EFFECTS OF SUPPLEMENTATION WITH DIFFERENT TYPES OF BOECs DERIVED-EXTRACELLULAR VESICLES ON THE DEVELOPMENT AND QUALITY OF *IN VITRO* PRODUCED BOVINE EMBRYOS

APISIT POLRACHOM

A Thesis Submitted in Partial Fulfillment of the Requirements for the
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ผลของการเสริม Extracellular vesicles ที่ได้มาจากเซลล์ BOECs ที่ต่างชนิดต่อการพัฒนาและคุณภาพของตัวอ่อนโคในหลอดแก้ว

นายอภิสิทธิ์ พลราชม

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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อภิสิทธิ์ พลราชม: ผลของการเสริม Extracellular vesicles ที่ได้มาจากเซลล์ BOECs ที่ต่างชนิดต่อการพัฒนาและคุณภาพของตัวอ่อนโคในหลอดแก้ว (EFFECTS OF SUPPLEMENTATION WITH DIFFERENT TYPES OF BOECs DERIVED EXTRACELLULAR VESICLES ON THE DEVELOPMENT AND QUALITY OF *IN VITRO* PRODUCED BOVINE EMBRYOS) อาจารย์ที่ปรึกษา: ศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 55 หน้า.

คำสำคัญ: ถุงนอกเซลล์/การพัฒนาของตัวอ่อน/เซลล์เยื่อบุผิวท่อนำไข่โค/การปฏิสนธิในหลอดแก้ว/การโคลนนิ่ง

การผลิตตัวอ่อนในหลอดทดลอง (*In vitro* embryo production: IVEP) เป็นเทคโนโลยีสำคัญที่ช่วยส่งเสริมความก้าวหน้าทางพันธุกรรมและประสิทธิภาพการสืบพันธุ์ในปศุสัตว์ อย่างไรก็ตาม สภาพแวดล้อมในหลอดทดลองไม่สามารถจำลองสัญญาณชีวภาพที่ซับซ้อนของระบบสืบพันธุ์เพศเมียได้อย่างสมบูรณ์ จึงถูกจำกัดคุณภาพและศักยภาพในการพัฒนาคุณภาพของตัวอ่อน โดยเฉพาะในตัวอ่อนที่ได้จากเทคนิคโคลนนิ่งด้วยการย้ายฝากนิวเคลียสด้วยเซลล์ร่างกาย (Somatic cell nuclear transfer: SCNT) ซึ่งโดยธรรมชาติมักมีศักยภาพในการพัฒนาต่ำ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของถุงส่งออกนอกเซลล์ (Extracellular vesicles: EVs) ที่ได้จากเซลล์เยื่อบุท่อนำไข่ของโค (BOECs) ซึ่งเพาะเลี้ยงสองระบบ ได้แก่ระบบเซลล์ชั้นเดียว (BOECs-M-EVs) และระบบถุงแขวนลอย (BOECs-V-EVs) ต่อการพัฒนาและคุณภาพของตัวอ่อนที่ผลิตโดยเทคนิค *in vitro* fertilization (IVF) และ SCNT โดย EVs ถูกแยกจากน้ำยาเลี้ยง BOECs ด้วยวิธีอัลตราเซ็นตริฟิวจ์ และตรวจสอบด้วยการวิเคราะห์ขนาดอนุภาค (nanoparticle tracking analysis , NTA) ซึ่งพบว่า EVs มีขนาดระหว่าง 118-131 nm จากนั้น EVs ถูกเติมในน้ำยาเลี้ยงตัวอ่อนที่ความเข้มข้น 2×10^6, 4×10^6 และ 8×10^6 อนุภาค/มิลลิลิตร ผลการทดลองพบว่า การเติม EVs โดยเฉพาะในความเข้มข้นสูง มีผลเพิ่มอัตราการได้ตัวอ่อนระยะ บลาสโตซิสต์ การขยายตัวของ blastocoel และจำนวนเซลล์ทั้งหมดในตัวอ่อน โดยมีความสัมพันธ์เชิงบวกระหว่างเส้นผ่านศูนย์กลางของตัวอ่อนกับจำนวนเซลล์ นอกจากนี้ การแสดงออกของยีนที่เกี่ยวข้องกับความเป็น พลูริโพเทนท์ (*OCT4, NANOG*) และการยอมรับการฝังตัวของแม่ (*IFN-tau*) เพิ่มขึ้น ในขณะที่ยีน *BAX* ซึ่งเกี่ยวข้องกับการตายของเซลล์ลดลง โดยเฉพาะในกลุ่ม SCNT ผลการศึกษานี้แสดงให้เห็นว่า EVs จาก BOECs-M สามารถเลียนแบบสัญญาณจากแม่ ส่งเสริมคุณภาพของตัวอ่อน ซึ่งจะลดการตายของเซลล์ และสนับสนุนการพัฒนาของตัวอ่อนในหลอดทดลอง โดยเฉพาะผลิตด้วยเทคนิค SCNT ที่ตัวอ่อนมักมีศักยภาพในการพัฒนาต่ำ

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา.....

APISIT POLRACHOM: EFFECTS OF SUPPLEMENTATION WITH DIFFERENT TYPES OF BOECs DERIVED-EXTRACELLULAR VESICLES ON THE DEVELOPMENT AND QUALITY OF *IN VITRO* PRODUCED BOVINE EMBRYOS. THESIS ADVISOR: PROF. RANGSUN PARNPAI, Ph.D., 55 PP.

Keyword: Extracellular vesicles/Embryonic development/Oviduct epithelial cells/ *In vitro* fertilization/Somatic cell nuclear transfer

In vitro embryo production (IVEP) is a key biotechnology tool for enhancing genetic progress and reproductive efficiency in livestock. However, conventional in vitro systems lack the complex signaling environment of the female reproductive, which limits embryo quality and developmental potential, particularly in somatic cell nuclear transfer (SCNT) embryos. Extracellular vesicles (EVs), nano-sized membrane-bound particles secreted by cells, play important roles in cell communication and early embryonic development. This study aimed to evaluate the effects of EVs derived from bovine oviduct epithelial cells (BOECs), cultured in either monolayer (BOECs-M-EVs) or vesicle-like suspension (BOECs-V-EVs), on the development and quality of embryos produced via *in vitro* fertilization (IVF) and SCNT. EVs isolated by ultracentrifugation and characterized using nanoparticle tracking analysis (NTA), confirming a size ranging between 118 to 131 nm. The isolated EVs were supplemented into embryo culture media at 2×10^6, 4×10^6, and 8×10^6 particles/mL. Results showed that EV supplementation significantly improved blastocyst formation rates, blastocoel expansion, and total cell numbers, particularly at higher concentrations. A positive correlation was observed between embryo diameter and total cell number, indicating enhanced proliferation. Moreover, gene expression analysis revealed upregulation of pluripotency (*OCT4*, *NANOG*) and maternal recognition (*IFNT-tau*) genes and downregulation of the pro-apoptotic gene *BAX*, especially in SCNT embryos. These findings suggest that BOECs-M-derived EVs can mimic maternal signals, enhance embryo quality by reducing cell death and supporting in vitro embryo development.

The study highlights the potential of BOECs-EVs as effective supplements to improve IVEP efficiency, particularly in SCNT systems where developmental potential is low.

School of Biotechnology Student's Signature .....

Academic Year 2024 Advisor's Signature .....

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Apisit Polrachom

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

ART	=	Assisted Reproductive Technology
BAX	=	BCL 2 Associated X Protein
BCL2	=	B-cell Lymphoma 2
BOECs	=	Bovine oviductal epithelial cells
BOECs-M	=	Bovine oviductal epithelial cells from monolayer
BOECs-V	=	Bovine oviductal epithelial cells from vesicle
BOECs-M-EVs	=	Extracellular vesicles from monolayer-cultured BOECs
BOECs-V-EVs	=	Extracellular vesicles from vesicle-shaped cultured BOECs
CB	=	Cytochalasin B
CHX	=	Cycloheximide
CD	=	Cytochalasin D
cDNA	=	Complementary Deoxyribonucleic Acid
CO_2	=	Carbon Dioxide
COCs	=	Cumulus-Oocyte Complexes
CR1aa	=	Charles Rosenkrans 1 Amino Acid Medium
DNA	=	Deoxyribonucleic Acid
DMSO	=	Dimethyl Sulfoxide
dFBS	=	Depleted EVs Fetal Bovine Serum
E2	=	Estradiol
EGF	=	Epidermal Growth Factor
EVs	=	Extracellular Vesicles
EVC	=	EVs from cultured endometrial cells
FBS	=	Fetal Bovine Serum
FCS	=	Fetal Calf Serum
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GDF-9	=	Growth Differentiation Factor 9
GV	=	Germinal Vesicle

LIST OF ABBREVIATIONS (Continued)

GVBD	=	Germinal Vesicle Breakdown
hCG	=	Human Chorionic Gonadotropin
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	=	Hepatocyte Growth Factor
ICM	=	Inner Cell Mass
IFN-tau	=	Interferon Tau
IVP / IVEP	=	*In Vitro* Embryo Production
IVM	=	*In Vitro* Maturation
IVF	=	*In Vitro* Fertilization
IVC	=	*In Vitro* Cultur
LH	=	Luteinizing Hormone
LOS	=	Large offspring syndrome
MAPK	=	Mitogen-Activated Protein Kinase
MII	=	Metaphase II
mRNA	=	Messenger Ribonucleic Acid
mDPBS	=	modified Dulbecco's phosphate-buffered saline
MVBs	=	Multivesicular Bodies
NTA	=	Nanoparticle tracking analysis
NANOG	=	Homeobox protein NANOG
OCT4	=	Octamer-Binding Transcription Factor 4
OPU	=	Ovum Pick-Up
O_2	=	Oxygen
OV-EVs	=	EVs from Oviduct fluid
PBS	=	Phosphate-buffered saline
PI	=	Propidium Iodide
PVP	=	Polyvinylpyrrolidone
qPCR	=	Quantitative Polymerase Chain Reaction
RNA	=	Ribonucleic Acid
ROS	=	Reactive Oxygen Species

LIST OF ABBREVIATIONS (Continued)

SCNT	=	Somatic Cell Nuclear Transfer
SOF	=	Synthetic Oviduct Fluid
SOX2	=	SRY (Sex-determining Region Y)-Box 2
TALP	=	Tyrode's Albumin Lactate Pyruvate
TCM	=	Tissue culture medium
TE	=	Trophectoderm
TGF	=	Transforming Growth Factor
Ut-EVs	=	EVs from Uterus
ZP	=	Zona Pellucida

CHAPTER I
INTRODUCTION

1.1 Background and Significance

In vitro embryo production (IVEP) represents a cornerstone technology in livestock genetic improvement programs by enabling the large-scale generation of embryos from genetically superior animals. Despite its advantages, embryos produced *in vitro* typically exhibit reduced developmental competence and lower survival rates compared to their *in vivo* counterparts. The reduced developmental competence of *in vitro*-produced embryos is largely due to the limitations of artificial culture systems in recapitulating the biochemical and cellular complexity of the maternal reproductive environment. The quality of embryos may be affected by *in vitro* culture (IVC) conditions since they have been shown to trigger cellular stress responses, interfere with apoptotic pathways. To overcome these limitations, several approaches have been investigated to optimize IVC systems, including the incorporation of bioactive molecules into culture media. One of the most enduring difficulties is

the inability to replicate the complicated maternal signaling environment required for appropriate embryonic development; this is especially true of embryos created by somatic cell nuclear transfer (SCNT), which are particularly sensitive to depressed environmental conditions. SCNT-derived embryos commonly exhibit incomplete nuclear reprogramming, reduced developmental potential, and abnormal cellular architecture. Notably, supplementation with certain bioactive compounds, such as resveratrol, has demonstrated beneficial effects by alleviating oxidative stress and enhancing cellular viability.

Interestingly, several studies have highlighted extracellular vesicle (EVs as promising supplements for improving embryo development in species such as mice and sheep; however, research in cattle remains limited. EVs are known to facilitate intercellular communication by transporting bioactive molecules critical for early

embryonic development, implantation, and pregnancy (Feliciano et al., 2014; Qiao et al., 2018). In ruminant livestock, EVs supplementation has been shown to improve embryo development and quality. Qu et al. (2017), for example, isolated EVs from the bovine uterus and reported an increased inner cell mass (ICM) to trophectoderm (TE) ratio and upregulation of genes related to proliferation, growth, and implantation. Similarly, Wei et al. (2022) demonstrated that EV supplementation during *in vitro* maturation (IVM) enhanced bovine embryo development by improving ICM and TE quality. EVs derived from bovine oviduct epithelial cells (BOECs) have also been reported to upregulate genes associated with blastocyst development (Qiao et al., 2018). Moreover, Leal et al. (2022) showed that EVs from the oviduct and uterus improved mitochondrial activity and reduced lipid accumulation in bovine IVF embryos, potentially enhancing embryo quality by downregulating apoptosis-related genes. Lopera-Vasquez et al. (2017) further demonstrated that EVs from monolayer-cultured BOECs (BOECs-M-EVs) enhanced embryo quality by upregulating genes associated with developmental competence, outperforming even co-culture with fresh BOECs. Despite these advances, no studies to date have evaluated the impact of EVs derived from vesicle-shaped or spheroid-cultured BOECs (BOECs-V-EVs) on bovine embryo development. The SCNT, a technique for cloning in which a somatic cell nucleus is transferred into an enucleated oocyte to produce cloned embryos. While common in research and biotechnology, this method is generally carried out with poor efficiency and embryo quality compared to IVF, and as such, represents an excellent model for testing of culture conditions. Given the inherent limitations in SCNT efficiency and embryonic development, EVs represent a promising strategy to bridge this gap by providing physiological cues and regulatory molecules that may compensate for deficiencies in cytoplasmic reprogramming and cell lineage allocation. Thus, the present study aims to investigate the effects of BOECs-M-EVs and BOECs-V-EVs supplementation in IVC medium on the development and quality of bovine embryos produced via IVF and SCNT.

EVs derived from BOECs were chosen for this study because of their exceptional ability to replicate the physiological conditions in the oviductal microenvironment, which may improve embryo development by transferring bioactive molecules necessary for embryogenesis. Furthermore, compared to embryos obtained

from *in vivo* and IVF sources, SCNT is still hindered by low developmental competence and poor embryonic quality. Therefore, evaluating the impact of BOECs-derived EVs on both IVF and SCNT systems is essential for enhancing embryo quality and developmental efficiency, particularly in the context of improving the viability of cloned embryos for livestock ruminant breeding.

1.2 Research Objectives

1.2.1 To evaluate the effects of BOECs-M-EVs and BOECs-V-EVs supplementation in IVC medium on the developmental rate and quality of embryos produced by IVF and SCNT techniques.

1.2.2 To examine the effects of BOECs-M-EVs and BOECs-V-EVs supplementation in IVC medium on the expression of genes associated with embryo development.

1.3 Research Hypotheses

1.3.1 Supplementation with optimal concentrations of BOECs-M-EVs and BOECs-V-EVs in IVC medium can enhance the developmental rate and total cell number of bovine embryos produced by IVF and SCNT.

1.3.2 Supplementation with optimal concentrations of BOECs-M-EVs and BOECs-V-EVs in IVC medium can upregulate the expression of genes associated with embryo development in IVF- and SCNT-derived bovine embryos compared to untreated groups.

