattle breeds. Beefmaster donors also demonstrated high pregnancy rates similar to Wagyu cattle, but it was slightly higher pregnancy loss rates, which were not significantly different. The average pregnancy rate for Beefmaster was 35.39%, which was comparable to the report by Flores Rodriguez et al. (2023). They reported embryos derived from Beefmaster donors were transferred freshly to heifers with pregnancy rates of 40%. This suggested the potential for enhancing the reproductive efficiency of Beefmaster cattle in the future. The results indicated that Beefmaster cattle were superior in terms of oocyte production and embryo development. However, the slightly increase in pregnancy loss rates observed in Beefmaster cattle warrants further investigation to understand the underlying causes and improve the overall reproductive success and efficiency of Beefmaster cattle, making them a more viable option for breeding programs.

The results of this study demonstrated pregnancy rates and pregnancy loss from different donor breeds. Cattle breeds e.g. Wagyu, Brahman, and Beefmaster did not

affect pregnancy rates and pregnancy loss. The results of this study were in contrast to Arreseigor et al. (2016) study. They investigated pregnancy rates of different donor breeds, including Angus, Nelore, Brangus, Holstein, Gyr, and Girolando, and demonstrated that the breed of the embryo significantly influenced the pregnancy rate in crossbreed recipients. Discrepancy may be explained by difference of cattle breed. Arreseigor et al. (2016) focused on two cattle groups, namely beef and dairy breeds while only beef breed was used to investigate their effects on pregnancy rates and pregnancy loss in the present study. Furthermore, Arreseigor (2016) examined embryo mortality among different cattle breeds and found that Angus, Nelore, Brangus, Holstein, Gyr, and Girolando breeds significantly affect embryo mortality. Moreover, embryos from *Bos indicus* and their crossbreeds show lower embryo mortality than embryos from pure *Bos taurus* breeds. This underscores the importance of considering breed-specific factors and genetic backgrounds when evaluating reproductive outcomes in cattle. In this study, there was no significant difference in pregnancy loss among different donors and recipient breeds and recipients receiving slow freezing and vitrified embryos with resveratrol supplement possibly due to an insufficient sample size. In general, the production and transfer of embryos depend largely on the popularity of cattle breeds in different regions. Wagyu and Beefmaster cattle have only recently gained popularity resulting in lower number of donors compared to Brahman, which has been extensively bred in Thailand for a long time. Therefore, larger sample size of Wagyu and Beefmaster is further required to ensure whether donors and recipient breeds affect pregnancy rates and pregnancy loss of *in vitro* produced embryos.

The present study examined the effect of resveratrol supplementation in IVC medium on the developmental potential. The analysis focused on cleavage and blastocyst formation rates under two conditions, untreated (-Res) and treated (+Res). The significant differences observed between the treated and untreated groups in both cleavage and blastocyst rates underscore the potential of resveratrol as a beneficial supplement in IVC medium. The results were accordant to several previous studies. For example, Madrid Gaviria et al. (2019) demonstrated that high concentration (10 μM) of resveratrol was detrimental to embryos, while low concentrations (0.2-0.5 μM) were provided beneficial effects on embryo development including improved blastocyst rate

and total cell number. The concentration-dependent effect of resveratrol is consistent with report by Podgrajsek et al. (2024). They observed that higher concentrations could induce apoptosis, whereas lower concentrations exhibited anti-apoptotic effects. Similar to the present study, 0.5 μM resveratrol was utilized and conferred desired effects on embryo development. In addition to supplementation into IVC medium, resveratrol has also been applied into IVM medium in order to increase blastocyst rates. Wang et al. (2013) reported that resveratrol in IVM medium improved blastocyst rates, hatching blastocyst rates, and the mean number of cells per blastocyst after oocyte maturation and fertilization *in vitro*. These results are in agreement with the present study. Wang et al. (2013) described that the improvement was associated with increased expression levels of antioxidant genes in cumulus cells, elevated intracellular GSH levels, and decreased ROS levels in oocytes. Moreover, resveratrol was shown to enhance the levels of *SIRT1* gene expression, a crucial regulator in cellular metabolism and stress responses (Uddin et al., 2021). It generally known that resveratrol improves mitochondrial function by activating *SIRT1* gene expression (Alcaín & Villalba, 2009) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). For instance, Robb et al. (2008) observed that resveratrol induced a 14-fold increase in superoxide dismutase 2 (SOD2) activity through the Res>*SIRT1*/NAD+>*FoxO3A*>SOD2 pathway. SOD2 activity is essential for reducing superoxide levels, thereby providing resistance to mitochondrial dysfunction, permeability changes, and apoptotic death in various diseases (LA, 2001). These beneficial actions of resveratrol may account for increase in cleavage and blastocyst rates of bovine embryos observed in this study.

