Improving equine oocyte quality to increase the efficiency of embryo in vitro production

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STATEMENT OF ORIGINALITY

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and its sources have been acknowledged.

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ARTIFICIAL INTELLIGENCE

During the preparation of this thesis, the author used Grammarly solely to improve the spelling, grammar, formatting, and consistency of the review of the literature.

The author confirms that any AI-assisted suggestions were carefully reviewed and edited to ensure accuracy, appropriateness, and compliance with academic standards. The author takes full responsibility for the content of the submitted thesis, confirms that the work is original, and affirms that the use of generative AI complied with University guidelines and policies.

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SUMMARY

Recent advances in reproductive biotechnologies, along with improvements in the various stages involved in the in vitro production of equine embryos from immature oocytes obtained via aspiration from donor mares, have established this technique as a highly promising and economically viable strategy for the equine breeding industry. The stages involved in this process include oocyte aspiration, in vitro maturation (IVM) of oocytes, intracytoplasmic sperm injection (ICSI), and in vitro embryo culture. In addition, somatic cell nuclear transfer (SCNT) has been successfully employed for the generation of cloned embryos and the production of foals. The main objective of the thesis studies was to systematically evaluate the effects of different treatments aimed at improving oocyte quality. For this purpose, immature equine oocytes collected from donor mares and abattoir-derived ovaries were used. The results demonstrated that oocytes obtained from donor mares exhibited superior quality, reflected in higher embryonic development rates and an increased proportion of viable offspring (Chapter 2).

Oocyte developmental potential represents a key limiting factor in assisted reproduction processes; therefore, establishing an optimal in vitro maturation (IVM) system is essential for the efficient and reliable production of viable embryos. Given that embryo quality directly depends on the quality of the originating oocytes, it is crucial to employ the most advanced IVM systems to achieve the best outcomes. Although current protocols for equine oocyte in vitro maturation have proven effective at a commercially acceptable level, studies in other species have identified promising areas for improvement, particularly the use of pre-IVM culture systems and the application of specific treatments during maturation that enhance both oocyte growth and survival (Chapters 3–4).

In this context, different pre-in vitro maturation (pre-IVM) and IVM treatments were directly compared through a series of experiments designed to generate mature oocytes, which were subsequently used for embryo production through conventional somatic cell nuclear transfer (SCNT) techniques. The evidence obtained shows that pre-IVM treatments employing IVM modulators, aimed at preventing spontaneous resumption of meiosis, did not enhance the developmental potential of equine oocytes (Chapter 3). Additionally, supplementation of the pre-IVM and IVM media with nicotinic acid (NA) showed a highly beneficial effect on the capacity of oocytes to develop to the blastocyst stage (Chapter 4). Although further research is needed to conclusively confirm the influence of meiosis-inhibiting treatments on equine oocytes, as well as to assess the positive impact of nicotinic acid (NA) supplementation, an agent capable of increasing NAD^+ levels, the studies presented in this thesis have significantly contributed to our understanding of the acquisition of oocyte developmental competence in this species. In conclusion, this thesis provides valuable evidence on the refinement of in vitro maturation systems for equine oocytes and lays the groundwork for the development of improved protocols in the in vitro production of embryos in equids.

LIST OF ABBREVIATIONS

°C	degrees Celsius
µ	(prefix) micro (x 10^{-6})
6-DMAP	6-dimethylaminopurine
FSK	Forskolin
IBMX	3-isobutyl-1-methylxanthine
BSA	bovine serum albumin
COC	cumulus-oocyte complex
ARTs	assisted reproductive technologies
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
ICSI	intracytoplasmic sperm injection
SCNT	somatic cell nuclear transfer
OPU	Ovum Pick Up
hCG	human chorionic gonadotropin
LH	luteinizing hormone
FSH	follicle stimulating hormone
TSH	thyroid stimulating hormone
eCG	equine chorionic gonadotropin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
MPF	maturation-promoting factor
CDK-1	cyclin-dependent kinase 1
LHR	luteinizing hormone receptor

EGFβ	Epidermal Growth Factor β
MTOCs	microtubule-organizing centers
GVBD	germinal vesicle breakdown
GnRH	gonadotropin releasing hormone
ERβ	estrogen receptor β
LHCGR	luteinizing human chorionic gonadotropin receptor
GPCRs	G protein-coupled receptors
TCs	theca cells
TCM-199	tissue culture medium 199
IGF-1	insulin-like growth factor 1
BM-MSC	bone marrow mesenchymal stem cells
SPOM	simulated physiological oocyte maturation
H-SOF	hepes-buffered synthetic oviductal fluid
FCS	fetal calf serum
ET	embryo transfer
NA	nicotinic acid
ROS	reactive oxygen species
DMSO	dimethyl sulfoxide
L	litre (s)
M	Molar
MII	metaphase-II
min	minute(s)
NaAD	nicotinic acid adenine dinucleotide
NAD/NAD+/NADH	nicotinamide adenine dinucleotide
NADS	nicotinamide adenine dinucleotide synthase

NAM	nicotinamide
NaMN	nicotinic acid mononucleotide
NAMPT/NamPRT	nicotinamide phosphoribosyltransferase
NaPRT	nicotinic acid phosphoribosyl transferase
NaR	nicotinic acid riboside

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LIST OF PUBLICATIONS BY THE AUTHOR

Journal Publications

*Published papers of thesis chapters

Cortez JV, Vajta G, Valderrama NM, Portocarrero GS, Quintana JM (2018) High Pregnancy and Calving Rates with a Limited Number of Transferred Handmade Cloned Bovine Embryos, Cellular Reprogramming, 20(1):4-8. https://doi.org/10.1089/cell.2017.0024

Magalhães LC, **Cortez JV**, Bhat MH, Sampaio ACNPC, Freitas JLS, Duarte JMB, Melo LM, Freitas VJF (2020) In Vitro Development and Mitochondrial Gene Expression in Brown Brocket Deer (Mazama gouazoubira) Embryos Obtained by Interspecific Somatic Cell Nuclear Transfer, Cellular Reprogramming, 22(4):208-216. https://doi.org/10.1089/cell.2019.0069

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J.V. Cortez, K. Hardwicke, J. Cuervo-Arango and C.G. Grupen (2022) Effect of Oocyte Source on the Development of Equine Embryos Produced by Somatic Cell Nuclear Transfer, Proceedings of the 19th International Congress on Animal Reproduction, Bologna, Italy. [**Oral** presentation; published abstract].

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the Development of Equine Embryos Produced by Somatic Cell Nuclear Transfer, Journal of Equine Veterinary Science, 113:103971. [**Oral** presentation; published abstract].

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J.V. Cortez, Kylie Hardwicke, Danton Cervi, Christopher G. Grupen, (2024) Normal Rates of Pregnancy and Foaling Achieved Following Transfer of Large Expanded Equine Blastocysts Vitrified After Blastocoele Collapse, Society for the Study of Reproduction, Annual meeting, 57. [**Poster** presentation].

J.V. Cortez, D. Cervi and C.G. Grupen (2025) Enhanced neonatal viability in equine cloning using bone marrow-derived mesenchymal stem cells as nuclear donors, Proceedings of the Annual Meeting of the Society for Reproductive Biology, 69. [**Oral** presentation].

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CHAPTER 1

Review of the Literature

PROLOGUE

The primary objective of this thesis was to enhance oocyte quality in equines, with the ultimate aim of improving fertility in the mare. Chapter 1 presents a comprehensive review of the scientific literature concerning the most recent advances in fertility across various species, with a particular focus on horses. In cases where information on horses was limited, studies on livestock species were considered first, followed by research involving humans and murine models. The chapter begins with the characterization of the equine oocyte, emphasizing its fundamental role in assisted reproductive technologies, its physiological functions, and the latest advancements in improving its quality and embryonic development. The review continues with an analysis of the main reproductive issues affecting mares. Subsequently, the developmental potential of oocytes retrieved from both live and post-mortem mares is examined, with the aim of identifying more reliable sources of high-quality oocytes. Additionally, the effects of maturation modulators are analyzed with the goal of replicating biological processes within in vitro culture systems. Special attention is given to the role of vitamins, particularly niacin, with a detailed exploration of its function as a cofactor in the synthesis of nicotinamide adenine dinucleotide (NAD+), as well as the emerging effects of NAD+ on various aspects of female fertility. The chapter concludes with the formulated hypotheses and specific objectives of this thesis and a structural overview of the subsequent chapters.

1.1 Introduction

In horses, as in other species, the application of assisted reproductive technologies (ARTs), such as intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT), is fundamentally reliant on access to high quality oocytes. The mature oocyte is the most important component in the endeavor to produce viable offspring because it provides all the cytoplasm that will form the early embryo. At fertilization, the oocyte cytoplasm contains the factors necessary for processing the penetrating spermatozoon, progressing the activation-induced events, and supporting the early embryonic cleavage divisions. Remarkably, following entry of the donor nucleus at SCNT, the oocyte cytoplasm also provides the reprogramming machinery needed to reset a differentiated nucleus to the embryonic state (Hinrichs, 2018).

The in vivo maturation of the equine oocyte occurs within the preovulatory follicle as a process coordinated with follicular growth and the periovulatory hormonal surge with the LH signal, such that meiotic resumption, cumulus cell expansion, and cytoplasmic modifications take place within a highly regulated follicular microenvironment (Ginther et al., 2007). Morphological and functional studies show that, in addition to nuclear maturation, the acquisition of embryonic competence critically depends on cytoplasmic maturation involving organelle redistribution, accumulation of metabolic reserves, and redox regulation processes, which differ in timing and metabolic requirements from those of other species (Hinrichs, 2010). The composition of the follicular fluid and the paracrine signaling of granulosa cells modulate oocyte quality; proteins, metabolites, and secreted factors present in the preovulatory environment influence the oocyte's ability to complete cytoplasmic maturation and sustain activation and subsequent embryonic development

(Luis-Calero et al., 2024). These physiological and metabolic characteristics of the equine cumulus oocyte complex partly explain why current in vitro maturation systems still fail to fully replicate in vivo maturation and therefore limit the efficiency of embryo production technologies in this species (Fernandez-Hernandez et al., 2020).

Oocyte maturation is a complex process in which the oocyte undergoes a series of nuclear and cytoplasmic changes to acquire the ability to be fertilized and sustain embryonic development (Albertini et al., 2001; Mermillod & Marchal, 1999). The completion of nuclear maturation involves the breakdown of the germinal vesicle and the reorganization and segregation of chromosomes to form the meiotic spindle, culminating in the extrusion of the first polar body and the formation of a metaphase II plate. These nuclear changes occur separately from the acquisition of developmental competence, which involves molecular and structural changes within the oocyte that is referred to as cytoplasmic maturation (Tremoleda et al., 2001; Trounson et al., 2001). Furthermore, it is essential that nuclear and cytoplasmic maturation progress in a coordinated, synchronous manner. This can be achieved in vitro using chemical agents that control the synthesis and activity of key regulatory proteins (Lonergan et al., 2000).

Recently, greater attention has been paid to oocyte cytoplasmic maturation and its correlation with developmental competence. A study carried out by (Arlotto et al., 1996), revealed that the quality of nuclear and cytoplasmic maturation was directly related to the size of the oocyte, with larger oocytes extruding the first polar body faster than smaller oocytes. Moreover, (Rodriguez et al., 2019), reported that oocytes that mature earlier give rise to embryos with greater developmental potential. This is relevant because the *in vitro* production of equine embryos has increased significantly in the last decade and has

become a routine procedure in several countries. Recent improvements in the various steps needed to produce equine embryos in vitro using immature oocytes aspirated from donor mares have made this an appealing and economically viable option for horse breeders (Galli et al., 2007; Stout, 2020). However, the efficiency of ICSI remains highly variable, with reported blastocyst rates between 10% and 41% (Rodriguez et al., 2019). Similarly, SCNT has grown in recent years, but blastocyst production rates range from 18% to 40% and pregnancy rates range from 20% to 40% (Gambini & Maserati, 2017).

While significant advances have been made to equine oocyte IVM procedures (Galli et al., 2007), many improvements reported in other species have yet to be investigated in the horse. The use of pre-IVM treatments to prevent spontaneous meiotic resumption has improved oocyte developmental potential in several species (Park et al., 2016; Soto-Heras et al., 2019; Sugimura et al., 2018; Tukur et al., 2020). Also, supplementing the IVM medium with antioxidants, growth promoters and oocyte-secreted factors has been shown to enhance the developmental potential of oocytes (An et al., 2019; Kafi et al., 2019; Metcalf et al., 2020; Richani et al., 2019; Stocker et al., 2020; Zou et al., 2020). Therefore, this review will concentrate on systematically evaluating the effects of the most promising treatments found to improve oocyte quality. In addition, oocyte quality will be discussed through a detailed evaluation of embryo development and fetal development after embryo transfer.

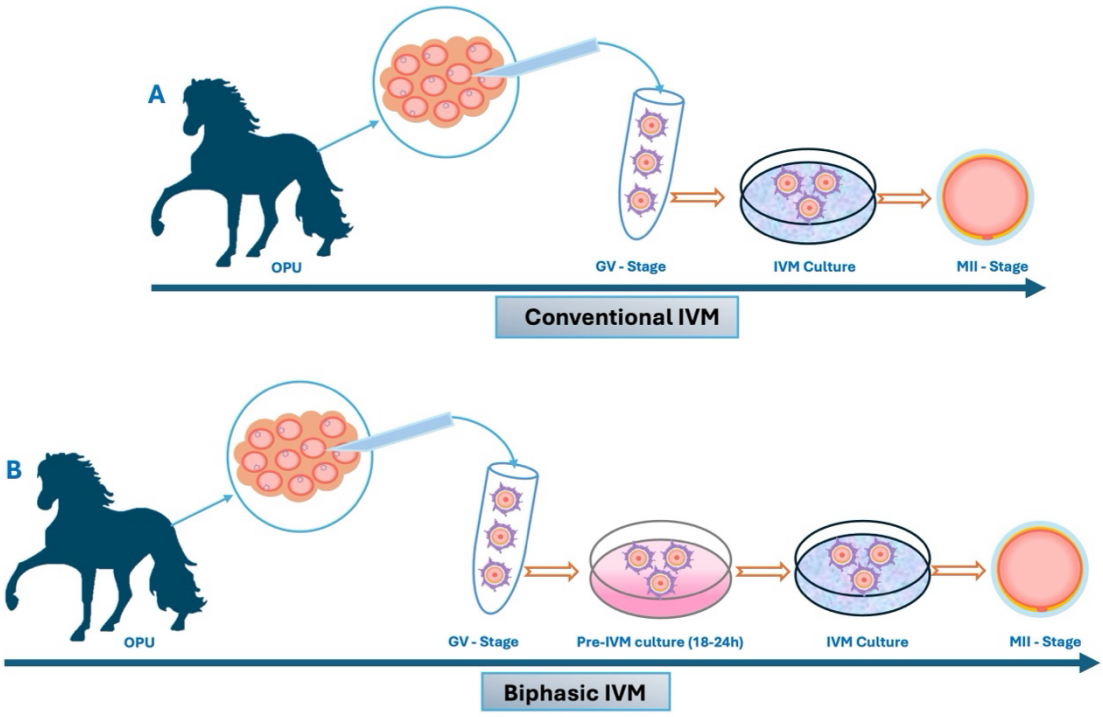

Figure 1.1 Horse conventional and biphasic IVM culture systems used to obtain metaphase-II (MII) stage oocytes for embryo production. (A) Conventional IVM system includes the ovum pick-up (OPU) procedure to collect immature oocytes at the germinal vesicle (GV) stage and the IVM culture phase. (B) Biphasic IVM system, includes the OPU procedure, and separate pre-IVM culture and IVM culture phases.

1.2 Reproduction in mares

Mares are seasonally polyestrous long day breeders, meaning they cycle and are fertile primarily during the months with the longest daylight hours, an adaptation ensuring foals are born in favorable weather. The key regulatory mechanism is the photoperiod. Long days reduce melatonin secretion from the pineal gland, which normally inhibits the reproductive axis. This allows for increased secretion of GnRH from the hypothalamus. GnRH stimulates the pituitary to release FSH (Follicle Stimulating Hormone) for follicular growth, and LH (Luteinizing Hormone) for follicular maturation and ovulation (Ginther, 1992).

The mare's reproductive cycle averages 21.9 days (range 19–23 days) and is divided into phases. During estrus, which lasts 4–7 days, the mare is receptive to the stallion, exhibiting signs like tail raising, squatting, and clitoral “winking”. Follicles grow to 30–50 mm in diameter, producing estradiol. Ovulation occurs uniquely at the ovulation fossa of the kidney bean shaped ovary, typically 24–48 hours before the end of estrus (Ginther, 1992).

During diestrus, which averages 14.9 days, the mare is non-receptive to the stallion. The ovulated follicle forms a Corpus Luteum (CL), which secretes Progesterone (P4) to prepare the uterine endometrium for implantation. If the maternal recognition of pregnancy signal from the developing embryo is not detected by day 14, the uterus releases Prostaglandin F2α (PGF2α), causing the CL to regress (luteolysis) and a new cycle is initiated (Aurich, 2011; Ginther, 1992). In mares, constant transuterine migration of the conceptus between days 10 and 14 is essential for maternal recognition of pregnancy, but the precise molecular mechanism suppressing PGF2α release is yet to be fully elucidated (Swegen, 2021).

The average gestation period in mares is 320–360 days. Secondary follicles develop around days 35–40, and specialized fetal trophoblast cells invade the endometrium to form endometrial cups that secrete Equine Chorionic Gonadotropin (eCG). This hormone acts like LH, causing the secondary follicles to luteinize and form accessory CLs, which produce additional P4 until the placenta takes over P4 production from weeks 12 to 20, ensuring pregnancy is maintained (Aurich, 2011; Ginther, 1992).

1.2.1 Estrous cycle

The estrous cycle is defined as the physiological interval providing females a repeated opportunity for conception, with its duration and seasonal timing varying across species. It spans from the onset of sexual receptivity (estrus) to the start of the subsequent estrus. Mares are classified as seasonally polyestrous (Aurich, 2011) and are characteristic long-day breeders; their reproductive activity, which involves multiple cycles within a breeding season, is initiated by increasing daylight hours during the spring and summer months (September–April in the Southern Hemisphere). This contrasts with short-day breeders, such as ewes, whose cyclicity is triggered by the shortening daylight hours of autumn and winter (Aurich, 2011).

The mare's estrous cycle, which averages 21 days (range 15–26 days) (Aurich, 2011; Ginther, 1992) is structurally divided into two primary phases. The follicular phase (estrus) is the shortest, representing approximately 25% of the cycle, beginning with the regression of the CL. During early estrus, the dominant period of P4 ends, initiating the maturation of antral follicles under the influence of FSH and LH, leading to estradiol (E2) dominance (Ginther, 1992). This transitions into estrus, the period of sexual receptivity marked by characteristic physical and behavioral changes, culminating in the ovulation of a single oocyte. The much longer luteal phase (diestrus), accounting for approximately 75% of the cycle, begins post-ovulation. The cycle concludes with Diestrus, the longest stage, during which the CL achieves maximum function and P4 secretion peaks, maintaining the uterus in preparation for potential pregnancy. Follicle growth itself follows a wave-like pattern, influenced by factors such as age and season (Ginther, 1992).

1.3 Folliculogenesis

Basal levels of FSH and LH are sufficient for follicular cell proliferation, which in turn highly depends on GnRH secretions. The completion of follicular differentiation is triggered by the large increase in circulating LH, which leads to rupture of the dominant follicle and expulsion of the mature oocyte (Ginther, 1992). In summary, the changes induced by the LH-surge include the differentiation of granulosa, and thecal cells in preparation for ovulation and formation of a functional corpus luteum, expansion of the cumulus cells that surround the oocyte, and concomitant oocyte maturation. This chain of events has to happen in a coordinated way so that a mature and fertilizable oocyte is released and a corpus luteum can support pregnancy.

Unlike other species, the ovulatory "surge" of LH in mares is in fact a prolonged, gradual increase in the level of LH over several days, starting before the onset of estrus and reaching a peak 24-48 h *after* ovulation (Bergfelt et al., 1991; Whitmore et al., 1973). This unique pattern of LH release is associated with the presence of a dominant follicle that produces high levels of estradiol. In addition, the change in the level of FSH is very different in mares compared to other species, as the highest level of FSH occurs during the luteal phase (Ginther et al., 2007).

1.3.1 Hormonal regulation of folliculogenesis

The anterior pituitary gland secretes gonadotropins, which have an impact on the ovary and testis. They are heterodimeric glycoproteins with an alpha subunit and a beta subunit. A component of FSH, LH, thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG) is the α-subunit (Ginther & Bergfelt, 1992). Gonadotropins work with intraovarian factors in the ovary to control steroidogenesis, oocyte maturation, ovulation, corpus luteum formation, and follicle development. Estrogens synthesized in the ovary during folliculogenesis influence the hypothalamic-pituitary (H-P) axis to control gonadotropin secretion. A high level of estrogen during the preovulatory period causes increased gonadotropin secretion, which is necessary for oocyte maturation and ovulation induction, although estrogens generally have a negative regulatory effect on gonadotropin secretion. The hypothalamic-pituitary-gonadal axis has positive and negative feedback loops that coordinate follicle maturation with sexual behavior and preparation for pregnancy. The ovary produces growth factors such as activin, inhibin, and follistatin to control FSH secretion and regulate local follicle development. FSH induces the development of early antral follicles to small antral follicles (McGee & Hsueh, 2000; Orisaka et al., 2021; Richards & Ascoli, 2018), while LH directs the development of antral follicles at the Graafian stage (Figure 1.2).

The LH and FSH α subunits are identical, but both have different β subunits. Each hormone binds to its cognate receptor uniquely because of this difference. However, receptor binding is not exclusive to the β subunit because the α subunit also interacts with gonadotropin receptors (Pierce & Parsons, 1981). FSH binds to specific membrane receptors on granulosa cells (GCs) to activate the G-protein complex and subsequently

adenylate cyclase, the enzyme responsible for the synthesis of cAMP from ATP. In the presence of cAMP, protein kinase A (PKA) phosphorylates and activates transcription factors that regulate gene activity, the products of which include aromatase, inhibin, FSH and LH receptors (FSHR/LHCGR), and other enzymes, hormones and receptors. Mural GCs express both FSHR and LHCGR and respond to both gonadotropins, whereas theca cells (TCs) express LHCGR and respond to LH stimulation. The gonadotropin response of TCs or GCs is then transmitted to the oocyte via gap junctions because oocytes do not express gonadotropin receptors (Gromoll et al., 1996; Richards & Ascoli, 2018). As a result, the PI3K-AKT and MEK1-ERK1/2 pathways are activated during oocyte maturation and follicle activation.

The LH surge activates genes responsible for the formation and stabilization of the extracellular matrix of the cumulus oophorus which is the region of the antral follicle wall that surrounds the oocyte. The expression of genes from the epidermal growth factor (EGF) family (amphiregulin, epiregulin, betacellulin) in cumulus cells increases rapidly during the LH surge. These ligands are transmembrane proteins that are released from the cell surface through ectodomain cleavage. These compounds bind to EGF receptors (EGFRs), leading to activation of mitogen-activated protein kinase 1 (MAPK1) and mitogen-activated protein kinase 3 (MAPK3), which are also known as extracellular signal-regulated kinases 1 and 2 (ERK1/2). While ERK1/2 promotes oocyte maturation, the specific roles of the EFG and ERK1/2 networks in regulating ovulation, oocyte maturation, and subsequent follicular luteinization are still not fully understood (Fan et al., 2009; Zhang et al., 2010)

1.3.2 Response of granulosa cells and theca cells to gonadotropins

Granulosa cells (GCs) and theca cells (TCs) are two types of somatic cells that surround the oocytes that form ovarian follicles. From the latent phase to ovulation, these somatic cells control oocyte development and steroidogenesis. GCs are responsible for both steroidogenesis and regulation of oocyte maturation, whereas TCs are primarily responsible for steroidogenesis. Somatic cells can express gonadotropin receptors, such as the FSH receptor (FSHR) and the LH/chorionic gonadotropin (CG) receptor (LHCGR), but oocytes do not express these receptors. Therefore, signaling present in somatic cells regulates the gonadotropin response leading to oocyte maturation (Richards & Ascoli, 2018). Estrogen signaling regulates gonadotropin secretion and gonadotropin functions in the ovary (Richards, 1980). Somatic cells of the ovary and the H-P axis have highly expressed estrogen receptors. GC cells do not express the estrogen receptor (ER), while TCs do (Jefferson et al., 2000). The predominant estrogen receptor in the ovary is the ERα (Drummond & Fuller, 2010). Loss of the ER is associated with a decreased estrogen level and an attenuated preovulatory gonadotropin rise, as well as complete ovulation failure (Jayes et al., 2014; Rumi et al., 2017) .

During folliculogenesis, the cohort of recruited primary follicles contain growing oocytes and proliferating granulosa cells that subsequently form secondary follicles. Tertiary follicles are characterised by the formation of an antrum with two distinct populations of granulosa cells: (1) cumulus cells that completely cover the oocyte, including the corona cells in the innermost layers; and (2) mural granulosa cells that line the entire follicle wall (Buccione et al., 1990; Cortvrindt & Smitz, 2001). The oocyte, cumulus cells, and mural granulosa cells interact through gap junctions. The follicle's vascular supply is situated in

the theca interna, and in this way, the granulosa cells supply nutrients to the oocytes and connect them to the circulating blood (Redmer & Reynolds, 1996). In mares, the Graffian follicles grow to diameters greater than 20 mm, such that the preovulatory oocytes are at a relatively great distance from the vascular supply of oxygen, nutrients, and signals.

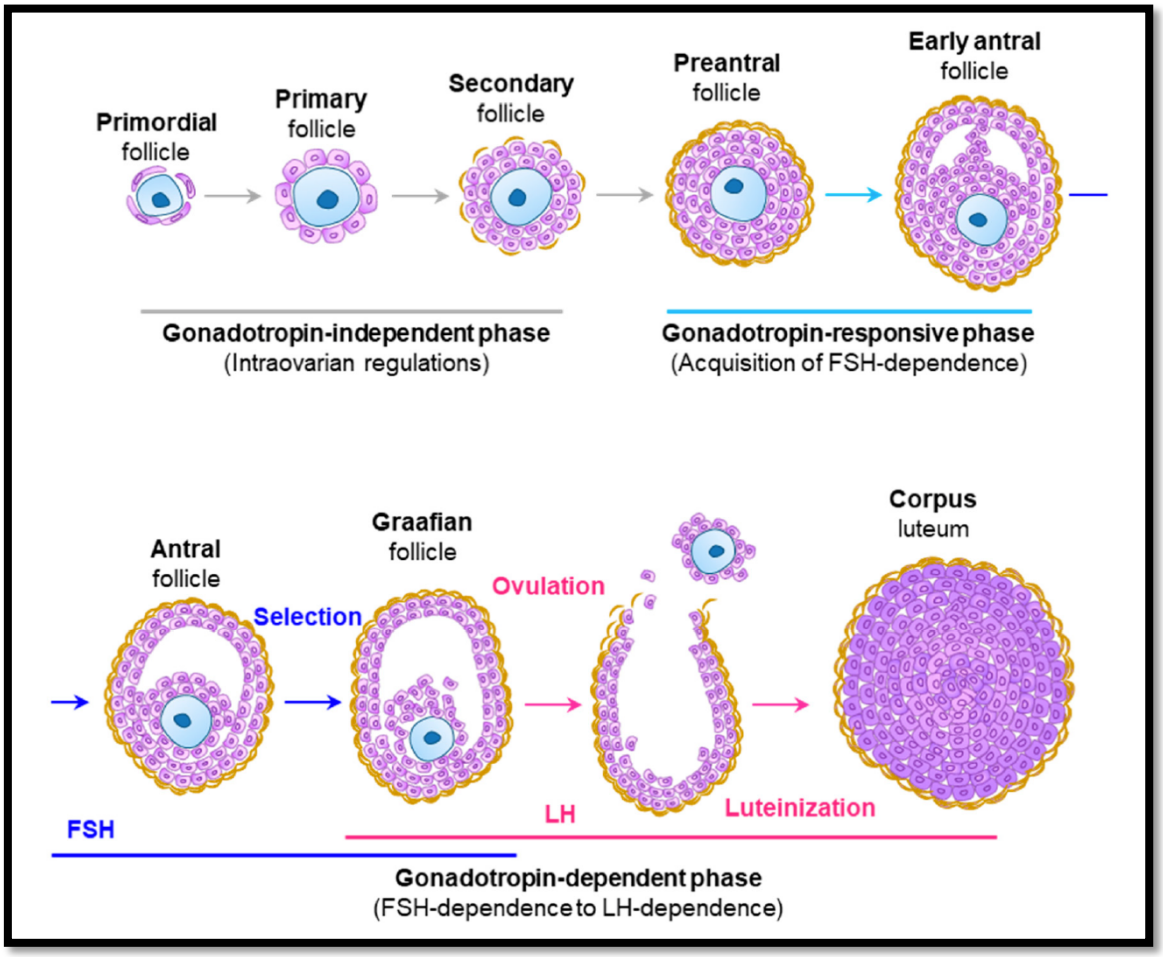

Figure 1.2 Follicular development begins under intraovarian. The gonadotropin independent control and, from the preantral stage onward, becomes dependent on FSH and subsequently LH for final maturation and ovulation. (from Lee et al., 2021).