1.4 Scope and limitations of the study

This study investigates the effects of BOECs-M-EVs and BOECs-V-EVs supplementation in IVC medium on the developmental outcomes and expression of development-related genes in bovine embryos produced via IVF and SCNT. Limitations include the variability inherent to *in vitro* embryo culture and the specificity of the EVs source and preparation meth

CHAPTER II
LITERATURE REVIEW

2.1 *In vitro* embryo production (IVEP)

One of the most important biotechnologies in animal reproduction is *in vitro* embryo production (IVEP), which greatly advances genetic improvement initiatives and boosts reproductive efficiency. *In vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of embryos are the three essential processes that make up IVEP. By raising reproductive success rates and decreasing generation intervals, this reproductive biotechnology has facilitated fast genetic improvement, leading to a notable increase in livestock output, especially in cattle and buffalo (Currin et al., 2021). The main benefit of IVEP is the effective use of female genetic potential, which enables the production of many embryos from females, even those that are not often used, such as aged cattle, prepubertal heifers, and even pregnant animals (ref). To maximize the genetic value from superior animals, oocytes for IVEP can be obtained from slaughterhouse ovaries or live animals utilizing methods such as ultrasound-guided follicular aspiration (ovum pick up; OPU) (Viana et al., 2010). Additionally, IVEP is cost-effective since it drastically lowers the quantity of semen needed for fertilization. More than 200 oocytes may be fertilized by a single semen straw, allowing for the inexpensive and extensive transmission of better genetics. By enabling focused breeding and quick propagation of chosen sex progeny, the combination of IVEP and semen sexing technologies further boosts efficiency and significantly improves dairy and beef production systems (Viana et al., 2010). In comparison to embryos created *in vivo*, IVEP still has problems with embryo quality and subsequent pregnancy success rates, despite its widespread use. According to Camargo et al. (2006), IVEP usually changed shape, decreased cryotolerance, and increased susceptibility to environmental stress. IVEP results are greatly influenced by variables such breed trait donor animal age, culture media composition, and laboratory procedures (Camargo et al., 2006).

To guarantee high-quality embryo generation and better pregnancy outcomes, these constraints emphasize the continuous need to refine IVEP procedures, especially with relation to media supplementation and donor animal management. Recent improvements in IVEP techniques and culture medium have improved overall embryo viability, blastocyst rates, and cryopreservation success, highlighting the technology's promise and ongoing development. Thus, IVEP continues to be a fundamental biotechnology in the reproductive sciences, promoting faster genetic gain and enhancing the sustainability and general production of animals.

Although IVEP has become a fundamental technique in animal biotechnology, several limitations remain unresolved. Embryos produced *in vitro* often display lower developmental competence, altered gene expression profiles, and reduced cryotolerance when compared to their *in vivo* counterparts (ref). The *in vitro* environment fails to fully replicate the maternal reproductive tract, which naturally provides dynamic biochemical signals, antioxidants, and tightly regulated oxygen tension. Moreover, the use of undefined supplements such as fetal calf serum (FCS) may result in increased lipid accumulation, mitochondrial immaturity, and epigenetic dysregulation factors known to impair implantation and postnatal health (ref). These limitations highlight the need for novel supplementation strategies that better mimic conditions of *in vivo*, such as the incorporation of EVs, which have emerged as promising tools for improving embryo development and quality *in vitro*.

2.1.1 Importance of media for IVEP

One common approach is the supplementation of culture media with additives such as fetal bovine serum (FBS), serum replacements, or bovine serum albumin (BSA). While FBS has been shown to support embryo development (Holm et al., 1999; Thompson et al., 1998), its use has been linked to reduced cryotolerance (Rizos et al., 2003). BSA, on the other hand, has been found to produce comparable or even superior blastocyst rates and embryo quality when compared to FBS (Stroebech et al., 2018). BSA can bind low molecular weight compounds, including heavy metal ions, free radicals, citrate, and steroids. This binding capacity allows it to protect cellular components from toxic substances while also playing a role in regulating redox potential, pH, and osmolarity (George et al., 2008). Although serum

contains beneficial substances for embryo development, such as antioxidants, growth factors, and metal chelators, it can negatively impact embryo quality by promoting the accumulation of lipid droplets (Abe et al., 2002; Ferrer-Roda et al., 2024).

2.1.2 *In vitro* maturation (IVM)

Oocytes can be collected through various methods, including OPU, ovariectomy, and postmortem collection. Following the aspiration of oocytes from ovarian follicles, cumulus-oocyte complexes (COCs) are retrieved, which significantly influences embryo formation and pregnancy outcomes (Leal et al., 2022; Sidrat et al., 2022) Watanabe et al., 2018). Initially, oocytes are in the germinal vesicle (GV) stage and mature to the meiosis-II (MII) stage during IVM. The first observable event in this maturation process is germinal vesicle breakdown (GVBD), characterized by the nuclear membrane breaking down. Research by Gliedt et al. (1996) and Rose & Bavister (1992) indicates that the medium TCM199 yields optimal maturation results. Supplements such as FCS, FBS, estrus cow serum (ECS), and BSA are commonly added to the IVM medium, affecting embryo development from cleavage to blastocyst stages (Blanco & Simonetti, 2002; Lee et al., 1996). Similar to the luteinizing hormone (LH) surge occurring naturally before ovulation, follicle-stimulating hormone (FSH) also plays a critical role in the *in vitro* development of COCs, contributing significantly to both nuclear and cytoplasmic maturation (McGee & Hsueh, 2000). COCs produce various substances and cytokines essential for fertilization, optimizing the ovulation environment, and activating important growth factors, including growth differentiation factor-9 (GDF-9), neurotrophins, inhibins, and tumor necrosis factor-alpha (TNF-α) (Morrison & Marcinkiewicz, 2002; Wang & Roy, 2006). Additionally, oocytes respond to kit ligand (KL) stimulation, secreting critical developmental factors such as bone morphogenetic factor-15 (BMP-15), GDF-9, and basic fibroblast growth factor (bFGF) (Skinner, 2005). Theca cells further contribute to oocyte development by producing keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) (Hyttel et al., 1997). In bovine species, oocytes can undergo GVBD when follicles reach 2-3 mm in diameter, and nuclei capable of maturation to the MII stage typically measure around 110 μm in diameter. Cytoplasmic maturation also occurs, involving critical organelles such as mitochondria (providing energy) and cortical granules (preventing polyspermy

post-fertilization). Additionally, the rough endoplasmic reticulum (RER) synthesizes proteins, and the smooth endoplasmic reticulum (SER) is involved in the synthesis and transport of fatty acids, lipoproteins, RNA, and steroid hormones (Hyttel et al., 1997). These intracellular changes are essential for the successful completion of maturation. Specific proteins, including male pronucleus growth factor (MPGF) and maturation-promoting factor (MPF), significantly influence cytoplasmic maturation and initiate cell cycle progression, preparing oocytes for fertilization (Hyttel et al., 1997). Essential substances such as sodium pyruvate (Arlotto et al., 1996), sodium lactate, glutamine (Fukui, 1990), glucose, sodium bicarbonate (Younis et al., 1989), and epidermal growth factor (EGF) (Blanco et al., 2003) further support and accelerate nuclear maturation processes, ultimately enhancing the developmental competence of the oocyte.

2.1.3 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 undergo critical changes for fertilization to occur. Sperm undergo a crucial process called capacitation, which enhances their motility and metabolism, facilitating their journey to the fallopian tube. Capacitation occurs due to the acidic vaginal environment, activating adenosine triphosphate (ATP) enzymes in the sperm's cytoplasm. This capacitation process involves significant modifications, including alterations in the lipid and glycoprotein content of the sperm plasma membrane (De Jonge & Barratt, 2017). Another essential modification during capacitation prepares the sperm to penetrate the extracellular matrix of the oocyte. The sperm acrosome contains lysosomal enzymes necessary for penetrating the oocyte, released during exocytosis (Okabe et al., 2014). Specifically, the acrosomal enzyme hyaluronidase breaks down the hyaluronic acid surrounding the oocyte, exposing acrosin located in the sperm's inner membrane. Acrosin then facilitates the breakdown of the zona pellucida (ZP) (Coy et al., 2012). To prevent polyspermy, once a sperm successfully penetrates the ZP, an increase in intracellular calcium triggers the release of cortical granules (CGs). These CGs release enzymes that modify the extracellular matrix, making the ZP impermeable to additional sperm by degrading the sperm receptor glycoproteins ZP2 and ZP3, thus ensuring a proper chromosome count in the resulting zygote (Georgadaki et al., 2016).

In the context of IVF, capacitation is essential. Glycosaminoglycans serve as energy sources that stimulate sperm motility and facilitate conditions necessary for fertilization. Additionally, agents such as caffeine and heparin enhance capacitation. Commonly used IVF media formulas include TALP and Brackett and Oliphant (BO), with an optimal capacitation period of 9 to 18 hours (Ward et al., 2003).

2.1.4 *In vitro* culture (IVC)

IVC of bovine embryos is a crucial component in the IVEP system and directly impacts the development, quality, and viability of resulting embryos. Despite progress in assisted reproductive technologies, the IVC phase remains one of the most influential yet challenging steps in achieving consistent blastocyst development comparable to that seen *in vivo*. The composition of culture media plays a central role in embryonic success, as demonstrated by Takahashi and First (1992), who found that while glucose is traditionally present in culture media, it adversely affects early embryo development. Instead, pyruvate and lactate serve as primary energy substrates essential for the progression of bovine embryos to the morula and blastocyst stages (ref). Moreover, supplementation with essential and non-essential amino acids significantly enhances developmental competence, although vitamins alone appear insufficient to support development beyond the morula stage (ref). The inclusion of FCS in culture media has historically increased blastocyst formation rates; however, this comes at the expense of embryo quality. Studies have revealed that serum induces lipid accumulation in embryos, with lipid droplets measuring between 2 to 6 µm, along with an increased presence of immature mitochondria and altered ultrastructure, which negatively affect cryotolerance and post-thaw survival (Abe et al., 2002). Additionally, long-term concerns include large offspring syndrome and abnormal fetal development, often linked to aberrant DNA methylation of genes regulating growth and metabolism (Hasler, 2000; Niemann & Wrenzycki, 2000), Gene expression studies further support the fact that culture conditions significantly influence developmental outcomes. *In vitro*-derived embryos tend to exhibit altered expressions of key regulatory genes such as Connexin43, LIF, Hsp70.1, and Glut-1 compared to their *in vivo* derived counterparts. These modifications may be associated with epigenetic dysregulation, raising concerns about the long-term developmental competence of

embryos cultured under suboptimal conditions. To mitigate these issues, co-culture systems using somatic cells like oviductal epithelial cells or cumulus cells have been developed to provide embryo trophic support and detoxify the environment. While effective, these systems often lack reproducibility and raise biosafety concerns due to the undefined nature of the co-culture medium. Morphologically, embryos cultured *in vitro*, particularly in serum-supplemented media tend to have darker cytoplasm, swollen blastomeres, and atypical lipid profiles, which correlate with poor cryosurvival, impaired mitochondrial structure, and chromosomal instability (Rizos et al., 2003). Despite achieving similar blastocyst yields, embryos produced *in vivo* consistently demonstrate higher quality than those generated *in vitro*.

Given these findings, current recommendations emphasize the use of chemically defined, serum-free media supplemented with selected amino acids, growth factors, and antioxidants. Ongoing improvements in media formulation and a deeper understanding of embryo–maternal communication are essential for optimizing IVC conditions and reducing the risks of epigenetic and developmental abnormalities. The successful refinement of IVC systems will ultimately enhance the reliability and safety of bovine embryo production for both research and commercial applications.

2.2 Introduction to EVs

Extracellular vesicle or EVs are nano-sized, membrane-bound particles secreted by almost all cell types into the extracellular environment. These vesicles play a pivotal role in intercellular communication by delivering biologically active cargo, including proteins, lipids, mRNAs, and non-coding RNAs, thereby modulating physiological and pathological functions of recipient cells (Ludwig & Giebel, 2012; Sheta et al., 2023; Zou et al., 2023). According to their size and biogenesis, EVs are often divided into three primary subtypes: apoptotic bodies (500–2000 nm), microvesicles (MVs, 100–1000 nm), and exosomes (30–150 nm) (György et al., 2011). Multivesicular bodies (MVBs), which are created when endosomal membranes bud inward, merge with the plasma membrane to release intraluminal vesicles as exosomes. While apoptotic bodies are produced after planned cell death, microvesicles are created directly from the plasma membrane by outward budding (Ludwig & Giebel, 2012). Blood plasma, urine, saliva, amniotic fluid, and reproductive

tract secretions are among the bodily fluids that contain EVs in large quantities. Critical processes such as oocyte maturation, fertilization, and embryo development have been demonstrated to be supported by EVs produced from follicular fluid, oviductal fluid, and uterine secretions in reproductive biology (Zarà et al., 2019). Oviductal EVs are important for maintaining embryonic competence because they are implicated in the regulation of oxidative stress, mitochondrial function, and transcriptome reprogramming of early embryos. In addition to reproduction, EVs are being aggressively investigated for their potential therapeutic uses in medication delivery, tissue regeneration, and diagnostics (ref). They are desirable candidates for cell-free therapeutic approaches because of their inherent stability, biocompatibility, and natural targeting capabilities (Zou et al., 2023). To fully utilize EVs in reproductive biotechnology and regenerative medicine, it is crucial to comprehend their biogenesis, cargo composition, and function.

EVs are small, membrane-bound vesicles released by cells into the extracellular environment. They play a crucial role in intercellular communication, contributing to various biological processes, including cell development and maintenance. EVs are characterized by specific marker proteins on their surface, such as CD9, CD63, and CD81. These vesicles are commonly classified into three main subtypes: exosomes, MVs, and apoptotic bodies.

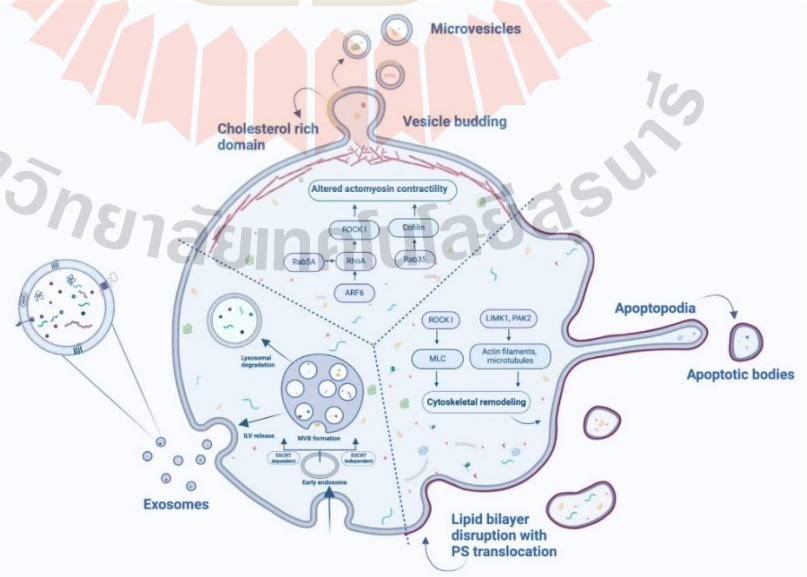


Figure 1. Schematic overview of the biogenesis of EVs and associated regulatory (Ovčar & Kovačič, 2022).

2.2.1 Exosomes

Exosomes are small vesicles, typically 30-150 nm in size, that carry a diverse array of biomolecules, including proteins, nucleic acids, lipids, and various signaling molecules. Their cargo is selectively packaged and transferred to recipient cells, allowing them to influence the behavior and functions of those cells. Exosome exchange between cells facilitates the transfer of biological information and enables intercellular communication (Yang et al., 2020). Once inside recipient cells, exosome cargo can alter functions such as gene expression, protein synthesis, immune signaling, and tissue repair.

2.2.2 Microvesicles (MVs)

Microvesicles range in size from 50 nm to 1000 nm and have a spherical shape. Their formation results from cytosolic cargo being enclosed in a membrane and released through cytoskeletal remodeling and calcium influx (Butler et al., 2018). MVs are typically produced in response to elevated intracellular calcium levels, stress, or cellular activation.