The present study exhibited that embryos cryopreserved through vitrification had higher pregnancy rates compared to those using the slow freezing method. These results were consistent with earlier studies. Vitrification, a rapid freezing technique, conferred higher survival rates of human embryos compared to the slow freezing approach (Balaban et al., 2008; Debrock et al., 2015; Huang et al., 2005; Kasai & Mukaida, 2004; Kim et al., 2001). Cryopreservation, in general, can cause negative impact on the quality of blastocysts due to various reasons, such as structural damage to cell membranes, impaired cellular functions, osmotic shock, toxicity from CPAs, and ice

crystal formation. It is obvious that ice crystallization is incompatible with any developmental stage embryos and must be avoided as far as possible (Shaw & Jones, 2003). The slow freezing approach cannot effectively avoid ice crystal formation, resulting in lower survival rates of *in vitro* produced embryos and, consequently, lower pregnancy rates compared to vitrification. However, the slow freezing direct transfer method still requires development because vitrification necessitates skilled professionals to thaw the embryos in a laboratory setting, posing a limitation for commercial exchange. Improving the slow freezing technique could enhance efficiency and accessibility in commercial application.

Additionally, ROS produced during the cryopreservation process at low temperatures can cause further damage (Gupta et al., 2010). It is widely accepted that slow freezing and vitrification can impair embryo quality, leading to decreased developmental potential and ATP content (Brevini et al., 2005; Chen et al., 2016). Hayashi et al. (2019) found that the embryo after slow freezing reduces mitochondrial function and mtDNA integrity, resulting in fewer mitochondria in blastocysts. Moreover, ROS levels tend to increase during embryo culture due to various environmental stressors such as temperature fluctuations, light exposure, pH variations, atmospheric oxygen levels, suboptimal culture media, and cryopreservation procedures (Keane & Ealy, 2024; Ramos-Ibeas et al., 2019). Exposure to ROS can significantly reduce the efficacy of *in vitro* embryo production, leading to decreased post-ET pregnancy rates, increased pregnancy loss, chromosomal abnormalities, and large offspring syndrome (Ealy et al., 2019; Hansen, 2020; Kępka et al., 2023; Tšuiko et al., 2017). The results of this study demonstrated that fresh embryos derived from supplemented with resveratrol had the highest pregnancy rates with statistical significance and an apparent reduction in pregnancy loss. These results were in line with previous reports. These are likely due to resveratrol's capacity to lower ROS levels as suggested by Sovernigo et al. (2017). Furthermore, Abe et al. (2017) reported that resveratrol supplementation in the IVC medium not only reduced ROS content but also decreased lipid accumulation.

In general, FBS is one of important ingredients in IVC medium that play a vital role in not only enhancing embryo development, but also increasing lipid accumulation within the embryos (Abe & Hoshi, 2003; Fukui et al., 2000). It has been shown that high

lipid content in embryos reduces their survival rates following cryopreservation (George et al., 2008). The cytoplasmic lipid content of mammalian embryos is related to their cryosensitivity (Nagashima et al., 1995; Nagashima et al., 1994; Pereira et al., 2007; Shehab-El-Deen et al., 2009). Resveratrol activates *SIRT1*, leading to AMPK activation, which increases lipid utilization in embryos through beta-oxidation, thereby reducing lipid content. This mechanism could be explained the higher pregnancy rates observed in recipients who received slow frozen and vitrified embryos supplemented with resveratrol compared to those without supplementation in the present study. Resveratrol may increase the resistance of embryos to damage during cryopreservation as reported by Abdel-Wahab et al., 2012. Resveratrol, a potent activator of *SIRT1*, enhances mitochondrial functions, which is necessary for regulating and maintaining cellular homeostasis (Price et al., 2012). Previous study conducted by Hayashi et al. (2018) revealed that pretreating embryos with resveratrol aided in the recovery of mitochondrial numbers from cryoinjury through de novo synthesis and the degradation of damaged mitochondria via *SIRT1* activation. The present study showed higher pregnancy rates in recipients received with slow freezing and vitrified embryos derived from supplemented with resveratrol, although the differences were not significant different. The slightly increase in pregnancy rate is likely due to the embryos derived from supplemented with resveratrol enhanced survival rates of cryopreserved embryos and/or recovery of mitochondrial numbers from cryoinjury. In human studies, embryos with high pregnancy potential had higher mtDNA copy numbers, compared to those of low pregnancy potential embryos (Stigliani et al., 2014).