Granulosa cells play an essential role in equine oocyte maturation by providing a supportive environment for development and regulating various processes critical for the acquisition of nuclear and cytoplasmic competence. These cells maintain close communication with the oocyte through gap junctions, allowing the exchange of signals and nutrients, such as amino acids, glucose, and nucleotides (Albertini et al., 2001; Sutton et al., 2003). During follicle development, when granulosa cells become responsive to gonadotropins, follicle-stimulating hormone (FSH) promotes their proliferation and differentiation into cumulus cells, leading to the production of estrogens, which are fundamental for regulating the follicular hormonal environment (Ginther et al., 2007). Additionally, granulosa cells express receptors for growth factors like insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF), which also modulate oocyte maturation and cumulus cell function (Hinrichs, 2010).

1.3.3 The role of cumulus cells

Cumulus cells are differentiated granulosa cells that exclusively surround mammalian oocytes and are essential for supporting the final oocyte growth and maturation processes (Olivera et al., 2018). Cellular connections between the cumulus cells and the oocyte, via gap junctions, allow the transfer of small metabolites and regulatory molecules (Tanghe et al., 2002). Thus, cumulus cells maintain the oocyte under meiotic arrest (Dekel, 1988; Eppig, 1989) and participate in the resumption of meiosis by conveying the LH signal to the oocyte (Mattioli & Barboni, 2000). Cumulus cells “expand” immediately in response to the ovulation-inducing surge of LH and results from the secretion of a mucoelastic matrix of proteoglycans primarily comprised of hyaluronic acid (Ball et al., 1984; Salustri et al., 1990). Cumulus expansion is one of the most important events in oocyte maturation.

This process involves cumulus cells secreting hyaluronic acid and other extracellular matrix components, which facilitates the separation of the oocyte from the rest of the follicle in preparation for ovulation (Hyttel et al., 1997). The presence of intact cumulus cells during oocyte in vitro maturation (IVM) greatly enhances nuclear and cytoplasmic maturation, highlighting their indispensable role in creating an optimal microenvironment for oocyte maturation (Choi et al., 2006). Despite the importance of cumulus-oocyte interaction during growth and maturation, there is no consensus regarding the exact role of the cumulus oophorus during fertilization. At fertilization, the connection between the oocyte and the cumulus cells degenerates but the corona cells remain attached to the proteoglycan matrix (Van Soom et al., 2003). For fertilization to occur successfully, a series of events must take place, including 1) sperm capacitation, 2) penetration of the zona pellucida by the sperm, 3) union and fusion of the sperm plasma membrane with the oolema, 4) activation of the oocyte, and 5) decondensation of the chromatin to form the male and female pronuclei (Topfer-Petersen, 1999)

1.3.4 Follicular fluid

Follicular fluid plays an essential role in equine oocyte maturation by providing an optimal biochemical and hormonal environment for development. This fluid, which fills the antral space of the follicle, is a complex mixture of hormones, growth factors, proteins, metabolites, and other bioactive molecules that regulate both the nuclear and cytoplasmic maturation of the oocyte (Eppig, 1996). One of the primary functions of follicular fluid is to facilitate the transport of nutrients and hormones, such as LH and FSH, to the granulosa cells and the oocyte. Growth factors within follicular fluid include insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF), which modulate

processes essential for ovulation and oocyte maturation (Hinrichs, 2010). Additionally, follicular fluid regulates the osmotic balance and pH of the follicular microenvironment, and acts as a buffer, protecting the oocyte from sudden environmental changes like heat shock and oxidative stress (Chen et al., 2023).

Follicular fluid provides various nutrients that directly influence the metabolism of the oocyte, including amino acids, glucose, and fatty acids. These components are essential for energy production and the accumulation of cellular reserves needed to support the processes occurring after fertilization (McNatty et al., 2005). Follicular fluid also contains hypoxanthine, a phosphodiesterase inhibitor, which maintains intra-oocyte cyclic AMP (cAMP) levels, the key second messenger controlling meiotic resumption (Conti & Franciosi, 2018). Supplementing IVM medium with follicular fluid has been shown to improve the meiotic and cytoplasmic competence of equine oocytes, underscoring its beneficial influence on oocyte maturation processes (Choi et al., 2006).

1.3.4.1 Activins and inhibins

Activins and inhibins are two groups of glycoproteins belonging to the transforming growth factor β (TGF-β) superfamily. These molecules are primarily produced by granulosa cells in ovarian follicles and have a significant impact on follicular development and hormonal regulation within the reproductive cycle.

Activins promote granulosa cell proliferation and survival, as well as steroid hormone production, particularly estrogens, by stimulating aromatase expression. In the context of oocyte maturation, activins influence granulosa cell apoptosis regulation, creating a

favorable environment for oocyte development (Turathum et al., 2021). Additionally, activins play an important role in communication between cumulus cells and the oocyte. These molecules are thought to mediate follicular growth and oocyte maturation by activating intracellular signaling pathways that foster the synthesis of proteins necessary for oocyte maturation (Turathum et al., 2021).

Conversely, inhibins act as negative regulators within the hypothalamic-pituitary-gonadal (HPG) axis. There are two main forms, inhibin A and inhibin B, produced by granulosa cells. Inhibins regulate the secretion of follicle-stimulating hormone (FSH) from the pituitary gland, which in turn affects ovarian follicle growth and maturation (Gregory & Kaiser, 2004). During follicular development, inhibins provide negative feedback on FSH production, ensuring that only the most competent follicles continue to develop. This mechanism is essential for preventing excessive follicle production and for ensuring that a single dominant follicle ovulates in each estrous cycle (Scaramuzzi et al., 2010).

The balance between activins and inhibins is crucial for follicular development and oocyte maturation. While activins promote granulosa cell growth and function, inhibins act as negative regulators to limit gonadotropin action. This delicate balance is essential for proper oocyte maturation and regulation of the estrous cycle in mares (Knight et al., 2012).

1.3.4.2 Kisspeptin

Kisspeptin is a neuropeptide derived from the KISS1 gene and has been identified as a key regulator of the hypothalamic-pituitary-gonadal (HPG) axis across various species, including equines. Kisspeptin is known for its ability to activate the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, a process fundamental to reproductive function and oocyte maturation. The levels of kisspeptin fluctuate throughout the reproductive cycle, peaking before ovulation to trigger the release of GnRH and subsequent LH surge. Kisspeptin does not function in isolation; its role is closely linked with other hormones and factors regulating oocyte maturation. For instance, estrogens, produced by developing follicles in response to FSH, have been shown to influence kisspeptin expression, creating a feedback loop that regulates HPG axis activity (Dubois et al., 2015).

1.3.4.3 Prostaglandins

Produced in the ovary, prostaglandin E2 (PGE2) acts as a local mediator in the regulation of follicular function and oocyte maturation. It is essential for inducing ovulation across many species, including equines. During the preovulatory phase of the estrous cycle, PGE2 is produced in response to the LH surge. The resulting increase in PGE2 levels stimulates follicular rupture and the release of the mature oocyte. PGE2 acts by activating specific receptors on follicular cells, leading to increased tissue permeability and extracellular matrix remodeling, thereby facilitating ovulation (Damasceno Teixeira et al., 2019). PGE2 also has a direct role in oocyte maturation. Studies have shown that

PGE2 influences the resumption of meiosis in equine oocytes, facilitating the transition to the metaphase II stage (Hinrichs, 2010). The administration of PGE2 during IVM has been found to improve several indicators of oocyte quality (Boruszewska et al., 2020). The action of PGE2 in oocyte maturation and ovulation is interrelated with other hormones, such as LH and estrogens. Estrogens can induce PGE2 production in granulosa cells, establishing a positive feedback loop that supports follicular development and oocyte maturation (McCracken et al., 1999).

1.4 Oocyte Maturation In Vivo

As in other mammals, equine oocyte maturation is a dynamic and highly regulated process involving complex interactions within the follicular environment and between the oocyte and its surrounding cumulus cells. This process depends on the precise coordination of hormonal, protein, and molecular factors that act sequentially to ensure proper maturation. For most of its development, equine oocytes remain in meiotic arrest at the diplotene stage of prophase I, and the resumption of meiosis requires a complex orchestration of signals from the hypothalamic-pituitary-gonadal axis, along with the influence of intrafollicular factors and cell-to-cell communication (Hinrichs, 2010). The progression to metaphase II, the stage at which the oocyte is competent for fertilization, is regulated by gonadotropins such as FSH and LH, steroid hormones, growth factors, and autocrine and paracrine signals generated by the follicle itself (Aurich, 2011). These modulators work closely to synchronize nuclear and cytoplasmic maturation of the oocyte. Proper cytoplasmic maturation is crucial because the oocyte must accumulate maternal mRNAs, stage-specific proteins and other components required for fertilization, chromatin processing, and early embryo development (Hinrichs, 2018).

A detailed understanding of the mechanisms and modulators involved in equine oocyte maturation has significant implications for improving the efficiency of assisted reproductive technologies in this species, including in vitro fertilization (IVF) and embryo in vitro production (IVP).

1.4.1 Regulation of oocyte maturation

The meiotic and developmental capacities of oocytes are acquired gradually and sequentially during folliculogenesis, regulated via interactions with the accompanying somatic cells. Two signalling molecules participate in the regulation of oocyte meiosis at the level of intercellular communication: cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Strączyńska et al., 2022). They play an important role in maintaining the inhibition of meiosis of oocytes, regulating their maturation by ensuring timely meiotic progression.

Graafian follicles retain oocytes during prophase I from the first meiotic division until the preovulatory LH surge in mammals (Mehlmann et al., 2004). Reactivation of meiosis is possible due to processes triggered by the binding of LH to LHR present on granulosa and cumulus cells. Elevated LH levels increase the transient and multiplicative synthesis of cAMP in granulosa cells, leading to local activation of growth factors of the EGFβ family (amphiregulin, epiregulin, betacellulin) (Gilchrist et al., 2016 , Hsieh et al., 2009, Richani et al., 2018) and reduced cell growth. An increase in the synthesis and/or hydrolysis of cGMP reduces the flux of cGMP from the granulosa cells to the oocyte.

Depletion of cGMP in turn impairs the inhibition of phosphodiesterase 3A (PDE3A) activity, which leads to a decrease in oocyte cAMP levels (Straczynska et al., 2022). A reduction in intra-oocyte cAMP levels leads to the inactivation of Protein Kinase A (PKA), which normally phosphorylates and inhibits the Maturation Promoting Factor (MPF) components of cyclin B and Cyclin-Dependent Kinase 1 (CDK1) (Chesnel & Eppig, 1995). This decrease in PKA activity results in activation of MPF, enabling the resumption of meiosis (Mehlmann et al., 2004).

In addition, the increase in LH triggers a well-defined mechanism based on the interruption of communication between the oocyte and the cumulus cells, which stops the transfer of cAMP to the oocyte (Dekel, 1988; Eppig, 1989). This activating effect of LH has been shown to consist of two sequential steps: an immediate transient increase in the phosphorylation state of Cx43 and a subsequent decrease in CX43 protein levels through transcriptional or translational modifications (Granot & Dekel, 1994). This disruption of the gap junctions can lead to the resumption of meiosis (Straczynska et al., 2022). Together, cAMP and cGMP signaling are involved in the regulation of meiotic maturation. A decrease in the content of either cyclic nucleotide signals the initiation of meiotic maturation in oocytes (Norris et al., 2009; Vaccari et al., 2009).

The PKA signaling pathway, activated by cAMP, initiates the phosphorylation of various proteins within the oocyte and cumulus cells. This includes activating transcription factors that promote the expression of genes necessary for oocyte maturation and development (Mehlmann et al., 2004). Additionally, PKA is involved in regulating apoptosis in cumulus cells, helping to maintain the integrity of the follicular microenvironment. Studies have demonstrated that manipulating cAMP levels during

IVM can significantly influence the oocyte's ability to undergo fertilization and embryonic development. The transient addition of cAMP analogs to the culture medium can enhance the synchronisation of nuclear and cytoplasmic maturation, indicating that a proper balance of cAMP is crucial for obtaining developmentally competent oocytes (Choi et al., 2006).

1.4.2 Modulators of oocyte maturation

1.4.2.1 Gonadotropins

In the context of IVM, FSH and LH are commonly used to replicate the endocrine environment necessary for proper oocyte maturation, thereby improving nuclear maturation rates and fertilization competence (Choi et al., 2006). FSH upregulates the expression of LH receptors on granulosa cells, as well as several growth factors, such as insulin-like growth factor 1 (IGF-1), which enhances FSH action and supports follicular cell differentiation (Hinrichs, 2010). Activation of the FSHR/cAMP/PKA signaling pathway in granulosa cells triggers cAMP production, which maintains the oocyte in meiotic arrest until the luteinizing signal induces the resumption of meiosis (Richards & Ascoli, 2018). Upregulation of LH receptors allows LH to stimulate the production of steroids and other paracrine signals that promote nuclear and cytoplasmic maturation of the oocyte (Hinrichs, 2010). LH also triggers the breakdown of connections between the oocyte and cumulus cells, facilitating the resumption of meiosis and progression to metaphase II (Ginther, 1992).

1.4.2.2 Estrogens

One of the primary functions of estrogens is modulating the expression of gonadotropin receptors, particularly LH receptors, on granulosa and theca cells. This modulation is essential for the follicle's responsiveness to the preovulatory LH surge. Estrogens also influence cumulus expansion, a critical process that facilitates oocyte release and subsequent fertilization (Hinrichs, 2010). This cumulus expansion is mediated by the stimulation of hyaluronic acid synthesis, allowing the oocyte to separate from the surrounding follicular cells. Additionally, estrogens play a significant role in regulating the secretion of growth factors and other local modulators within the follicle, contributing to both nuclear and cytoplasmic maturation of the oocyte (Albertini et al., 2001). In the context of IVM, the addition of estrogens has been shown to improve oocyte quality and increase maturation and fertilization efficiency, highlighting their importance as regulators of the follicular environment (Choi et al., 2006).

1.4.2.3 Progesterone

Although progesterone's role in oocyte maturation is not as prominent as that of LH or estrogens, it has been shown to significantly impact the coordination of processes that enable the oocyte to achieve meiotic competence and readiness for fertilization (Ginther, 1992). Studies on the IVM of equine oocytes suggest that the presence of progesterone in the culture medium may improve the quality of mature oocytes, promoting their subsequent development (Choi et al., 2006).

1.4.2.4 Growth Factors

Insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) are key intra-follicular regulators that act synergistically with gonadotropins to support granulosa and cumulus cell function during oocyte maturation. Produced locally within the follicle, IGF-1 binds to receptors on granulosa cells and the oocyte to activate the PI3K/AKT pathway, promoting cell survival, protein synthesis, and the acquisition of oocyte developmental competence. Equine IVM studies have shown that IGF-1 supplementation enhances nuclear maturation and embryo development (Ginther et al., 2004; Hinrichs, 2010; Sirotkin, 2011). Likewise, EGF and its EGF-like ligands activate EGFR in granulosa and cumulus cells to stimulate the PI3K/AKT and MAPK pathways, driving cumulus expansion and meiotic progression. Adding EGF to equine IVM medium has been found to improve meiotic resumption and oocyte quality (Conti et al., 2006).

Other key oocyte maturation factors include growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). Secreted by the oocyte, GDF9 and BMP15 play vital roles in cumulus cell function and oocyte-cumulus cell communication. These factors support cumulus cell proliferation, differentiation, and expansion, all of which are essential for creating a supportive microenvironment for oocyte maturation (McNatty et al., 2005).

1.5 Oocyte in vitro maturation (IVM)

Oocyte in vitro maturation is carried out in culture media intended to provide physiologically relevant nutritional and physicochemical support to the cumulus-oocyte complex (COC). However, the media selected for equine IVM have been based on those developed in other species. Tissue culture medium (TCM)-199 (Zhang et al., 1989) and Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 Nutrient Mixture (DMEM/F12) (Galli et al., 2007), are the most commonly used base media for equine IVM. The composition of such media is likely not optimal because it differs from the in vivo environment. For example, pyruvate concentrations in equine follicular fluid are 0.03 to 0.13 mM, whereas TCM-199 does not provide it. Pyruvate plays an important role in both oocyte metabolism and scavenging reactive oxygen species (ROS) (O'Donnell-Tormey et al., 1987). It is important to note that the specific requirements of equine COCs during IVM are largely unknown and a better understanding is needed to define the metabolic requirements for optimal acquisition of developmental competence (Fleming et al., 2018).

Measuring oxygen consumption, along with other substrates, like glucose, pyruvate, and lactate, can be used to estimate how much ATP COCs produce. In the most studied model, the mouse, cumulus cells perform glycolysis to produce ATP and provide pyruvate to the oocyte (Harris et al., 2009). The profiles of pigs and cattle are similar, with the oocyte oxidizing pyruvate along with other endogenous energy sources to produce ATP in the mitochondria via the TCA cycle and oxidative phosphorylation (OXPHOS) (Steeves & Gardner, 1999; Sturmey & Leese, 2003; Sutton et al., 2003). The anaerobic consumption of one molecule of glucose via glycolysis yields a net production of two molecules of

ATP, while the aerobic consumption of one molecule of glucose via oxidative phosphorylation produces approximately 30 molecules of ATP (Mookerjee et al., 2017). The most detailed description of glucose, lactate and pyruvate metabolism, oxygen consumption and mitochondrial efficiency in equine COCs was provided by Lewis et al. (Lewis et al., 2020). Importantly, they revealed that equine COC metabolism is distinct from that of COCs in other mammals. During IVM, aerobic glycolysis is the major pathway of glucose metabolism in equine COCs, and lactate production in COCs is responsible for 95% of the glucose consumed. The concurrent high oxygen consumption rate (OCR) demonstrates that oxidative phosphorylation plays a major role in the energy metabolism of equine COCs, although most of the glucose consumed could be attributed to lactate production. The identity of the substrates for oxidative phosphorylation is likely to include amino acids and fatty acids, as well as a portion of glucose not accounted for in terms of lactate production. However, further research is needed to confirm this. In addition, Lewis et al. observed a progressive decline in mitochondrial function as IVM progressed.

Not only do we lack knowledge about the ideal culture media for equine IVM, but we also know little about the ideal incubation conditions. In this regard, it is widely accepted that a low oxygen concentration during embryo culture is advantageous, and the use of 5% oxygen is virtually universal for embryo culture in both animals and humans (Wale & Gardner, 2016). The ideal oxygen tension for IVM is still under debate, but in practice, IVM is frequently performed under atmospheric oxygen tension in most species (Filali et al., 2008). Despite the fact that the O_2 tension in horse ovarian follicles is unknown, current equine IVM protocols are typically performed using the atmospheric concentration of O_2 (~21%) (Foss et al., 2013; Galli et al., 2007; Hinrichs et al., 2005).

Determining the optimum conditions for oocyte IVM is crucial for the success of assisted reproduction.

It is well established that in vitro-matured oocytes have lower developmental competence compared to in vivo-matured oocytes, which is also true for horses (Buckett et al., 2008; Foss et al., 2013). The conditions to which a cell is exposed can significantly affect its biochemical and metabolic regulation. In the case of equine oocytes, a study conducted by Walter et al. (Walter et al., 2019) found that protein and metabolite profiles in cumulus cells changed significantly when oocytes were matured in vitro compared to those matured in vivo. Walter and colleagues identified various biochemical pathways in cumulus cells that were affected by in vitro exposure, supporting the proposal that changes in metabolism can cause variations in developmental competence (Hollywood et al., 2006).

1.6 Development of ARTs for Equine Breeding

The implementation of ARTs in horse breeding programs is still lagging behind that in other livestock species, despite the high economic value of elite individuals (Choi et al., 2014; Lazzari et al., 2020; Olivera et al., 2018).

The first report of the in vitro maturation (IVM) of equine oocytes was made by Fulka and Okolski in 1981 (Fulka & Okolski, 1981). Amazingly, equine embryo production using IVM oocytes was successfully carried out for the first time in 1989 (Zhang et al., 1989). In this early work, oocytes were collected from slaughterhouse-sourced ovaries, matured in vitro, and, after transferring the matured oocytes to the oviducts of

inseminated mares, an embryo was recovered by uterine lavage 7 days later. During that time, the oocyte IVM systems of other domestic livestock animals were undergoing constant development. Oocytes obtained from slaughterhouse ovaries were mostly used in IVM studies due to their ready availability and practically unlimited supply, especially in species such as cattle, pigs, goats, and sheep (Galli et al., 2007). The development of this technology was driven not only by the pursuit of scientific knowledge but also by the declared interest in embryo production by the livestock industries. As a consequence, countless scientific publications were generated and, in a relatively short time, reliable embryo in vitro production (IVP) procedures, including oocyte IVM, in vitro fertilization (IVF), and embryo culture, were established.

In horses, the development of ARTs has taken much longer to progress compared to other domestic livestock species. The limited availability of slaughterhouse ovaries from mares has greatly restricted the study of oocyte IVM and embryo IVP. Even when a lab has access to mare ovaries from a slaughterhouse, the low numbers of oocytes recovered from each ovary pair, compared to other species, is a further constraint. Furthermore, the equine oocyte extraction process is particularly difficult because the cumulus-oocyte complex (COC) is very tightly adhered to the inner wall of the antral follicle. Consequently, following the early successes (Zhang et al., 1989) in equine oocyte IVMs, relatively few improvements were achieved using slaughterhouse oocytes (Hinrichs, 2018), forcing researchers to focus on the use of in vivo-matured oocytes for embryo IVP.

Horses serve as useful models for the study of comparative reproductive biology. However, the clinical translation of assisted reproductive technologies to the horse remains limited, largely due to the lack of success of conventional in vitro fertilization

(IVF) (Leemans et al., 2016). Intracytoplasmic sperm injection (ICSI), which is effective in producing equine embryos in vitro, and subsequently foals (Choi et al., 2002; Galli et al., 2007), has been used in part to circumvent this limitation and has gained significant commercial and research interest as a result (Claes et al., 2019; Hinrichs et al., 2005). However, the limitations of assisted reproduction in the horse are compounded because horses are monovular and do not respond well to superovulation protocols (Roser et al., 2020; Squires & McCue, 2007). As a result, oocytes used for ICSI are typically obtained from pre-ovulatory follicles and need to be matured in vitro.

1.6.1 Embryo Transfer

Embryo Transfer (ET) is a widely utilized reproductive biotechnology in the equine industry, serving to maximize the offspring production from genetically superior mares within a single breeding season, and to circumvent certain issues of subfertility (Hinrichs & Choi, 2005). The success of ET is fundamentally dependent on the quality and uterine environment provided by the recipient mare. Therefore, the selection of an appropriate recipient is considered the most critical challenge prior to the transfer procedure. To maximize the probability of selecting a recipient with a high potential for maintaining a pregnancy, it is generally recommended to have a pool of at least three suitable recipients available for each embryo donor (Squires, 2013).

Uterine and cervical characteristics pertinent to conception are primarily regulated by the effects of P4, such that a functionally active CL is essential for endometrial preparation and pregnancy maintenance (Ginther, 1992). While directly measuring the circulating concentrations of P4 would be an efficient gauge of CL activity, the high cost

and delayed results of assays limit its clinical utility (Squires, 2013). Furthermore, the CL's functional status cannot be accurately assessed through conventional transrectal palpation or B-mode ultrasonography (Squires, 2020), as its morpho-echogenic characteristics are not reliably correlated with its luteal output in horses. Therefore, mares are selected as suitable recipients for embryo transfer based on uterine tone, with emphasis on a well-contracted, firm uterus that indicates optimal reproductive tract health and readiness for pregnancy (Carnevale et al., 2000).

1.6.2 In Vitro Fertilisation

While IVF has been successfully developed in almost all species of domestic animals, the routine implementation of IVF in horses has yet to be realised. The first report of *in vitro* blastocyst production following standard IVF in the horse, as well as the generation of three foals resulting from the transfer of these blastocysts, was recently published (Felix et al., 2022). Moreover, the authors used exclusively *in vitro*-matured oocytes and achieved fertilization (>50%), blastocyst development, and foal production from oocytes recovered both via OPU and post-mortem ovary collection (Felix et al., 2022). These represent the only foals produced through standard IVF, aside from the two foals reported by Eric Palmer's laboratory in 1991 and 1992, which were generated using *in vivo*-matured oocytes, A23187-treated sperm, and the surgical transfer of cleaved embryos into the oviduct (Palmer et al., 1991).

In the absence of an effective conventional IVF system, researchers have established the methods used to produce equine embryos by ICSI. Although commercially acceptable rates of embryo production have been achieved in multiple laboratories (1.5 blastocysts

per OPU session) (Galli et al., 2018; Hinrichs, 2018; Stout, 2020), further improvements are needed (Briski & Salamone, 2022; Hinrichs, 2020; Lazzari et al., 2020). Several factors affecting equine embryo in vitro production have been identified, including incomplete sperm capacitation and alterations in the zona pellucida (Hinrichs, 2010; Palmer et al., 1991). Furthermore, adoption of equine ICSI requires considerable expertise to master the technical challenges of the sperm injection procedure (Galli et al., 2018; Metcalf et al., 2020).

1.6.3 Intra-Cytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI), defined as the injection of a single spermatozoon into the ooplasm of a metaphase II oocyte (Palermo et al., 1992), has become a mainstay of human ARTs over the last two and a half decades. Its widespread use in humans stems from its ability to address numerous issues related to male infertility such as lack of sperm motility and globozoospermia (Palermo et al., 1992) as well as certain female infertility factors, including suboptimal oocyte quality or low oocyte yield. Furthermore, ICSI mitigates reproductive challenges like polyspermy and fertilization failure associated with aged oocytes or the zona hardening resulting from the premature exocytosis of cortical granules in cryopreserved oocytes (Galli et al., 2007; Hinrichs & Choi, 2005). The technique also offers the important benefit of reducing the transmission of infectious diseases such as HBV, HCV, and HIV (Palermo et al., 2017).

The development of ARTs in horses has historically been hampered by the inability to consistently achieve successful fertilization via conventional IVF, notwithstanding the recent IVF achievements already mentioned (Choi et al., 2002; Felix et al., 2022). This

limitation persists despite the documented success of embryo generation following the transfer of *in vitro* matured oocytes into inseminated recipient mares (Hinrichs et al., 1990). Consequently, the primary obstacles identified for equine ARTs include zona pellucida hardening during oocyte maturation (Choi et al., 2002; Choi et al., 2006), the consistent failure of sperm to acquire the necessary capacitation to penetrate *in vitro* matured oocytes (Choi et al., 2006), and the inherent difficulties associated with achieving blastocyst development through embryo culture (Leemans et al., 2016).

1.6.4 Somatic Cell Nuclear Transfer

Equine cloning, compared to other domestic species, developed relatively late after the birth of *Dolly* the sheep, the world's most famous cloned animal (Wilmut et al., 1997). The first equid cloned through SCNT was a mule, generated from the cell of a 45 day old fetususing in vivo matured oocytes and immediate oviductal transfer (Woods et al., 2003). That same year, the group led by Cesare Galli in Italy reported the cloning of an adult horse using a completely in vitro procedure, from oocyte collection to blastocyst development (Galli et al., 2003). The resulting foal, named *Prometea*, was genetically identical to the mare that carried her to term, challenging the presumed necessity of maternal immunological recognition of fetal antigens for successful pregnancy (Szekeres-Bartho, 2002).

Subsequently, Katrin Hinrichs' research group produced the first cloned horse in the United States (Hinrichs & Choi, 2005). Since then, equine cloning has expanded worldwide, with South America emerging as a key center of innovation. Argentina was the first country in the region to achieve equine cloning (Gambini et al., 2012; Miragaya

et al., 2011), followed by Brazil, which reported its first cloned horse in 2012 (Vitro Brasil Clonagem). Colombia achieved this milestone in 2016, and Peru in 2024. In Asia and Europe, between two and five cloned foals were produced annually in 2015 (Lee et al., 2015). In Australia, the birth of a cloned foal was reported in 2019 using an oocyte aggregation method to produce the cloned embryos (Damasceno Teixeira et al., 2019). *Catalina Genetics* was established in 2018, and, following the death of a valuable mare called *Easter*, a cloned foal was produced using traditional cloning methods to rescue her genetics.

Traditional cloning, the method used to generate *Dolly* the sheep (Wilmut et al., 1997), involves the enucleation of a mature oocyte, the insertion of a donor cell into the perivitelline space, and subsequent electrofusion, activation, and in vitro culture. Electrofusion can be omitted if the donor cell is directly injected into the enucleated ooplasm (Wakayama et al., 1998). A variation of this approach, known as zona pellucida-free (ZP-free) SCNT, or "hand-made" cloning, involves removing the zona pellucida to facilitate enucleation and cell fusion. However, this method requires individual manipulation of reconstructed embryos and specialized culture systems to maintain blastomere cohesion during development (Vajta et al., 2008). A more recent commercial strategy employs G2 phase synchronized donor fibroblasts paired with telophase II activated oocytes obtained via OPU (Maserati & Mutto, 2016). Several studies have demonstrated that traditional cloning yields higher and more consistent blastocyst and pregnancy rates compared to ZP-free cloning (Choi et al., 2014; Gambini et al., 2014; Gambini et al., 2012; Lagutina et al., 2005; Olivera et al., 2016).