2.2.3 Apoptotic Bodies

Apoptotic bodies form during programmed cell death (apoptosis), when nuclear karyorrhexis occurs, leading to cytoplasmic blebbing. These vesicles, larger than 1000 nm, consist of an intact plasma membrane that encloses cytosolic components, including organelles and nuclear fragments (Taylor et al., 2008).

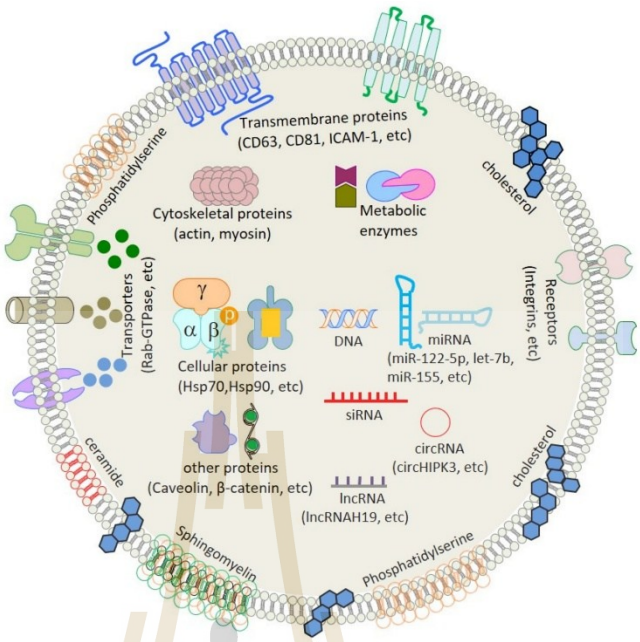


Figure 2. General representation of the exosome structure (Jan et al., 2021).

EVs with high purity and biological activity may be isolated using one of the most popular and dependable techniques, OptiPrepTM density gradient ultracentrifugation (ODG UC). This technique effectively removes protein aggregates, lipoproteins, and other non-vesicular pollutants by separating vesicles according to their buoyant density. Pavani et al. (2019) used a discontinuous iodixanol (OptiPrep™) density gradient to separate EVs from *in vitro* embryo-conditioned medium. Four density increments (5%, 10%, 20%, and 40%) were layered in ultracentrifuge tubes as part of the procedure, and then the tubes were centrifuged at 100,000 × g for eighteen hours at 4°C. Keep the pellets determined to be EVs, concentrate on their density and the presence of certain EVs markers. To confirm EVs identity and purity, the researchers employed several characterization technique as follow;

1) Western blotting to detect canonical EVs markers such as CD9, CD63, and TSG101, and to confirm the absence of contaminants such as ApoA-I and Ago-2.
2) Transmission electron microscopy (TEM) to visualize vesicle morphology.
3) Nanoparticle tracking analysis (NTA) to determine particle size and concentration.

The particle population isolated ranged between 25 to 230 nm, with a mean diameter of approximately 123.7 ± 7.8 nm, which is consistent with the expected size range of exosomes. Moreover, supplementation in embryo culture and fluorescent labeling (PKH67) were used to confirm the biological activity of isolated EVs. The functional importance of EVs acquired using ODG UC was proven by the internalization of labeled EVs by zona-intact embryos, as well as by enhanced blastocyst development and decreased apoptotic cell ratios. Overall, this method provides high-quality EVs preparation suitable for *in vitro* applications in embryo production and reproductive biotechnology.

2.2.4 The Role of EVs in Reproduction

In the oviduct, which is the location of important early reproductive processes such as oocyte maturation, fertilization, and preimplantation embryo development, EVs have become important mediators of cell-to-cell communication in the reproductive tract (Bastos et al., 2022). The oviductal fluid contains these nano-sized vesicles, which transport bioactive substances, like proteins, lipids, mRNAs, and miRNAs that alter cellular reactions in the maternal and embryonic compartments. The developing embryos, follicular fluid, gametes, and oviductal epithelial cells are some of the origins of oviductal EVs. The precise sources of these EVs are difficult to determine, but it is believed that they assist oocyte maturation, sperm capacitation, embryo genome activation, and early embryonic development, among other physiological activities (Almiñana & Bauersachs, 2020). Throughout the estrous cycle, their concentration varies dynamically, indicating that s composition and function are influenced by hormonal control (Almiñana et al., 2018). In the oviduct, EVs mediate the early maternal-embryonic crosstalk in addition to facilitating communication between gametes and the maternal environment. For instance, it has been demonstrated that embryos living in the isthmus for 4–5 days after conception change the miRNA profile of the epithelial cells there, indicating a feedback process facilitated by EVs (Mazzarella et al., 2021). By altering signaling pathways associated with metabolism, immunological response, and pluripotency, these miRNAs can control the expression of certain genes in recipient cells. Improved embryo survival, cryotolerance, and lipid composition have been linked to the addition of oviductal EVs or those

generated from BOECs to IVC (Banliat et al., 2020; Lopera-Vasquez et al., 2016). It's interesting to note that the lipid cargo of oviductal EVs and the lipid profile of blastocysts matched, indicating that EVs contents were actively incorporated into embryos. Moreover, EVs have an impact on early development's epigenetic regulation. Research has shown that embryos exposed to oviductal EVs have altered DNA methylation and hydroxy methylation, suggesting a possible function in chromatin remodeling. These findings support the notion that EVs not only transmit passive information but also actively modulate early embryonic development. EVs are promising bioactive components for enhancing assisted reproductive technologies because of their diverse activities. This includes perfect candidates for improving IVEP systems because of their capacity to replicate maternal signals and promote embryo development. Altogether, these findings emphasize that EVs are not merely passive messengers but active modulators of reproductive processes. Their incorporation into IVEP systems may hold the key to improving embryo quality and mimicking *in vivo* conditions more effectively.

2.2.5 Application of BOECs-EVs in IVC

The complex and dynamic maternal environment necessary for the best possible embryonic development is frequently not replicated by IVC methods. EVs generated from bovine oviductal epithelial cells (BOECs-EVs) have become a potential bioactive supplement for enhancing the quality and growth of embryos *in vitro*. Proteins, lipids, RNAs, and regulatory molecules that mimic maternal signals and improve developmental competence are carried by these vesicles. Wei et al. (2022) showed that adding 3% BOECs-EVs to IVM conditions significantly improved cumulus cell growth, mitochondrial function, and oocyte maturation. This was linked to lower levels of reactive oxygen species (ROS), higher expression of oocyte maturation genes (GDF9, CPEB1), and gap junction markers (CX43 and CX37). Moreover, oocytes treated with BOECs-EVs produced embryos with increased blastocyst rates and enhanced implantation potential, indicating that BOECs-EVs had a beneficial effect on both nuclear and cytoplasmic maturation processes. The addition of BOECs-EVs during IVC significantly enhanced embryo growth in later phases. Research has shown that embryos cultivated with BOECs-EVs had better ICM to TE ratios, higher blastocyst

formation rates, and more total cells (Leal et al., 2022; Sidrat et al., 2022). With processes connected to tight junction integrity, water channel control (e.g., aquaporins), and trophectoderm structure maintenance, these enhancements were followed by lower apoptosis and higher cryotolerance (Sidrat et al., 2022). Importantly, EVs appear to promote embryo-maternal signaling even under *in vitro* conditions. Aguilera et al. (2024) found that EVs isolated from uterine fluid (EVs-UF) significantly enhanced Interferon tau (*IFNT)* expression and blastocyst expansion, while EVs from cultured endometrial cells (EVC) influenced the expression of embryo quality-related genes. These findings suggest that EVs' tissue source and physiological context impact their effectiveness in supporting embryonic development. Increased resistance to cryopreservation stress, greater embryo quality, and faster development rates are just a few advantages shown when BOECs-EVs are used in IVC systems. The molecular payload of EVs is responsible for these actions, supporting gene expression, mitochondrial function, membrane integrity, and epigenetic modification. Additional investigation is necessary to improve EVs dose, supplementation timing, and EVs subtype characterization for translational use in embryo biotechnology and bovine reproduction.

2.3 Problems in Cloning

Reproductive biotechnology has greatly benefited from SCNT, yet enduring problems with embryo quality continue to restrict its practical application. Even when cloned embryos reach the blastocyst stage of development, they frequently exhibit morphological and developmental defects that jeopardize their viability and survival after transfer. To important problem with embryos created from SCNTs is the aberrant distribution of cells between the TE and ICM. According to Wells (2005) and Tsunoda & Kato, 2002, cloned embryos frequently show reduced total cell counts, an unbalanced ICM-to-TE ratio, and elevated blastocyst apoptotic rates. Due to their detrimental effects on later developmental stages, these cellular abnormalities raise the risk of embryonic death and impair implantation rates. Furthermore, placental dysfunction frequently accompanies cloned embryos, manifesting in abnormal placenta formation, enlargement, and inadequate vascularization. Such placental defects are major contributors to prenatal losses, affecting fetal nutrient exchange and

oxygen supply, ultimately reducing embryo viability and increasing fetal mortality rates (Hill et al., 2002; Wells, 2005). Moreover, compared to embryos created using traditional techniques like IVF, cloned embryos are more likely to experience developmental arrest at different pre-implantation stages. Research has shown that problems, including incomplete cytoplasmic reprogramming and mitochondrial malfunction in recipient oocytes, severely hinder the growth rate, metabolism, and overall developmental potential of the embryo (Tachibana et al., 2009). Another major issue with cloned embryos is the large offspring syndrome (LOS), which is defined by neonatal structural abnormalities and abnormal birth weights. Immune deficiencies, respiratory discomfort, and decreased motor function are frequent neonatal problems. Inadequate cytoplasmic maturation and ineffective nuclear-cytoplasmic connections during SCNT operations are primarily responsible for these phenotypic defects (Hill, 2014). Enhancing oocyte quality evaluation, optimizing nuclear transfer methods, and improving embryo culture conditions are crucial to overcoming these constraints. Cloning procedures may potentially benefit from the development of strong prediction indicators for embryonic competency before embryo transfer. The overall effectiveness and dependability of cloning technologies would be enhanced by such developments, which would drastically lower embryonic and newborn losses (Oback, 2008).

2.4 EVs in IVEP

EVs, particularly exosomes, have shown significant promise in enhancing the efficiency and quality of embryos produced by assisted reproductive technologies, such as SCNT and parthenogenetic activation (PA). Recent studies in bovine and porcine models demonstrate that EVs supplementation into embryo culture media can mimic critical *in vivo* conditions, promoting developmental competence of cloned embryos through improved cellular physiology and reduced apoptosis. Qu et al. (2017) investigated the significance of embryo-derived exosomes during IVC using a bovine SCNT model. According to their research, regular culture medium renewal, which eliminates exosomes generated by the embryo, impairs embryo growth. On the other hand, blastocyst development, total cell quantity, decreased apoptotic index were all markedly improved by continued culture without medium renewal. These adverse

effects were mitigated by adding exosomes back into the renewed media, which significantly restored developmental parameters including blastocyst formation and calving rates to levels equivalent to those of nonrenewed culture settings. This data highlights the critical function of exosomes produced from embryos in preserving developmental signaling and embryonic homeostasis in cloned embryos. EVs produced from steroid-primed porcine oviductal epithelial cells (POECs) were shown by Bang et al. (2023) to greatly improve the development of both PA and SCNT embryos in pig models. Estradiol and progesterone were used to induce POECs to replicate the diestrus and estrus phases of the estrous cycle. EVs derived from these cells demonstrated improved pig embryo developmental potential. In particular, the addition of hormone-primed EVs to embryos resulted in enhanced expression of pluripotency-associated genes (*OCT4, NANOG, SALL4*) and anti-apoptotic BCL2, as well as increases in blastocyst formation rates, total cell counts, and decreased apoptosis. These findings imply that EVs composition may be influenced by hormonal factors and point to their potential application as a bioactive supplement to enhance the quality of embryos *in vitro*. Han et al. (2025) extended these results by extracting EVs straight from uterine fluids (Ut-EVs) and pig oviductal fluids (Ov-EVs) to mimic the natural embryo migration environment. Sequentially cultivated PA and SCNT embryos using Ov-EVs and Ut-EVs showed decreased apoptosis, increased cell counts, and markedly enhanced mitochondrial activity at the blastocyst stage. Crucially, SCNT embryos' blastocyst growth rate significantly increased upon successive culture with Ov-EVs and Ut-EVs. Increased expression of pluripotency markers *(OCT4, SOX2)* and a significant decline in pro-apoptotic gene expression were linked to these benefits. *In vitro* embryo culture conditions may be made more physiological by using EVs made from reproductive tract fluids, which will eventually improve embryonic competence and implantation potential. All these investigations together offer strong proof that EVs supplementation may greatly improve the developmental competence of cloned and IVEP, especially when produced under physiologically appropriate settings. As important regulators of mitochondrial activity, apoptotic regulation, and embryonic gene expression, EVs provide a potent strategy for enhancing the results of reproductive biotechnology. Standardized separation techniques, the ideal dose, and

the intricate biochemical pathways driving EVs-mediated enhancements in embryo development should all be investigated in future research.

2.5 EVs-Induced Molecular and Morphological Improvements in Embryos

EVs-Induced molecular and morphological improvements in embryos by mediating intercellular communication and delivering molecular cues, EVs, especially exosomes derived from reproductive tissues, have become essential bioactive components that enhance embryonic development. Studies on humans and animals have shown that EVs have a positive effect on gene regulation and embryo shape. Compared to control groups, oviductal or endometrial EVs-cultured embryos show better blastocyst quality morphologically, with bigger diameters, more total cells, and greater ICM to TE ratios. For example, EVs have been demonstrated to dramatically boost cellular proliferation and blastocyst diameter, indicating improved cell survival and decreased apoptosis inside the embryo (Qu et al., 2020). At the molecular level, EVs supplementation alters the expression of genes linked to mitochondrial function, anti-apoptotic markers (*BCL2*), and pluripotency (e.g., *OCT4, SOX2, NANOG*). Melatonin-loaded EVs improved energy metabolism and decreased oxidative stress by stabilizing mitochondrial membrane potential and increasing *OCT4* and *NANOG* expression, as shown by Qu et al. (2020). Additionally, it has been demonstrated that EVs produced from BOECs preserve tight junction integrity and increase aquaporin expression in TE cells, both of which are critical for the development of blastocoels and the growth of blastocysts (Sidrat et al., 2022; Leal et al., 2022). Mechanistically, EVs act by delivering small RNAs, proteins, and lipids that influence signal transduction, epigenetic remodeling, and cytoskeletal organization within embryonic cells. As outlined by Jan et al. (2021), EVs uptake by embryos can result in transcriptional reprogramming through miRNA and lncRNA delivery, influencing key developmental pathways such as MAPK, PI3K/Akt, and TGF-β. Collectively, these results provide credence to the use of EVs as a physiological, non-invasive method of enhancing embryonic competence through the imitation of maternal signals. This developmental enhancement is particularly crucial in somatic cell nuclear transfer (SCNT) and other assisted reproductive technologies, where in vitro conditions often compromise embryo quality

and viability. By mimicking the physiological cues naturally present in the female reproductive tract, EVs provide a bioactive microenvironment that supports cellular communication, reduces oxidative stress, and modulates gene expression involved in survival, implantation, and pluripotency. Notably, the upregulation of implantation-related genes such as IFN-tau, and the downregulation of apoptotic markers like BAX, highlight the EVs' functional relevance in improving embryo competence. Consequently, integrating EVs into IVEP protocols not only promotes more consistent and reliable embryonic development but also paves the way for replacing undefined supplements like serum or BSA with a standardized, cell-origin-specific additive, aligning with the long-term goal of developing fully defined culture systems.

CHAPTER III

MATERIALS AND METHODS

3.1 Reagents and Ethics statement

All reagents described in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA), except otherwise noted.