Hayashi et al. (2018) also reported significantly higher pregnancy rates of resveratrol-pretreated embryos compared to untreated ones. Similarly, the present study found significantly higher pregnancy rates in recipients receiving fresh embryos supplemented with resveratrol compared to those without supplementation. Moreover, the effect of resveratrol treatment on increasing pregnancy rates, likely attributed to its mechanisms of action on p53, a protein that plays a vital role in reproduction and regulation of embryo implantation. Hu et al. (2007) found that p53 deficiency in female mice resulted in reduced implantation rates, pregnancy rates, and litter sizes. In humans, genetic variations in p53 have been associated with reproductive

outcomes, particularly implantation and pregnancy success (Fraga et al., 2014; Palomares et al., 2021). The activity of p53 is regulated by NAD-dependent deacetylase *SIRT1* (Vaziri et al., 2001), which is involved in metabolism, responses to environmental stresses, and cellular senescence (Duntas, 2011; Rahman & Islam, 2011). McBurney et al. (2003) reported that *SIRT1* plays a crucial role in reproduction, as evidenced by infertility in mice lacking two *SIRT1* alleles. These results indicates that resveratrol plays a crucial role in improving the initial success rate of embryo implantation.

The study showed slight variations in pregnancy rates and pregnancy loss rates across different recipient breeds, but no significant differences were observed among Holstein Friesian, Brahman, Charolais, Angus, and Brangus recipient crossbreeds. These results were consistent with Hasler et al. (1987) , who investigated the effects of 23 factors related to time, embryos, donors, and recipients in a commercial bovine embryo transfer program over six years. They also found that the pregnancy rate was not affected by the recipient breed. Interactions between embryo quality and the stage of development of the embryo relative to the uterine age of the recipients significantly influenced the pregnancy rate. These factors underscore the complex interaction between the embryo and the recipient environment, suggesting that optimal timing and embryo quality are crucial for successful pregnancies, regardless of the donor breed.

The cattle production industry continuously strives to enhance reproductive efficiency. Experimental trials conducted on donor breeds such as Wagyu, Brahman, and Beefmaster, which are crucial in the cattle production industry for their aesthetics and high-quality meat production, have shown that resveratrol can improve both the quality and quantity of embryos. This suggests that resveratrol may also be beneficial for other breeds. Furthermore, resveratrol can enhance the quality and quantity of blastocysts for embryo transfer, leading to increased pregnancy rates and reduced pregnancy loss. This improvement enables better production of genetically superior livestock while reducing costs associated with failed implantation and pregnancy loss. Cost-effectiveness calculation in this study indicated that the additional cost derived from resveratrol per production cycle was minimal compared to the increased likelihood of producing a calf. This demonstrates that continued use of resveratrol is recommended for future breeding programs. During the study, it was found that the

pregnancy rate was also influenced by many other external factors that should be further investigated. For instance, the impact of donor diet on oocyte quality should be explored, as it was observed that excessive donor nutrition can lead to high lipid accumulation in oocytes. This high lipid content can reduce embryo survival rates after freezing, even though supplements like resveratrol might help reduce lipid content, addressing this issue at its root would be more effective. This highlights the need for comprehensive approaches to improving reproductive outcomes, considering both supplementation and dietary management of donor cattle.

Future research should focus on cryopreservation techniques are needed, particularly in improving slow freezing methods to make the process more accessible and efficient for commercial purposes. Research should also explore strategies to mitigate oxidative stress and cellular damage caused by cryopreservation, thereby improving embryo viability and pregnancy outcomes. A comprehensive approach to improve reproductive efficiency should be adopted, combining supplementation with optimal management practices. This includes exploring the effects of donor diet, environmental factors, and genetic influences on reproductive outcomes. By addressing these factors, future research can enhance the production of genetically superior livestock, reduce costs associated with failed implantations and pregnancy loss, and ultimately improve the sustainability of cattle breeding programs.

5.2 Conclusions

Resveratrol supplementation in IVC medium was found to significantly enhance cleavage and blastocyst formation rates. Embryos treated with resveratrol showed higher pregnancy rates and reduced pregnancy loss. These findings suggest that resveratrol is a beneficial supplement for improving the quality and viability of embryos. Cryopreservation techniques were also evaluated, with vitrification emerging as more effective than slow freezing in preserving embryo viability. The present study revealed that pregnancy rates and pregnancy loss did not significantly differ among slow freezing and vitrified embryos. However, vitrified embryos had higher pregnancy rates than slow freezing embryos, highlighting the critical role of embryo quality. This finding underscores the complexity of reproductive outcomes, influenced by multiple factors beyond the breed of the donor or recipient.

The study revealed significant breed variations in reproductive performance among Wagyu, Brahman, and Beefmaster cattle. Wagyu cattle were found to produce fewer oocytes and have lower embryo developmental rates compared to Brahman and Beefmaster. However, Wagyu cattle demonstrated higher pregnancy rates and lower pregnancy loss, suggesting that they produce lower number of embryos, but the breed had higher likelihood of successful pregnancy. Brahman cattle, on the other hand, exhibited efficient oocyte and embryo production but faced challenges in maintaining pregnancies. Beefmaster cattle was superior in terms of oocyte production and embryo development, although they showed slightly higher pregnancy loss rates, indicating potential areas for improvement.

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