The acceptance of cloning and other ARTs varies substantially among equine associations and studbooks worldwide. International Thoroughbred studbooks prohibit cloning and any form of ART. Similarly, the American Quarter Horse Association (AQHA), the largest horse breed association in the United States, does not allow the registration of clones (Squires, 2019). In December 2013, the European Commission proposed banning the cloning of farm animals and the importation of their products within the European Union. Although an exemption for sport animals was initially considered, the European Parliament removed this exception in October 2015 (Campbell, 2018). In contrast, most Warmblood studbooks and the World Breeding Federation for Sport Horses accept cloned horses for registration. Likewise, the Fédération Équestre Internationale (FEI) determined that cloning does not compromise fair competition and authorized the participation of clones and their offspring in official events, recognizing that such competitions evaluate the combined performance of both horse and rider (Reis, 2015). In Argentina, the Argentine Association of Polo Horse Breeders (AACCP) actively promotes research and the application of equine reproductive technologies.

The overall efficiency of equine cloning has improved markedly in recent years, resulting in an increased production of cloned horses. The abnormalities observed in cloned foals appear to be less severe and less frequent than those reported in other species, particularly cattle, where large offspring syndrome, severe placental pathology, and high perinatal mortality are common (Johnson et al., 2010). In contrast, cloned foals typically present with milder neonatal issues, such as transient maladjustment, enlarged umbilicus, or angular limb deformities, that often resolve with supportive care (Johnson & Hinrichs, 2015). These defects are attributed to molecular level deficiencies, particularly

incomplete genomic reprogramming, aberrant epigenetic remodeling of donor nuclei, and inefficient artificial activation during SCNT. Consequently, further research is required to optimize these aspects and enhance the success rates of equine SCNT. From a commercial and regulatory perspective, there is an urgent need to harmonize international regulations governing the registration of cloned horses across countries and breeding associations. As new tools for equine genetic improvement continue to evolve, regulatory frameworks should prioritize animal health and welfare, grounded in sound biological principles that promote the sustainable growth and competitiveness of international equine markets (Gambini & Maserati, 2017).

1.6.5 Oocyte Collection

Compared to other domestic livestock species, the number of equine oocytes available for commercial and research purposes is low. Thus far, the mono-ovular physiology of equines continues to be the major limitation, together with the fact that there are no commercially available gonadotropins capable of reliably stimulating the development of multiple preovulatory follicles in the mare. This means that only 1 or 2 preovulatory follicles per natural cycle are available for the retrieval of mature oocytes (Carnevale, 2008; McCue, 1996; Stout, 2020), despite much work being done to find an effective combination of hormones, using either homologous or heterologous hormones (eCG, eFSH, GnRH, EPE), or recombinant hormones (reFSH, reLH). Recombinant hormones, which are characterized by being free of contaminants and prion diseases, have shown promising results, but the success rate has been inconsistent (Jablonka-Shariff et al., 2007; Jennings et al., 2009). It has been proposed that the administration of these hormones is not timed appropriately relative to the follicular waves of each mare. A more detailed

evaluation of the endogenous levels of eFSH, eLH, estradiol, inhibin, activin, and eIGFI is needed to better control the response to treatment at a given time (Roser & Meyers-Brown, 2012; Squires, 2006). As a result, oocytes are typically retrieved from immature ovarian follicles by ovum pick-up (OPU) and matured in the laboratory by in vitro maturation (IVM). Oocyte recovery may be performed through follicular aspiration, either transvaginally or via the flank approach. Alternatively, oocytes can be obtained post-mortem from the ovaries of donor mares (Hinrichs & Choi, 2005).

1.6.5.1 Ovum Pick-Up

The use of ovum pick-up (OPU) for the recovery of oocytes intended for embryo in vitro production was first developed in humans in the early 1980s and was rapidly adopted by the cattle industry, achieving oocyte recovery rates exceeding 70% per follicle (Galli, Colleoni, et al., 2014). In horses, a flank approach was implemented in the late 1980s (Palmer et al., 1987), yielding oocyte recovery rates of 63–72% from pre-ovulatory follicles 36 hours after gonadotropin treatment. In the early 1990s, the development of the transvaginal approach allowed serial follicle puncture, resulting in pregnancies and births without compromising mare fertility. Currently, ultrasound-guided transvaginal OPU is the standard method used worldwide for collecting oocytes in horses (Bols & Stout, 2018).

Over time, the OPU technique has been optimized through the use of double-lumen needles, which increased the recovery rates of immature oocytes to over 50% (Jacobson et al., 2010). Today, a variety of commercial flushing media are available, all supplemented with heparin to prevent coagulation of follicular fluid and blood (Foss et

al., 2013). During the procedure, the fluid is maintained at 37 °C, and each follicle is flushed and scraped 8–10 times to maximize oocyte recovery (Galli, Duchi, et al., 2014).

Current oocyte recovery rates are approximately 50-70%, with an average of 5–12 oocytes retrieved per OPU session, depending on the number of follicles present and individual mare factors such as age and breed (Lazzari et al., 2020). In OPU–ICSI programs, donor mares are evaluated by transrectal ultrasonography one to three days before the procedure to assess follicle number and size. Typically, OPU is performed when at least ten follicles with a diameter ≥10 mm are present. This number of follicles is not always attainable in older mares, such that OPU is sometimes performed even if only a single suitable follicle is available.

The interval between OPU sessions in the same mare usually ranges from 3 to 5 weeks, although in some cases it may extend to 6–8 weeks, depending on the donor (Claes & Stout, 2022; Galli et al., 2007). While oocyte recovery efficiency has improved significantly, this is partially offset by reduced maturation rates, with approximately 50% of oocytes from immature follicles reaching the MII stage (Morris, 2018).

1.6.5.2 Ovaries Recovered Post-mortem

The development of effective methods for transporting and processing abattoir-sourced ovaries while preserving oocyte viability is crucial for studies on equine oocyte maturation and the advancement of ARTs in horses. Such methodologies are also useful following the accidental death of a mare with high genetic value, as the recovery of oocytes from her ovaries could enable the production of additional offspring (Carnevale

et al., 2003). However, progress in this field has been constrained by the limited availability of biological material, particularly as the number of horse abattoirs in many countries, including Australia, is declining.

Scott et al. (2001) reported an embryonic development rate of approximately 10% when oocytes were collected from ovaries transported at 28–30 °C for 6–8 hours. These oocytes were matured in vitro and subsequently transferred into the oviducts of inseminated mares (Matsukawa et al., 2007; Scott et al., 2001) . In 2001, the birth of a healthy foal was documented from oocytes recovered from five euthanized mares whose ovaries had been shipped by air at ambient temperature (Carnevale et al., 2003). In the same year, Li et al. (2001) successfully produced foals after IVM and ICSI of oocytes obtained from ovaries transported at 12–20 °C for 4–24 hours (Li et al., 2001). While these studies demonstrated the feasibility of obtaining viable pregnancies using oocytes from transported ovaries, the generally low and variable efficiency reported highlights the need to further improve the conditions used to transport and process ovaries recovered post-mortem.

1.6.6 Oocyte IVM

The quality of the oocytes following IVM is one of the most important factors determining the success of embryo production in vitro because the oocyte contains all the necessary factors for sperm processing and the initial embryonic cleavage divisions. Also, in SCNT-produced embryos, the remodeling and reprogramming of donor nuclei are controlled by factors present in the recipient oocyte cytoplasm (Choi et al., 2013; Galli et al., 2003; Hinrichs, 2018; Olivera et al., 2018). It is well established in all species studied, including the horse, that in vitro-matured oocytes have decreased developmental

competence compared to their in vivo-matured counterparts (Hinrichs, 2018; Lewis et al., 2020). The metabolism of oocytes matured in vitro is typically dysregulated, due to IVM systems being carried out under atmospheric levels of oxygen and higher than physiological concentrations of glucose (Leese et al., 2008).

The culture media currently used in equine IVM systems (TCM-199, B2, Ham's F10, and Ham's F12) are derived from those developed for other species, which are normally formulated based on the composition of the reproductive tract fluid in those species (Galli, Colleoni, et al., 2014; Hinrichs, 2010). Few attempts have been made to modify equine IVM medium based on the components of the mare's own reproductive tract, although a recent study examined adding preovulatory follicular fluid secretome to improve oocyte maturation (Luis-Calero et al., 2024). Interestingly, the proteome of mare oviductal fluid was evaluated and found to differ consistently before and after ovulation (Fernandez-Hernandez et al., 2020). Such analyses provide valuable information that will inform ongoing improvements to equine oocyte IVM media. Despite the media deficiencies, nuclear maturation rates of about 60% are routinely achieved for both abattoir- and OPU-derived equine oocytes (Claes & Stout, 2022; Gambini & Maserati, 2017; Merlo et al., 2018; Rodriguez et al., 2019; Stout, 2020).

1.6.7 Transport and Holding Period

The development of methods for maintaining equine oocytes at the germinal vesicle (GV) stage originated as an approach to optimize the scheduling of ICSI procedures. This strategy is essential, as it facilitates the coordination of ICSI with OPU, a time-consuming procedure that is often performed at facilities geographically distant from the

micromanipulation laboratory. To address the initial logistical challenges, various methods of meiotic suppression were evaluated to preserve oocytes overnight prior to maturation. While the use of cell cycle inhibitory agents such as butyrolactone, 6-dimethylaminopurine, and roscovitine effectively maintained oocytes at the GV stage, these treatments reduced developmental competence (Choi et al., 2006; Hinrichs et al., 2002).

Conversely, it was demonstrated that successful preservation primarily depends on the transport and maintenance of immature oocytes at ambient temperature. Equine oocytes can remain viable for at least 24 hours (Choi et al., 2006; Diaw et al., 2018) when stored in commercial embryo-holding media or synthetic oviductal fluid (Foss et al., 2013; Galli, Colleoni, et al., 2014). Remarkably, maintaining equine oocytes at ambient temperature appears to promote chromatin condensation at the GV stage without reducing developmental competence (Galli, Colleoni, et al., 2014; Hinrichs, 2020). This mechanism becomes compromised when temperatures are too low (4–10 °C) or too high (~37 °C), as premature meiotic resumption has been found to occur under such conditions, negatively affecting subsequent embryonic development (Foss et al., 2013).

Although an approximate optimal temperature range has been suggested, the ideal temperature for maintaining immature equine oocytes has not yet been firmly established, and significant variation persists among laboratories. Therefore, further research is required to standardize not only the temperature conditions but also the specific formulations of holding media and handling protocols (Metcalf et al., 2020). Until these parameters are clearly defined, current recommendations are to transport oocytes at 20–

22 °C and limit their storage to no more than 36 hours before transferring them into maturation medium (Briski & Salamone, 2022).

1.7 Strategies to Improve Oocyte Quality

Advances in IVM of equine oocytes have driven the development of comprehensive strategies aimed at optimizing oocyte competence for subsequent embryonic development, a critical determinant of the efficiency of assisted reproduction programs such as ICSI and cloning (Hinrichs, 2018). The quality of mature oocytes critically depends on their cytoplasmic content, which provides mRNA, regulatory proteins, and enzymatic machinery necessary for sperm chromatin remodeling and early embryonic activation. In this context, biphasic protocols that include a pre-maturation (pre-IVM) phase with cAMP-modulating treatments, have been shown to significantly improve nuclear–cytoplasmic synchronization and cytoplasmic maturation (Leal et al., 2022).

Concurrently, modulation of the culture microenvironment has emerged as an essential component. The incorporation of preovulatory follicular secretomes or paracrine factors derived from granulosa cells optimizes the metabolism of COCs, affecting glucose and amino acid uptake as well as the activity of key enzymes, which translates into improved cytoplasmic quality and reduced oxidative stress (Bertoldo et al., 2020; Luis-Calero et al., 2024). Moreover, supplementation of IVM media with antioxidants and redox cofactors, such as NAD+/NADH, contributes to energy and epigenetic homeostasis, both critical for embryonic viability (Hinrichs et al., 2002). From a logistical perspective, strategies such as holding oocytes at controlled temperatures (20–22 °C) support cytoplasmic maturation prior to IVM without compromising developmental competence

or pregnancy rates, thereby offering opportunities to optimize procedural planning and extend the culture time window (Broothaers et al., 2025).

1.7.1 cAMP-elevating treatments

As already described, high cAMP levels maintain the oocyte's meiotic machinery in an inactive state, and disruption of the cumulus-oocyte bidirectional communication, such as that occurring after follicular aspiration for in vitro maturation (IVM), leads to a rapid decline in cAMP levels, which triggers spontaneous meiotic resumption (Gilchrist et al., 2016). This results in premature nuclear maturation and asynchrony between nuclear and cytoplasmic events, ultimately compromising oocyte competence and embryonic developmental potential (Hinrichs, 2018).

To maintain meiotic arrest in vitro, various biphasic systems have been developed that employ pharmacological modulators of the cAMP/cGMP signaling pathways. The so called "Simulated Physiological Oocyte Maturation" (SPOM) system, which uses a combination of cAMP-elevating agents including forskolin (FSK) (an adenylyl cyclase activator), 3-isobutyl-1-methylxanthine (IBMX) (a non-specific phosphodiesterase inhibitor), and cilostamide (a selective PDE3A inhibitor), has been applied effectively to temporarily preserve meiotic arrest and promote more efficient cytoplasmic maturation before the controlled resumption of meiosis (Albuz et al., 2010; Leal et al., 2022). In mice, initial trials demonstrated improvements in embryonic development, total cell number, and pregnancy rates (Albuz et al., 2010). Similarly, in cattle, SPOM yielded a blastocyst production rate of 69%, compared to the global average of 30–40% at that time (Bilodeau-Goeseels, 2011). Notably, the embryos exhibited a higher total cell count,

comparable to those derived in vivo, highlighting the positive impact of this system on both embryo quality and in vitro production efficiency (Albuz et al., 2010). Subsequently, Metcalf et al., 2020 (Metcalf et al., 2020) evaluated a modified SPOM protocol in horses and ovserved an increase in blastocyst rates, suggesting a potential improvement in oocyte developmental competence.

Recently, the CAPA-IVM protocol, which is based on the action of C-type natriuretic peptide (CNP) through its receptor NPR2, has shown promising results across species by transiently maintaining meiotic arrest and improving nuclear–cytoplasmic synchrony. In the equine model, Fakhar-I-Adil et al. 2025 (Fakhar et al., 2025) reported that brief pre-IVM treatment with CNP significantly increased the proportion of oocytes that reached the MII stage and developed to the blastocyst stage, supporting the concept of an "optimal meiotic arrest window" in this species.

Overall, inhibitory pre-IVM in equine oocytes emerges as a key strategy to synchronize nuclear and cytoplasmic maturation, enhance oocyte quality, and improve the efficiency of advanced reproductive biotechnologies such as ICSI and SCNT.

1.7.2 NAD^+-elevating treatments

Nicotinamide adenine dinucleotide (NAD^+) is an essential coenzyme, initially recognized for its central role in energy metabolism as an electron acceptor in the mitochondrial electron transport chain, a function indispensable for adenosine triphosphate (ATP) synthesis (Canto et al., 2015; Lehninger, 1960). In recent decades, NAD^+ has been redefined as a key regulatory molecule involved in fundamental cellular processes,

including stress response, inflammation, and cellular longevity (Howitz et al., 2003; Lin et al., 2000). Maintaining the balance between NAD^+ consumption and regeneration is crucial for preserving cellular homeostasis.

In addition to its role as an energy carrier in its oxidized form, NAD^+ serves as an essential substrate for three major families of regulatory enzymes: poly(ADP-ribose) polymerases (PARPs), cyclic ADP-ribose synthases (cADPr synthases), and sirtuins (SIRTs) (Belenky et al., 2007). Since these enzymes consume NAD^+ during processes such as energy metabolism, oxidative stress response, and DNA repair, continuous NAD^+ replenishment is vital for maintaining adequate intracellular pools (Covarrubias et al., 2020; El Sheikh et al., 2020; Pollard, 2024).

The age-associated decline in NAD^+ levels is a well-documented phenomenon linked to cellular dysfunction and the metabolic deterioration characteristic of aging (Covarrubias et al., 2021). Consequently, restoring NAD^+ levels has emerged as a promising therapeutic strategy. Among the most studied approaches is the administration of nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR), which enhance the production of NAD^+ via the salvage pathway (Figure 4) (Chini et al., 2021). Studies in aged mice have demonstrated that NMN supplementation in vivo can improve the quality of their oocytes by increasing the intra-oocyte levels of NAD^+, restoring mitochondrial function, and enhancing subsequent embryonic development (Bertoldo et al., 2020; Miao et al., 2020).

Nicotinic acid (NA), a form of vitamin B_3 (niacin), also serves as a precursor of NAD^+ through the Preiss–Handler biosynthetic pathway (Figure 4). Supplementing IVM

medium with NA has been found to increase nuclear maturation rates in bovine oocytes (Kafi et al., 2019). Similar studies in porcine oocytes have also demonstrated that NA supplementation during IVM enhances nuclear maturation and development to the blastocyst stage (Almubarak et al., 2021; Pollard et al., 2021c). In addition, NA has demonstrated a partial protective effect during IVM against toxic environmental agents in mouse and human oocytes (Gao et al., 2025). This protective effect is attributed to its role in maintaining the NAD^+/SIRT1 axis and mitigating oxidative stress, which are both critical for proper meiotic progression and mitochondrial function (Lee et al., 2019). In mares, dietary supplementation with NA has been shown to increase the bioavailability of NAD^+ precursors in the fluid of the dominant follicle and has been proposed as an intervention to improve oocyte quality in vivo, particularly in aged mares (Pollard et al., 2021b).

Given the essential role that NAD^+ plays in supporting oocyte maturation, the supplementation of NAD^+ precursors, both in vivo and in vitro, represents a promising intervention for enhancing oocyte quality and increasing the efficiencies of embryo IVP systems. Further research is vital to standardize dosage, establish long term safety, and fully evaluate the therapeutic potential of these treatments in both humans and animals.

1.8 Conclusion

In summary, the acquisition of complete oocyte developmental competence is fundamental to ensuring the viability of embryos to term, and the birth of healthy offspring. The growth and maturation of the oocyte within the follicle are highly dynamic processes, and any intervention whether physiological, hormonal, or in vitro can

profoundly influence oocyte quality and subsequent embryo development. Despite substantial research efforts to delineate the key determinants of full developmental competence, many intrinsic factors regulating equine oocyte quality remain poorly understood (Hinrichs, 2010). Studies in bovine models have provided compelling evidence that embryos derived from oocytes collected via OPU exhibit superior in vitro and in vivo developmental outcomes compared with embryos generated from abattoir derived oocytes, highlighting the impact of oocyte source on developmental potential (Lee et al., 2015). Complementing these findings, Choi (2013) demonstrated that oocytes retrieved from live mares display enhanced developmental competence, yielding higher embryo production rates and successful foaling outcomes, emphasizing the importance of physiological oocyte maturation conditions.

A critical aspect of maintaining oocyte competence during in vitro handling is the prevention of premature meiotic resumption. Transient exposure to pharmacological agents that increase intra-oocyte cAMP levels is commonly employed to synchronize nuclear and cytoplasmic maturation, thereby enhancing oocyte developmental potential (Leal et al., 2022; Metcalf et al., 2020).

The emerging evidence across multiple mammalian species including mice, pigs, and cattle, suggests that treatments designed to elevate NAD+ levels during IVM can enhance mitochondrial function, increase cellular energy production, and restore key metabolic and signaling pathways compromised by aging or environmental stressors. These interventions collectively contribute to improvements in oocyte quality, fertilization outcomes, and embryo developmental competence (Bertoldo et al., 2020; Miao et al., 2020; Pollard et al., 2021b). Together, these findings underscore the complex interplay

of intrinsic and extrinsic factors that govern oocyte quality and highlight the potential of targeted interventions to optimize embryo production efficiency in equine assisted reproduction.

1.9 Aims of the Thesis

The oocyte cytoplasm contains and accumulates factors that are needed to prepare the paternal and maternal chromatin following sperm penetration and support the development of a viable embryo. Amazingly, the capacity of these cytoplasmic factors to generate an embryonic nucleus extends to the reprogramming of a somatic cell nucleus to a totipotent state, such that the epigenetic marks defing the cell's differentiation are reset. The unifying hypothesis of the thesis is that oocyte factors and IVM treatments that improve cytoplasmic maturation will increase the efficiency of equine cloning. The overall aim of this thesis work was to examine the effects of oocyte factors and treatments known to influence the acquisition of oocyte developmental competence on cloned embryo production. Working within the constraints of a commercial equine breeding program, the capacity of oocytes to support embryo development in vitro and give rise to pregnancies and foals following embryo transfer was evaluated.

The specific objectives of this study were to:

Determine the effect of oocyte source on the development of cloned equine embryos (Chapter 2)

The effects of oocyte origin, whether obtained from abattoir-sourced ovaries or from live mares, on developmental competence have been previously assessed. However, direct comparative studies remain surprisingly scarce, and sample sizes are generally limited. The available evidence indicates there are significant differences in the developmental potential of oocytes, depending on their source. Current equine IVM systems achieve nuclear maturation rates of approximately 60% for both abattoir-derived oocytes and those obtained from live mares (Claes & Stout, 2022). Embryos derived from oocytes harvested by ovum pick-up (OPU) appear to exhibit superior in vitro and in vivo developmental potential compared to those derived from abattoir-derived oocytes (Brom-de-Luna et al., 2021; Galli et al., 2007). While the influence of oocyte source is unquestionable, our understanding of the extent of the difference that intrinsic oocyte factors exert on embryonic and fetal development remains very limited.

To better understand the impact of oocyte source, the development of equine SCNT embryos produced using immature oocytes retrieved either from live mares via OPU or from ovaries collected at abattoirs was examined in Chapter 2. The effects of oocyte origin, specifically in the context of cloning procedures and nuclear reprogramming, on embryo viability to foaling, have not yet been comprehensively investigated. It was hypothesised that oocytes harvested from live mares have superior developmental potential and epigenetic reprogramming capacity compared with oocytes recovered from ovaries collected post-mortem at abattoirs.

Determine the effect of cAMP modulators during pre-IVM on the developmental potential of equine oocytes (Chapter 3)

Spontaneous meiotic resumption occurs immediately after oocytes are physically removed from antral follicles. It is well established in the oocytes of several species, including horses, that artificially sustaining high intra-oocyte cAMP levels maintains meiotic arrest (Leal et al., 2018). Numerous compounds have been used to elevate cAMP levels, either through the stimulation of adenylate cyclase to increase cAMP synthesis, or via inhibition of phosphodiesterases to decrease cAMP degradation. Additionally, analogues of cAMP, such as the membrane permeable dibutyryl cAMP, can be effectively employed to achieve a similar result (Gilchrist et al., 2016). A system based on the use of cAMP modulators during oocyte IVM, referred to as the Simulated Physiological Oocyte Maturation (SPOM) system, seeks to replicate the physiological conditions of the in vivo environment, with the objective of improving the synchrony of nuclear and cytoplasmic maturation (Albuz et al., 2010). In the only equine study of the SPOM system to date, an exposure period of 18 h was applied. However, SPOM treatment durations of 2 and 6 h have been found to exert beneficial effects in bovine oocytes.

In Chapter 3, the influence of the SPOM system on the developmental potential of equine oocytes was explored. Specifically, using oocytes recovered from abattoir-sourced ovaries, the effect of the duration (4 and 18 h) that the oocytes were treated with the cAMP modulators was determined. This was achieved by assessing the in vitro and in vivo development of cloned embryos produced using the treated and untreated (control) oocytes. It was hypothesised that the oocytes treated with the cAMP modulators would have superior developmental potential compared with untreated oocytes.

Determine the effect of nicotinic acid supplementation during pre-IVM and IVM on the developmental competence of equine oocytes (Chapter 4)

Nicotinic acid (NA) is a precursor of NAD^+, a molecule that plays essential roles in energy metabolism, regulation of apoptosis, and DNA repair. In addition, NA has multiple therapeutic applications, acting as an antioxidant, anti-inflammatory agent, and lipid modulator (Wang et al., 2018; Zhang et al., 2012). The addition of NA to IVM media has been shown to improve oocyte quality in several domestic species. However, no studies to date have examined the effects of NA supplementation during the pre-IVM and IVM periods on the acquisition of developmental competence in equine oocytes.

To gain a better understanding of the effects of NA on equine oocyte maturation, the study presented in Chapter 4 first assessed the impact of supplementing the pre-IVM media with different concentrations of NA (0, 50, and 200 μM) using oocytes recovered from abattoir-sourced ovaries. Having found that the 200 μM NA dose provided the greatest benefit, the effect of the treatment during the pre-IVM and IVM periods was determined using oocytes collected from live mares by OPU. The development of cloned embryos produced using the treated and untreated oocytes was examined after in vitro culture. It was hypothesised that the addition of NA to media during the pre-IVM and IVM periods would enhance the developmental competence of equine oocytes.

CHAPTER 2

Cloning horses by somatic cell nuclear transfer: effects of oocyte source on development to foaling

Authorship Attribution Statement

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J.V. Cortez: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft; **K. Hardwicke:** methodology, investigation, data curation; **J. Cuervo-Arango:** methodology, investigation, writing – review and editing; **C.G. Grupen:** conceptualization, formal analysis, writing – original draft, visualization, supervision.

The corresponding author has granted permission to include the published material in this thesis.

Jenin V. Cortez

May 2026

As supervisor for the candidature upon which this thesis is based, I confirm that the authorship attribution statement above is correct.

Christopher G. Grupen

May 2026

Abstract

The cloning of horses is a commercial reality, yet the availability of oocytes for cloned embryo production remains a major limitation. Immature oocytes collected from abattoir-sourced ovaries or from live mares by ovum pick-up (OPU) have both been used to generate cloned foals. However, the reported cloning efficiencies are difficult to compare due to the different somatic cell nuclear transfer (SCNT) techniques and conditions used. The objective of this study was to compare the in vitro and in vivo development of equine SCNT embryos produced using oocytes recovered from abattoir-sourced ovaries and from live mares by OPU. A total of 1,128 oocytes were obtained, of which 668 were abattoir-derived and 460 were OPU-derived. The methods used for in vitro maturation and SCNT were identical for both oocyte groups, and the embryos were cultured in Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 Ham medium supplemented with 10% fetal calf serum. Embryo development in vitro was assessed, and Day 7 blastocysts were transferred to recipient mares. The embryos were transferred fresh when possible, and a cohort of vitrified-thawed OPU-derived blastocysts was also transferred. Pregnancy outcomes were recorded at Days 14, 42 and 90 of gestation and at foaling. The fusion rate tended to be greater for OPU-derived couplets than for abattoir-derived couplets (91.0 ± 2.5% vs 82.1 ± 4.9%; P=0.069), and the rates of cleavage (68.7 ± 3.9% vs 62.4 ± 4.7%) and development to the blastocyst stage (34.6 ± 3.3% vs 25.6 ± 2.0%) were superior for OPU-derived embryos compared with abattoir-derived embryos (P<0.05). The proportions of recipient mares pregnant at Days 14 and 42 were similar for both groups. However, beyond Day 42, the percentages of recipient mares that still had a viable conceptus at Day 90 (84.6% vs 37.5%) and gave birth to a healthy foal (61.5% vs 12.5%) were greater for the OPU group compared with the abattoir group (P<0.05). Surprisingly, more favourable pregnancy outcomes were achieved when blastocysts were

vitrified for later transfer, probably because the uterine receptivity of the recipient mares was more ideal. A total of 12 cloned foals were born, 9 of which were viable. Given the differences observed between the two oocyte groups, the use of OPU-harvested oocytes for generating cloned foals is clearly advantageous. Continued research is essential to better understand the oocyte deficiencies and increase the efficiency of equine cloning.

2.1 Introduction

Twenty years ago, the first horse foal cloned from somatic cells was born (Galli et al., 2003). The possibilities that somatic cell nuclear transfer (SCNT) presented for disseminating, perpetuating, and salvaging the genetics of rare and valuable horses were immediately obvious. The cloning of exceptional geldings would facilitate the use of the resulting male foals as breeders once sexually mature. Likewise, SCNT would allow the replication of champion mares that have no breeding opportunities during their most fertile years due to demanding competition schedules. Further, the genetic reconstitution of unbred individuals from biopsied tissue would now be possible following unexpected or accidental death. Despite the SCNT inefficiencies and associated challenges particular to equids, early studies showed that most cloned foals developed normally, and horse clone production was soon commercialized (Hinrichs et al., 2006).

Many hundreds of horse clones have now been produced around the world, and the SCNT procedures have been refined to the stage where they can be readily applied in equine clinical practice (Gambini & Maserati, 2017). Polo, the so-called sport of kings, houses the largest number of cloned performance horses (Hinrichs, 2018). The popularity of the use of clones in this highly competitive professional sport is because few females provide

offspring due to the demands of the sport and males are usually castrated for ease of management. While the equine cloning achievements to date are remarkable, the efficiency of SCNT remains low, with losses at each step of cloned embryo production and throughout gestation following embryo transfer. Hence, as large numbers of oocytes are needed to produce a cloned foal, a major constraint of equine SCNT is the supply of oocytes (Galli & Lazzari, 2021).