3.2 Ethical statement

Ethical approval for this study was obtained from the Institutional Animal Care and Use Committee of Suranaree University of Technology, Thailand (Approval Number: IACUC-67-42).

3.3 Experiment design

Experiment 1

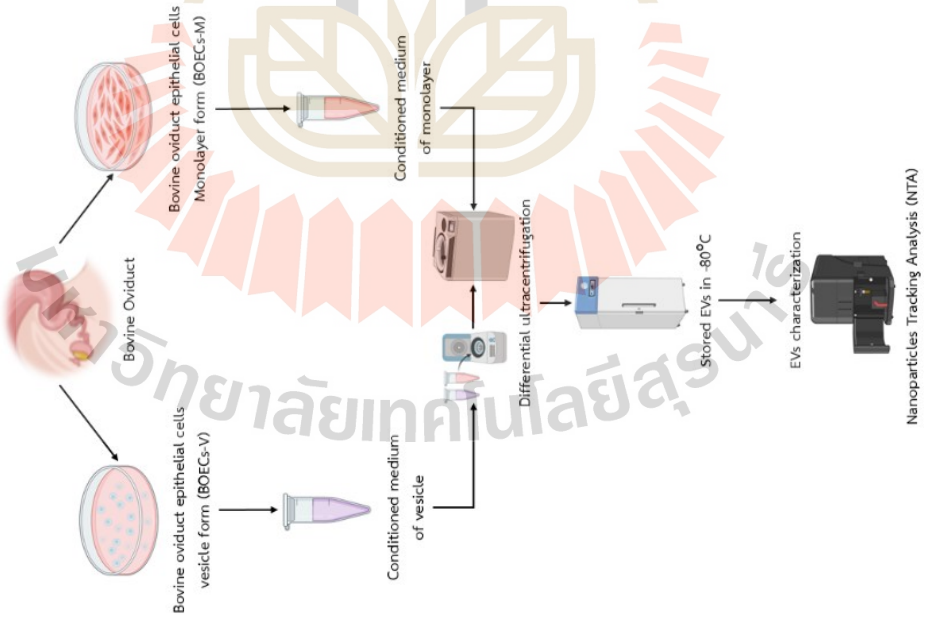


Figure 3. Workflow for isolating extracellular vesicles (EVs) from bovine oviduct epithelial cells cultured in vesicle (BOECs-V) or monolayer (BOECs-M) forms. EVs were collected by differential ultracentrifugation and analyzed by nanoparticle tracking analysis (NTA)

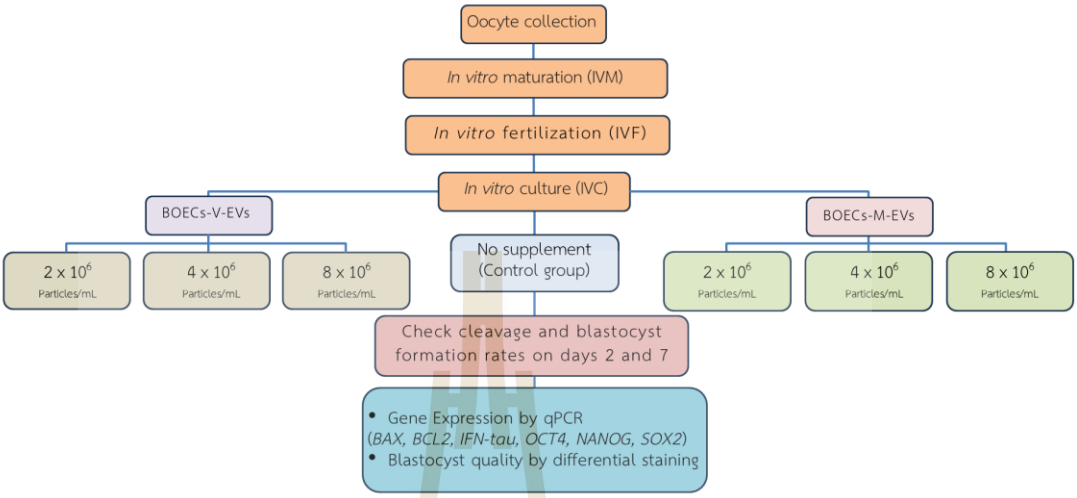


Figure 4. Experimental design of the production of *in vitro* fertilized embryo IVF supplemented with BOECs-derived EVs at varying concentrations. Embryo development and quality were assessed by cleavage and blastocyst rates, qPCR, and differential staining

Experiment 2

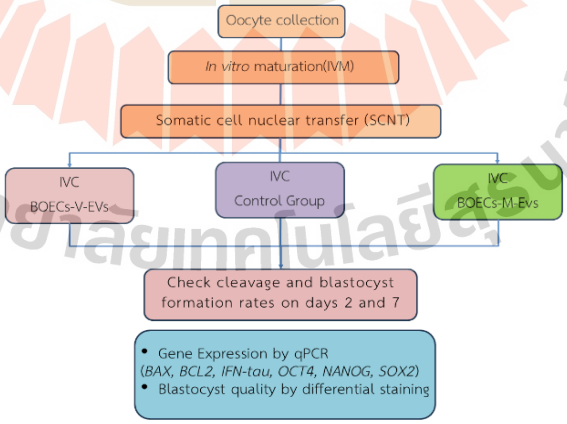


Figure 5. Experimental design of somatic cell nuclear transfer (SCNT) embryo culture supplemented with BOECs-derived EVs. Embryo development and quality were evaluated by cleavage and blastocyst rates, gene expression, and differential staining

3.4 Preparation of EVs-depleted fetal bovine serum (dFBS)

EVs were separated from heat-inactivated FBS as previously detailed (Aswad, Jalabert, & Rome, 2016). In brief, FBS was heat-inactivated (30 min at 56°C in a water bath), followed by two rounds of ultracentrifugation (90 min at 120,000 × g at 4°C), and the supernatant was collected, aliquoted (in conical tubes), and stored at −20°C until usage.

3.5 Preparation of bovine oviduct epithelial cells (BOECs)

Healthy bovine oviducts at the Post-ovulatory stage that presented day 1-5 Cl on ovary were collected from a slaughterhouse, as described by Almiñana et al. (2017). Briefly, the oviducts were thoroughly rinsed with 0.9% (w/v) NaCl solution, placed in sterile plastic bottles containing the same solution, and stored at 4°C. Upon arrival at the laboratory within 4-6 h after slaughtered, they were rinsed again with 0.9% NaCl to remove blood and debris, then soaked in phosphate-buffered saline (PBS). To reduce contamination, the oviducts were briefly submerged in 70% ethanol, followed by additional rinses with PBS and washed twice in 70% ethanol. Connective tissues and blood vessels were carefully removed inside a biosafety cabinet. Subsequently, the oviducts were rinsed with a washing solution consisting of TCM199-HEPES supplemented with 0.2% (w/v) bovine serum albumin (BSA). Oviductal epithelial cells were collected by pressing the oviductal segments into 1.5 mL microcentrifuge tubes using sterile forceps. At least three oviducts were pooled. The harvested cells were dissociated by repeatedly aspirating and expelling the cell suspension ten times using a 1 mL syringe fitted with 21-gauge needles. The resulting cell suspension was transferred to a 15 mL conical tube containing 10 mL of TCM199 with 10% FBS and incubated at 38.5°C for 5 minutes. After incubation, the supernatant was discarded, and the cells were washed twice with 10 mL of washing in culture medium. Subsequently, 100 µL of the recovered cells were diluted in 10 mL of culture medium (TCM199 supplemented with 10% FBS) and seeded in 100 mm culture dishes (Nunc, Denmark; 10 mL per dish). The cultures were incubated at 38.5°C in a humidified atmosphere containing 5% CO_2 for 48 hours. The BOECs were then cryopreserved in liquid nitrogen until further use.

3.5.1 Preparation of conditioned medium from monolayered bovine oviduct epithelial cells (BOECs-M)

BOECs from 3.5 were seeded in 100 mm culture dishes containing TCM199 medium -the cell-free medium was discarded and replaced with 10 mL of fresh TCM199 medium supplemented with 10% dFBS. The cells were maintained at 38.5°C in a humidified atmosphere with 5% CO_2 in air until they reached confluence. Conditioned media were collected on days 2, 4, and 6 post-incubation for EVs isolation. After each collection, 10 mL of fresh TCM199 medium supplemented with 10% dFBS was added to the culture dishes. The harvested conditioned media were stored at –80°C until further use.

3.5.2 Preparation of conditioned medium from vesicle-shaped bovine oviduct epithelial cells (BOECs-V)

BOECs from 3.5 were propagated in culture medium in 100 mm culture dishes and incubated at 38.5°C for 48 hours, as previously described. To obtain EVs exclusively from suspended BOECs-V, the conditioned medium was transferred to a new 100 mm culture dish and incubated at 38.5°C for another 48 hours. Subsequently, the conditioned medium containing suspended BOECs-V was pipetted into 5 mL of TCM199 medium supplemented with 10% dFBS and incubated at 38.5°C for 48 hours. The suspended BOECs-V were washed twice in TCM199 washing solution containing 10% dFBS. After washing, the cells were cultured in 60 mm dishes with 5 mL of TCM199 supplemented with 10% dFBS. Conditioned media were collected on days 2, 4, and 6 post-incubation and stored at –80°C, as previously described.

3.5.3 EVs isolation

EVs were isolated from the conditioned media collected from BOECs monolayers (BOECs-M-EVs) and suspended vesicle-derived BOECs (BOECs-V-EVs) using a protocol based on Théry et al. (2006), with minor modifications. The conditioned media containing BOECs-M-EVs and BOECs-V-EVs were separately centrifuged at 300 × g for 15 minutes at 4°C to remove cells and debris. The resulting supernatants were collected and centrifuged again at 2,000 × g for 15 minutes at 4°C, followed by a third centrifugation step at 12,000 × g for 15 minutes at 4°C to eliminate large vesicles and

apoptotic bodies. The final supernatants were subjected to ultracentrifugation at 100,000 × g for 90 minutes at 4°C to pellet the EVs. The pellets were resuspended in PBS (without calcium and magnesium; PBS–) and filtered through a 0.22 µm filter to remove remaining contaminants. Both BOECs-M-EVs and BOECs-V-EVs were stored at –80°C until further use.

3.5.4 The particle size distribution by nanoparticle size analyzer

Particle size distribution of the isolated EVs was analyzed using NTA with the NanoSight Pro instrument (Malvern Panalytical, UK), following the manufacturer's instructions. Briefly, EVs pellets were resuspended in PBS(–). The EVs suspension was then transferred into a clean glass cuvette and loaded into the instrument for particle size and concentration analysis using the NTA system.

3.6 *In vitro* embryo production (IVEP)

3.6.1 Oocyte collection and IVM

Bovine ovaries were collected from a local abattoir and transported to the laboratory in 0.9% (w/v) NaCl solution at room temperature. Upon arrival to laboratory within X h, the ovaries were rinsed thoroughly with 0.9% NaCl solution. Cumulus–oocyte complexes (COCs) were aspirated from 2–8 mm follicles using an 18-gauge needle attached to a 10-mL syringe. The retrieved COCs were examined under a stereomicroscope, and those exhibiting at least three layers of compact cumulus cells and homogeneous cytoplasm were selected. Selected COCs were washed in modified Dulbecco's phosphate-buffered saline (mDPBS) and subsequently transferred into a 60 mm culture dish containing IVM medium (20 COCs per 100 µL drop), covered with mineral oil. The IVM medium consisted of TCM-199 supplemented with 10% FBS, 1 IU/mL human chorionic gonadotropin (hCG; Intervet, Netherlands), 0.02 IU/mL follicle-stimulating hormone (FSH, Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan), and 1 µg/mL 17ß-estradiol. Oocytes were cultured for 23 h at 38.5°C in a humidified atmosphere 5% CO_2 in air.

3.6.2 Sperm preparation and IVF

Frozen semen from a proven Wagyu bull (Pornchai Intertrade Ltd., Ratchaburi, Thailand) stored in 0.25 ml straws was thawed in air for 10 seconds and subsequently immersed in a 37.5°C water bath for 1 minute. The thawed semen was transferred to the bottom of a 5 ml snap-cap tube (Corning, Glendale, AZ, USA) containing 2 ml of TALP medium (Lu et al., 1987) and incubated at 38°C under a humidified atmosphere of 5% CO_2 for 30 minutes. After incubation, the upper 1.8 ml layer was gently collected and transferred to a 15 ml conical tube (SPL Life Sciences) containing 5 ml of TALP medium. The sperm suspension was centrifuged at 400 × g for 5 minutes, and the supernatant was discarded. The sperm pellet was resuspended and adjusted to a final concentration of 2×10^6 sperm/ml using TALP medium. A 50 µl aliquot of the sperm suspension was placed into 35 mm culture dishes overlaid with mineral oil and incubated at 38°C in a humidified atmosphere of 5% CO_2 for 10 hours. Following co-incubation, presumptive zygotes were denuded of cumulus cells and excess sperm. The zygotes were then cultured in CR1aa medium (Rosenkrans et al., 1993) supplemented with 5% fetal bovine serum (FBS) under a gas phase of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C for 7 days. Embryo development was assessed on Day 2 and Day 7 to evaluate cleavage (2–8 cell stage) and blastocyst formation, respectively.

3.6.3 Donor cell preparation

Ear skin tissues were obtained by biopsy from male cattle. The collected tissues were minced into small fragments and cultured in alpha modification of minimum essential medium (αMEM) supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 8–10 days. Upon reaching sub-confluence, fibroblasts were harvested using 0.25% trypsin-EDTA and subcultured in αMEM supplemented with 10% FBS for expansion up to the third passage. For cryopreservation, fibroblasts were suspended in αMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO), then stored in liquid nitrogen. Prior to use, the frozen-thawed fibroblasts were cultured for an additional 2–3 days. Only cells at the fourth passage were used as nuclear donor cells for subsequent procedures.

3.6.4 SCNT and embryo culture

Following a 23-hour culture of COCs in IVM medium, denudation of the cumulus cells was achieved by treatment with a 0.1% hyaluronidase solution. Metaphase II (MII) oocytes, characterized by the presence of the first polar body, were selected for enucleation. The selected MII oocytes were placed in 5 µg/mL cytochalasin B for 5 minutes to inhibit microtubule formation. The zona Pellucida (ZP) above the first polar body was carefully dissected with a glass needle, and approximately 5–10% of the cytoplasm beneath the first polar body was squeezed out. The success of enucleation was confirmed by staining the extracted cytoplasm with 5 µg/mL Hoechst 33342 and visualizing the stained nucleus under an inverted fluorescence microscope (IX71, Olympus, Tokyo, Japan). A single donor fibroblast was then inserted into the perivitelline space of the enucleated oocyte. The donor cell-cytoplast couplet (DCCC) was fused in Zimmermann fusion medium (Zimmermann and Vienken, 1982) using fusion electrodes. Fusion was induced by two direct current pulses (24 V, 15 µsec). The fused DCCCs were washed four times in TCM199 HEPES supplemented with 10% FBS. Fusion success was assessed one hour after electrostimulation. The reconstructed were subsequently activated by exposure to 7% ethanol in TCM199 HEPES supplemented with 10% FBS for 5 minutes at room temperature. Activation was followed by culture in CR1aa medium supplemented with 1.25 µg/mL cytochalasin D (CD) and 10 µg/mL cycloheximide (CHX) at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 5 hours. After activation, the reconstructed embryos were cultured in CR1aa medium supplemented with 5% dFBS and either BOECs-M-EVs or BOECs-V-EVs at optimum concentration from IVF experiment at a density of 12 oocytes per 80 µL culture medium under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C for 7 days. Embryo development was assessed on days 2 and 7 post-culture.