One source of immature oocytes that has been used widely by researchers to produce foals by SCNT and intracytoplasmic sperm injection (ICSI) involves the post-mortem recovery of ovaries from slaughtered mares (Choi et al., 2009; Galli et al., 2003; Lagutina et al., 2005). Extensively flushing and scraping the antral follicles of ovaries collected post-mortem has been reported to yield a mean of 14 oocytes per mare(Hinrichs et al., 2012), though typically about 4 cumulus-oocyte complexes (COCs) are recovered per abattoir-sourced ovary (Galli et al., 2007). A regular and reliable supply of post-mortem ovaries is often difficult to gain access to, and horse abattoirs are scarce in most countries. In Australia, small numbers of mares are euthanized sporadically, and the importation of abattoir-derived oocytes is not a viable option due to biosecurity issues Asseged et al., 2012. Furthermore, in some countries, like the United States, the slaughter of horses is banned (Nolen, 2006).

Immature oocytes have also been collected from live mares by transvaginal ultrasound-guided follicle aspiration, or ovum pick-up (OPU) to produce cloned foals (Choi et al., 2013; Lee et al., 2015). The use of oocytes from the same maternal line as the nucleus donor animal avoids the presence of heterogenous mitochondrial DNA in the foal (Choi et al., 2013). Although OPU is costly and technically challenging in mares, and there is

some risk associated with the procedure, this oocyte source is increasingly being used for the production of ICSI embryos (Stout, 2020). With the various refinements made to the equine OPU technique (Morris, 2018), experienced practitioners can now recover a mean of 9 to 14 oocytes per mare following aspiration of antral follicles 6 to 30 mm in diameter (Claes et al., 2016; Hinrichs et al., 2014). Mare age, breed and season have been found to influence the recovery of oocytes by OPU (Claes et al., 2016; Galli et al., 2014).

Following the collection and transportation of immature oocytes to the laboratory, current equine in vitro maturation (IVM) systems achieve nuclear maturation rates of around 60% for both abattoir- and OPU-derived oocytes (Merlo et al., 2018; Stout, 2020). Whilst there are few comparative studies, and the sample sizes are often small, the accumulating evidence suggests that OPU-derived embryos have superior in vitro and in vivo developmental potential compared with abattoir-derived embryos (Brom-de-Luna et al., 2021; Galli et al., 2007; Lee et al., 2015). To the best of our knowledge, only one equine SCNT study has directly compared the developmental competence of both types of oocytes, and in that study, there was no assessment of in vitro development because the embryos were transferred to recipient mares immediately after couplet activation (Lee et al., 2015).

Therefore, the objective of this study was to compare the efficiency of equine SCNT using immature oocytes retrieved from live mares by OPU and from abattoir-sourced ovaries. The rates of oocyte maturation, couplet fusion, embryonic cleavage, and blastocyst formation were assessed. Following the transfer of Day 7 blastocysts to recipient mares, pregnancies and the birth of cloned foals were evaluated.

2.2 Materials and methods

2.2.1 Mares

The study was performed in the 2020 and 2021 breeding seasons (September to March in the southern hemisphere) at the Catalina Equine Reproduction Centre (North Richmond, NSW Australia). A total of 35 standardbred mares, aged 3 to 15 years, were used as oocyte donors and embryo transfer recipients. An additional 13 standardbred mares, of a similar age range, were used as embryo transfer recipients only. All procedures were carried out with informed consent from the owners and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013), the NSW Animal Research Act (1985), the NSW Animal Research Regulations (2010), and other relevant legislation.

2.2.2 Chemicals and media

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Australia). Hepes-buffered Synthetic Oviductal Fluid (H-SOF) (Thompson et al., 1990) was used for procedures performed outside of the CO_2 incubator. Unless otherwise stated, H-SOF contained 10% foetal calf serum (FCS; Cellsera, Rutherford, NSW, Australia). The maturation medium consisted of DMEM/F-12 medium supplemented with 0.1 IU/mL follicle-stimulating hormone and 0.1 IU/mL luteinizing hormone (Menopur, Ferring Pharmaceuticals, Copenhagen, Denmark), 50 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 1 mM sodium pyruvate, insulin-transferrin-sodium selenium mixture (1×), and 10% FCS. The fusion medium consisted of 3.0 M D-sorbitol,

0.05 mM $CaCl_2$, 0.10 mM $MgCl_2$ and 0.05% (w/v) fatty acid-free bovine serum albumin (BSA). Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) medium supplemented with 10% FCS was used as the embryo culture medium.

2.2.3 Collection of Immature Oocytes by Ovum Pick Up (OPU)

The OPU procedure was performed as described previously (Cuervo-Arango et al., 2019d). The day before OPU, mares were scanned transrectally using an ultrasound machine (Mindray M9; Mindray, Shenzhen, China) equipped with a 5-8 MHz linear-array transducer (6LE5Vs) to determine the number and size of the follicles present on the ovaries. Only mares that had at least 15 follicles, with the largest follicle <25 mm in diameter, were scheduled for OPU. In a total of 20 sessions, with one or two sessions carried out in any single week, 64 donor mare retrievals were performed (mean of 3.2 retrievals per session). Some of the mares underwent OPU on multiple occasions with a minimum of two weeks between sessions.

In preparation for OPU, mares were sedated using a combination of detomidine hydrochloride (4 mg iv) and butorphanol tartrate (6 mg iv). Immature oocytes were collected by transvaginal ultra-sound guided aspiration of follicles 5 to 30 mm in diameter using a 12G double-lumen needle attached to a vacuum pump. After aspirating the follicular fluid, each follicle was flushed 10 times with 0.5 to 5 mL (depending on follicle size) of embryo flushing medium (BoviFlush; Minitube Australia Pty. Ltd., Smythesdale, VIC, Australia) supplemented with sodium heparin (5 IU/mL, Pharma, Denmark) and pre-warmed to 37°C. The follicular fluid and lavage medium were collected in 500 mL flasks kept at 37°C. The collected fluids were poured through a sterile embryo collection

filter (EmCon filter; Immuno Systems Inc., Spring Valley, WI, USA) immediately after the end of the OPU procedure, and the residual fluid and follicular material were then rinsed into a sterile Petri dish. Subsequently, immature oocytes were identified using a stereomicroscope, washed three times with H-SOF and transferred to a cryovial filled with a 1:1 mix of DMEM/F-12 and TCM-199 supplemented with 10% FCS at 20 to 22°C. The cryovial was tightly capped and the oocytes were kept in a polystyrene container at 20 to 22°C overnight (18 to 24 h) prior to in vitro maturation (IVM).

2.2.4 Collection of Immature Oocytes from Ovaries Harvested Post-Mortem

Equine ovaries were obtained from an abattoir and processed within 1-2 h of slaughter. Cumulus-oocyte complexes (COC) were recovered from follicles <30 mm in diameter by aspirating with an 18G needle and extensively scraping the follicle walls with a bone curette. The liquid obtained was transferred to a 100 mm Petri dish containing H-SOF at 37°C. Immature oocytes were identified using a dissection microscope, washed three times with H-SOF and transferred to a cryovial filled with a 1:1 mix of DMEM-F12 and TCM-199 containing 10% FCS. The cryovial was tightly capped and transported to the laboratory in a polystyrene container at 20-22°C overnight (18-24 h) prior to IVM the next day.

2.2.5 In Vitro Maturation (IVM)

Immature oocytes retrieved from both groups (abattoir- and OPU-derived) were washed 3 times with H-SOF, and then transferred to maturation medium and incubated for 18 to 24 h at 38°C in a humidified atmosphere of 5% CO_2 in air.

2.2.6 Somatic Cell Nuclear Transfer (SCNT)

A total of 14 different fibroblast cell lines were derived from the subcutaneous tissue of 14 adult horses (12 females and 2 males). Two of the fibroblast cell lines were purchased from Avantea (Cremona, Italy). To culture the fibroblast cells, DMEM/F-12 medium supplemented with 1 mM glutamine, 0.2 mM pyruvate, 10 ng/mL EGF and 10% FCS was used. Briefly, after plating and initial expansion, cells were passaged twice, frozen at −80°C in culture medium containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Upon thawing, the donor cells were cultured for at least two days to ensure confluence-induced cell cycle arrest for a minimum of 24 h prior to use. Immediately before SCNT, the donor cells were harvested by trypsinization, washed and suspended in H-SOF containing 2% FCS, and kept at room temperature (RT) until use (approximately 10 min).

Following IVM, cumulus cells were removed from the oocytes by gentle pipetting in H-SOF supplemented with 1mg/mL hyaluronidase. Denuded oocytes and donor cells were transferred to a droplet of H-SOF covered with mineral oil. The polar body and the metaphase plate of each mature oocyte were aspirated using an enucleation pipette attached to a Piezo drill (PMAS-CT150; Prime Tech Ltd., Ibaraki, Japan), which assisted

penetration of the zona pellucida with speed and intensity set to 7 and 8, respectively. The donor cells were then loaded into an injection pipette and a single cell was deposited within the perivitelline space of each cytoplast. The couplets were held for 1 h in H-SOF at 38°C, before being transferred to fusion medium on a fusion chamber slide between electrodes 0.5 mm apart. A direct current (DC) pulse of 2.2 kV/cm strength and 15 µs duration was immediately applied to the couplets using the Voltain™ EP-1 system (CryoLogic, Mulgrave, VIC, Australia). The pulsed couplets were washed 3 times in H-SOF, transferred to embryo culture medium, and incubated for 2 h. Activation was carried out by exposing the fused couplets to 5 µM ionomycin for 5 min in H-SOF. After several washes in H-SOF, the fused and activated couplets were treated with 1 mM 6-dimethylaminopurine and 5 µg cycloheximide in embryo culture medium for 4 h. Finally, the cloned embryos produced were washed several times, transferred to droplets of culture medium, and incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. The cloned embryos were transferred to fresh culture medium every 2 days. Embryonic cleavage was assessed at the first change of culture medium and blastocyst development was assessed on Day 7 of in vitro culture, using well described morphological features to identify blastocyst formation (Leal et al., 2018). For the fresh embryo transfers, Day 7 blastocysts were transferred to fresh culture media, loaded immediately into 0.5 mL straws, and transported in a container held at 37°C to the site of embryo transfer (<30 min).

2.2.7 Blastocyst vitrification and thawing

Blastocysts were vitrified using the Cryotop method according to the manufacturer's instructions (Kitazato BioPharma, Shizuoka, Japan). In brief, embryos were transferred to the top of a 300 µL droplet of equilibration solution (ES) at RT for up to 15 min until a cycle of shrinkage (dehydration) and re-expansion (ES infiltration) was observed. This took 12 to 15 min, depending on the initial quality and size of the blastocyst. Equilibrated embryos with a minimum volume of ES were then transferred to the top of a 300 µL droplet of vitrification solution 1 (VS1) at RT for 30 s, during which the embryos were displaced three times within the VS1 droplet to completely wash out ES. Embryos with a minimum volume of VS1 were then transferred to a 300 µL droplet of VS2 for another 30 s with twice stirring and displacing of embryos within VS2 until complete dehydration was observed. As soon as the embryos were placed onto the thin polypropylene strip of the Cryotop, the excess VS2 around the embryos was removed and the device was immediately submerged vertically into liquid nitrogen. For thawing, the Cryotop was immersed directly into 1 mL of pre-warmed (37°C) thawing solution (TS) for 1 min. Thawed embryos in TS were gently deposited at the bottom of a 300 µL droplet of dilution solution (DS) for a gradual displacement of TS to DS for 3 min at RT. Then embryos with a 2 mm column of DS in the pipette were gently deposited at the bottom of a 300 µL droplet of WS1 for gradual displacement of DS to WS1 for 5 min at RT. Embryos with a minimal volume of WS1 were washed by twice submerging in WS2 for 1 min. Finally, the embryos were deposited in culture media (37°C), loaded immediately into 0.5 mL straws, and transported in a container held at 37°C to the site of embryo transfer (<30 min).

2.2.8 Embryo Transfer (ET)

Once in oestrus, the recipient mares were scanned daily by transrectal ultrasound to determine the day of ovulation, observing the disappearance of the preovulatory follicle and the appearance of the corpus luteum (CL). Day 7 blastocysts were transferred transcervically to recipients on Day 5 post-ovulation. One day before ET, potential recipients were examined by transrectal ultrasound to confirm the absence of endometrial oedema and excessive intrauterine fluid accumulation, and the presence of adequate uterine tone, a tight cervix, and at least one CL of expected echogenicity. If considered suitable for ET, the mares were sedated with detomidine hydrochloride (4 mg iv) and the procedure was performed. Following the procedure, mares were administered progesterone (1.5 g per dose) according to E. Rojas (personal communication) to establish and maintain pregnancy. The mares were examined by transrectal ultrasonography on Days 14 (Day 9 after ET), 42 and 90 of gestation to determine their pregnancy status. Additional scans were subsequently performed to monitor the progress of ongoing pregnancies.

The numbers of embryos transferred per recipient (multiple vs single) and the type of embryos transferred (fresh only vs fresh and vitrified-thawed) differed between the groups. In most of the replicates that utilised abattoir-derived oocytes, the number of blastocysts produced was much greater than the number of recipients suitable on the day of fresh ET. Based on a previous report (Galli et al., 2007), multiple (up to four) fresh abattoir-derived blastocysts were transferred to each mare. In this way, all abattoir-derived blastocysts were transferred fresh (89 blastocysts transferred to 29 recipients). In some of the replicates that utilised OPU-derived oocytes, the number of blastocysts

produced was greater than the number of recipients suitable on the day of fresh ET. Those mares that were suitable on the day of fresh ET received a single OPU-derived blastocyst. The remaining OPU-derived blastocysts were vitrified and stored in liquid nitrogen as described in subsection 2.7. Later, when a recipient mare was at the appropriate stage for ET, a single vitrified OPU-derived blastocyst was thawed and transferred. In this way, all OPU-derived blastocysts were transferred singly (48 blastocysts transferred to 48 recipients).

2.2.9 Statistical analyses

Data were analysed using the Genstat statistical software package (18th edition; VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The embryo in vitro production data (oocytes matured, couplets fused, embryos cleaved, and blastocysts formed) were subjected to logistic regression analysis with oocyte source and cell line as factors. The pregnancy and foaling data were analysed using chi-square tests. Kendall's rank correlation was used to test the similarities in the ordering of the in vitro and in vivo data for the different donor cell lines (the probability of tau was not adjusted for ties). A P value of less than 0.05 designated a significant difference.

2.3 Results

2.3.1 In vitro production of cloned embryos

The effects of oocyte source on the in vitro production and development of cloned embryos are shown in Table 2.1. For the abattoir-derived oocytes, a total of 668 oocytes were collected in the 15 replicates (mean of 44.5 oocytes per replicate). For the OPU-derived oocytes, a total of 460 oocytes were collected from a total of 64 donor mare retrievals (mean of 7.2 oocytes per retrieval) in the 20 replicates (mean of 23.0 oocytes per replicate). A greater proportion of the abattoir-derived oocytes matured to the metaphase II stage by the end of IVM compared with the OPU-derived oocytes (61.6 ± 3.1% vs 49.9 ± 2.6%; $P<0.001$). The OPU-derived couplets tended to fuse at a higher rate than the abattoir-derived couplets, but the difference was not significant (91.0 ± 2.5% vs 82.1 ± 4.9%; $P=0.069$). The cleavage and blastocyst formation rates of the OPU-derived embryos were both superior to those of the abattoir-derived embryos (68.7 ± 3.9% vs 62.4 ± 4.7% and 34.6 ± 3.3% vs 25.6 ± 2.0%, respectively; $P<0.05$). Using abattoir-derived oocytes, blastocysts were produced in every replicate (mean of 6.2 blastocysts per replicate). Using OPU-derived oocytes, blastocysts were produced in 18 of the 20 replicates (mean of 3.5 blastocysts per replicate).

2.3.2 Pregnancies and foals from cloned embryos

The results of pregnancy diagnosis and foaling following the transfer of cloned embryos to recipient mares are shown in Fig. 2.1. The proportions of mares in which embryonic vesicles were detected at Day 14 of gestation were similar for the abattoir and OPU groups (12/29 and 17/48, respectively; $P=0.601$). In three of the mares of the abattoir

group that were diagnosed as pregnant at Day 14, two embryonic vesicles were detected. One of the embryonic vesicles in each of these mares was subsequently ablated to maximize the likelihood of the remaining conceptus surviving. Therefore, of the 89 abattoir-derived blastocysts transferred, 15 were viable at Day 14. Calculated per transferred embryo, the Day 14 viability of abattoir-derived blastocysts was lower than that of OPU-derived blastocysts (16.9% vs 35.4%; P=0.016).

The proportions of mares pregnant at Day 14 that were still pregnant at Day 42 did not differ between the abattoir and OPU groups (8/12 and 13/17, respectively; P=0.561). When considering the post-attachment viability of conceptuses (beyond Day 42 of gestation), a smaller proportion of mares remained pregnant at Day 90 in the abattoir group compared with the OPU group (3/8 vs 11/13; P=0.026). Moreover, the proportion of viable foals born was lower in the abattoir group compared with the OPU group (1/8 vs 8/13; P=0.027). Of the 3 foals born in the abattoir group, one had a markedly enlarged umbilicus and died, and one had severe angular limb deformities and died. Of the 9 foals born in the OPU group, one had severe craniofacial malformations and was euthanised, and one was declared healthy after successful treatment of minor forelimb and umbilical cord problems. All the other foals were born normal and healthy.

2.3.3 Vitrified-thawed vs fresh OPU-derived cloned embryos

When cloned embryos were produced there was often insufficient suitable recipient mares to transfer all Day 7 blastocysts as single fresh embryos. Therefore, the remaining Day 7 blastocysts were vitrified, stored, and transferred later as single vitrified-thawed embryos. Of the 50 blastocysts transferred singly to recipient mares, 35 were transferred as fresh

embryos, and 15 were transferred as vitrified-thawed embryos. Of the nine donor cell lines used to produce blastocysts for the fresh vs vitrified-thawed comparison, seven yielded blastocysts that were transferred both fresh and vitrified-thawed, and two yielded blastocysts that were transferred fresh only. The results of pregnancy diagnosis and foaling following the transfer of fresh and vitrified-thawed cloned embryos to recipient mares are shown in Table 2.2. The proportions of mares detected as pregnant following transfer of vitrified-thawed blastocysts were greater than those of mares detected as pregnant following transfer of fresh blastocysts at Day 14 (60.0% vs 28.6%; $P<0.05$) and Day 90 (40.0% vs 14.3%; $P<0.05$). The proportions of mares detected as pregnant at Day 42, carried the pregnancy full term, and gave birth to a healthy foal did not differ between the two groups of blastocysts ($P>0.05$).

2.3.4 In vitro and in vivo development from different fibroblast cell lines

A total of 14 different fibroblast cell lines were used to provide the donor nuclei for cloned embryo production. Three of the cell lines were used to produce embryos from both abattoir- and OPU-derived oocytes. Four of the cell lines were used to produce embryos from abattoir-derived oocytes only. Seven of the cell lines were used to produce embryos from OPU-derived oocytes only. The distributions of couplet fusion, embryonic cleavage, and blastocyst formation rates obtained for the cell lines are shown in Fig. 2.2. The mean couplet fusion rate was 89.8% and there was no effect of cell line on the rate achieved ($P>0.05$). However, the cell line used to provide the donor nuclei affected the rates of embryonic cleavage and blastocyst formation ($P<0.05$). The mean cleavage rate was 65.2% and the mean blastocyst formation rate was 31.2%.

The pregnancy outcomes obtained for the different fibroblast cell lines following transfer of the cloned blastocysts to recipient mares are shown in Table 2.3. Blastocysts obtained from one cell line that was used in only one SCNT replicate were not transferred to recipient mares. Therefore, this cell line was excluded from the analysis of pregnancy outcomes. Blastocysts produced from 11 of the 13 cell lines formed embryonic vesicles that were detected at the Day 14 pregnancy scan. The two cell lines from which no embryonic vesicles developed were only used once or twice to produce cloned embryos. Of the 11 cell lines from which embryonic vesicles developed, 8 resulted in conceptuses at Day 90, a critical milestone of pregnancy, when attachment is complete. While some post-attachment losses were observed, all 8 cell lines from which Day 90 conceptuses developed resulted in the birth of a foal. Only one of the cell lines that resulted in full term development did not produce a viable foal. Hence, healthy foals were obtained from 7 of the 13 cell lines used.

Further analysis showed there was a correlation between the rates of embryo development in vitro and the rates of foaling (Fig. 3.3). The cell line ranking of healthy foals born from the number of embryo transfers performed was similar to the cell line ranking of cleavage and blastocyst formation rates ($P<0.05$). Despite low cleavage and blastocyst formation rates (41.3% and 12.5%, respectively), one of the donor cell lines resulted in the birth of a healthy foal following transfer of two OPU-derived blastocysts. This apparent outlier was not excluded from the analysis (Fig. 3.3). The other cell lines that resulted in the births of healthy foals had cleavage rates greater than 65% and blastocyst formation rates greater than 35%.

Table 2.1. The effect of oocyte source on the rates of oocyte maturation, couplet fusion, embryonic cleavage, and blastocyst formation.

Oocyte source	Oocytes n	Maturation (n)	Fusion (n)	Cleavage* (n)	Blastocysts* (n)
Abattoir	633	61.9 ± 3.4%[a] (396)	81.9 ± 5.2% (336)	61.9 ± 5.0%[a] (209)	25.4 ± 2.1%[a] (88)
OPU	495	50.3 ± 2.5%[b] (238)	90.7 ± 2.4% (214)	68.8 ± 3.7%[b] (153)	34.3 ± 3.2%[b] (75)

Percentage values are presented as the mean ± SEM.

[a,b]Within columns, values labelled with different letters are significantly different ($P<0.05$).

*Cleavage and blastocyst formation rates were calculated from couplets fused.

Table 2.2. The percentages of recipient mares that were diagnosed as pregnant at Days 14, 42 and 90 of gestation, carried the pregnancy full term, and gave birth to a healthy foal following transfer of fresh and vitrified-thawed OPU-derived cloned embryos.

Embryo group	Embryos n	Day 14 (n)	Day 42 (n)	Day 90 (n)	Full term (n)	Healthy (n)
Fresh	35	28.6%[a] (10)	20.0% (7)	14.3%[a] (5)	11.4% (4)	11.4% (4)
Vitrified-thawed	15	60.0%[b] (9)	40.0% (6)	40.0%[b] (6)	33.3% (5)	26.7% (4)

Percentages are the number of positive recipient mares divided by the total number of embryos.

[a,b]Within columns, values labelled with different letters are significantly different ($P<0.05$).

Table 2.3. Summary of the pregnancy outcomes obtained for the different fibroblast cell lines used to provide the donor nuclei.

Cell line	SCNT reps[a]	Oocyte source	Recipient mares n	Recipient mares positive n[b]				
				Day 14	Day 42	Day 90	Full term	Healthy
#01	5	Both	14	5	3	1	1	1[c]
#02	5	Both	11	3	3	3	2	2[d]
#03	5	Abattoir	10	5	5	2	2	0
#04	4	OPU	10	5	3	3	2	2
#05	4	Abattoir	8	2	1	0		
#06	1	OPU	6	1	1	1	1	1
#07	1	OPU	5	2	1	1	1	1
#08	1	OPU	5	2	2	2	2	1
#09	2	OPU	2	2	0			
#10	2	OPU	2	1	1	1	1	1
#11	2	OPU	2	0				
#12	1	Abattoir	1	1	1	0		
#13	1	Abattoir	1	0				

[a]Number of replicates cloned embryos were produced.

[b]Number of recipient mares diagnosed as pregnant at Days 14, 42 and 90 of gestation, carried the pregnancy full term, and gave birth to a healthy foal.

[c]The healthy foal developed from an abattoir-derived blastocyst.

[d]Both healthy foals developed from OPU-derived blastocysts.

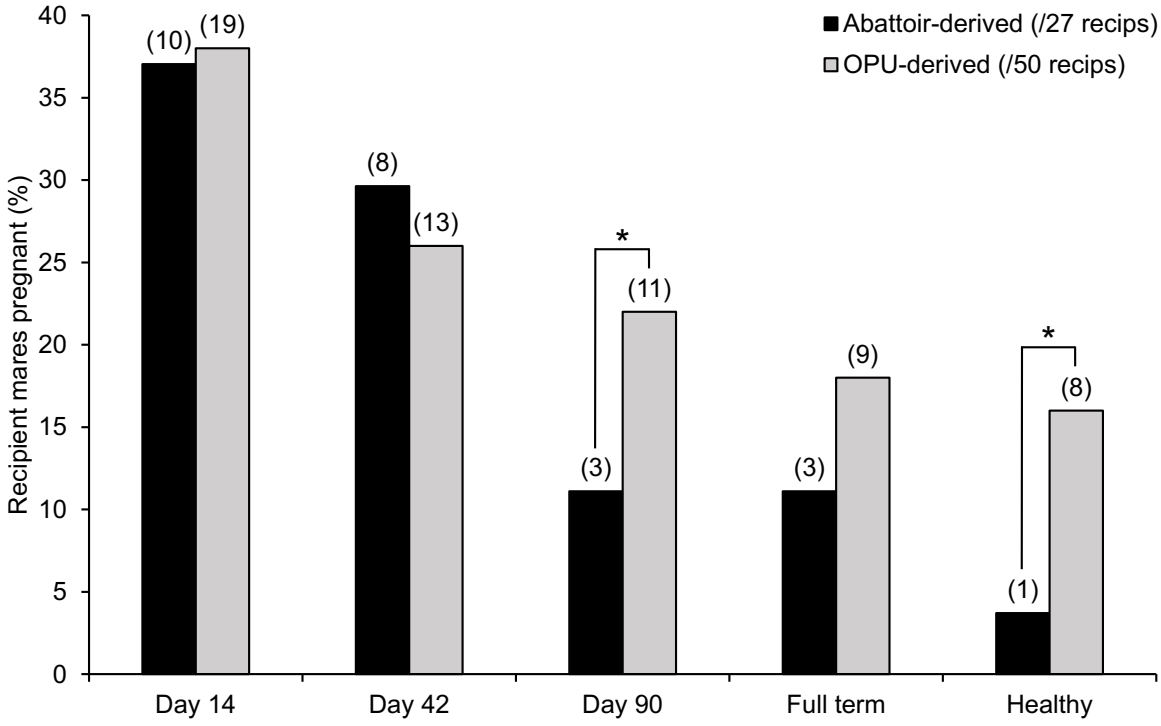

Figure 2.1. The percentages of recipient mares that were diagnosed as pregnant at Days 14, 42 and 90 of gestation, carried the pregnancy full term, and gave birth to a healthy foal following transfer of cloned embryos. Day 7 abattoir- and OPU-derived blastocysts were transferred to 27 and 50 recipient mares, respectively. The numbers in parentheses above each bar indicate the number of recipient mares positive for that parameter. The asterisk brackets indicate that conceptus and foal viability after Day 42 of gestation is significantly different between groups ($P<0.05$).

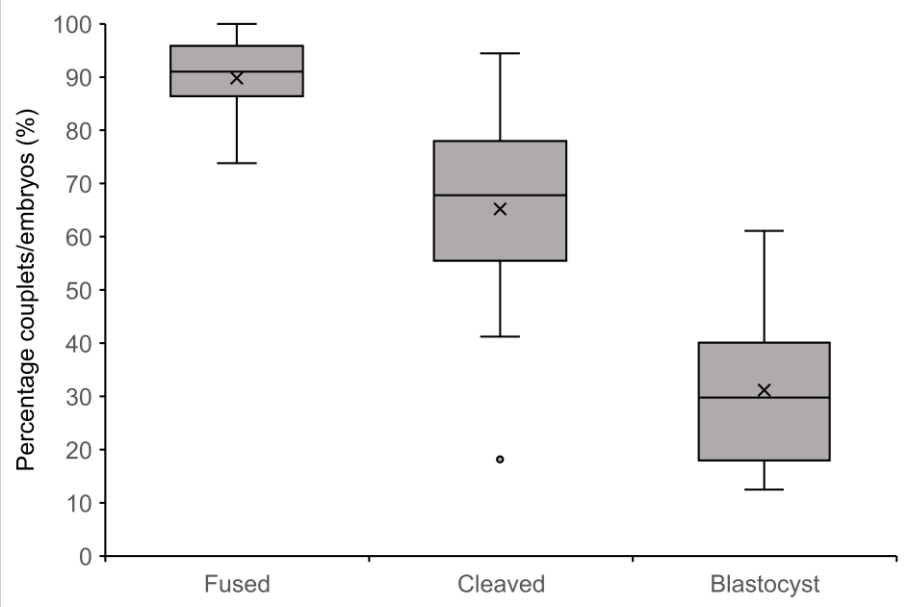

Figure 2.2. Box plots of the percentages of couplets that fused, and cloned embryos that cleaved and developed to the blastocyst stage, showing the distributions due to the fibroblast cell lines used to provide the donor nuclei. The mean values are indicated by crosses and an outlier is marked by a point. While the fusion rate was not affected by the cell line used ($P>0.05$), the cleavage and blastocyst formation rates were ($P<0.05$).

Figure 2.3. Cloned foals born from full-term pregnancies following transfers of SCNT embryos produced using oocytes obtained either slaughterhouse ovaries or from live mares.