3.6.5 IVC of presumptive zygotes

After 10 hours of co-incubation of sperm and COCs in the fertilization medium, the presumptive zygotes were denuded by gentle pipetting in TCM199 HEPES supplemented with 10% FBS. The denuded zygotes were then cultured in CR1aa

medium supplemented with 5% dFBS and either BOECs-M-EVs or BOECs-V-EVs at three different concentrations (2×10^6, 4×10^6, and 8×10^6 particles/mL). The CR1aa medium supplemented with 5% dFBS, but without EVs supplementation, served as the negative control. The culture medium was covered with mineral oil, and the zygotes were cultured in 35 mm culture dishes at a ratio of 12 zygotes per 80 µL of culture medium. Incubation was carried out under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C for 7 days. Embryo development was assessed on day 2 and day 7 post-culture.

3.6.6 Embryo quality

On day 7 post-insemination and cloning, blastocysts were first measured for embryo diameter before fixation and staining. Bright-field images were captured at 200× magnification using an inverted microscope. Embryo diameter was measured using ImageJ software (NIH, USA) by drawing a straight line across the widest axis of the blastocyst, excluding the ZP. Each measurement was calibrated using a built-in scale bar, and each group's average diameter was recorded.

Following size measurement, the embryos were stained with 0.1 mg/ml propidium iodide (PI) and 0.2% Triton X-100 in mDPBS supplemented with 0.1% PVP for 1 min. Afterward, embryos were placed into Hoechst 33342 solution dissolved in 99.5% ethanol for 5 min before being mounted with glycerol on a glass slide. Using an inverted fluorescent microscope, counts of TE and ICM were conducted. ICM cells appeared blue due to Hoechst uptake, while TE cells were stained pink red.

3.7 Quantitative real-time polymerase chain reaction (qPCR)

Twenty blastocysts from each group were washed three times with PBS (-) and stored at -80°C until further use. The manufacturer extracted total mRNA using the FavorPrep™ Tissue total RNA Mini Kit (Favorgen Biotech Crop., Pingtung, Taiwan). cDNA synthesis was performed using biotechrabbit™ cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany), and the expression of specific genes was assessed using the KAPA SYBR FAST qPCR Master Mix (Applied Biosystems) on the CFX Opus 96 real-time PCR system (Biorad, Hercules, California, USA). Melting curve analysis was performed for all primers, which were optimized. The primer sequences are provided in Table 3.9.1.

GAPDH was used as a housekeeping gene to normalize the expression of target genes. qPCR was performed in triplicate, and statistical analysis was

conducted using the 2^-ΔΔCt method. GAPDH was used as the housekeeping gene to normalize the target genes.

Table 1. Genes used for real-time qPCR of blastocysts.

Genes	Primer sequences	Product length (bp)	Accession numbers
Bax	F:(5´–3´) TCTGACGGCAACTTCAACTG R:(5´–3´) TCGAAGGAAGTCCAATGTCC	135	NM_173894.1
BCL2	F:(5´–3´) ATGCGGCCCCTGTTTGATTT R:(5´–3´) GCCTGTGGGCTTCACTTATG	116	NM_001166486.1
IFN-tua	F:(5´–3´) CTGGCCCGAATGAACAGACT R:(5´–3´) AGAGGTTGAAGCACTGCTGG	151	XM_024989143.2
POU5F1/Oct4	F:(5´–3´) GAGTGTGGTTTTGCAAGCGT R:(5´–3´) ATACGGGTCCCCCTGTGAA	108	NM_174580.3
SOX2	F:(5´–3´) TCTTGGTTCCATGGGTTCGG R:(5´–3´) CTGGAGTGGGAGGAAGAGGT	160	NM_174365.4
NANOG	F:(5´–3´) AGTCCTGCTTGCAGTTCCAG R:(5´–3´) TCAGGTTGCATGTTCGTGGA	175	XM_013471593.1
GAPDH	F: (5´–3´) CTCCCAACGTGTCTGTTGTG R: (5´–3´) TGAGCTTGACAAAGTGGTCG	222	NM_001034034.2

3.8 Statistical analysis

Data are expressed as mean ± SEM. Numbers of embryo development and total cell number were tested for normality and homoscedasticity before statistical analysis. Differences in embryo development and total cell number were analyzed using a one-way ANOVA followed by Dunnett's Multiple Comparison for post-hoc comparison Gene expression data were obtained from at least three biological replicates, each performed in triplicate, and analyzed using one-way ANOVA, followed by Tukey's multiple comparison test to determine statistical differences among groups. Differences were considered statistically significant at a probability level of $P < 0.05$. All statistical analyses were performed using GraphPad Prism software

(GraphPad Prism 5.01 Inc., La Jolla, CA, USA). The level of significance for all analyses was $P < 0.05$.

CHAPTER IV

RESULTS

4.1 EVs isolated from oviduct epithelial cells in this study

As shown in Fig. 6, EVs were isolated from monolayer cells and vesicle forms. The two culture strategies used in this study could produce two forms of EVs: BOECs-M and suspended BOECS-V. The isolated BOECs-M, sized ranging 50-100 μm in diameter, was attached to the dish. Meanwhile, suspended BOECs-V sized ranging 100-200 μm in diameter were floated in the dish.

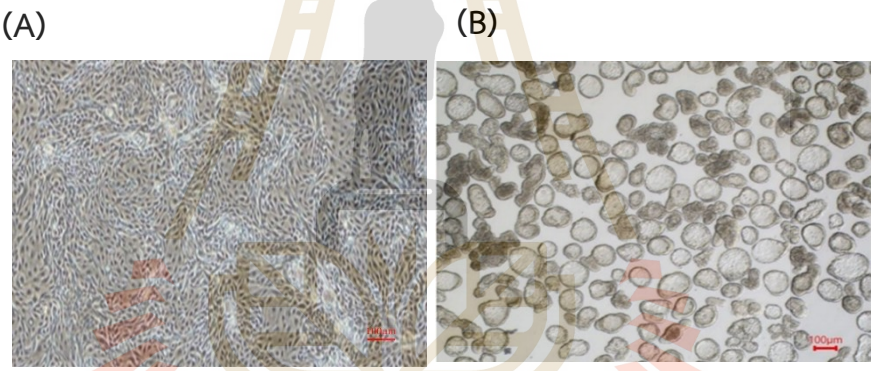


Figure 6. Morphology of oviduct epithelial cells grown in TCM199+10 % dFBS in this study: A) monolayer culture (BOECs-M), and B) vesicle culture (BOECs-V). A: Scale bar 100 μm, B: Scale bar 100 μm.

EVs could be isolated from both types of cells: BOECs-M and BOECs-V (Figure 6). Based on NTA, the concentration of BOECs-M EVs was 1.89×10^{12} particles/mL, while BOECs-V EVs had a concentration of 0.24×10^{12} particles/mL. The BOECs-M EVs and BOECs-V EVs were spherical particles with varied sizes ranging from 118 to 684 nm, respectively. The predominant sizes of BOECs-M EVs and BOECs-V EVs were 118 and 131 nm, respectively (Fig. 7A and 7B)

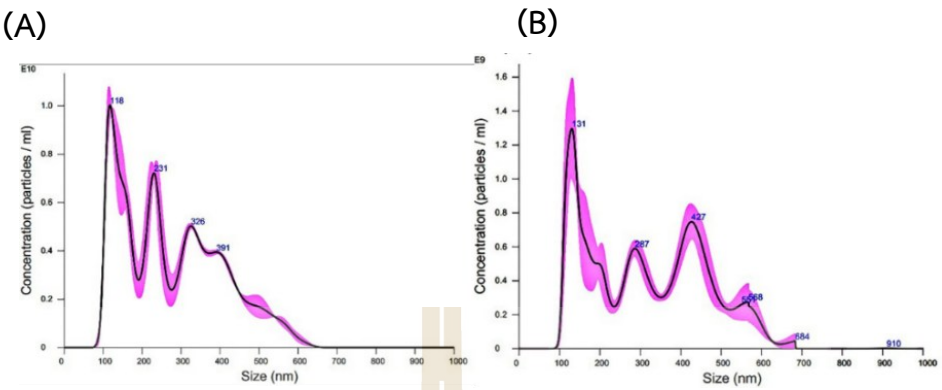


Figure 7. Average particle size of BOECs-M-EVs (A) and BOECs-V-EVs (B) using the NTA technique

4.2 Embryo development

The cleavage rates of IVF embryos did not significantly differ among treatment groups, ranging from 69.10% to 78.91%. However, blastocyst formation rates on day 7 were significantly increased in embryos treated with BOECs-M-EVs and BOECs-V-EVs, particularly at concentrations of 4×10^6 and 8×10^6 particles/mL, when compared to the control group ($P < 0.05$). Embryos treated with 4×10^6 particles/mL BOECs-M-EVs (M4) showed the highest blastocyst rate at 36.12%, followed closely by the BOECs-V-EVs at 8×10^6 (V8) and BOECs-M-EVs at 8×10^6 (M8) groups. In addition, the hatching and hatched blastocyst rates significantly improved in M 2×10^6, M 8×10^6, and V 4×10^6 groups ($P < 0.05$), with the M8 group exhibiting the highest hatching rate (50.92%).

Table 2. Effect of BOECs-derived extracellular vesicles and monolayer co-culture on bovine embryo development in *in vitro* fertilization.

Group/Type of cells	Treatment (particles/mL)	No. IVC	Cleaved D.2 (%)	Blastocyst D.7 (%)	Hatching D.7 (%)
Control	0	468	340/468 (72.65)	121/468 (25.85)	53/121 (43.80)^{b}
Monolayer	$2x10^6$	468	338/468 (72.22)	150/468 (31.32)	72/150 (48.00)^{ab}
	$4x10^6$	468	378/468 (80.76)	173/468 (36.12)	77/173 (44.51)^{a}
	$8x10^6$	468	331/468 (72.01)	163/468 (34.03)	83/168 (50.92)^{a}
Vesicles	$2x10^6$	468	344/468 (73.50)	142/468 (29.65)	58/142 (40.85^{ab}
	$4x10^6$	468	339/468 (72.43)	151/468 (31.52)	73/151 (48.34)^{a}
	$8x10^6$	468	348/468 (74.35)	167/468 (34.86)	71/167 (42.51)^{a}

ANOVA –test (10 replicates) %: Mean ± SEM. $^{a\ b}$ values with different superscripts are significantly different at $P < 0.05$

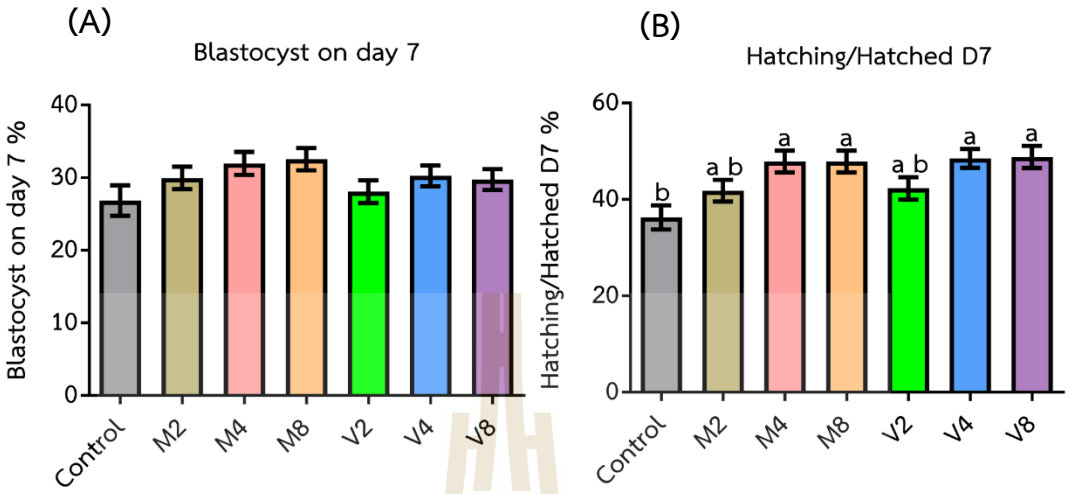


Figure 8. Effects of BOECs-derived EVs supplementation on blastocyst formation ($P > 0.05$) and hatching rates of IVF-derived embryos. Data are presented as mean ± SEM, a b values with different superscripts are significantly different at $P < 0.05$ (ANOVA test), M2 = BOECs-M-EVs 2×10^6 Particle/ml, M4 = BOECs-M-EVs 4×10^6 Particle/ml, M8 = BOECs-M-EVs 8×10^6 Particle/ml, and V2 = BOECs-V-EVs 2×10^6 Particle/ml, V4 = BOECs-V-EVs 4×10^6 Particle/ml, V8 = BOECs-V-EVs 8×10^6 Particle/ml

4.3 Embryo quality in IVF-derived blastocysts

The average diameter of day-7 blastocysts in the BOECs-EVs group was significantly larger in the BOECs-M-EVs and BOECs-V-EVs groups, respectively (190.5 ± 16.59 and 179.3 ± 15.93 μm) compared to the control group (151.7 μm) ($p < 0.05$). Similarly, embryo quality was further assessed based on total cell number, TE, and ICM in IVF-derived blastocysts following EVs supplementation. All EVs-treated groups (2×10^6, 4×10^6, and 8×10^6 particles/mL of both BOECs-M-EVs and BOECs-V-EVs demonstrated significantly higher total cell numbers than the control group ($P < 0.05$). Notably, no significant differences were observed among the EVs-treated groups. For TE cell number, embryos derived from 4×10^6 and 8×10^6 particles/mL of Monolayer EVs, as well as 8×10^6 particles/mL of Vesicle EVs, exhibited a significant increase compared to the control group ($P < 0.05$), whereas 2×10^6 particles/mL treatments from both sources showed intermediate values. Interestingly, supplementation with 8×10^6 particles/mL of Vesicle EVs resulted in the highest TE cell number among all groups. In contrast, ICM cell numbers did not significantly differ among all groups. However,

embryos supplemented with EVs, especially at concentrations of 4×10^6 and 8×10^6 particles/mL, tended to show slightly higher ICM values than the control group. These results suggest that EVs supplementation, particularly at higher concentrations ($4–8\times10^6$ particles/mL), improves blastocyst quality by enhancing total cell number and TE

Tables 3. Effects of BOECs-M EVs and BOECs-V EVs on numbers of trophectoderm and inner cell mass in IVF embryos.

Types of EVs	Treatment (particles/mL)	Mean Total cells	Mean ICM	Mean TE
Control	-	119.4 (±5.833)^{b}	39.42 (±2.604)	80.40 (±2.418)^{c}
BOECs-M-EVs	2×10^6	136.8 (±9.975)^{ab}	43.25 (±4.392)	101.5 (±7.195)^{ab}
	4×10^6	150.2 (±6.725)^{a}	50.08 (±2.981)	107.5 (±5.924)^{a}
	8×10^6	155.5 (±6.697)^{a}	49.42 (±2.548)	116.6 (±3.357)^{a}
BOECs-V-EVs	2×10^6	126.9 (±7.976)^{ab}	39.83 (±2.570)	82.3 (±2.937)^{bc}
	4×10^6	154.3 (±5.267)^{a}	45.75 (±3.369)	101.4 (±2.187)^{ab}
	8×10^6	153.9 (±2.773)^{a}	43.58 (±2.109)	117.0 (±6.759)^{a}

ANOVA –test (10 Blastocysts) %: Mean ± SEM. $^{a\ b}$ values with different superscripts are significantly different at $P < 0.05$

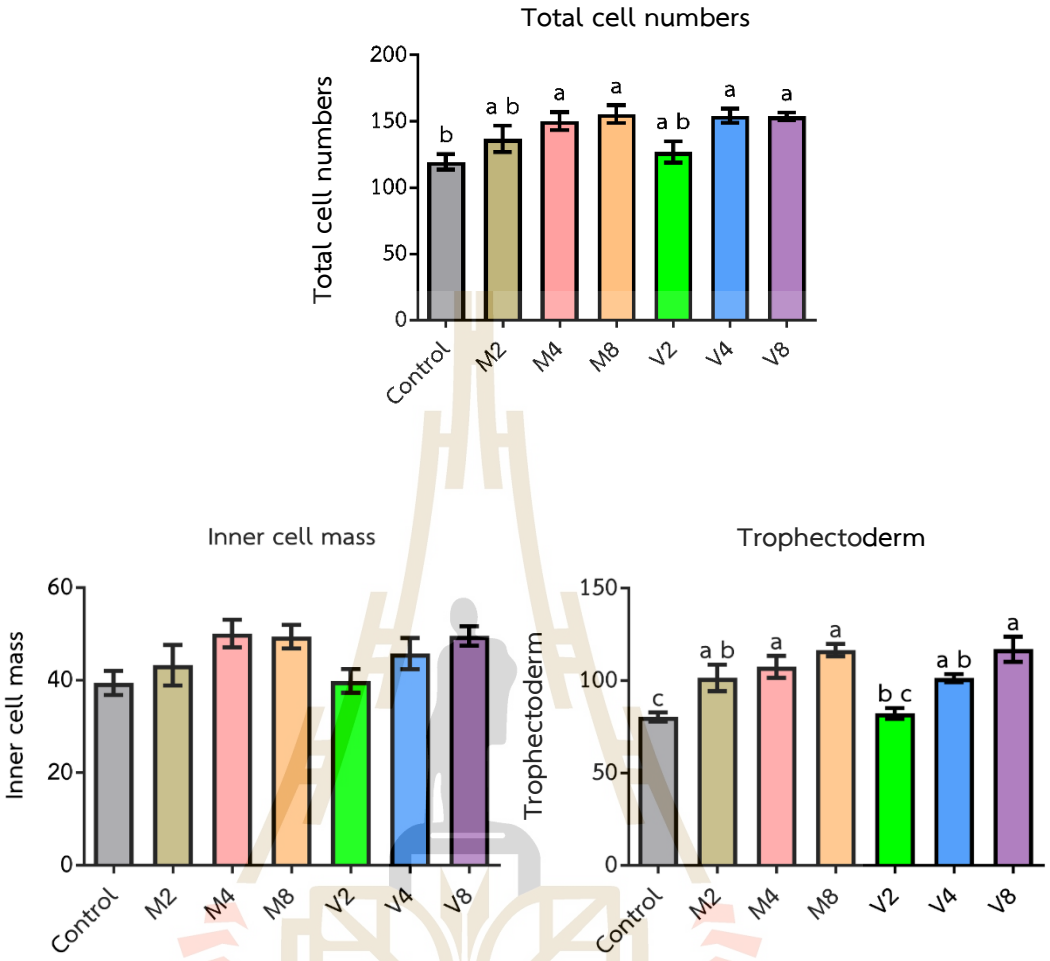


Figure 9. Analysis of the TE, ICM, and total cell number of IVF embryos cultured in IVC medium supplemented with or without BOECs-M-EVs and BOECs-V-EVs. A total of 10 embryos per group were analyzed. Data are presented as mean ± SEM, $P < 0.05$ (ANOVA test). M2 = BOECs-M-EVs 2 x 10^6 particles/ml, M4 = BOECs-M-EVs 4 x 10^6 particles/ml, M8 = BOECs-M-EVs 8 x 10^6 particles/ml, and V2 = BOECs-V-EVs 2 x 10^6 particles/ml, V4 = BOECs-V-EVs 4 x 10^6 particles/ml, V8 = BOECs-V-EVs 8 x 10^6 particles/ml

4.4 Gene Expression in IVF-derived Blastocysts

We investigated the expression of genes related to apoptosis (*BAX, BCL2*), implantation (*IFN-tau*), and pluripotency (*POU5F1, SOX2, NANOG*) in day-7 of IVF-derived blastocysts to assess the molecular impacts of BOECs-derived EVs supplementation during *in vitro* embryo development. While *BAX*, a pro-apoptotic

gene, had the lowest expression in the BOECs-V-EVs group ($P < 0.05$), indicating less apoptotic activity following EVs treatment, the anti-apoptotic gene *BCL2* was considerably downregulated in both BOECs-M-EVs and BOECs-V-EVs groups compared to the control ($P<0.05$). Expression of *IFN-tau*, which is associated with maternal recognition of pregnancy, was significantly upregulated in both EVs-treated groups compared to the control ($P < 0.05$), suggesting enhanced implantation potential.

For pluripotency-related genes, *POU5F1 (OCT4)* expression was significantly increased in both BOECs-M-EVs and BOECs-V-EVs groups compared to the control ($P < 0.05$), whereas *SOX2* expression was significantly lower in the BOECs-V-EVs group ($P < 0.05$). *NANOG* expression was significantly reduced in the BOECs-M-EVs group compared to the control, while the vesicle group showed intermediate levels. These findings suggest that BOECs-M-EVs and BOECs-V-EV supplementation during IVF culture can positively modulate embryonic gene expression by reducing apoptosis, enhancing implantation potential, and supporting pluripotency maintenance, particularly through the upregulation of *POU5F1 (OCT4)* and IFN-tau related to embryo. quality, particularly by reducing apoptosis and enhancing implantation and pluripotency potential.

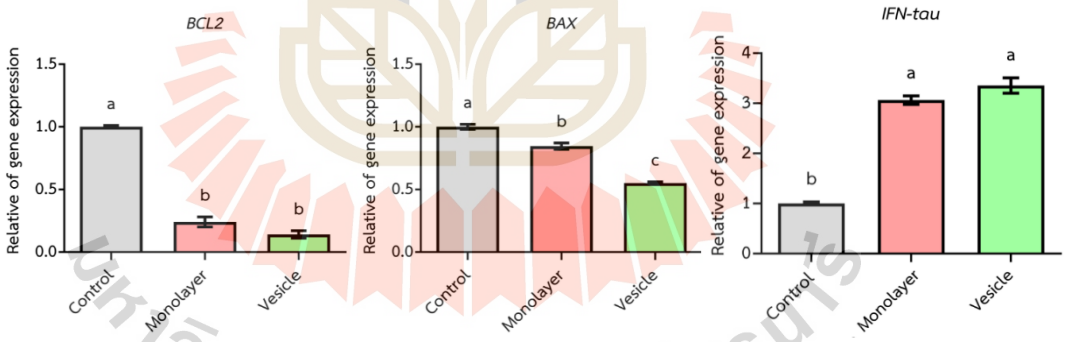


Figure 10. Expression levels of *BCL2*, *BAX*, and *IFN-tau* genes in bovine embryos produced *in vitro*. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. a, b and c Values with different superscripts are significantly different at $P < 0.05$ (ANOVA test). Monolayer (BOECs-M-EVs) and Vesicle (BOECs-V-EVs) concentrated 4 x 10^6 particles/ml.

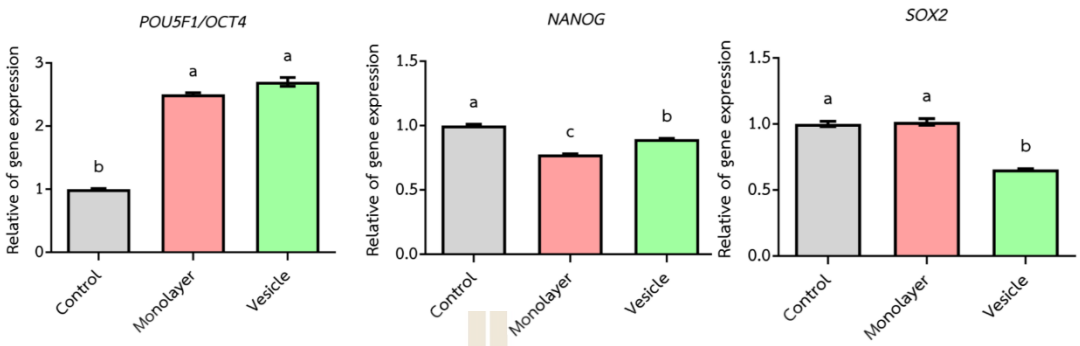


Figure 11. Expression levels of *POUF1/OCT4*, *NANOG*, and *SOX2* genes in bovine IVF embryos produced. Data are presented as mean ± SEM. a, b and c Values with different superscripts are significantly different at $P < 0.05$ (ANOVA test). Monolayer (BOECs-M-EVs) and Vesicle (BOECs-V-EVs) concentrated 4×10^6 particles/ml.

4.5 Embryo Development in SCNT-derived Embryos

Embryo development following SCNT was significantly improved by supplementation with BOECS-M-EVs and BOECS-V-EVs groups at an optimized concentration of 4×10^6 particles/mL, which was previously determined based on IVF experiments. The cleavage rate was slightly increased in both the BOECs-M-EVs (81.53%) and BOECs-V-EVs (82.17%) groups compared to the control group (78.32%). Notably, a significantly higher blastocyst formation rate on day 7 was observed in embryos supplemented with BOECs-M-EVs (39.81%) and BOECs-V-EVs (38.85%) than in the control group (27.18%) ($P < 0.05$). Likewise, the percentage of cleaved embryos developing into blastocysts (blastocysts per cleavage) was significantly higher in both the BOECs-M-EVs (48.83%) and BOECs-V-EVs (47.29%) groups compared to the control group (34.71%). However, no statistically significant differences were observed between the two EVs treatment groups in any of the evaluated parameters. These findings indicate that supplementation with either form of BOECs-M-EVs and BOECs-V-EVs during SCNT embryo culture at the optimized concentration enhances embryonic developmental competence and promotes blastocyst formation.

Table 4. Developmental competence of SCNT bovine embryos produced with or without EVs produced from BOECs.

Treatment	No. IVC	Cleaved (%)	Blastocyst D.7 (%)	Bls/Cleavage (%)
Control	309	242/309 (78.32)	84/309 (27.18[b])	84/242 (34.71[b])
BOECs-M-EVs	314	256/314 (81.53)	125/314 (39.81[a])	125/256 (48.83[a])
BOECs-V-EVs	314	258/314 (82.17)	122/314 (38.85[a])	122/258 (47.29[a])

ANOVA –test (15 replicates) %: [a b] Values with different superscripts are significantly different at $P < 0.05$

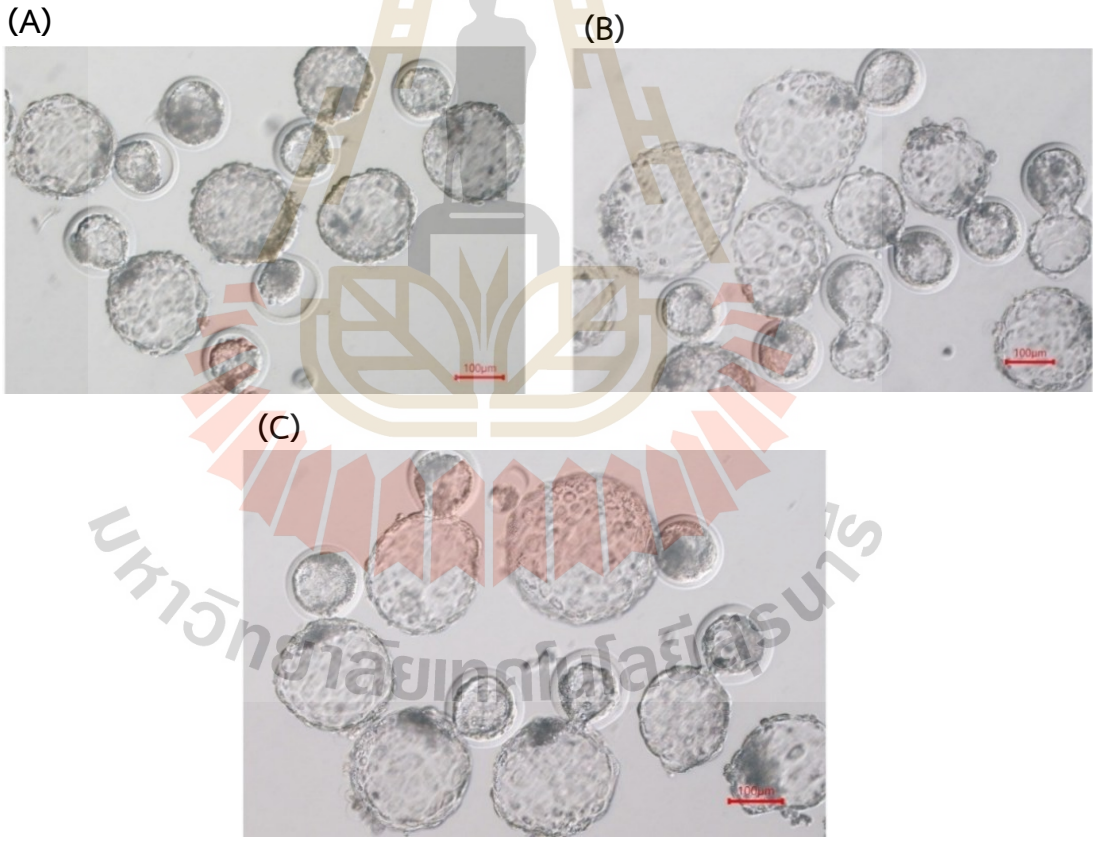


Figure 12. Representative image of embryo at hatching blastocyst stage in the control group (A), supplemented BOECs-M-EVs (B) and BOECs-V-EVs groups (C) on day 7, respectively. Scale bar = 100 µm

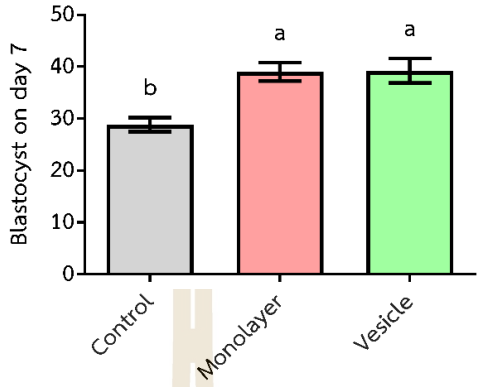


Figure 13. Blastocyst formation rate of SCNT embryos supplemented with BOECS-M-EVs and BOECS-V-EVs. Data are presented as mean ± SEM. a b Values with different superscripts are significantly different at $P < 0.05$ (ANOVA test). Monolayer (BOECs-M-EVs) and Vesicle (BOECs-V-EVs) concentrated 4×10^6 particles/ml.

4.6 Embryo quality in SCNT-derived blastocysts

Embryo quality was assessed by evaluating total cell numbers, ICM, and TE cells of day-7-blastocysts. The results demonstrated that embryos derived from the BOECs-M-EVs group had significantly greater total cell numbers ($P < 0.05$) compared to the control group, whereas no significant difference was observed between the BOECs-V-EVs and control groups. A similar pattern was observed in the ICM counts, with significantly greater numbers in the BOECs-M-EVs group ($P < 0.05$), while the BOECs-V-EVs group did not significantly differ from the control group. No significant differences were detected in the number of TE cells among the three groups.

Table 4. Effects of BOECs-M EVs and BOECs-V EVs on the numbers of trophectoderm and inner cell mass in SCNT embryos.