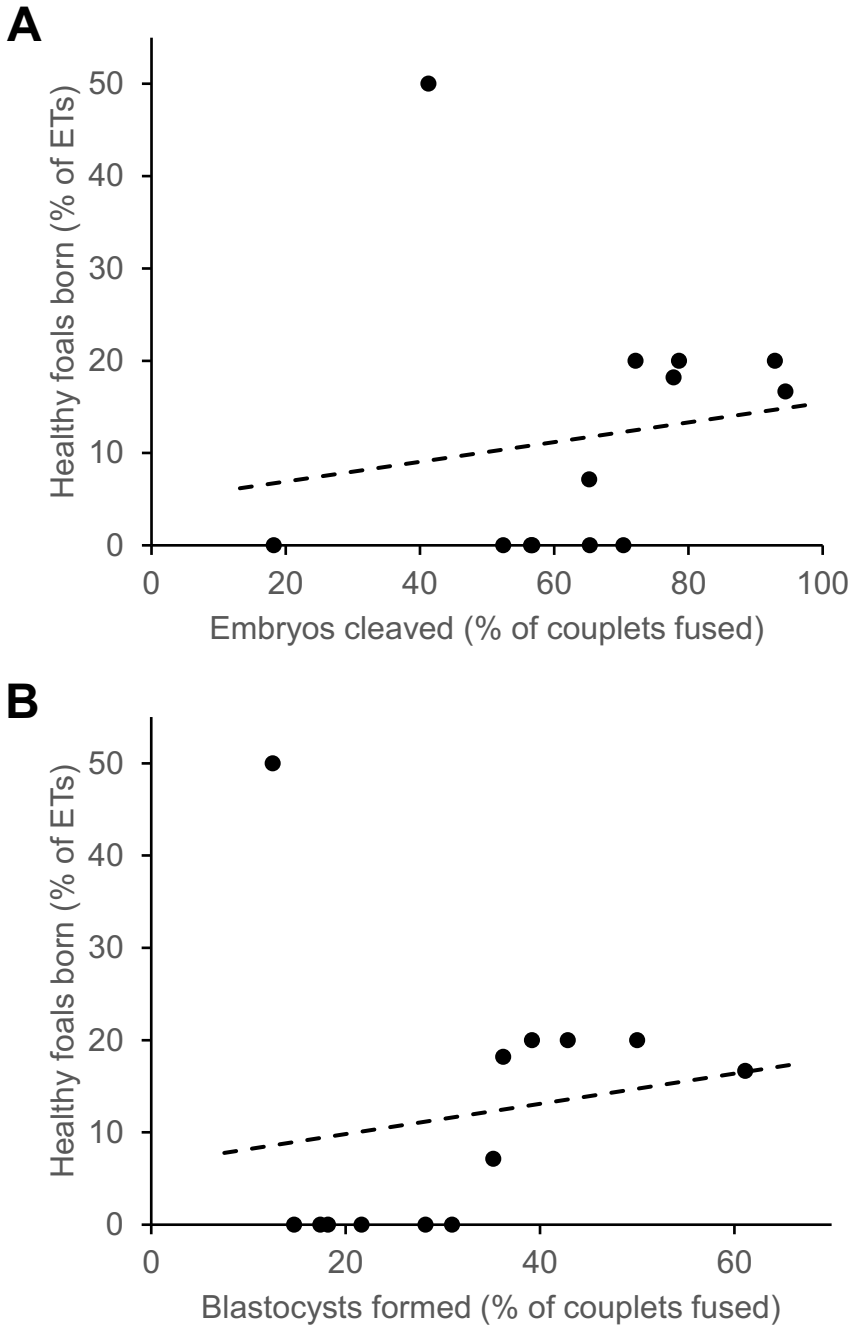

Figure 2.4. Scatter plots showing the relationship between the percentage of embryo transfers resulting in the birth of a healthy cloned foal and the in vitro embryo development results for each donor cell line. The linear trendline is indicated by the dashed line. Kendall's rank correlation revealed that the healthy foaling rate was ranked in a similar order to the rates of (A) cleavage (P=0.038) and (B) blastocyst formation (P=0.021).

2.4 Discussion

The results of this somatic cell nuclear transfer (SCNT) study, carried out over two breeding seasons at a commercial stud in Australia, reveal insights into several factors that influence the efficiency of equine cloning. A total of 77 embryo transfers were performed from 35 replicates in which cloned embryos were produced using abattoir- or OPU-derived oocytes and 14 different nuclear donor cell lines. A total of 12 cloned foals were born, of which 9 were healthy and 3 were not viable. Firstly, the sources of oocytes used to provide the recipient cytoplasts differed greatly in their capacity to support positive pregnancy outcomes. Secondly, vitrification of OPU-derived Day 7 blastocysts, a necessary additional procedure that addresses the unavoidable shortage of suitable recipient mares on the day of single fresh ET, did not reduce their potential to generate healthy foals. Thirdly, the fibroblast cell line used to provide the donor nuclei affected the efficiency of cloned embryo production, which was reflected in the pregnancy outcomes achieved.

A major limitation of equine cloning is the restricted number of oocytes available for cloned embryo production. Immature oocytes can be recovered from ovaries collected post-mortem, but this supply is often limited and sporadic, even when the laboratory has access to slaughterhouse material. Alternatively, immature oocytes can be harvested from live mares, but this supply requires significant resources and effort. With Day 7 blastocysts produced in nearly every replicate, we found the rates of cleavage and blastocyst formation were higher for OPU-derived embryos than for abattoir-derived embryos. To the best of our knowledge, this is the first such reported comparison of equine cloned embryo development in vitro. A previous study by Lee et al. (2015)

compared the in vivo development of equine cloned embryos produced using abattoir- and OPU-derived oocytes, but in vitro development was not assessed because the embryos were transferred to recipient mares immediately after couplet activation (Lee et al., 2015). In other equine SCNT studies that produced embryos from oocytes of either source, the reported rates of cleavage and blastocyst formation vary greatly. The differences in equine cloned embryo in vitro development between studies is likely due to the many differences in the SCNT technique and treatments, the nucleus donor cells, and the conditions for oocyte maturation and embryo culture. When used to produce equine embryos by intracytoplasmic sperm injection (ICSI), OPU-derived oocytes supported higher rates of development to the blastocyst stage compared with abattoir-derived oocytes (Brom-de-Luna et al., 2021).

Following transfer of Day 7 blastocysts to recipient mares, similar pregnancy rates were observed at Day 14 of gestation in both groups. However, per blastocyst transferred, abattoir-derived embryos had a reduced ability to form embryonic vesicles compared with OPU-derived embryos (16.9% vs 35.4%, respectively). Of the vesicles that developed, most had a viable embryo proper at Day 42 of gestation. Pregnancy loss beyond Day 42, when conceptus attachment is established (Allen, 2001), was greater at Day 90 for the abattoir group than for the OPU group. Such loss suggests placental dysfunction involving inadequate release of equine chorionic gonadotrophin (eCG), which stimulates the formation of secondary CL beyond Day 42 to maintain pregnancy(Allen, 2001; Vanderwall et al., 2006). Despite the administration of exogenous progesterone, failure of secondary CL formation may cause the level of progesterone to be insufficient. The difference between groups was also reflected in the viability of foals, with 3.4% and 16.7% of embryo transfers resulting in the birth of a healthy foal in the abattoir and OPU

groups, respectively. To date, equine SCNT studies have mostly utilised abattoir-derived oocytes for cloned embryo production. The pregnancy and foaling rates for abattoir-derived embryos reported here compare favourably with those reported previously (Choi et al., 2009; Galli et al., 2003; Lagutina et al., 2005; Lee et al., 2015). Relatively few studies have used OPU-derived oocytes for SCNT and obtained live foals after ET (Choi et al., 2013; Lee et al., 2015). In the comparison by Lee et al. (2015), one foal was obtained in the in vivo (OPU) group from 26 fused couplets transferred to 13 recipients, and no foals were obtained in the in vitro (abattoir) group from 42 fused couplets transferred to 11 recipients (Lee et al., 2015).

Of the three non-viable foals born, one had a markedly enlarged umbilicus, one had severe angular limb deformities, and one had severe craniofacial malformations. Another foal had minor forelimb and umbilical cord problems that were resolved. Developmental abnormalities are frequently observed in foals generated by SCNT (Lagutina et al., 2005; Pozor et al., 2016; Vanderwall et al., 2006). In a detailed assessment of 14 cloned foals born alive by Johnson et al. (Johnson et al., 2010), seven had an umbilical cord abnormality, and eight had a limb abnormality. Craniofacial malformations are a commonly observed equine congenital defect (Crowe & Swerczek, 1985). It is interesting to note that the cloned foals assessed in the Johnson et al. (Johnson et al., 2010) study were generated using abattoir-derived oocytes (Choi et al., 2009; Hinrichs et al., 2006; Hinrichs et al., 2007; Johnson et al., 2010). Our results indicate that using oocytes collected from live mares reduced the incidence of cloning-associated abnormalities, referred to as “cloned offspring syndrome” (Cross, 2001). Recently, a similar improvement in live cloned offspring efficiency was reported in dromedary camels, although the oocytes obtained by OPU were matured in vivo following gonadotrophin

stimulation (Son et al., 2022). Studies in cattle and buffalo have also demonstrated the superior developmental competence of OPU-derived oocytes compared with abattoir-derived oocytes (Manjunatha et al., 2008; Yang et al., 2021). Given the high incidence of abnormalities seen in the cloned offspring of livestock species, particularly in cloned calves, we recommend the use of oocytes collected by OPU for SCNT embryo production.

The pregnancy loss and compromised neonatal health associated with equine cloning have been attributed to defective epigenetic reprogramming that results in aberrant gene expression (Hinrichs, 2006). A recent transcriptomic analysis of equine placentas identified 1,651 differentially regulated genes between control artificial insemination (AI) pregnancies and cloned pregnancies that yielded non-viable foals; pathway analysis indicated that angiogenesis was disrupted in the cloned placentas (Verstraete et al., 2022). Our results suggest that the epigenetic reprogramming ability of oocytes from slaughtered mares is inherently worse than that of oocytes harvested from live mares. Populations of slaughtered horses usually include a greater proportion of old and sub-fertile mares, so the quality of oocytes in the abattoir group would be expected to be poorer (Derisoud et al., 2021). Oocytes from mares of advanced maternal age have been found to have impaired metabolic activity and a compromised ability to align chromosomes compared with oocytes from young mares (Catandi et al., 2021; Rizzo et al., 2019). Therefore, aging-induced depletion of oocyte factors involved in spindle formation, which is accompanied by aneuploidy and developmental defects in bovine and murine SCNT embryos (Cheng et al., 2013; Han et al., 2010), may have contributed to the greater incidence of pregnancy loss observed in the abattoir group. Additionally, the phase of the oestrous cycle was unknown at the time of slaughter, whereas the wave of developing

follicles was closely monitored in preparation for OPU. Therefore, differences in follicular wave development between the two groups of donor mares very likely influenced oocyte quality (Vernunft et al., 2013).

Not all OPU-derived blastocysts were transferred fresh because there were not enough recipient mares at the desired stage (Day 5 post-ovulation) on the day of fresh ET. Hence, a proportion of blastocysts were vitrified and then thawed and transferred when recipient mares attained the desired stage. Unexpectedly, the pregnancy outcomes achieved for the vitrified-thawed blastocysts (four healthy foals from 15 transfers) were more favourable than for the fresh blastocysts (four healthy foals from 35 transfers). This finding highlights the importance of a synchronous embryo-uterine interaction and suggests that some of the mares used for the fresh ETs did not have optimal uterine receptivity. Previously, Galli et al. (Galli et al., 2007) observed no differences in pregnancy and foaling rates between fresh and frozen cloned embryos. The clear advantage of transferring a vitrified-thawed embryo is that the transfer can be delayed until a mare is deemed to be at the "perfect" stage. Cuervo-Arango et al. (Cuervo-Arango et al., 2019a) found that the number of days after ovulation and the number of CL at ET influenced the likelihood of pregnancy in mares following transfer of in vitro produced (IVP) embryos.

The retrospective analysis of frozen ICSI ET cycles showed that the optimal recipient mare stage for transfer of Day 7-8 IVP blastocysts was Day 4 post-ovulation (Cuervo-Arango et al., 2019a). Interestingly, the rate of ongoing pregnancies was lower in mares with two CL on Day 5 post-ovulation compared to mares with one CL on Day 5 post-ovulation (Cuervo-Arango et al., 2019a). Therefore, the number of CL that the recipient mares had on Day 5 post-ovulation may have influenced the pregnancy rates obtained in

the present study. Recently, excessive heat conditions on the day of and/or in the week after ET, a relevant issue in the context of global warming (Arias et al., 2021), has been associated with early embryonic loss in horses (Yu et al., 2022). While the results of prospective cohort studies are needed to support the previous findings, transferring equine SCNT embryos on Day 4 post-ovulation, and scheduling to avoid extreme heat on the day of and in the week after ET, is proposed to improve pregnancy rates.

Fourteen nuclear donor cell lines were used to produce the cloned embryos in this study, and blastocysts from 13 of these cell lines were transferred to recipient mares. All the fibroblast cell lines were generated from biopsied skin tissue using the same procedure and were treated in the same way to prepare the donor cells for embryo reconstruction. Even though the same protocols were used, the cell line influenced the rates of cleavage and development to the blastocyst stage, presumably because of different epigenetic characteristics that affected nuclear reprogramming. It is well established that the reprogramming plasticity of donor cells, even those derived from the same tissue, can vary significantly (Lagutina et al., 2005; Powell et al., 2004; Zhou et al., 2006). In a horse cloning study that compared the use of adult fibroblast cells and bone marrow-mesenchymal stem cells (BM-MSC) as nuclear donors, preimplantation embryo development and foal viability were superior from BM-MSC (Olivera et al., 2018), reinforcing the assertion that less differentiated donor cells increase cloning efficiency. However, acquiring BM-MSC from donor animals involves an invasive procedure that comes with some risk of complications (Mocchi et al., 2020), such that horse owners prefer the collection of cells from a superficial skin biopsy. An alternative strategy to improve the developmental potential of SCNT embryos is to apply treatments that overcome the epigenetic reprogramming barriers (Glanzner et al., 2022). The findings of

recent pig cloning studies suggest that dual inhibition of DNA and histone methyltransferases may be the most effective approach to improve SCNT efficiency (Liu et al., 2021; Weng et al., 2020).

Pregnancies were established from 11 of the cell lines and healthy cloned foals were obtained from 7 of these, demonstrating that most gained totipotency following SCNT reprogramming in at least a proportion of the embryos produced. Interestingly, as the cleavage and blastocyst formation rates achieved for a cell line increased, so too did the percentage of embryo transfers that resulted in the birth of a healthy foal. Apart from one outlier, the cell lines that yielded a healthy foal also produced blastocysts at rates greater than 35% (of couplets fused). This relationship may be attributed to the capacity of the cell line to be reprogrammed to a totipotent state. Alternatively, this finding may reflect that the generation of cloned offspring is just "a numbers game". Put simply, the more Day 7 blastocysts produced and transferred, the more likely a foaling will be. For the cell lines that produced a healthy foal, the foaling rate ranged from 7.1% (1 foal from 14 transfers) to 50.0% (1 foal from 2 transfers), with a mean of 21.7%, which equates to about 5 embryo transfers for each healthy foal born. Based on our in vitro and in vivo development results, we believe a useful strategy to increase the likelihood of foaling is to first determine the chosen cell line's capacity to form Day 7 blastocysts, and to only perform ET, with or without blastocyst vitrification, if the blastocyst rate achieved is greater than 35% using OPU-derived oocytes, or greater than 25% using abattoir-derived oocytes.

While the results show a clear difference in cloning efficiency between abattoir- and OPU-derived oocytes, some limitations of the study should be acknowledged. Firstly, the

production of cloned embryos in each replicate involved the use of either abattoir- or OPU-derived oocytes. Ideally, oocytes from both sources should be utilised in each replicate to account for any variation between the separate days of embryo production. Such an immense undertaking was simply not possible due to the logistical constraints. Secondly, the glaring procedural difference between the groups was the number of blastocysts transferred to each recipient mare; abattoir-derived blastocysts were transferred as multiples and OPU-derived blastocysts were transferred singly. As the number of blastocysts produced using abattoir-derived oocytes (mean of 6.2 blastocysts per replicate) exceeded the number of recipients suitable on the day of fresh ET, the decision was made to transfer the embryos of the abattoir group as multiples based on the results of previous equine SCNT studies (Galli et al., 2007). Galli et al. (2007) transferred a total of 118 SCNT embryos to 46 mares (up to four embryos per recipient) and obtained 13 pregnancies with one embryonic vesicle each. Given that mare factors contribute to pregnancy loss, the embryonic vesicle development results of the present study should be viewed with some caution. Finally, commercial constraints prevented the use of all the donor cell lines in both oocyte groups. To investigate the effects of oocyte source on in vitro and in vivo development it would be ideal to use only a single donor cell line.

2.5 Conclusions

In conclusion, here we report that standard equine SCNT procedures were successfully applied in a commercial horse breeding operation to generate 12 cloned foals using abattoir- and OPU-derived oocytes. Although the quality of oocytes would be expected to be poorer from slaughtered mares compared with live mares monitored for OPU, the results provide a measure of the difference in SCNT reprogramming efficiency between

the two oocyte sources. Despite the considerable resourcing, expense and effort involved, we find the efficiency gains achieved with OPU-harvested oocytes make this source of oocytes undeniably preferrable for producing healthy cloned foals. Vitrification had no detrimental impact on the developmental potential of OPU-derived Day 7 blastocysts, and enabled embryo transfers to be performed when the uterine receptivity of the recipient mares was more ideal. Healthy cloned foals were obtained from seven different adult fibroblast cell lines used to provide the donor nuclei, demonstrating the reliability of the SCNT method used. These findings will inform strategies to further improve the efficiency of SCNT programs and help direct the focus of future cloning research efforts. Clearly, further studies are needed to better understand the oocyte maturational requirements that facilitate complete epigenetic reprogramming. As the birth of live offspring is the only definitive measure of embryo viability, the generation of cloned foals provides an invaluable model for addressing fundamental questions in reproductive and developmental biology.

CHAPTER 3

Effect of pre-IVM duration with cAMP modulators on the production of cloned equine embryos and foals

Authorship Attribution Statement

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As documented using the journal's author contributions framework, each author's specific contributions to the research and manuscript were:

J.V. Cortez: conceptualization, methodology, validation, formal analysis, data curation, investigation, writing – original draft, visualization, project administration; K. Hardwicke: methodology, investigation, resources, data curation, project administration, supervision; C.E. Méndez-Calderón: investigation; C.G. Grupen: conceptualization, methodology, formal analysis, resources, writing – review and editing, visualization, supervision.

The corresponding author has granted permission to include the published material in this thesis.

Jenin V. Cortez

May 2026

As supervisor for the candidature upon which this thesis is based, I confirm that the authorship attribution statement above is correct.

Christopher G. Grupen

May 2026

Abstract

The asynchrony of cytoplasmic and nuclear maturation in cumulus-oocyte complexes (COCs) due to prematurely declining concentrations of cyclic adenosine monophosphate (cAMP) has been shown to result in reduced oocyte developmental competence. The objective of this study was to evaluate the effect of pre-IVM treatment with cAMP modulators for different durations on the developmental potential of equine oocytes used for cloned embryo production. Collected COCs were transferred to cryovials filled with transport medium at 20-22°C. Within the cryovials, the COCs were either untreated (Control) for 18 h or treated with 50 µM forskolin and 100 µM 3-isobutyl-1-methylxanthine for the first 4 h (Pre-IVM 4h) or the entire 18 h (Pre-IVM 18h). Oocytes were then transferred to maturation medium, and incubated for a further 22–24 h at 38.5°C in 5% CO_2 in air. Somatic cell nuclear transfer embryos were then produced using the meiotically mature oocytes and donor cells from six different fibroblast cell lines. The rates of maturation and embryo development did not differ significantly between the groups, though blastocyst formation tended to be inferior in the Pre-IVM 4h group compared with the Control group (p = 0.06). Of 67 blastocysts produced, 23 were transferred to recipient mares on Day 4 or 5 post-ovulation. Regarding the pregnancy outcomes, no significant differences were found between the groups, and 4 viable foals were born, each derived from a different donor cell line. The findings expand on those from previous evaluations of this biphasic IVM system, and indicate that the cAMP-modulating treatments exert limited effects under the pre-IVM conditions used here.

3.1 Introduction

In horses, assisted reproductive technologies (ARTs) are constantly being refined to enhance or extend the reproductive potential of valuable animals. While oocyte in vitro maturation (IVM) can successfully generate viable embryos after transfer (Squires, 2020), a major focus in recent years has been on improving oocyte IVM systems. In vivo, oocyte maturation is regulated by signals from the somatic compartment of the follicles, including granulosa and cumulus cells, which coordinate the acquisition of developmental competence, such that the oocyte cytoplasmic components correctly direct the intricate events following fertilization and oocyte activation (Gilchrist & Thompson, 2007; Hinrichs, 2018).

The development of IVM for equine embryo production has been limited due to the significantly lower success rate achieved compared to that in other domestic animals, and the constraints in obtaining immature oocytes (Hinrichs, 2018). Asynchrony of nuclear and cytoplasmic maturation results when immature oocytes are removed from antral follicles, contributing to the poor developmental potential of IVM oocytes (Gilchrist, 2011). A commonly used maturational synchronizing strategy is to simulate the conditions that maintain oocyte meiotic arrest at the prophase I stage by elevating the intra-oocyte concentrations of cAMP, a key regulator of meiotic progression (Appeltant et al., 2016; Rose et al., 2013). Transient exposure of oocytes to cAMP-elevating agents inhibits the degradation of germinal vesicles through the activation of protein kinase A, and prevents the spontaneous resumption of meiosis, thereby reducing the asynchrony of cytoplasmic and nuclear maturation (Downs, 2010; Richani & Gilchrist, 2022).

The cAMP modulating treatments used in the so called Simulated Physiological Oocyte Maturation (SPOM) system have been shown to exert beneficial effects in several species, including cattle (Bernal-Ulloa et al., 2016; Li et al., 2016), goats (Suresh et al., 2021), horses (Metcalf et al., 2020), and mice (Albuz et al., 2010; Zeng et al., 2014). However, the effectiveness of the SPOM system remains contentious, owing to the inconsistent results achieved by different research groups (Leal et al., 2022). This pre-IVM approach uses a combination of intra-oocyte cAMP modulators, specifically forskolin (FSK), which activates adenylate cyclase and increases cAMP synthesis, and isobutyl-1-methylxanthine (IBMX), which acts as a phosphodiesterase (PDE) inhibitor and prevents cAMP catabolism. The combined activities stimulate greater elevation of cAMP levels than either activity alone, and supposedly maintains oocyte meiotic arrest in a way that more closely mimics that in an in vivo environment (Albuz et al., 2010). Interestingly, the duration of the SPOM pre-IVM incubation varies considerably between studies, with an overnight duration being used successfully in horses (Metcalf et al., 2020), and shorter durations (2–6 h) being beneficial in cattle (Bernal-Ulloa et al., 2016; Li et al., 2016). Overnight pre-IVM treatment of equine oocytes is logistically convenient, as this coincides with the period typically needed to transport them from the site of collection to the laboratory for subsequent maturation and embryo production.

The objective of this study was to evaluate the effectiveness of the pre-IVM treatment (FSK and IBMX combined) applied for different durations (4 and 18 h) on the developmental competence of equine oocytes recovered from abattoir-sourced ovaries. After IVM (with or without the pre-IVM treatments), matured oocytes were used to produce cloned embryos by conventional somatic cell nuclear transfer, and the rates of maturation, couplet fusion, embryonic cleavage, and blastocyst formation were assessed.

Following the transfer of blastocysts to recipient mares, pregnancy outcomes were also monitored.

3.2 Materials and methods

3.2.1 Mares

This study was performed at the Catalina Equine Reproduction Centre (North Richmond, NSW Australia). A total of 23 standardbred mares, aged 3 to 15 years, were used as embryo transfer recipients. All procedures were carried out with informed consent from the owners of the animals and in compliance with the NSW Animal Research Act (1985), which incorporates the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

3.2.2 Chemicals and media

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Australia). Hepes-buffered Synthetic Oviductal Fluid (H-SOF) (Thompson et al., 1990) supplemented with 10% fetal calf serum (FCS; AU-FBS/PG; Cellsera, Rutherford, NSW, Australia) was used for procedures performed outside of the CO_2 incubator. The transport medium used to hold oocytes prior to in vitro maturation (including the pre-IVM treatments) consisted of a 1:1 mix of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; D8437) and Medium 199 (M3769) supplemented with 10% FCS. The oocyte maturation medium and the couplet fusion medium were prepared as previously described (Cortez et al., 2023).

3.2.3 Collection of Immature Oocytes

Mare ovaries were obtained and processed at a slaughterhouse. The process of cumulus-oocyte complex (COC) recovery has been described in detail previously (Stout et al., 2020). Briefly, antral follicles <30 mm in diameter were aspirated using an 18G needle and the follicle walls were scraped extensively with a bone curette. Following transfer to a 100 mm Petri dish containing H-SOF at 37°C to facilitate searching, selected COCs were washed three times with H-SOF. A total of 715 oocytes were collected in 6 replicates. In each replicate, the COCs were randomly allocated to one of three pre-IVM treatment groups such that each group was transferred to a separate 1.5 mL cryovial filled with transport medium at 20–22°C. The cryovials were tightly capped and the COCs were transported to the laboratory in a polystyrene container at 20–22°C overnight prior to IVM 18 h later. The three cryovials contained either i) non-supplemented transport medium for the entire 18 h duration (Control), ii) transport medium supplemented with 50 μM forskolin (FSK) and 100 μM 3-isobutyl-1-methylxanthine (IBMX) for the first 4 h, and then non-supplemented transport medium for the next 14 h (Pre-IVM 4h), and iii) transport medium supplemented with FSK and IBMX for the entire 18 h duration (Pre-IVM 18h). The concentrations of FSK and IBMX were selected based on the findings of Metcalf and co-workers (Metcalf et al., 2016; Metcalf et al., 2020).

3.2.4 Oocyte In Vitro Maturation (IVM)

At the end of the 18 h transport and holding period, the COCs of each group were washed 3 times with H-SOF, and then transferred to wells of 4-well dishes (144444; Nunc, Roskilde, Denmark) containing 500 μL of pre-equilibrated maturation medium (20–50

COCs per well). The COCs were incubated for 22–24 h at 38.5°C in a humidified atmosphere of 5% CO2 in air.

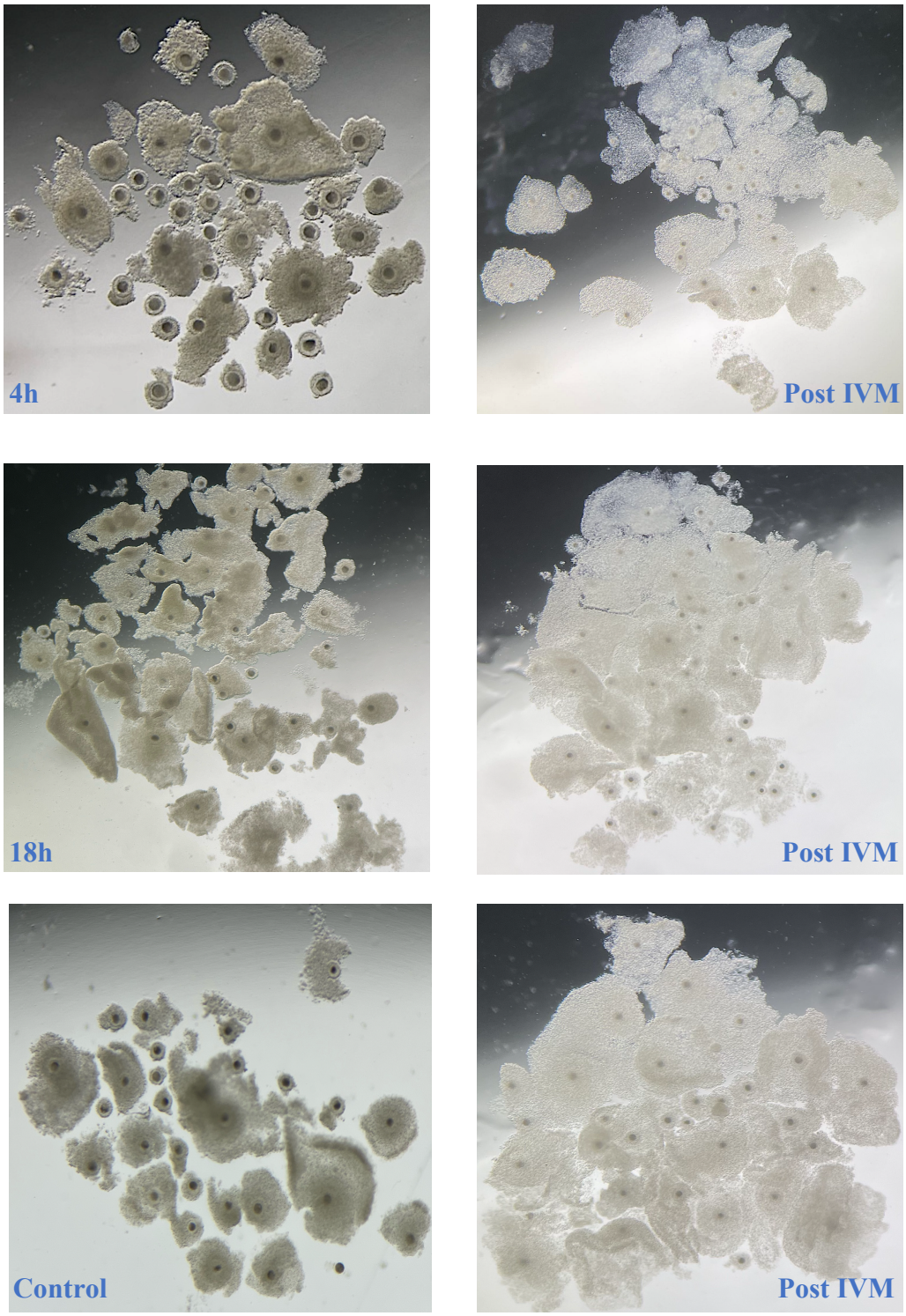

Figure 3.1. Representative images of in vitro matured oocytes treated with cAMP modulators for different exposure time (4 h, 18 h), and untreated oocytes (Control).