Types of EVs	Mean total cells	Mean ICM	Mean TE
Control (IVF)	213.9 (±4.542)[a]	79 (±2.049)[a]	160.7 (±3.774)
Untreat	186.5 (±3.267)[b]	60.6 (±2.500)[b]	135.1 (±8.649)
BOECs-M-EVs	210.7 (±6.136)[a]	66.50 (±1.916)[b]	143.5 (±9.283)
BOECs-V-EVs	200.2 (±4.117)[a]	60.70 (±1.967)[b]	149.4 (±5.918)

ANOVA –test (10 Blastocysts) %: Mean ± SEM. [a b] values with different superscripts are significantly different at $P < 0.05$

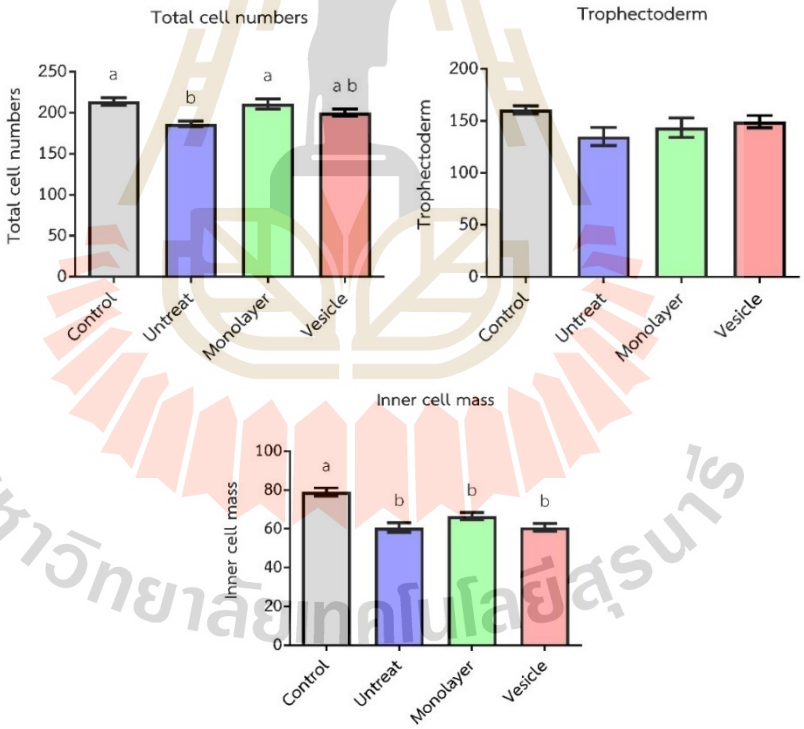


Figure 14. Embryo quality assessment of SCNT blastocysts cultured with BOECs-derived EVs supplementation. Data are presented as mean ± SEM. a and b Values with different superscripts are significantly different at $P < 0.05$ (ANOVA test). Monolayer (BOECs-M-EVs) and Vesicle (BOECs-V-EVs) concentrated 4 x 10^6 particle/ml.

4.7 Gene expression in SCNT-derived blastocysts

To assess the impact of BOECs-M-EVs and BOECs-V-EVs groups on the molecular quality of SCNT embryos, the expression levels of key developmental (IFN-tau, *POU5F1/OCT4*, *NANOG*, and *SOX2*) and apoptotic (*BAX* and *BCL2*) genes were analyzed in day 7 blastocysts. SCNT embryos without EVs treatment (Untreated) exhibited significantly lower expression of *IFN-tau*, *POU5F1/OCT4*, *NANOG*, *SOX2*, and *BCL2*, while *BAX* expression was elevated compared to IVF-derived embryos (Control), suggesting impaired developmental potential and increased apoptotic signaling ($P < 0.05$). Supplementation with optimized concentrations of BOECs-derived EVs (4×10^6 particles/mL) from both Monolayer and Vesicle sources partially restored gene expression. Notably, the Monolayer-EVs group showed significantly upregulated expression of *IFN-tau* and *BCL2* and downregulated *BAX* expression compared to the Untreated group, indicating improved embryo quality and reduced apoptosis. *NANOG* expression was also significantly enhanced in both EVs-treated groups, reaching levels comparable to those in the IVF group. However, expressions of *POU5F1/OCT4* and *SOX2* remained significantly lower than in the Control group across all SCNT conditions. These findings demonstrate that BOECS-derived EVs supplementation during SCNT embryo culture can improve molecular quality by modulating gene expression toward a more developmentally favorable profile.

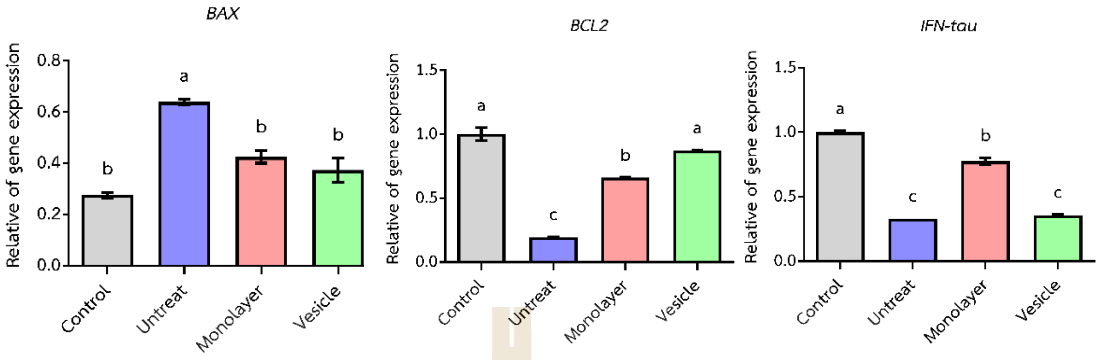


Figure 15. Expression levels of *BCL2*, *BAX*, and *IFN-tau* genes in bovine SCNT embryos were analyzed and compared to control (IVF embryos). A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. a, b and c Values with different superscripts are significantly different at $P < 0.05$ (ANOVA test). Monolayer (BOECs-M-EVs) and Vesicle (BOECs-V-EVs) concentrated 4 x 10^6 particle/ml.

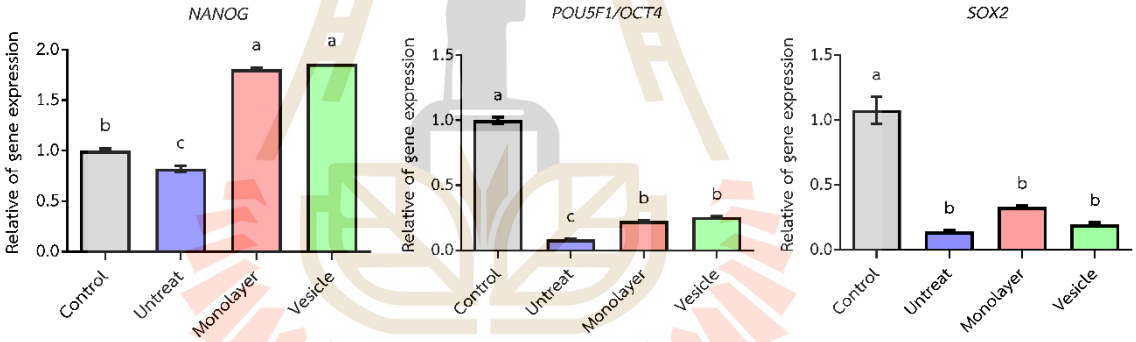


Figure 16. Expression levels of *POUF1/OCT4*, *NANOG*, and *SOX2 genes* in bovine SCNT embryos were analyzed and compared to control (IVF embryos). A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. Values with different superscripts (a, b and c) are significantly different at $P < 0.05$ (ANOVA test). Monolayer (BOECs-M-EVs) and Vesicle (BOECs-V-EVs) concentrated 4 x 10^6 particles/ml.

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 DISCUSSION

This study is the first investigation of effects of EVs derived from vesicle-shaped bovine oviduct epithelial cells (BOECs-V-EVs) on bovine embryo development. To our knowledge, no previous research has applied BOECs-V-EVs in either IVF or SCNT embryo culture systems in cattle. This novel approach expands the potential application of reproductive tract-derived EVs in assisted reproductive technologies. Our findings demonstrate that supplementation of IVC medium with BOECs-derived EVs significantly enhances embryo developmental competence and quality in both IVF and SCNT systems. The optimal supplementation levels led to improvements in blastocyst formation, hatching rates, and embryo quality parameters, including total cell number and balanced allocation between the ICM and TE lineages. EVs were effectively separated from attached monolayer cells (BOECs-M) and floating vesicle-like cells (BOECs-V), two morphologically different types of BOECs. NTA revealed that while BOECs-M produced a higher concentration of EVs, both types exhibited sizes consistent with small EVs (around 118–131 nm) as shown in Figure 7, supporting their classification based on biophysical properties (Bastos et al., 2022; Lopera-Vasquez et al., 2016). Although surface marker profiling (e.g., CD9, CD63, CD81) was not performed, the combination of ultracentrifugation and NTA characterization was considered sufficient for functional evaluation in embryo culture. Nevertheless, future studies should include molecular profiling following the Minimal Information for Studies of Extracellular Vesicles guidelines to enhance EVs characterization (Leal et al., 2022; Sidrat et al., 2022; Wei et al., 2022). Supplementation with BOECs-M-EVs and BOECs-V-EVs groups during IVF culture significantly increased blastocyst formation and hatching rates, particularly at 4×10^6 and 8×10^6 particles/mL. These results align with previous reports demonstrating that reproductive tract-derived EVs can improve embryonic development and mimic physiological conditions (Leal et al., 2022; Sidrat et al., 2022;

Wei et al., 2022). Consistently, Wei et al. (2022) confirmed that supplementation with BOECs-derived exosomes enhanced the developmental competence and implantation potential of bovine embryos. The average diameter of the embryos supplemented with EVs produced from BOECs was noticeably greater than that of the control group. This Increased size may indicate of increased total cell number. Interestingly, Hoechst 33342 nuclear staining revealed a correlation between an increase in diameter and a greater total number of cells. These results are in line with earlier research that suggested embryo size is a good indicator of viability and developmental potential (Lonergan et al., 2003). The findings are consistent with the theory that EVs increase embryonic development by delivering bioactive chemicals that promote cell division and metabolic support during the culture period. These enhancements could be explained by the bioactive cargo that EVs carry, such as proteins and miRNAs that can affect intracellular pathways, including MAPK and PI3K/AKT. These signaling cascades control mitochondrial function, apoptosis suppression, and cell proliferation, all of which are important factors in blastocyst growth and a rise in the overall number of cells. Therefore, during IVC, EVs probably function as modulators of embryonic physiology, improving cellular and morphological development (Gurung et al., 2021; Qu et al., 2020; Sidrat et al., 2022). This impact is particularly significant when it comes to SCNT, since cloned embryos often experience developmental halt, aberrant blastocyst form, and a decreased total cell number. These limitations reflect poor cellular dynamics and inadequate reprogramming, rather than epigenetic mistakes (Wells, 2005; Tsunoda & Kato, 2002). EVs may be able to assist get over these restrictions by imitating maternal signals and enhancing the culture environment, as evidenced by their capacity to enhance cell proliferation and encourage blastocoel growth. Previous studies have shown evidence that EVs supplementation improves cellular survival, membrane integrity, and mitochondrial activity in both SCNT and IVF embryos (Bang et al., 2023; Qu et al., 2017). Thus, the observed increases in embryo width and cell quantity in EVs-treated groups may represent both better morphology and greater developmental potential, both of which are essential for increasing the success rates of cloning and IVEP. To convert

these discoveries into useful reproductive biotechnology applications, more research is necessary to optimize the time and dosage of EVs supplementation in SCNT systems.

Although *IFN-tau* expression was not directly measured, the improvement in embryo quality and implantation outcomes reported in previous study supports the notion that reproductive tract-derived EVs may promote maternal recognition signaling pathways, including *IFN-tau* upregulation. Embryo quality analysis further confirmed the beneficial effects of EVs, as total cell numbers and TE cell counts were significantly higher in EVs-treated groups compared to the control (Figure 9). Although ICM cell numbers did not differ substantially, there was a trend toward higher ICM values with increasing EVs concentrations, suggesting that EVs supplementation supports balanced lineage allocation (Lopera-Vasquez et al., 2017). Gene expression profiling revealed that EVs supplementation modulated key apoptosis, implantation, and pluripotency genes. The pro-apoptotic gene *BAX* was downregulated in BOECs-V-EVs supplemented embryos, indicating reduced cellular stress, whereas the anti-apoptotic gene *BCL2* also showed lower expression across EVs-treated groups, reflecting a less stressful environment *in vitro*. Moreover, *IFN-tau*, critical for maternal recognition of pregnancy, was upregulated, suggesting enhanced implantation potential. Pluripotency markers *OCT4* and *NANOG* were positively modulated, further supporting the role of EVs in maintaining embryonic developmental potential (Wei et al., 2022). The observed upregulation of these pluripotency-related genes aligns with recent findings that emphasize the critical cooperative activity of *OCT4* and *SOX2* in activating transcriptional programs essential for early embryonic pluripotency (Hou et al., 2024). Additionally, comparative transcriptomic analyses in mammalian embryos have highlighted the conserved importance of *OCT4* and *NANOG* across species for establishing and maintaining the pluripotent state (Bernardo et al., 2018).

Although both BOECs-M-EVs and BOECs-V-EVs significantly improved embryo development and quality compared to the control, subtle functional differences were observed between the two EVs sources. BOECs-M-EVs tended to promote higher blastocyst and hatching rates and upregulated pluripotency markers (*OCT4*, *NANOG*), whereas BOECs-V-EVs demonstrated a more pronounced anti-apoptotic effect through stronger *BAX* suppression. These findings suggest that the bioactive cargo composition

of EVs may differ depending on the origin of cellular architecture. In the SCNT system, BOECs-M-EVs and BOECs-V-EVs supplementation also led to significant improvements. Cleavage and blastocyst formation rates were higher in EVs-treated groups compared to controls. Importantly, gene expression in SCNT-derived blastocysts reflected similar trends to those observed in IVF embryos. Supplementation with either BOECs-M-EVs or BOECs-V-EVs led to a significant reduction in *BAX* expression, suggesting a mitigation of apoptotic stress. *BCL2* expression was markedly higher in EVs-supplemented groups compared to the untreated SCNT group, approaching levels found in IVF embryos. Moreover, *IFN-tau* expression was significantly elevated, highlighting enhanced implantation potential. The upregulation of pluripotency-related genes, such as *NANOG* and *OCT4*, observed in EVs-supplemented SCNT embryos, supports the notion that EVs contribute to the enhancement of pluripotency (Bernardo et al., 2018). Taken together, these findings support the hypothesis that BOECs-derived EVs can enhance nuclear reprogramming, alleviate apoptosis, and improve post-activation development in cloned embryos. The beneficial effects observed here are consistent with previous reports demonstrating that reproductive tract-derived EVs carry bioactive cargos capable of modulating cellular stress responses and reactivating essential developmental pathways (Lopera-Vásquez et al., 2015; Sidrat et al., 2022). The differential outcomes observed between BOECs-M-EVs and BOECs-V-EVs raise the possibility that these vesicles carry distinct molecular cargos, such as microRNAs, tRNAs, or proteins, which influence developmental signaling pathways. Further proteomic and transcriptomic analyses are warranted to elucidate the underlying mechanisms (Sheta et al., 2023).