3.2.5 Somatic Cell Nuclear Transfer (SCNT)

A total of 6 different fibroblast cell lines were derived from the subcutaneous tissue of 6 adult female horses. A different cell line was used in each of the 6 replicates. DMEM/F-12 medium supplemented with 1 mM glutamine, 0.2 mM pyruvate, 10 ng/ml EGF, and 10% FCS was used to culture the fibroblast cells. After plating and initial expansion, cells were passaged twice, frozen overnight at −80°C in culture medium containing 10% dimethyl sulfoxide (DMSO), and then stored in liquid nitrogen. After thawing, the cells were cultured for no less than two days and grown to confluence to induce cell cycle arrest at least 24 h before use as donor cells for SCNT. Immediately before SCNT, the cells were trypsinized, washed, and suspended in H-SOF containing 2% FCS, and kept at room temperature (RT) until use (approximately 10 min).

After IVM, the cumulus cells were removed from the oocytes by gentle pipetting in H-SOF supplemented with 1 mg/ml hyaluronidase. The micromanipulation process involved the removal of the polar body and the metaphase plate from each oocyte using an enucleation pipette attached to a Piezo drill (PMAS-CT150; Prime Tech Ltd., Ibaraki, Japan), and placement of a donor cell in the perivitelline space of each cytoplast. The couplets were held for 1 h in H-SOF at 38°C, before being transferred to fusion medium on a fusion chamber slide between electrodes 0.5 mm apart. A direct current (DC) pulse of 2.2 kV/cm strength and 15 µsec duration was immediately applied to the couplets. The pulsed couplets were washed 3 times in H-SOF, transferred to the embryo culture medium, and incubated for 2 h. Activation was carried out by exposing the fused couplets to 5 µM ionomycin in H-SOF for precisely 5 min. After several washes in H-SOF, the fused and activated couplets were treated with 1 mM 6-dimethylaminopurine and 5 µg/ml cycloheximide in embryo culture medium for 4 h. Finally, the resulting cloned embryos

were washed several times, transferred to 10 µl droplets of culture medium (maximum of 7 embryos in each droplet), and incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. The SCNT embryos were transferred to fresh culture medium every 2 days. Embryonic cleavage was assessed at the first change of culture medium and blastocyst development was assessed at Days 7 and 8, using well described morphological features to classify blastocyst quality (Carnevale & Metcalf, 2019).

3.2.6 Blastocyst vitrification and thawing

Blastocysts were vitrified using the Cryotop method according to the manufacturer's instructions (Kitazato BioPharma, Shizuoka, Japan). In summary, 300 µl of equilibration solution (ES) and vitrification solution 1 (VS1) were deposited in separate wells of a 3-well plate at RT. Embryos were transferred to the ES drop for 12-15 min and a cycle of contraction (dehydration) and re-expansion (ES infiltration) was observed. Then, the equilibrated embryos were transferred to VS1 for 1 min, after which time each embryo was placed with a minimal amount of medium on the thin polypropylene strip of the Cryotop, and the device was immediately immersed vertically in liquid nitrogen. To thaw, the Cryotop was directly immersed in 1 ml of prewarmed (37°C) thawing solution (TS) for 1 min. Embryos thawed in TS were gently placed at the bottom of a 300 µl drop of dilution solution (DS) for a gradual shift from TS to DS over 3 minutes at RT. Embryos were drawn up into a pipette tip with a 2 mm column of DS and then gently deposited at the bottom of a 300 µl drop of warming solution 1 (WS1) for gradual transference from DS to WS1 over 5 min at RT. Embryos with a minimal volume of WS1 were washed twice in warming solution 2 (WS2) for 1 min. Finally, the embryos were placed in culture media (37°C) for embryo transfer.

3.2.7 Embryo Transfer (ET)

Non-surgical embryo transfers were performed in 23 standardbred mares as described previously (Cortez et al., 2023). Vitrified and warmed blastocysts were transferred trans-cervically to recipients on Day 4 or 5 post-ovulation. Pregnancies were diagnosed by transrectal ultrasonography on Days 14 (Day 9 after ET), 45 and 90 of gestation. During pregnancy, fetal movements, placental quality, and heart rate were monitored. Additional scans were subsequently performed once a month to monitor the progress of ongoing pregnancies.

3.2.8 Statistical Analysis

Data were analyzed using the Genstat statistical software package (18th edition; VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The embryo in vitro production data (oocytes matured, couplets fused, embryos cleaved, and blastocysts formed) were subjected to logistic regression analysis with treatment group and cell line as factors. When no significant differences were detected, the variance between groups was determined using ANOVA and Fisher's unprotected pairwise comparison. The pregnancy and foaling data were analyzed using chi-square tests to evaluate the null hypothesis that there was no difference between the groups. A p value of less than 0.05 designated a significant difference.

3.3 Results

3.3.1 In Vitro Development of SCNT Embryos

3.3.1.1 Effect of cAMP-Modulating Pre-IVM Treatments

The effects of the cAMP-modulating pre-IVM treatments on the in vitro production and development of SCNT embryos are shown in Figure 1. In the 6 replicates, a total of 266 oocytes were allocated to the Control group (mean of 44.3 oocytes per replicate), a total of 191 oocytes were allocated to the Pre-IVM 4h group (mean of 31.8 oocytes per replicate), and a total of 258 oocytes were allocated to the Pre-IVM 18h group (mean of 43.0 oocytes per replicate). The rates of maturation, couplet fusion, embryonic cleavage and overall cloning efficiency did not differ between the groups ($p > 0.05$). However, the rates of blastocyst formation, as a proportion of fused couplets and cleaved embryos, tended to be lower in the Pre-IVM 4h group than in the Control group ($p < 0.1$). The blastocyst rates of the Pre-IVM 18h group did not differ from those of the Control and Pre-IVM 4 h groups ($p > 0.05$). Using untreated Control oocytes, blastocysts were produced in all 6 replicates (mean of 4.5 blastocysts per replicate). Conversely, blastocysts were produced in 5 of the 6 replicates using the Pre-IVM 18h-treated oocytes (mean of 4.5 blastocysts per replicate), and in 4 of the 6 replicates using the Pre-IVM 4h-treated oocytes (mean of 2.2 blastocysts per replicate).

3.3.1.2 Effect of Donor Cell Lines

A total of six different fibroblast cell lines, generated from separate skin biopsies, were used to provide the donor nuclei used to produce the SCNT embryos. Each cell line was used in all three experimental groups (Control, Pre-IVM 4h and Pre-IVM 18h), such that a different cell line was used in each of the 6 replicates. There was no effect of donor cell line on the rates of couplet fusion, embryonic cleavage, or development to the blastocyst stage ($p > 0.05$; Figure 3.2).

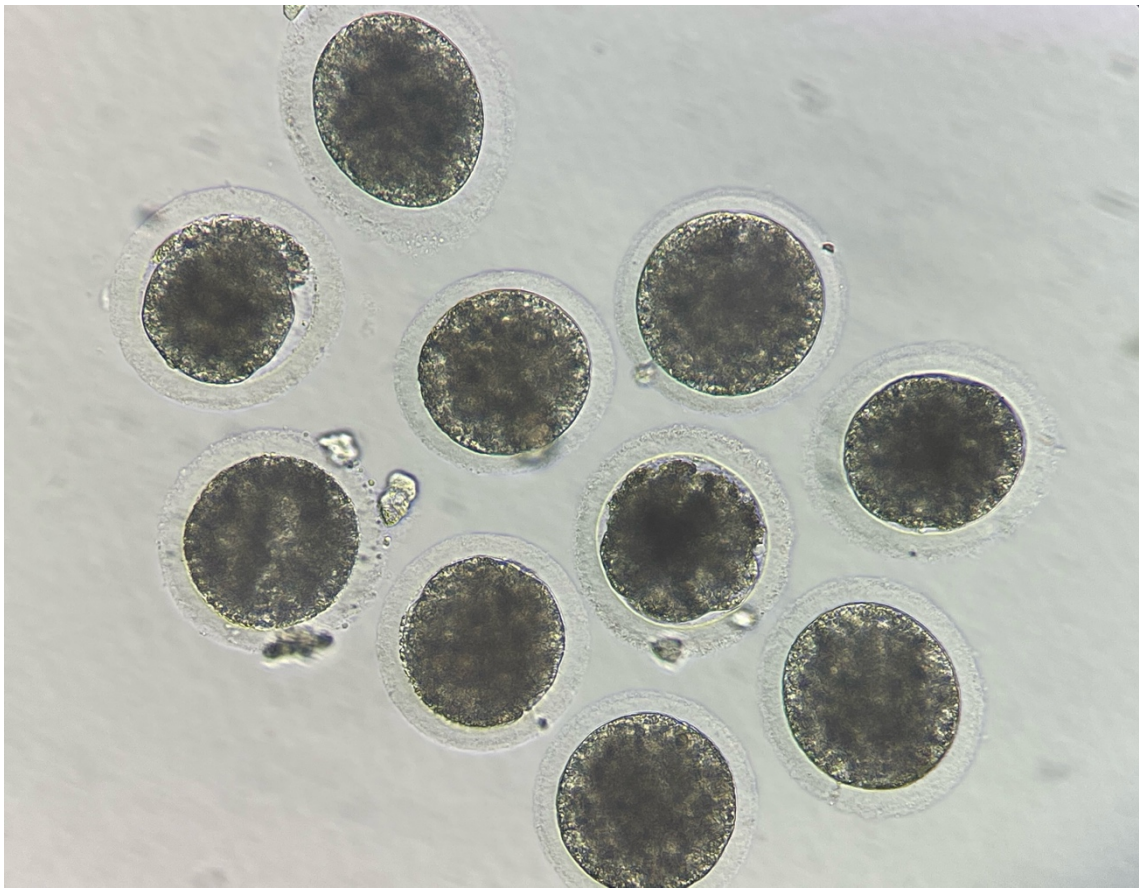

Figure 3.2. Embryos generated via SCNT using oocytes treated with cAMP modulator during the 18 h pre-IVM period.

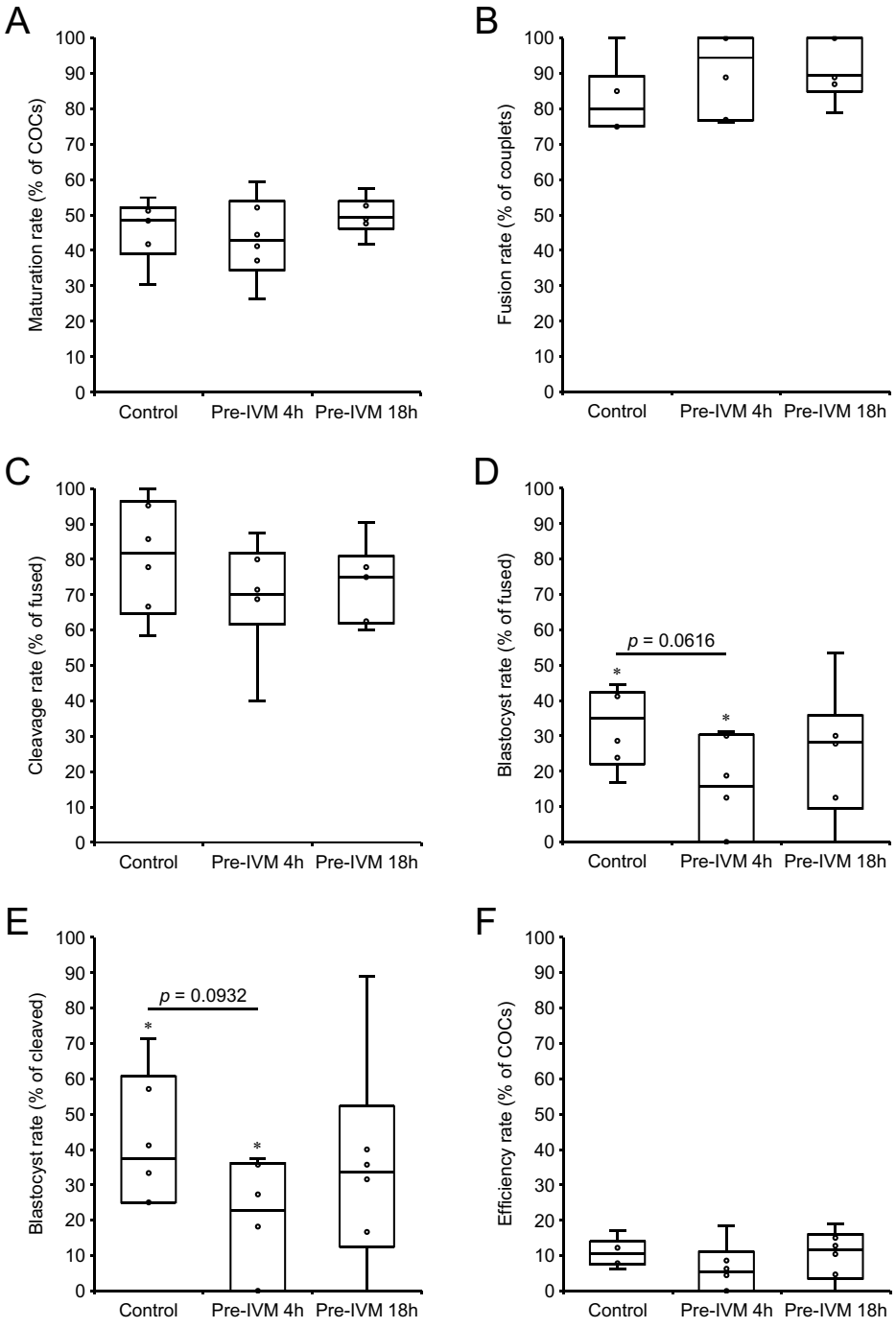

Figure 3.3. Effect of the cAMP-modulating pre-IVM treatments on SCNT embryo production (Control, Pre-IVM 4h, and Pre-IVM 18h groups). (A) The percentage of oocytes that matured after an additional 22–24 h of IVM, as determined by the presence of a first polar body. (B) The percentage of constructed couplets that fused. (C) The percentage of fused couplets that cleaved after 48 h of embryo culture. (D) The percentage of fused couplets that formed blastocysts. (E) The percentage of cleaved embryos that formed blastocysts. (F) The overall cloning efficiency rate, expressed as the percentage of cumulus-oocyte complexes (COCs) that resulted in blastocysts. Values are presented as the mean ± s.e.m. No significant differences were detected between the groups ($p > 0.05$). Values labelled with an asterisk tend to differ ($p < 0.1$).

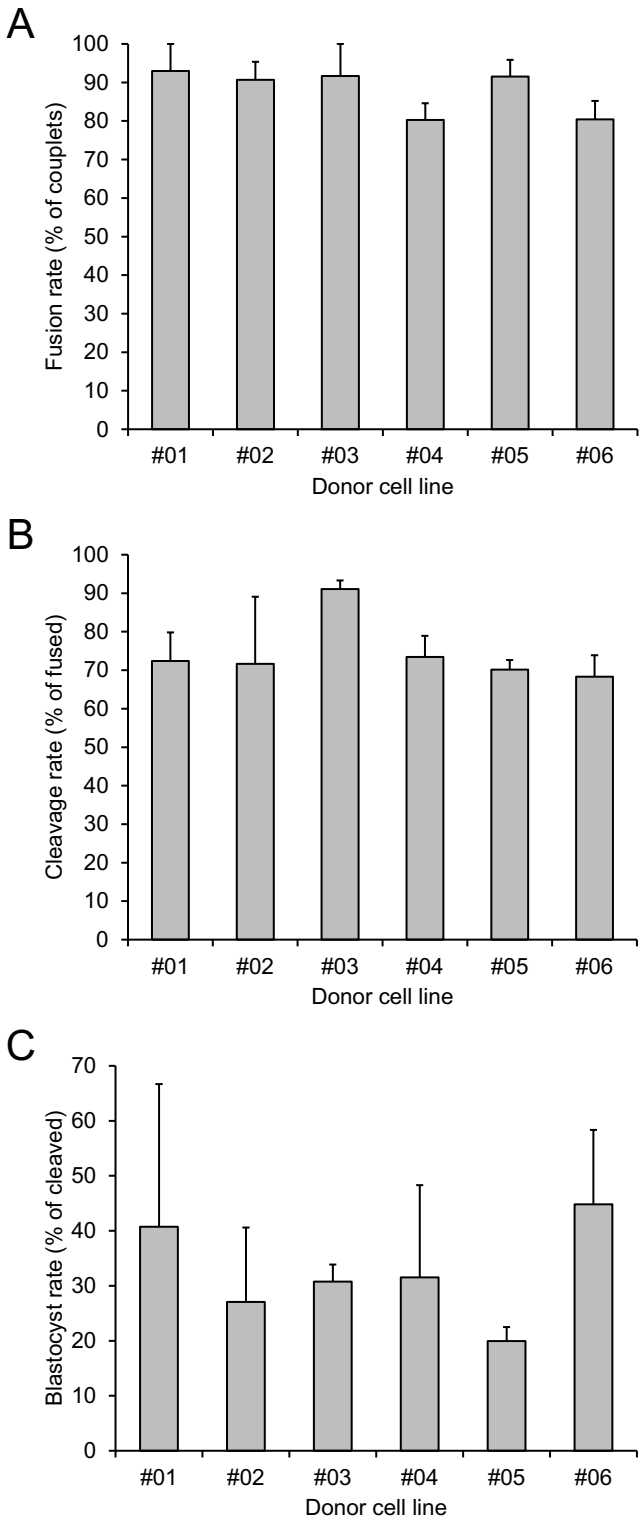

Figure 3.4. Effect of the donor cell lines on SCNT embryo production. (A) The percentage of constructed couplets that fused. (B) The percentage of fused couplets that cleaved. (C) The percentage of cleaved embryos that formed blastocysts. Values are presented as the mean ± s.e.m. No significant differences were detected between the groups ($p > 0.05$).

3.3.2 Pregnancy Outcomes After ET

3.3.2.1 Effect of cAMP-Modulating Pre-IVM Treatments

The results of pregnancy diagnosis after transfer of the SCNT embryos to recipient mares are shown in Table 3.1. The proportions of mares in which embryonic vesicles were detected on Day 14 of gestation were similar for the Control, Pre-IVM 4h and Pre-IVM 18h groups (1/4 and 1/2 and 8/17, respectively; $p > 0.05$). Embryo transfer was performed based on the breed of the donor animal and the size of the recipient mare. Therefore, of the 67 blastocysts produced (27, 13, and 27 for the Control, Pre-IVM 4h, and Pre-IVM 18h groups, respectively), a total of 23 blastocysts were transferred. As determined by ultrasonographic detection of an embryonic vesicle, 10 were viable on Day 14. Calculated per embryo transferred, the viability of the blastocysts on Day 14 of pregnancy did not differ significantly between the groups (25%, 50%, and 47% for the Control, Pre-IVM 4h, and Pre-IVM 18h groups, respectively; $p > 0.05$).

At Day 45 of pregnancy, the conceptuses of the Control and Pre-IVM 4h groups remained viable, while pregnancy losses occurred in the Pre-IVM 18h group, and two conceptuses remained viable. Beyond Day 45 of gestation, no pregnancy loss occured in any of the groups. A total of 4 foals were born (1, 1, and 2 in the Control, Pre-IVM 4h, and Pre-IVM 18h groups, respectively). Two of the foals were born normal and healthy, and the other two were declared healthy after successful treatment of minor forelimb and umbilical problems.

Table 3.1. Effect of cAMP-modulating pre-IVM treatments on pregnancy and foaling.

Group	Recipient mares total	Pregnant at Day 14[1]	Pregnant at Day 45[1]	Pregnant at Day 90	Mares foaling
Control	4	1 (25.0%)	1 (25.0%)	1	1
Pre-IVM4h	2	1 (50.0%)	1 (50.0%)	1	1
Pre-IVM18h	17	8 (47.1%)	2 (11.8%)	2	2
Total	23	10 (43.5%)	4 (17.4%)	4	4

[1]Values in parentheses are the percentage pregnant of total recipient mares.

3.3.2.2 Effect of Donor Cell Lines

The pregnancy results obtained for the different cell lines are shown in Table 3.2. A total of 23 blastocysts produced from 4 of the 6 cell lines were transferred to 23 recipient mares, and 10 of the transferred blastocysts formed embryonic vesicles at Day 14 of pregnancy. All of the resulting Day 45 conceptuses completed full-term development, such that 4 foals were born, one derived from each of the 4 donor cell lines involved.

Table 3.2. Effect of the donor cell lines on pregnancy and foaling.

Donor cell line	Recipient mares total	Pregnant at Day 14[1]	Pregnant at Day 45[1]	Pregnant at Day 90	Mares foaling
#01	10	3 (30.0%)	1 (10.0%)	1	1
#02	5	1 (20.0%)	1 (20.0%)	1	1
#03	4	3 (75.0%)	1 (25.0%)	1	1
#05	4	3 (75.0%)	1 (25.0%)	1	1

[1]Values in parentheses are the percentage pregnant of total recipient mares.

3.3.2.3 Effect of Embryo Grade

The effect of the quality of the transferred embryo (Grade 1 vs Grade 2) on pregnancy outcome is shown in Table 3.3. A total of 16 Grade 1 blastocysts and 7 Grade 2 blastocysts were transferred to the recipient mares. While pregnancies were detected at Day 14 for both embryo grades, only Grade 1 blastocysts established ongoing pregnancies and generated viable births (Grade 1: 4/16; Grade 2: 0/7). However, the apparent difference was not statistically significant ($p > 0.05$).

Table 3.3. Effect of embryo grade on pregnancy and foaling.

Embryo grade	Recipient mares total	Pregnant at Day 14[1]	Pregnant at Day 45[1]	Pregnant at Day 90	Mares foaling
Grade 1	16	8 (50.0%)	4 (25.0%)	4	4
Grade 2	7	2 (28.6%)	0 (0.0%)	0	0

[1]Values in parentheses are the percentage pregnant of total recipient mares.

3.3.2.4 Effect of Recipent Mare's Day Post-Ovulation

The effect of the recipient mare's day post-ovulation (Day 4 vs Day 5) at ET was also analyzed. As shown in Table 3.4, the transfer of embryos to recipients on Days 4 and 5 after ovulation resulted in similar Day 14 pregnancy rates. While more pregnancy losses occurred in the Day 5 post-ovulation group compared with the Day 4 post-ovulation group, the pregnancy rates at Days 45 and 90 and the foaling rates did not differ significantly (2/7 and 2/16, respectively).

Table 3.4. Effect of the recipient mare's day post-ovulation at embryo transfer on pregnancy and foaling.

Day post-ovulation	Recipient mares total	Pregnant at Day 14[1]	Pregnant at Day 45[1]	Pregnant at Day 90	Mares foaling
Day 4	7	3 (42.9%)	2 (28.6%)	2	2
Day 5	16	7 (43.8%)	2 (12.5%)	2	2

[1]Values in parentheses are the percentage pregnant of total recipient mares.

3.4 Discussion

The results of this study show that the developmental competence of equine oocytes used to produce embryos by somatic cell nuclear transfer (SCNT) was not improved following pre-IVM treatment with the cAMP modulators forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), a previously described biphasic IVM system referred to as Simulated Physiological Oocyte Maturation (SPOM) (Albuz et al., 2010; Leal et al., 2022). A total of 715 oocytes were recovered from slaughterhouse-sourced ovaries in 6 replicates, and a total of 67 blastocysts were produced. While no significant differences

between the treatment and control groups were found, the blastocyst formation rates tended to be lower in the the Pre-IVM 4h group than in the Control group. Within the constraints of a commercial equine cloning program, 23 blastocysts were transferred to recipient mares, resulting in the birth of 4 healthy foals derived from 4 different donor cell lines. There were no significant differences in pregnancy outcomes between the treatment and control groups, demonstrating for the first time that pre-IVM exposure of equine oocytes to FSK and IBMX is compatible with full term development of SCNT embryos. These findings expand the evaluation of the SPOM system in equine oocytes.

The developmental competence of in vitro matured oocytes is compromised due to the initiation of spontaneous meiotic resumption, which occurs in response to a decrease in the cAMP levels responsible for maintaining meiotic arrest (Conti et al., 2002; Gilchrist & Thompson, 2007). Adequate levels of cAMP in oocytes are generated and maintained by granulosa cells, cumulus cells, and metabolites of the follicular compartment to ensure that meiosis resumes in an orchestrated manner (Thomas et al., 2004). By disrupting the oocyte-follicle connection, the intra-oocyte cAMP levels decrease, such that the resumption of meiosis occurs in a more rapid and uncontrolled manner (Shu et al., 2008). In horses, the problem is exacerbated because the procedures used to recover oocytes via ovum pick up (OPU) or from slaughterhouse ovaries are more challenging than those used in other species (Hinrichs, 2010; Morris, 2018). Similar to the approaches described previously in horses and other species (Leal et al., 2022; Metcalf et al., 2016), the rationale for the pre-IVM treatment used here was to maintain the intra-oocyte cAMP concentrations immediately after collection, thus avoiding spontaneous meiotic resumption.

Research carried out in equine oocytes using treatments to inhibit meiosis is limited. Choi et al. (Choi et al., 2006) was one of the first to report the manipulation of meiotic resumption in this species using roscovitine, which is an analog of purine that specifically inhibits M-phase promoting factor activity. The addition of roscovitine to pre-IVM medium for 16–18 h effectively maintained oocytes at the germinal vesicle stage, but their developmental competence after subsequent IVM and intracytoplasmic sperm injection (ICSI) was not enhanced (Choi et al., 2006). Conversely, Metcalf et al. (Metcalf et al., 2016; Metcalf et al., 2020) found that the rates of maturation and blastocyst development were increased, compared with the control, when equine oocytes were held overnight in medium with 50 μM FSK and 100 μM IBMX (i.e. the SPOM system) (Metcalf et al., 2016; Metcalf et al., 2020). Using the same concentrations of FSK and IBMX as those used by Metcalf et al. (Metcalf et al., 2020), which were found to be optimal for equine oocytes in a pilot study (Metcalf et al., 2016), the results of the present study did not show any significant differences in the rates of maturation, couplet fusion, cleavage, and blastocyst formation between the Pre-IVM and Control groups. A major difference between the studies is that Metcalf et al. (Metcalf et al., 2016; Metcalf et al., 2020) produced ICSI embryos using oocytes harvested from live mares by OPU, whereas here we produced SCNT embryos using oocytes recovered from slaughterhouse-sourced ovaries. In a previous study we found that oocyte source (OPU-derived vs abattoir-derived) significantly impacted the development of SCNT embryos to the blastocyst stage (Cortez et al., 2023). Also, differences in the media used or other protocol discrepancies may account for the inconsistent findings.

The SPOM system has been applied to embryo production in numerous species, including cows (Albuz et al., 2010; Bernal-Ulloa et al., 2016; Ezoe et al., 2015; Razza et al., 2019),

mice (Albuz et al., 2010; Richani et al., 2014; Zeng et al., 2014), sheep (Buell et al., 2015; Rose et al., 2013), goats (Suresh et al., 2021), horses (Metcalf et al., 2020), and cats (Herrick, 2014). The effectiveness of the SPOM treatment has not been consistent, with only 34.7% (8/23) of studies achieving an improvement in blastocyst production (Leal et al., 2022). Further, in those studies conducted in cattle, only 25% (4/16) of them succeeded in improving blastocyst production (Leal et al., 2022). Differences between studies in the composition of the pre-IVM base medium and the supplements used suggest there are complex interactions with factors in the media that influence the effectiveness of the cAMP modulators (Leal et al., 2022).

A major variable of the SPOM system is the duration of the FSK and IBMX treatment. In horses, oocytes are often collected at a site distant to the laboratory, such that transportation overnight, as was the case in this study, is necessary. Hence, for the Pre-IVM 18h group, the oocytes were kept in transport medium supplemented with FSK and IBMX for the entire transportation period, whereas for the Pre-IVM 4h group, the oocytes were first held in transport medium supplemented with FSK and IBMX for 4 h before being transferred and kept in transport media without the cAMP modulators for the remaining 14 h. The oocytes of the control group were kept in transport medium without the cAMP modulators for 18 h. It is unclear why the Pre-IVM 4h-treated oocytes tended to have poorer developmental potential compared with the untreated control oocytes. A possible explanation is that the additional handling and medium change had a negative influence. In the only other horse studies of the SPOM system, an overnight pre-IVM duration was used with some success (Metcalf et al., 2016; Metcalf et al., 2020), while in cattle studies of the SPOM system, using pre-IVM durations of 2 and 6 h has achieved positive results (Albuz et al., 2010; Dall'Acqua et al., 2017; Leal et al., 2020; Li et al.,

2016). Further studies are needed to determine whether an alternative pre-IVM duration may be optimal. A key feature of equine oocyte maturation is the ability to hold immature oocytes at room temperature for 18 h, which appears to promote chromatin condensation at the germinal vesicle stage without reducing developmental competence (Galli, Colleoni, et al., 2014; Hinrichs, 2006). Another possibility is that the modulation of cAMP levels by the SPOM system is influenced by the pre-IVM holding temperature used.

The blastocysts produced were vitrified and then transferred into previously synchronized recipient mares. Due to the commercial imperative to obtain foals from particular donor cell lines, a similar number of blastocysts from each group could not be transferred. Pregnancy rates on Day 14 of gestation did not differ among the groups. Of the embryonic vesicles that developed, 40% (4/10) were viable conceptuses on Day 45 of gestation. Pregnancy loss and compromised neonatal health associated with equine cloning have been attributed to defective epigenetic reprogramming resulting in aberrant gene expression (Hinrichs, 2006) and the reproductive status of recipients carrying the pregnancy (Vanderwall et al., 2006). There was no pregnancy loss after Day 45 and viable foals were born in each group, demonstrating that the pre-IVM treatment with cAMP modulators did not have an adverse effect on fetal development. Similarly, in mice, Albuz et al. (Albuz et al., 2010) showed that viable offspring can be obtained following the transfer of embryos produced from oocytes subjected to the SPOM system.

The success of nuclear transfer depends on the ability of the donor cells to be reprogrammed to a totipotent state, guided by the reprogramming factors present in the recipient cytoplast (Fulka et al., 2023). The developmental plasticity of cells from

different lines is an important factor that determines the capacity of embryos to develop and give rise to healthy offspring (Niemann, 2016; Olivera et al., 2016). In this study, pregnancies were established, and healthy cloned foals were obtained from all the cell lines used to produce the transferred blastocysts, demonstrating that complete cellular reprogramming to a totipotent state was achieved. Overall, viable foals were obtained from 17.4% (4/23) of the blastocysts transferred, which equates to approximately 6 embryo transfers for each foal born. This foaling rate is comparable to that reported previously for SCNT embryos (Cortez et al., 2023).

The relationship between embryonic morphological features and pregnancy outcomes was also evaluated. Day 7 blastocysts classified as being of Grade 1 or 2 quality, according to well-defined criteria (Carnevale & Metcalf, 2019), were transferred to recipient mares. The morphological properties of in vitro produced equine blastocysts have been associated with their speed of development, and embryos that develop faster are more likely to be classified as Grade 1 and have a greater chance of generating births (Cuervo-Arango et al., 2019c; Lewis et al., 2023). In the present study, births were only obtained from Grade 1 embryos. Assessing the kinetics of embryonic development is now routine practice to predict the outcome of transferred embryos in domestic species and humans (Awadalla et al., 2021).

Regarding the recipient mare's day post-ovulation, similar pregnancy outcomes were achieved when ET was carried out 4 or 5 days after ovulation. In retrospective studies where many more ICSI-produced embryos were transferred to recipient mares on days 3–6 after ovulation, the best pregnancy rates were obtained when ET was performed on Day 4 (Cuervo-Arango et al., 2019a; Cuervo-Arango et al., 2019c), suggesting that the

mare's uterine environment and stage of IVP embryo development are optimally synchronized on that day. The success of equine ET is influenced by multiple factors, including other recipient mare factors such as age and uterine tone (Carnevale et al., 2000; Donato et al., 2023). Given the multitude of factors at play, the relatively small number of embryos transferred to recipient mares in the present study likely precluded the detection of any differences between groups.

3.5 Conclusions

In conclusion, the results show that pre-IVM treatment with cAMP modulators for 4 or 18 h did not enhance the quality of equine oocytes, as the rates of oocyte maturation, couplet fusion, embryonic cleavage, and development to the blastocyst stage did not differ significantly from those of the Control group. Indeed, the rates of blastocyst formation tended to be lower in the Pre-IVM 4h group, compared with the Control group. Following the transfer of blastocysts to recipient mares, four cloned foals were generated, including three from embryos produced using oocytes treated with the cAMP modulators, demonstrating for the first time that this treatment is compatible with full term development in horses. Furthermore, the four cloned foals were derived from four different cell lines, demonstrating the reliability of the SCNT methods used. While effects on pregnancy outcomes due to the various factors analyzed (pre-IVM treatment, donor cell line, embryo grade, and recipient mare's day post-ovulation) were not detected, transferring greater numbers of SCNT embryos may reveal significant differences. Regardless, as the cAMP modulators exerted no beneficial effects under the pre-IVM conditions described here, the findings do not support the use of the so called SPOM system for equine oocyte maturation, adding to the controversy in this area. Further

studies are needed to evaluate the merit of biphasic IVM approaches that modulate cAMP levels during equine oocyte maturation.

CHAPTER 4

Nicotinic acid treatment improves the developmental potential of equine oocytes for cloned embryo production

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May 2026

As supervisor for the candidature upon which this thesis is based, I confirm that the authorship attribution statement above is correct.

Christopher G. Grupen

May 2026

Abstract

Nicotinic acid (NA) treatment during in vitro maturation (IVM) has been shown to elevate nicotinamide adenine dinucleotide (NAD^+) levels and improve oocyte developmental competence. Suboptimal equine oocyte IVM systems currently limit the efficiency of viable embryo in vitro production. This study evaluated NA supplementation during IVM for cloned equine embryo production, using oocytes from abattoir-sourced ovaries and live mares via ovum pick-up (OPU). Abattoir-derived oocytes (n = 694) were treated without or with 50 or 200 μM NA during the 18 h holding period (Pre-IVM). Next, OPU-derived oocytes (n = 147) received either no treatment or 200 μM NA during Pre-IVM. Additionally, 285 OPU-derived oocytes were treated with 200 μM NA during Pre-IVM and then either without or with 200 μM NA during the final 22–24 h of IVM. While different cell lines provided the donor nuclei, all experimental groups used the same cell line in each replicate. In abattoir-derived embryos, the Pre-IVM NA treatment increased blastocyst rates compared with the control (50 μM: 27.1 ± 1.4%; 200 μM: 32.9 ± 3.0%; control: 19.9 ± 1.7%; $P < 0.05$). In OPU-derived embryos, the Pre-IVM NA treatment had no effect ($P > 0.05$), but NA supplementation during IVM improved the blastocyst rate (53.4 ± 9.6% vs 31.3 ± 8.1%; $P < 0.05$). The rates of nuclear maturation, couplet fusion, and cleavage were not influenced by NA supplementation. These results show that NA treatment during Pre-IVM and IVM enhanced equine oocyte developmental potential. Further research is needed to clarify the underlying mechanisms and assess embryo viability post-transfer.

4.1 Introduction

The complete acquisition of oocyte developmental competence is essential for successful fertilization, the development of viable embryos, and the birth of healthy offspring (Gilchrist & Thompson, 2007). Consequently, the in vitro production of embryos via somatic cell nuclear transfer (SCNT) or intracytoplasmic sperm injection (ICSI) is largely constrained by the effectiveness of the oocyte in vitro maturation (IVM) system used (Hinrichs, 2018; Luciano et al., 2018). In horses, embryo in vitro production is further constrained, compared with other livestock species, by the limited access to slaughterhouse ovaries, which are typically from mares of advanced maternal age, and are approaching reproductive senescence (Carnevale, 2008; Derisoud et al., 2021). Alternatively, equine oocytes can be collected from live mares by ovum pick-up (OPU), which effectively overcomes the lack of an efficient superovulation treatment (Morris, 2018; Squires & McCue, 2007). Also, many mares are bred into their older years, when the quantity and quality of their oocytes are declining (Cuervo-Arango et al., 2019b; Fonte et al., 2024). While the OPU technique has developed to the stage where highly trained practitioners can consistently harvest COCs, the numbers recovered can vary greatly between mares and collections, such that acceptable embryo yields may not be obtained from each oocyte retrieval (Cuervo-Arango et al., 2019b; Hinrichs, 2018). Hence, a major focus of research efforts in this area is to improve the success of equine IVM systems to make the most of the precious few oocytes obtained.

Numerous factors have been identified that influence the acquisition of oocyte developmental competence, including those related to energy metabolism, cell survival, and DNA repair (Baldini et al., 2024; Conti & Franciosi, 2018). An essential cofactor

implicated in many cellular processes during oocyte maturation is nicotinamide adenine dinucleotide (NAD^+) (Pollard et al., 2022a). Recent studies carried out in murine oocytes have shown that treatments that promote the production of NAD^+ can enhance mitochondrial function, reduce DNA damage, suppress apoptosis, and improve oocyte developmental competence, especially in cases of maternal and oocyte aging (Bertoldo et al., 2020; Miao et al., 2020; Wang et al., 2018; Wu et al., 2019). These processes are largely driven by NAD^+-dependent enzymes, including sirtuins (SIRTs) and poly-ADP-ribose polymerases (PARPs) (Pollard et al., 2022b). During meiosis, SIRT1 associates with spindle microtubules and facilitates cytoskeletal remodelling and epigenetic reprogramming, which are essential for establishing correct gene expression patterns in the resulting embryo (Nevoral et al., 2024; Nevoral et al., 2019). Synthesis of NAD^+ can occur through the catalytic conversion of the amino acid tryptophan (Trp) via the *de novo* synthesis pathway, recycling of NAD^+ metabolites via the salvage pathway, and metabolism of nicotinic acid via the Preiss-Handler pathway (Pollard et al., 2022b).

As a precursor of NAD^+, nicotinic acid (NA), also known as niacin or vitamin B3, plays a crucial role in a myriad of cellular processes (Covarrubias et al., 2021). In addition, NA has antioxidative and cell membrane protection properties (Lee et al., 2019; Tupe et al., 2011). Supplementation of IVM medium with NA has been reported to increase the rate of nuclear maturation in bovine and porcine oocytes (Kafi et al., 2019; Pollard et al., 2021b) and reduce the frequency of meiotic spindle defects in aged murine oocytes (Wu et al., 2019). It is worth noting that aberrant nuclear maturation and chromosome segregation errors associated with impaired meiotic spindle assembly are hallmarks of the maternal age-related decline in equine oocyte quality (Rizzo et al., 2019). To date, the effect of NA supplementation on the maturation of equine oocytes has not been reported.

Having successfully established a commercial equine breeding program that incorporates embryo in vitro production by SCNT, a major priority is to improve the effectiveness of the IVM system being used. The procedures and conditions applied in our laboratory to produce cloned equine embryos have been described previously (Cortez et al., 2023; Cortez et al., 2025), with embryos from OPU-derived oocytes found to have superior in vivo viability after transfer to recipient mares compared with embryos from abattoir-derived oocytes (Cortez et al., 2023). In the Australian context, access to ovaries from slaughtered mares is extremely limited, making the collection of oocytes from live mares by OPU highly preferable for commercial cloned embryo production. Additionally, refinement of the SCNT procedure has been informed by the finding that bone marrow mesenchymal stem cells (MSCs) are more efficiently reprogrammed compared with adult fibroblast cells (AFCs) (Olivera et al., 2018), which has led to their use as the preferred nuclear donor cell type.

Therefore, the aim of this study was to determine the effect of supplementing the oocyte maturation medium with NA on the capacity of equine oocytes to support cloned embryo production. As equine oocyte IVM typically involves a prolonged transport period (18 h) prior to maturation at the laboratory (22–24 h), NA supplementation during the transport period was first examined. After determining the optimal NA dose using oocytes from abattoir-sourced ovaries, the effect of this NA treatment was then assessed using oocytes harvested from live mares by OPU. Lastly, the effect of supplementing the final IVM medium with NA was evaluated using OPU-derived oocytes. Following oocyte maturation, SCNT embryos were produced using either MSCs or AFCs to compare the in vitro development of the different treatment groups.

4.2 Materials and methods

4.2.1 Mares

The study was performed at the Catalina Equine Reproduction Centre (North Richmond, NSW, Australia). A total of 23 standardbred mares, aged 3 to 15 years, were used as oocyte donors. All procedures were carried out with informed consent from the owners of the animals and in accordance with the NSW Animal Research Act (1985), which incorporates the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

4.2.2 Chemicals and media

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Australia). Hepes-buffered Synthetic Oviductal Fluid (H-SOF) (Thompson et al., 1990) supplemented with 10% fetal calf serum (FCS; AU-FBS/PG; Cellsera, Rutherford, NSW, Australia) was used for procedures performed outside of the CO_2 incubator. The medium used to transport and hold oocytes prior to IVM consisted of a 1:1 mix of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; D8437) and Medium 199 (M3769) supplemented with 10% FCS. A stock solution of 50 mM NA (N4126) was prepared by dissolving NA powder in ultra-pure water and 20 µL aliquots were stored at –20°C until use. The oocyte maturation medium and the couplet fusion medium were prepared as described previously (Cortez et al., 2023).

4.2.3 Retrieval of immature oocytes from ovaries collected post-mortem

Equine ovaries were obtained and processed at a slaughterhouse. The recovery of cumulus-oocyte complex (COC) from abattoir-sourced ovaries was performed as described previously (Cortez et al., 2023). Briefly, antral follicles <30 mm in diameter were aspirated with an 18G needle and the follicle walls were scraped extensively with a bone curette. Following transfer to a 100 mm Petri dish containing H-SOF at 37°C to facilitate searching, selected COCs were washed three times with H-SOF. In each replicate, ovaries were collected within a 2 h period and processed over an additional 2 h period, such that the maximum time from slaughter to transferring the retrieved oocytes to vials of transport medium was approximately 4.5 h. A total of 694 COCs were collected in 6 replicates (Experiment 1). In each replicate, the COCs were randomly allocated to one of three vials filled with transport medium at 20–22°C. In Experiment 1, the vials contained transport medium that was either unsupplemented or supplemented with 50 or 200 μM nicotinic acid (NA). These concentrations of NA were selected based on the findings of Pollard and co-workers (Pollard et al., 2021b). The vials were tightly capped and the COCs were transported to the laboratory in a polystyrene container at 20–22°C overnight, for IVM 18 h after COC recovery.

4.2.4 Retrieval of immature oocytes by ovum pick up (OPU)

The OPU procedure was performed as described previously (Cortez et al., 2023). The day before OPU, mares were scanned transrectally using an ultrasound machine (Mindray M9; Mindray, Shenzhen, China) equipped with a 5–8 MHz linear-array transducer

(6LE5Vs) to determine the number and size of the follicles present on the ovaries. Only mares that had at least 15 follicles, with the largest follicle <25 mm in diameter, were scheduled for OPU. In a total of 15 sessions, OPU was performed once a week on 2 or 3 scheduled mares. Some of the mares underwent OPU on multiple occasions with a minimum of two weeks between sessions.

In preparation for OPU, initial sedation consisted of 6 mg butorphanol tartrate i.v. (Butorgesic; 10 mg/mL; Troy Animal Health, Glendenning, NSW, Australia) and 4 mg detomidine hydrochloride i.v. (Sedator; 10 mg/mL; Randlab Australia, Revesby, NSW, Australia), administered in the stall regardless of body weight. Mares were then moved to stocks, where the rectum was emptied and the perineum cleansed with 2% chlorhexidine solution and water. The vaginal vestibule was scrubbed using sterile saline-soaked cotton wool. A sterile 22 Fr Foley catheter was inserted to empty the bladder and remained in place throughout the OPU procedure. After placement of a sterile IV catheter, mares received pre-medication consisting of 1.1 mg/kg flunixin meglumine i.v. (Ilium Flunixil, 50 mg/mL, Troy Animal Health), 22 mg/kg procaine benzylpenicillin i.m. (Benacillin, 300 mg/mL, Troy Animal Health), and 6.6 mg/kg gentamicin sulphate i.v. (Ilium Gentam, 100 mg/mL, Troy Animal Health). Approximately 5 min before commencing OPU, mares were given 0.1 mg/kg butylscopolamine bromide i.v. (Buscopan Compositum, Boehringer Ingelheim, NSW, Australia), with additional doses administered as required to reduce rectal straining. Immediately before starting OPU, a second sedation bolus (4 mg butorphanol, 3 mg detomidine) was administered via the IV catheter. Additional 2 mg detomidine boluses were administered as needed to maintain adequate sedation.

Immature oocytes were harvested by transvaginal ultra-sound guided aspiration of follicles 5 to 30 mm in diameter using a 12G double-lumen needle attached to a vacuum pump. After aspirating the follicular fluid, each follicle was flushed 10 times with 0.5–5 mL (depending on follicle size) of embryo flushing medium (BoviFlush; Minitube Australia Pty. Ltd., Smythesdale, VIC, Australia) supplemented with sodium heparin (2.5 IU/mL, Pharma, Denmark) and pre-warmed to 37°C. The follicular fluid and lavage medium were collected in 500 mL flasks kept at 37°C. The collected fluids were poured through a sterile embryo collection filter (EmCon filter; Immuno Systems Inc., Spring Valley, WI, USA) immediately after the end of the OPU procedure, and the residual fluid and follicular material were then rinsed into a sterile Petri dish. Subsequently, COCs were identified using a stereomicroscope, washed three times with H-SOF, and transferred to vials filled with transport medium at 20–22°C. Depending on the experimental design, the vials contained transport medium that was either unsupplemented or supplemented with 200 μM NA. The vials were tightly capped and the oocytes were kept overnight in a polystyrene container at 20–22°C, for IVM 18 h after COC recovery.

For each mare, the duration of the OPU procedure was 15 to 25 min. Following OPU, mares were moved to a box stall and monitored until fully recovered, remaining under observation for at least 48 h. During the two days after OPU, a daily physical examination was performed, and mares received flunixin meglumine 1.1 mg/kg i.v. SID, procaine benzylpenicillin 22 mg/kg i.m. BID, and gentamicin sulphate 6.6 mg/kg i.v. SID. If no abnormalities were detected, mares returned to pasture at the beginning of the third day after OPU.

4.2.5 In Vitro Maturation (IVM)

At the end of the 18 h transport and holding period (Pre-IVM), the COCs of each group were washed 3 times with H-SOF, and then transferred to wells containing 500 µL of pre-equilibrated maturation medium (maximum of 20–50 COCs per well). The oocyte maturation medium consisted of DMEM/F-12 medium supplemented with 0.1 IU/mL follicle-stimulating hormone and 0.1 IU/mL luteinizing hormone (Menopur, Ferring Pharmaceuticals, Copenhagen, Denmark), 50 ng/mL epidermal growth factor (EGF; E9644), 1 mM sodium pyruvate, insulin-transferrin-sodium selenium mixture (ITS; I1884), and 10% FCS. Depending on the experimental design, the maturation medium was either unsupplemented or supplemented with 200 µM NA. The COCs were incubated for 22–24 h at 38°C in a humidified atmosphere of 5% CO^2 in air.

4.2.6 Nuclear donor cell culture

A total of 17 different cell lines were derived from the tissues of 17 adult horses, 5 male and 12 female. For the establishment of primary adult fibroblast cell (AFC) lines, seven skin biopsies were obtained and processed as previously described (Cortez et al., 2024). Mesenchymal stem cells (MSCs) were isolated from ten sternal bone marrow aspirates following the protocol described by Sellon (Sellon, 2006). Briefly, horses were intravenously sedated with 0.01 mg/kg detomidine hydrochloride (10 mg/mL; Randlab Australia) and 0.01 mg/kg butorphanol tartrate (10 mg/mL; Troy Animal Health). The region between the 4^{th} and 5^{th} sternebrae was identified by ultrasonography, clipped, and surgically prepared. Approximately 6 mL of lignocaine hydrochloride (20 mg/mL; Troy

Animal Health) was infiltrated through the subcutis, muscle layers, and into the periosteum to provide local anesthesia. A stab incision was made using a #10 surgical blade and ~20 mL of bone marrow was aspirated using a 13-gauge 5 cm Jamshidi aspiration needle (BD; Becton Dickinson, NJ, USA) under negative pressure. Aspirates were collected into sodium citrate-containing vacutainers and transported to the laboratory at 5°C. At the laboratory, the samples were centrifuged at 1000 × g for 3 min, treated to selectively lyse erythrocytes (Gudleviciene et al., 2015), and washed twice using centrifugation under the same conditions (1000 × g for 3 min). The resulting cell pellet was resuspended with 5 mL of cell culture medium and the cell suspension was seeded in culture flasks. Both AFCs and MSCs were maintained in medium consisting of DMEM/F-12 medium supplemented with 1 mM glutamine, 0.2 mM pyruvate, 10 ng/mL EGF, and 10% FCS, and cultured in a humidified atmosphere of 5% CO_2 in air at 38.5°C. After 4–7 days, adherent cells were subcultured and expanded until reaching optimal confluency for cryopreservation in aliquots of culture medium containing 10% dimethyl sulfoxide (DMSO) (Cortez et al., 2023). All MSCs had a fibroblastic, spindle-like morphology characteristic of MSCs (Violini et al., 2009), with the expanded cell populations appearing homogeneous in all cases.

4.2.7 Somatic cell nuclear transfer (SCNT)

A different donor cell line was used in each of the six replicates of Experiments 1 and 2 (one cell line was used once in both experiments). In Experiment 3, seven different donor cell lines were used in the nine replicates, such that two of the cell lines were used twice (one of these had also been used once in Experiment 1). After thawing, the cells were cultured for no less than two days and grown to confluence to induce cell cycle arrest at

least 24 h before use as donor cells for SCNT. Immediately before SCNT, the cells were trypsinized, washed, and suspended in H-SOF containing 2% FCS, and kept at room temperature (RT) until use (approximately 10 min).

The cumulus cells were removed from the oocytes after IVM by gentle pipetting in H-SOF supplemented with 1 mg/mL hyaluronidase. The micromanipulation process involved the removal of the polar body and the metaphase plate from each oocyte using an enucleation pipette attached to a Piezo drill (PMAS-CT150; Prime Tech Ltd., Ibaraki, Japan), and placement of a donor cell in the perivitelline space of each cytoplast. The couplets were held for 1 h in H-SOF at 38°C, before being transferred to fusion medium, which contained 3.0 M D-sorbitol, 0.05 mM $CaCl_2$, 0.10 mM $MgCl_2$ and 0.05% (w/v) fatty acid-free bovine serum albumin (BSA; 700-102P; Gemini Bio-Products, West Sacramento, CA, USA), on a fusion chamber slide between electrodes 0.5 mm apart. A direct current (DC) pulse of 2.2 kV/cm strength and 15 μsec duration was immediately applied to the couplets. The pulsed couplets were washed 3 times in H-SOF, transferred to embryo culture medium, which consisted of DMEM/F-12 medium supplemented with 10% FCS, and incubated for 2 h. Activation was carried out by exposing the fused couplets to 5 μM ionomycin in H-SOF for 5 min. After several washes in H-SOF, the fused and activated couplets were treated with 1 mM 6-dimethylaminopurine and 5 μg/mL cycloheximide in embryo culture medium for 4 h. Finally, the reconstructed embryos were washed several times, transferred to droplets of culture medium, and incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. The SCNT embryos were transferred to fresh culture medium every 2 days. Embryonic cleavage was assessed at the first change of culture medium and blastocyst development was assessed

on Days 7 and 8 of in vitro culture, using well described morphological features to identify blastocyst features (Carnevale & Metcalf, 2019).

4.2.8 Experimental design

In Experiment 1, in which abattoir-derived oocytes were used, the recovered COCs were randomly allocated to tubes containing transport medium that was either unsupplemented (Control) or supplemented with 50 or 200 μM nicotinic acid (NA). After the 18 h transport and holding period (Pre-IVM), the COCs of the three groups were transferred to wells of IVM medium (containing no NA supplement). In Experiments 2 and 3, OPU-derived oocytes were used. In Experiment 2, the recovered COCs were randomly allocated to tubes containing transport medium that was either unsupplemented (Control) or supplemented with 200 μM NA. After the 18 h transport and holding period (Pre-IVM), the COCs of the two groups were transferred to wells of IVM medium (containing no NA supplement). In Experiment 3, all the recovered COCs were transferred to tubes containing transport medium supplemented with 200 μM NA. After the 18 h transport and holding period (Pre-IVM), the COCs were randomly allocated to wells of IVM medium that was either unsupplemented (Control) or supplemented with 200 μM NA. Following the IVM period in all three experiments, the meiotically mature oocytes of each group were used to produce SCNT embryos. Experiments 1, 2, and 3 were replicated 6, 6, and 9 times, respectively.

4.2.9 Statistical analysis

Data were analyzed using the Genstat statistical software package (18th edition; VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The embryo in vitro production data (oocytes matured, couplets fused, embryos cleaved, and blastocysts formed) were subjected to logistic regression analysis with treatment group, donor cell line, and donor cell type as factors. A P value of less than 0.05 designated a significant difference.

4.3 Results

4.3.1 Experiment 1: The Effect of Nicotinic Acid Supplementation During Pre-IVM on Abattoir-Derived Oocytes

A total of 694 oocytes were collected from abattoir-sourced ovaries in six replicates (mean of 115.7 oocytes per replicate). As shown in Table 4.1, the oocyte maturation rates did not differ significantly among the groups (control: 52.6%; 50 μM NA: 58.8%; 200 μM NA: 58.6%; $P > 0.05$). From the 391 MII-stage oocytes obtained, a total of 300 SCNT donor cell-ooplast couplets were constructed. The rates of couplet fusion and embryonic cleavage did not differ significantly among the groups ($P > 0.05$; Table 1). Treatment with 200 μM NA during Pre-IVM increased the proportion of fused couplets that formed blastocysts (32.9% vs 19.9%; $P < 0.05$), and the proportion of cleaved embryos that formed blastocysts (50.0% vs 30.7%; $P < 0.05$), compared with the control group (Table 1). Of fused couplets, the blastocyst formation rate of the 50 μM NA group was also superior to that of the control group (27.1% vs 19.9%; $P < 0.05$; Table 4.1). Blastocysts

were produced in all six replicates in the control, 50 μM NA, and 200 μM NA groups (mean of 3.0, 3.3, and 5.6 blastocysts per replicate, respectively).

Of the cell lines used to provide the donor nuclei for SCNT in Experiment 1, three were AFC lines (#1.3, #1.4, #1.6), and three were MSC lines (#1.1, #1.2, #1.5). No significant interaction between cell line and treatment was found. As shown in Figure 4.1, couplets constructed using two of the cell lines (#1.3 and #1.4) achieved a 100% fusion rate, which was significantly greater than that attained by couplets constructed using the other four cell lines (range of 82.4% to 86.4%). There was no effect of cell line on the rates of cleavage and blastocyst formation ($P > 0.05$; Figure 1). The fusion rate was greater for AFC-derived couplets than for MSC-derived couplets (94.8 ± 3.0% vs 84.6 ± 2.3%; $P < 0.05$), while the cleavage (70.1 ± 3.0% vs 63.1 ± 3.5%) and blastocyst formation (27.9 ± 3.3% vs 25.3 ± 1.3%) rates did not differ due to donor cell type ($P > 0.05$).

4.3.2 Experiment 2: The Effect of Nicotinic Acid Supplementation During Pre-IVM on OPU-Derived Oocytes

A total of 147 oocytes were collected by OPU in six replicates (mean of 24.5 oocytes per replicate). As shown in Table 4.2, the oocyte maturation rates did not differ significantly between the groups (Control: 63.9%; 200 μM NA: 54.9%; $p > 0.05$). From the 88 MII-stage oocytes obtained, a total of 81 SCNT donor cell-ooplast couplets were constructed. The rates of couplet fusion, embryonic cleavage, and blastocyst formation did not differ significantly between the groups ($p > 0.05$; Table 2). Blastocysts were produced in all six replicates in the control and 200 μM NA groups (mean of 2.2 and 2.3 blastocysts per replicate, respectively).

Of the cell lines used to provide the donor nuclei for SCNT, one was an AFC line (#2.2), and five were MSC lines (#2.1, #2.3, #2.4, #2.5, #2.6). No significant interaction between cell line and treatment was found. There was no effect of cell line on the rates of fusion, cleavage, and blastocyst formation ($P > 0.05$; Figure 2). Also, the rates of fusion (100 ± 0% vs 85.3 ± 5.0%), cleavage (64.4 ± 24.4% vs 80.2 ± 4.2%), and blastocyst formation (47.8 ± 7.8% vs 39.7 ± 5.9%) did not differ due to donor cell type ($P > 0.05$).

4.3.3 Experiment 3: The Effect of Nicotinic Acid Supplementation During IVM on OPU-Derived Oocytes

A total of 285 oocytes were collected by OPU in nine replicates (mean of 31.7 oocytes per replicate). As shown in Table 3, the oocyte maturation rates did not differ significantly between the groups (Control: 71.3%; 200 μM NA: 71.5%; $p > 0.05$). From the 200 MII-stage oocytes obtained, a total of 177 SCNT donor cell-ooplast couplets were constructed. The rates of couplet fusion and embryonic cleavage did not differ significantly between the groups ($p > 0.05$; Table 4.3). Treatment with 200 μM NA during IVM increased the proportion of fused couplets that formed blastocysts (41.0% vs 24.5%; $p < 0.05$), and the proportion of cleaved embryos that formed blastocysts (53.4% vs 31.3%; $p < 0.05$), compared with the control group (Table 4.3). Blastocysts were produced in all nine replicates in the control and 200 μM NA groups (mean of 2.6 and 3.3 blastocysts per replicate, respectively).