5.2 Conclusions

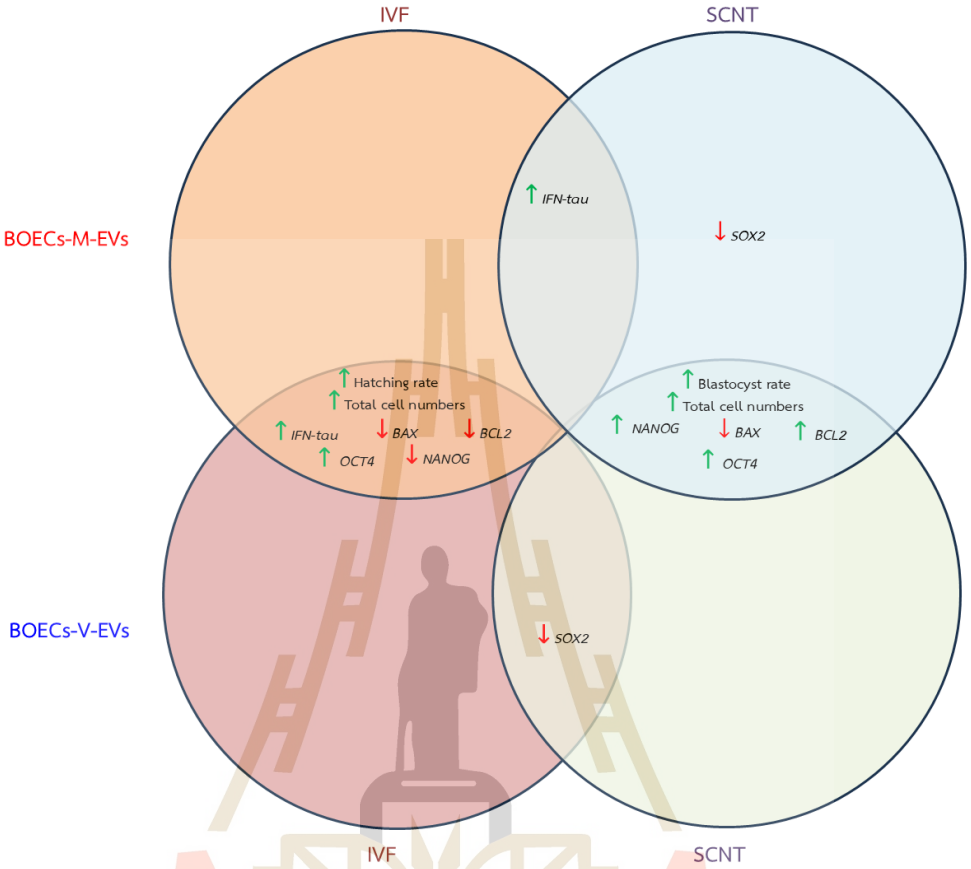
This research shows that the developmental potential of bovine embryos is greatly increased when extracellular vesicles (EVs) made from bovine oviductal epithelial cells (BOECs) are supplemented during *in vitro* culture. In comparison to controls, the embryos in the EV-treated groups showed increased diameters, higher blastocyst rates, and more total cells, all of which indicated better developmental competence. These findings support the hypothesis that EVs provide bioactive molecules, such as proteins, mRNAs, and miRNAs, that can regulate cell signaling

pathways involved in proliferation and survival, including the PI3K/AKT and MAPK pathways. In addition, BOECs-derived EVs enhanced embryo development and quality in the SCNT embryo model, indicating that they may make up for the reprogramming inefficiencies often seen in cloned embryos. These improvements demonstrate how EVs can mimic maternal signaling and provide a more physiological environment *in vitro*, which will improve embryonic outcomes. The importance of EVs as a prospective bioactive supplement in assisted reproductive technologies is shown by this study overall. Their incorporation into embryo culture systems may be an important step in improving the existing SCNT and IVEP methods, which might have additional consequences for reproductive biotechnology in cattle and livestock ruminant breeding.

Our findings indicate that EVs derived from bovine oviduct epithelial cells, particularly BOEC-M-EVs, have the potential to enhance embryonic development and regulate gene expression related to cell survival and implantation in both IVF and SCNT systems. These results highlight the feasibility of utilising EVs as bioactive supplements in embryo culture systems to improve the developmental competence and quality of in vitro-produced embryos, which is essential for the advancement of ruminant livestock production and ART. From a practical standpoint, EV supplementation could be incorporated into commercial IVP protocols to improve pregnancy rates and reduce embryonic loss. Given that current embryo culture media commonly rely on undefined components such as FBS, the application of EVs as a defined, cell-origin-specific supplement represents an innovative and potentially standardised alternative.

Future studies should aim to comprehensively characterise the molecular cargo of EVs such as small RNAs, proteins, and lipids and identify the key bioactive components responsible for the observed beneficial effects. Such efforts could facilitate the development of EV-based defined supplements or even fully defined culture media for bovine IVP systems, offering improved consistency, safety, and adaptability according to embryo type and production objectives.

Graphical conclusion



REFERENCES

Almiñana, C., & Bauersachs, S. (2020). Extracellular vesicles: Multi-signal messengers in the gametes/embryo-oviduct cross-talk. *Theriogenology*, *150*, 59–69.

Arlotto, T., Schwartz, J., First, N., & Leibfried-Rutledge, M. (1996). Aspects of follicle and oocyte stage that affect *in vitro* maturation and development of bovine oocytes. *Theriogenology*, *45*(5), 943–956.

Bang, S., Qamar, A. Y., Fang, X., Kim, H., Han, A., Kang, H., Cha, D., Shim, J., Kim, J.-H., Choi, K., Yun, S. H., Lee, H., Park, H. S., Kim, S. I., Kim, J.-Y., Saadeldin, I. M., Lee, S., & Cho, J. (2023). Effects of extracellular vesicles derived from steroids-primed oviductal epithelial cells on porcine *in vitro* embryonic development. *Theriogenology*, *209*, 213–223. https://doi.org/10.1016/j.theriogenology.2023.07.006

Banliat, C., Le Bourhis, D., Bernardi, O., Tomas, D., Labas, V., Salvetti, P., Guyonnet, B., Mermillod, P., & Saint-Dizier, M. (2020). Oviduct fluid extracellular vesicles change the phospholipid composition of bovine embryos developed *in vitro*. *International Journal of Molecular Sciences*, *21*(15), 5326.

Bastos, N. M., Ferst, J. G., Goulart, R. S., & Coelho da Silveira, J. (2022). The role of the oviduct and extracellular vesicles during early embryo development in bovine. *Animal Reproduction*, *19*.

Bernardo, A. S., Jouneau, A., Marks, H., Kensche, P., Kobolak, J., Freude, K., Hall, V., Feher, A., Polgar, Z., & Sartori, C. (2018). Mammalian embryo comparison identifies novel pluripotency genes associated with the naïve or primed state. *Biology Open*, *7*(8), bio033282

Blanco, M., Simonetti, L., & Rivera, O. (2003). Embryo production and progesterone profiles in ewes superovulated with different hormonal treatments. *Small Ruminant Research*, *47*(3), 183–191.

Coy, P., García-Vázquez, F., Visconti, P., & Avilés, M. (2012). Roles of the oviduct in mammalian fertilization. *Reproduction (Cambridge, England)*, *144*(6), 649.

Currin, L., Baldassarre, H., & Bordignon, V. (2021). *In Vitro* Production of Embryos from Prepubertal Holstein Cattle and Mediterranean Water Buffalo: Problems, Progress and Potential. *Animals*, *11*(8), 2275. https://doi.org/10.3390/ani11082275

De Jonge, C. J., & Barratt, C. L. (2017). *The sperm cell: Production, maturation, fertilization, regeneration*. Cambridge University Press.

Feliciano, D. M., Zhang, S., Nasrallah, C. M., Lisgo, S. N., & Bordey, A. (2014). Embryonic cerebrospinal fluid nanovesicles carry evolutionarily conserved molecules and promote neural stem cell amplification. *PloS One*, *9*(2), e88810.

Ferrer-Roda, M., Catalán, J., Izquierdo, D., Oliveira, M. E. F., & Paramio, M. (2024). Impact of semen cryopreservation season on *in vitro* embryo production of prepubertal goat oocytes. *Reproduction in Domestic Animals*, *59*, e14594.

Georgadaki, K., Khoury, N., Spandidos, D. A., & Zoumpourlis, V. (2016). The molecular basis of fertilization. *International Journal of Molecular Medicine*, *38*(4), 979–986.

Gurung, S., Perocheau, D., Touramanidou, L., & Baruteau, J. (2021). The exosome journey: From biogenesis to uptake and intracellular signalling. *Cell Communication and Signaling*, *19*(1), 47.

György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., László, V., Pállinger, E., Pap, E., & Kittel, A. (2011). Membrane vesicles, current state-of-the-art: Emerging

role of extracellular vesicles. *Cellular and Molecular Life Sciences*, *68*, 2667–2688.

Hasler, J. F. (2000). *In vitro* culture of bovine embryos in Ménézo's B2 medium with or without coculture and serum: The normalcy of pregnancies and calves resulting from transferred embryos. *Animal Reproduction Science*, *60–61*, 81–91. https://doi.org/10.1016/S0378-4320(00)00086-5

Hou, S., Liu, C., Yao, Y., Bai, Z., Gong, Y., Wang, C., He, J., You, G., Zhang, G., & Liu, B. (2024). Hematopoietic stem cell development in mammalian embryos. *Hematopoietic Stem Cells: Keystone of Tissue Development and Regenerative Medicine*, 1–16.

Hyttel, P., Fair, T., Callesen, H., & Greve, T. (1997). Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, *47*(1), 23–32.

Jan, A. T., Rahman, S., Badierah, R., Lee, E. J., Mattar, E. H., Redwan, E. M., & Choi, I. (2021). Expedition into Exosome Biology: A Perspective of Progress from Discovery to Therapeutic Development. *Cancers*, *13*(5), 1157. https://doi.org/10.3390/cancers13051157

Janini, L. C. Z., Dellaqua, T. T., Membrive, C. M. B., Oba, E., Nichi, M., Rizzoto, G., & da Cruz Landim-Alvarenga, F. (2023). Effects of anethole supplementation on bovine embryo production and quality. *Livestock Science*, *274*, 105262.

Leal, C. L. V., Karina Cañón-Beltrán, Yulia N Cajas, Hamdi, M., Yaryes, A., Blanca, M. G. M. de la, Beltrán-Breña, P., Mazzarella, R., Juliano Coelho da Silveira, & Alfonso Gutiérrez-Adán. (2022). Extracellular vesicles from oviductal and uterine fluids supplementation in sequential *in vitro* culture improves bovine embryo quality. *Journal of Animal Science and Biotechnology*, *13*(1), 116.

Lopera-Vasquez, R., Hamdi, M., Fernandez-Fuertes, B., Maillo, V., Beltran-Brena, P., Calle, A., Redruello, A., Lopez-Martin, S., Gutierrez-Adan, A., & Yañez-Mó, M.

(2016). Extracellular vesicles from BOECs in *in vitro* embryo development and quality. *PloS One*, *11*(2), e0148083.

Lopera-Vasquez, R., Hamdi, M., Maillo, V., Gutierrez-Adan, A., Bermejo-Alvarez, P., Ramírez, M. Á., Yáñez-Mó, M., & Rizos, D. (2017). Effect of bovine oviductal extracellular vesicles on embryo development and quality *in vitro*. *Reproduction*, *153*(4), 461–470.

Ludwig, A.-K., & Giebel, B. (2012). Exosomes: Small vesicles participating in intercellular communication. *The International Journal of Biochemistry & Cell Biology*, *44*(1), 11–15.

Mazzarella, R., Bastos, N. M., Bridi, A., Del Collado, M., Andrade, G. M., Pinzon, J., Prado, C. M., Silva, L. A., Meirelles, F. V., & Pugliesi, G. (2021). Changes in oviductal cells and small extracellular vesicles miRNAs in pregnant cows. *Frontiers in Veterinary Science*, *8*, 639752.

Morrison, L. J., & Marcinkiewicz, J. L. (2002). Tumor necrosis factor α enhances oocyte/follicle apoptosis in the neonatal rat ovary. *Biology of Reproduction*, *66*(2), 450–457.

Niemann, H., & Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: Implications for subsequent development. *Theriogenology*, *53*(1), 21–34. https://doi.org/10.1016/S0093-691X(99)00237-X

Okabe, K., Kobayashi, S., Yamada, T., Kurihara, T., Tai-Nagara, I., Miyamoto, T., Mukouyama, Y., Sato, T. N., Suda, T., & Ema, M. (2014). Neurons limit angiogenesis by titrating VEGF in retina. *Cell*, *159*(3), 584–596.

Qiao, F., Ge, H., Ma, X., Zhang, Y., Zuo, Z., Wang, M., Zhang, Y., & Wang, Y. (2018). Bovine uterus-derived exosomes improve developmental competence of somatic cell nuclear transfer embryos. *Theriogenology*, *114*, 199–205.

Qu, P., Luo, S., Du, Y., Zhang, Y., Song, X., Yuan, X., Lin, Z., Li, Y., & Liu, E. (2020). Extracellular vesicles and melatonin benefit embryonic develop by regulating reactive oxygen species and 5-methylcytosine. *Journal of Pineal Research*, *68*(3), e12635. https://doi.org/10.1111/jpi.12635

Qu, P., Qing, S., Liu, R., Qin, H., Wang, W., Qiao, F., Ge, H., Liu, J., Zhang, Y., Cui, W., & Wang, Y. (2017). Effects of embryo-derived exosomes on the development of bovine cloned embryos. *PLOS ONE*, *12*(3), e0174535.

Rizos, D., Gutiérrez-Adán, A., Perez-Garnelo, S., De la Fuente, J., Boland, M., & Lonergan, P. (2003). Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biology of Reproduction*, *68*(1), 236–243.

Sheta, M., Taha, E. A., Lu, Y., & Eguchi, T. (2023). Extracellular vesicles: New classification and tumor immunosuppression. *Biology*, *12*(1), 110.

Sidrat, T., Khan, A. A., Joo, M.-D., Xu, L., El-Sheikh, M., Ko, J.-H., & Kong, I.-K. (2022). Extracellular vesicles improve embryo cryotolerance by maintaining the tight junction integrity during blastocoel re-expansion. *Reproduction (Cambridge, England)*, *163*(4), 219.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: Controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology*, *9*(3), 231–241.

Tsunoda, Y., & Kato, Y. (2002). Recent progress and problems in animal cloning. *Differentiation*, *69*(4–5), 158–161. https://doi.org/10.1046/j.1432-0436.2002.690405.x

Wang, C., & Roy, S. K. (2006). Expression of growth differentiation factor 9 in the oocytes is essential for the development of primordial follicles in the hamster ovary. *Endocrinology*, *147*(4), 1725–1734.

Ward, N., Brown, M., Thompson, A., & Frackowiak, R. (2003). Neural correlates of motor recovery after stroke: A longitudinal fMRI study. *Brain*, *126*(11), 2476–2496.

Wei, Y., Idrees, M., Sidrat, T., Joo, M., Xu, L., Ko, J., & Kong, I. (2022). BOECS–Exo Addition Promotes *In Vitro* Maturation of Bovine Oocyte and Enhances the Developmental Competence of Early Embryos. *Animals*, *12*(4), 424. https://doi.org/10.3390/ani12040424

Yang, D., Zhang, W., Zhang, H., Zhang, F., Chen, L., Ma, L., Larcher, L. M., Chen, S., Liu, N., & Zhao, Q. (2020). Progress, opportunity, and perspective on exosome isolation-efforts for efficient exosome-based theranostics. *Theranostics*, *10*(8), 3684.

Younis, A., Brackett, B., & Fayrer‐Hosken, R. (1989). Influence of serum and hormones on bovine oocyte maturation and fertilization *in vitro*. *Gamete Research*, *23*(2), 189–201.

Zarà, M., Guidetti, G. F., Camera, M., Canobbio, I., Amadio, P., Torti, M., Tremoli, E., & Barbieri, S. S. (2019). Biology and role of extracellular vesicles (EVs) in the pathogenesis of thrombosis. *International Journal of Molecular Sciences*, *20*(11), 2840.

Zou, H., Kemper, J. M., Hammond, E. R., Xu, F., Liu, G., Xue, L., Bai, X., Liao, H., Xue, S., & Zhao, S. (2023). Blastocyst quality and reproductive and perinatal outcomes: A multinational multicentre observational study. *Human Reproduction*, *38*(12), 2391–2399.

BIOGRAPHY

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