Of the seven cell lines used to provide the donor nuclei for SCNT, four were AFC lines (#3.4, #3.5, #3.6, #3.7), and three were MSC lines (#3.1, #3.2, #3.3). No significant

interaction between cell line and treatment was found. There was no effect of cell line on the rates of couplet fusion and embryonic cleavage ($P > 0.05$; Figure 3). Also, the rates of fusion (92.4 ± 2.6% vs 84.2 ± 4.0%) and cleavage (83.3 ± 5.1% vs 78.4 ± 4.8%) did not differ due to donor cell type ($P > 0.05$). However, the rates of blastocyst formation differed significantly due to the donor cell line used and the donor cell type. Couplets constructed using cell line #3.3 formed blastocysts at a greater rate than those constructed using cell lines #3.4 and #3.6 ($P < 0.05$; Figure 3). Moreover, the blastocyst formation rate was greater for MSC-derived couplets than for AFC-derived couplets (43.5 ± 5.9% vs 24.1 ± 6.0%; $P < 0.05$).

Table 4.1. Effect of nicotinic acid (NA) supplementation during pre-IVM of abattoir-derived oocytes on the in vitro production of cloned embryos. Percentage values are expressed as the mean ± s.e.m.

	Control	50 μM NA	200 μM NA
Total COCs	245	189	260
Oocytes matured/COCs (%)	129/245	111/189	151/260
	(52.6 ± 4.4)	(58.8 ± 4.4)	(58.6 ± 3.4)
Total couplets constructed	104	82	114
Couplets fused/constructed (%)	91/104	74/82	103/114
	(87.8 ± 4.5)	(90.8 ± 3.5)	(90.5 ± 4.0)
Embryos cleaved/fused (%)	58/91	50/74	69/103
	(65.1 ± 2.0)	(67.9 ± 4.4)	(66.9 ± 6.0)
Blastocysts formed/fused (%)	18/91	20/74	34/103
	(19.9 ± 1.7)[a]	(27.1 ± 1.4)[b]	(32.9 ± 3.0)[b]
Blastocysts formed/cleaved (%)	18/58	20/50	34/69
	(30.7 ± 2.6)[a]	(40.1 ± 1.0)[ab]	(50.0 ± 4.1)[b]

[a,b]Within rows, values without a common letter differ significantly ($P < 0.05$).

Table 4.2. Effect of nicotinic acid (NA) supplementation during pre-IVM of OPU-harvested oocytes on the in vitro production of cloned embryos. Percentage values are expressed as the mean ± s.e.m.

	Control	200 μM NA
Total COCs	73	74
Oocytes matured/COCs (%)	46/73 (63.9 ± 6.4)	42/74 (54.9 ± 5.6)
Total couplets constructed	42	39
Couplets fused/constructed (%)	34/42 (83.8 ± 7.3)	37/39 (91.7 ± 5.3)
Embryos cleaved/fused (%)	28/34 (83.3 ± 4.2)	27/37 (71.9 ± 8.7)
Blastocysts formed/fused (%)	13/34 (39.0 ± 5.8)	14/37 (43.0 ± 8.7)
Blastocysts formed/cleaved (%)	13/28 (46.5 ± 6.3)	14/27 (62.8 ± 13.3)

Table 4.33. Effect of nicotinic acid (NA) supplementation during IVM of OPU-harvested oocytes on the in vitro production of cloned embryos. Percentage values are expressed as the mean ± s.e.m.

	Control	200 μM NA
Total COCs	146	139
Oocytes matured/COCs (%)	102/146 (71.3 ± 3.2)	98/139 (71.6 ± 3.1)
Total couplets constructed	93	84
Couplets fused/constructed (%)	82/93 (89.0 ± 2.9)	73/84 (86.6 ± 4.5)
Embryos cleaved/fused (%)	66/82 (79.6 ± 3.4)	59/73 (81.6 ± 6.2)
Blastocysts formed/fused (%)	21/82 (24.5 ± 6.0)[a]	30/73 (41.0 ± 6.5)[b]
Blastocysts formed/cleaved (%)	21/66 (31.3 ± 8.1)[a]	30/59 (53.4 ± 9.6)[b]

[a,b]Within rows, values without a common letter differ significantly ($P < 0.05$).

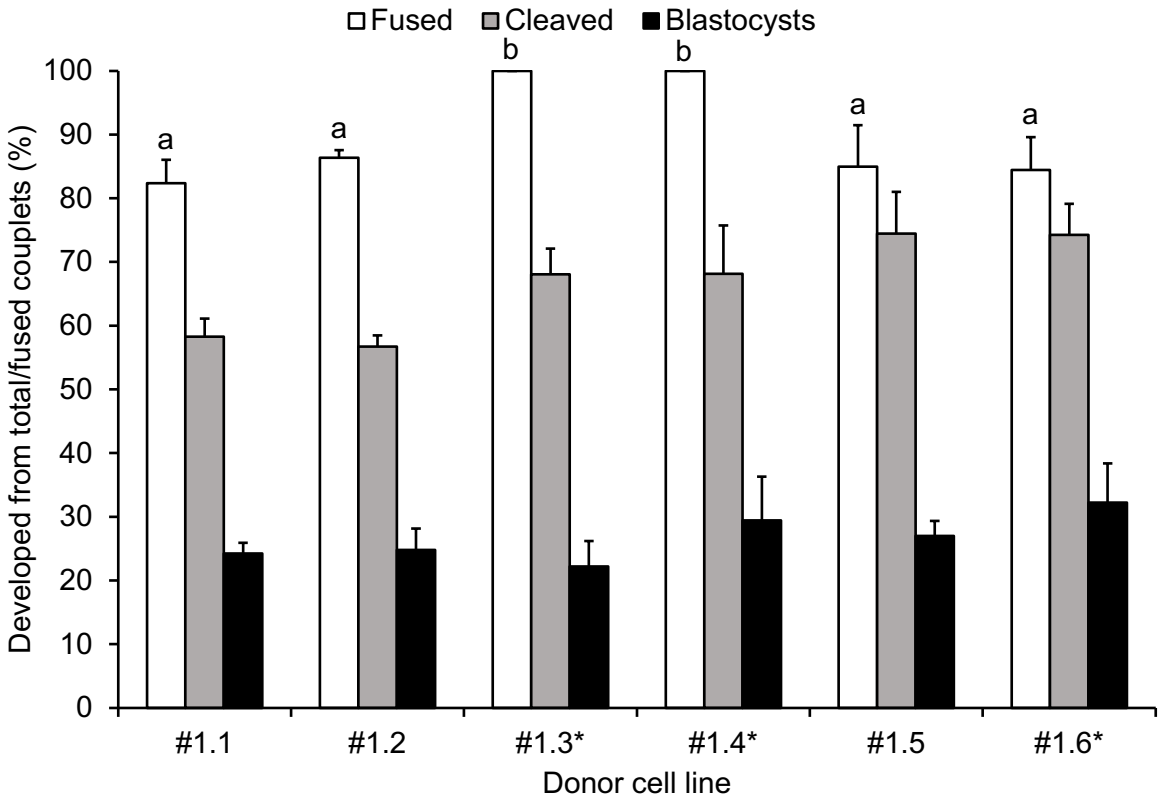

Figure 4.1. The effect of donor cell line (#1.1–#1.6) on the development of SCNT embryos in Experiment 1. Combining the data from the three experimental groups for each cell line, the values are expressed as the percentages (mean ± s.e.m.) of couplets that fused (from total couplets constructed; grey bars), embryos that cleaved (from fused couplets; blue bars), and blastocysts that formed (from fused couplets; orange bars). Bars without a common letter differ significantly ($P < 0.05$)

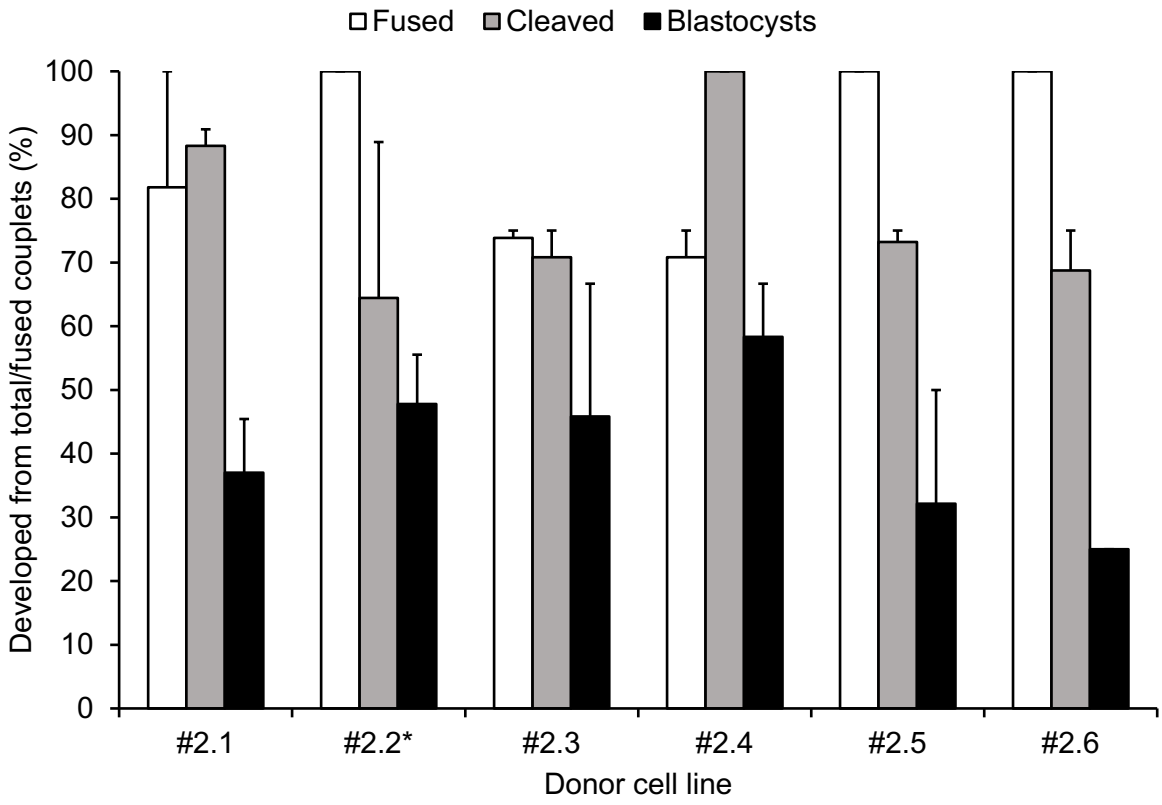

Figure 4.2. The effect of donor cell line (#2.1–#2.6) on the development of SCNT embryos in Experiment 2. Combining the data from the two experimental groups for each cell line, the values are expressed as the percentages (mean ± s.e.m.) of couplets that fused (from total couplets constructed; grey bars), embryos that cleaved (from fused couplets; blue bars), and blastocysts that formed (from fused couplets; orange bars). There were no significant differences among the cell lines.

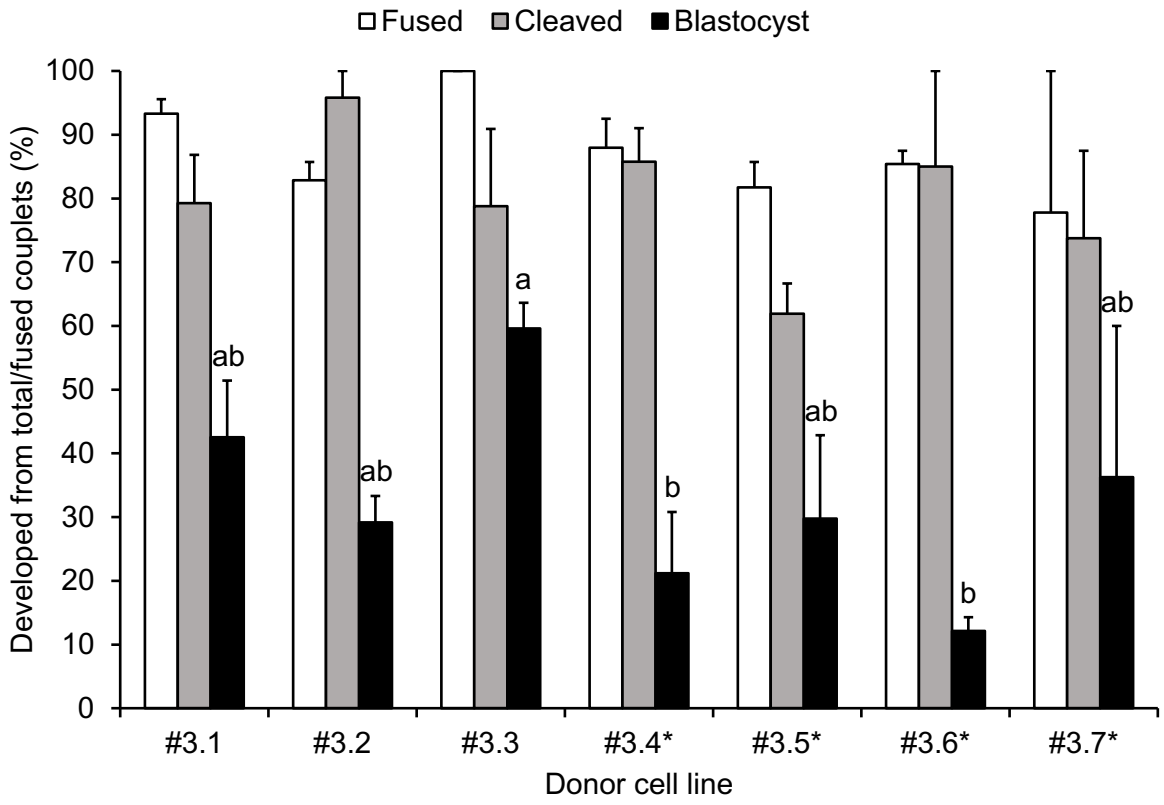

Figure 4.3. The effect of donor cell line (#3.1–#3.7) on the development of SCNT embryos in Experiment 3. Combining the data from the two experimental groups for each cell line, the values are expressed as the percentages (mean ± s.e.m.) of couplets that fused (from total couplets constructed; grey bars), embryos that cleaved (from fused couplets; blue bars), and blastocysts that formed (from fused couplets; orange bars). Bars without a common letter differ significantly ($P < 0.05$).

4.4 Discussion

The results of this SCNT study show that the developmental potential of equine oocytes matured in vitro can be significantly enhanced by supplementing the medium with nicotinic acid (NA). Treatment with NA during the 18 h transport and holding period (Pre-IVM) improved the developmental competence of abattoir-derived oocytes, markedly increasing the proportion of fused couplets that developed to the blastocyst stage. Likewise, treatment with NA during the 22-24 h IVM incubation enhanced the developmental competence of oocytes harvested from live mares by OPU, again, greatly increasing the proportion of fused couplets that developed to the blastocyst stage. Through the consistent production of blastocysts in a commercial equine cloning program, the beneficial effect of NA supplementation on oocyte developmental competence was found to be independent of the donor cell line and the type of cell used to provide the donor nuclei. These findings suggest that the provision of NA during Pre-IVM and/or IVM helps to meet an important metabolic requirement of equine cumulus-oocyte complexes (COCs), thereby increasing the efficiency of embryo in vitro production.

Nicotinic acid is a precursor of NAD^+, which is a key molecule involved in fundamental cellular processes such as energy production, apoptosis regulation, and DNA repair (Chini et al., 2021; Covarrubias et al., 2021). Moreover, the activity of NAD^+-consuming enzymes, including sirtuins (SIRTs) and poly-ADP-ribose polymerases (PARPs), is essential for oocyte maturation (Pollard et al., 2022b). Additionally, NA possesses antioxidant properties and functions as a lipid modulator (Romani et al., 2019), positioning it as a promising candidate treatment to improve oocyte quality. Recently,

reduced intra-oocyte NAD^+ levels have been linked with the age-related decline in female fertility (Bertoldo et al., 2020; Miao et al., 2020; Wu et al., 2019). In female mice of advanced age, treatments that elevated NAD^+ levels were found to improve oocyte developmental competence and pregnancy outcomes (Bertoldo et al., 2020; Miao et al., 2020; Wu et al., 2019). Furthermore, supplementing IVM medium with NA has been found to enhance nuclear and cytoplasmic properties of oocytes in pigs and cattle (Almubarak et al., 2021; Kafi et al., 2019; Pollard et al., 2021a; Pollard et al., 2022a).

Nuclear and cytoplasmic maturation of oocytes must proceed in a coordinated manner to ensure successful fertilization and subsequent embryonic development (Gilchrist & Thompson, 2007; Hinrichs, 2010). In equine oocytes, the time required to complete maturation, characterized by the extrusion of the first polar body, is directly related to the ability of the oocyte to support optimal embryonic development (Rodriguez et al., 2019). A unique feature of equine oocyte IVM systems is a commonly implemented 18 h (overnight) holding period at 20–22°C (Pre-IVM), which facilitates the transport of COCs from the site of collection to the laboratory where IVM is then carried out (Cortez et al., 2025; Hinrichs, 2020; Morris, 2018). In the present study, treatment with NA during the Pre-IVM phase (Experiments 1 and 2) did not influence the subsequent rates of maturation to the MII-stage. Similarly, the nuclear maturation rate of oocytes treated with 200 μM NA during IVM were comparable to that of the control group (Experiment 3). Similar results have been reported in studies with porcine oocytes, where no significant increases in nuclear maturation rates were observed following treatment with NA (Almubarak et al., 2021; Pollard et al., 2022a). In contrast, the addition of NA at a higher concentration (400 μM) during IVM increased nuclear maturation rates in bovine oocytes

(Kafi et al., 2019). The inconsistent nuclear maturation results observed among studies may be due to the different concentrations of NA used.

Regarding the acquisition of oocyte developmental competence, or cytoplasmic maturation, the Pre-IVM NA treatments (50 and 200 μM) enhanced the capacity of abattoir-derived oocytes to develop to the blastocyst stage following SCNT embryo production (Experiment 1). As the benefits to blastocyst production were more apparent at the higher NA dose in Experiment 1, the 200 μM concentration was used to assess the effects of NA on OPU-derived oocytes in Experiments 2 and 3. Interestingly, the Pre-IVM NA treatment did not enhance the capacity of OPU-derived oocytes to form blastocysts (Experiment 2), whereas the IVM NA treatment did (Experiment 3), compared with the untreated controls. The contrasting effect of NA during the pre-IVM period on the differently sourced oocytes suggests that the cytoplasmic deficiencies of abattoir-derived oocytes differ from those of OPU-derived oocytes. This finding is not surprising, given the previously reported effects of oocyte source on the development of equine cloned embryos (Cortez et al., 2023). The intrinsic quality of oocytes would be expected to be poorer from slaughtered mares than from live mares, because slaughtered mares are often older and less fertile, and their estrous cycle phase is unknown, compared with live mares monitored for OPU (Derisoud et al., 2021; Fonte et al., 2024; Vernunft et al., 2013). Further, abattoir-derived oocytes have compromised meiotic competence due to post-mortem changes (Luis-Calero et al., 2025), and their developmental potential declines as the interval between ovary excision and oocyte retrieval increases (Hinrichs, 2010; Martin-Maestro et al., 2020). The variation among the experiments in overall cleavage rates (Experiment 1: 66.6%; Experiment 2: 77.6%; Experiment 3: 80.6%) further highlights the effect of oocyte source on cloned embryo production. Nevertheless,

the oocytes from live mares benefited from the NA treatment when it was applied during the IVM period (Experiment 3). The effect of NA supplementation during IVM on abattoir-derived oocytes warrants additional investigation. Finally, it should be noted that the NA treatments had no effect on the rates of couplet fusion and development to the early cleavage stages in any of the experiments.

These findings are consistent with those from studies in pigs, cattle, and mice, where supplementation of IVM media with NA has been shown to improve the developmental competence of oocytes (Almubarak et al., 2021; Pollard et al., 2021a; Pollard et al., 2022a; Wu et al., 2019). In porcine oocytes from small antral follicles, Pollard et al. (2021c) also found that the addition of 200 μM NA to IVM medium increased the blastocyst formation rate, without affecting the cleavage rate, compared with the control (Pollard et al., 2021a). The conversion of NA to NAD^+ is thought to promote normal chromosome segregation and spindle assembly, because inhibition of the Preiss-Handler pathway during IVM increased the incidence of aberrant metaphase-II spindles in porcine oocytes (Pollard et al., 2022a). Also, NA treatment elevated the NAD^+ content and reduced the frequency of spindle defects in oocytes from old mice (Wu et al., 2019). At a higher concentration (600 μM), NA-enhanced embryo development was associated with higher levels of glutathione (GSH), lower levels of reactive oxygen species (ROS), and lower lipid droplet content, within porcine oocytes (Almubarak et al., 2021). More detailed assessments of equine oocytes are needed to determine the effects of NA treatments during Pre-IVM and IVM on GSH and ROS levels, lipid droplet content, and spindle morphology.

Given that the Pre-IVM NA treatment alone improved the developmental competence of abattoir-derived oocytes, but not OPU-derived oocytes, further investigation is needed to elucidate the specific deficiencies of these oocyte groups. As already mentioned, oocytes from abattoir-sourced ovaries are compromised due to post-mortem changes, and slaughtered mares are often older and less fertile than mares selected for OPU (Derisoud et al., 2021). Studies in mice have demonstrated that NAD^+ levels are lower in oocytes from females of advanced age, and that intra-oocyte NAD^+ levels can be restored by supplementing NAD^+ precursors in vivo, thereby improving fertility (Bertoldo et al., 2020; Miao et al., 2020). Interestingly, studies in mares have shown that oral administration of NA increases the concentrations of several NAD^+ precursors and metabolites in plasma and follicular fluid (Pollard et al., 2022a; Pollard et al., 2021a). Our in vitro findings support the proposal that supplementing the diet with NAD^+-elevating compounds may be a useful strategy to improve oocyte quality and pregnancy outcomes in mares, especially those of advanced maternal age (Pollard et al., 2022a). Moreover, our findings have important implications for the in vitro production of equine embryos using other techniques, such as intra-cytoplasmic sperm injection (ICSI) and conventional in vitro fertilization (IVF). An embryo transfer trial is currently in progress to evaluate the efficacy and safety of the NA treatment during Pre-IVM and IVM on conceptus development and foal health.

In the present study, a total of 17 different cell lines were used to provide the donor nuclei for cloned embryo production. Of these, 7 were AFC lines derived from skin biopsies, and 10 were MSC lines derived from aspirated sternal bone marrow. It is well known that the efficiency of SCNT embryo production is influenced by the donor cell type (Galli & Lazzari, 2021) and can vary considerably between donor cell lines derived from the same

tissue (Liu et al., 2013). Ideally, in a study examining the effects of an oocyte treatment on cloned embryo development, the same donor cell line would be used in all replicates. However, the use of different donor cell lines was unavoidable, due to the commercial imperative of the horse breeding enterprise. Importantly, the same donor cell line was used for all experimental groups in each replicate, and no interactions were detected between treatment and cell line, indicating that the reported effects of the NA treatments were independent of the donor cell line used. An effect of cell type on blastocyst formation was detected in Experiment 3 (replicated 9 times), but not in Experiments 1 and 2 (replicated 6 times each). A previous equine SCNT study has shown that the development of bone marrow MSC-derived embryos is superior to that of AFC-derived embryos (Olivera et al., 2018). In the present study, an inability to consistently detect significant differences due to the donor cell type used can be attributed to a lack of statistical power. Notably, blastocysts were produced in all experimental groups in every replicate, demonstrating the reliability and robustness of the SCNT procedures used.

4.5 Conclusions

These results demonstrate that exposure to 200 μM NA during the Pre-IVM and IVM periods significantly enhanced the developmental competence of equine oocytes. A substantial improvement to blastocyst production was achieved regardless of whether the oocytes were collected from abattoir-sourced ovaries or from live mares by OPU. This is the first study to evaluate the effect of NA supplementation on equine oocytes, and our results align with those from studies in other species, highlighting the positive impact of NAD+-elevating treatments on the acquisition of oocyte developmental competence. These findings pave the way for more detailed investigations into the molecular

mechanisms involved during the maturation of oocytes and inform future refinements to IVM systems that are key to improving the efficiencies and outcomes of assisted reproductive technologies in clinical practice.

CHAPTER 5

General Discussion

5.1 Thesis Summary

The quality of embryos is directly influenced by the quality of the oocytes from which they are derived (Hinrichs, 2018), making it essential to utilize the most efficient in vitro maturation (IVM) system available. Although current IVM systems for equine oocytes have proven to be commercially acceptable, research in other species has identified promising areas for improvement. These include the use of a pre-IVM culture and the application of various treatments during IVM that support oocyte growth and viability. The effectiveness of these pre-IVM and IVM treatments at generating mature oocytes was assessed through a series of experiments. The developmental competence of the oocytes was assessed by producing embryos through conventional somatic cell nuclear transfer (SCNT) procedures. The overall aim of this thesis was to systematically evaluate the effects of the most promising treatments found to improve oocyte quality. To achieve this, the initial analysis aimed to address the gap in knowledge regarding the influence of oocyte origin on embryo production and viability. Subsequently, the effects of pre-IVM treatments using cAMP modulators for different durations were evaluated. Next, the effects of supplementing the pre-IVM and IVM media with nicotinic acid (NA), a precursor of NAD+, were examined. Working within the constraints of a commercial equine breeding operation, all morphologically sound cloned blastocysts were either transferred fresh to recipient mares, or cryopreserved for later transfer. Importantly, the post-transfer outcomes of pregnancy and foaling enabled the ultimate measure of embryo viability to be evaluated.

In Chapter 2, the quality of oocytes obtained from abattoir-sourced ovaries or collected by OPU from live mares was compared. The effects of oocyte origin on embryonic

development have been poorly studied in any species. Few studies have evaluated the capacity of equine oocytes from different sources to support development through the embryonic stages and birth (Choi et al., 2013; Lee et al., 2015). Both oocyte sources have their advantages and limitations. The use of oocytes obtained from the same maternal lineage as the nuclear donor animal prevents the presence of heterogeneous mitochondrial DNA in the foal (Choi et al., 2013). In contrast, oocytes sourced from slaughterhouses typically lack information regarding the breed, age, and reproductive status of the animal. Since mitochondria are associated with health, energy metabolism, and overall performance, studies have highlighted the importance of maintaining maternal lineage continuity (Bray et al., 2009; Engel et al., 2022). Advancements in OPU techniques have enabled the retrieval of an average of 9 to 14 oocytes per mare by aspirating antral follicles ranging from 6 to 30 mm in diameter (Galli et al., 2014; Herrick, 2014). However, significant resources and expertise are required to obtain OPU-collected oocytes, and there are animal welfare considerations associated with the procedure. Oocyte recovery from post-mortem ovaries, achieved through follicular flushing and scraping, yields an average of about eight oocytes per mare (Galli et al., 2007). In laboratories where ovaries from slaughtered mares can be readily accessed, they are the primary source of immature oocytes for embryo generation. However, in recent years, access to a regular and reliable supply of slaughterhouse ovaries has diminished. In countries such as Australia and the United States, the slaughter of mares is an infrequent occurrence, and the importation of oocytes from slaughterhouses is not a viable option due to biosecurity restrictions (Asseged et al., 2012; Nolen, 2006). Despite the various experimental limitations, the results of the retrospective comparison showed that the developmental potential of OPU-collected oocytes was far superior to that of abattoir-derived oocytes. The blastocyst formation rate for OPU-derived embryos was almost 20%

greater than that for abattoir-derived embryos. Furthermore, the in vivo development of abattoir-derived embryos was markedly poorer than that of OPU-derived embryos. The pregnancy losses observed beyond Day 42 of gestation suggest that placental function was impaired to a greater degree in the abattoir-derived conceptuses. Calculated per healthy foal born, approximately 14-fold more abattoir-derived oocytes were needed as starting material for cloned embryo production compared with the OPU-derived oocytes. This highlights the magnitude to which these two oocyte groups differ in their ability to support embryonic development and/or reprogram the donor nucleus to an embryonic state. A surprising finding from this study was that the transfer of vitrified-warmed blastocysts resulted in better pregnancy outcomes than the transfer of fresh embryos. While recipient mares were deemed to be suitable on the day of fresh embryo transfer, it seems that waiting for a mare to be at the ideal stage for receiving an embryo, and then warming a vitrified blastocyst for immediate transfer, is preferable. Therefore, the use of OPU-collected oocytes is highly recommended for the production of healthy cloned foals, with vitrification of blastocysts advised to facilitate transfer when mares are optimal as recipients.

In Chapter 3, equine oocytes obtained from slaughterhouse ovaries were used to evaluate the effects of cAMP modulators during the pre-IVM interval. To this end, the oocytes were exposed to a combined forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX) treatment for periods of 4 or 18 h before initiating IVM. Previous studies in bovine, ovine, caprine, equine, murine, and feline models have used this cAMP-elevating strategy, referred to as Simulated Physiological Oocyte Maturation (SPOM), to promote synchronization of oocyte cytoplasmic and nuclear maturation, which has translated into improved maturation, embryonic development, and, in some cases, birth rate (Leal et al.,

2022). One study in horses showed that SPOM-treated oocytes had improved development to the blastocyst stage compared with oocytes of the control group (Metcalf et al., 2020). However, some studies have reported no improvement in oocyte quality following the application of the SPOM protocol (Leal et al., 2022). In our study, exposure to the cAMP modulators during pre-IVM exerted no beneficial effects; in fact, the 4 h exposure tended to reduce the rate of blastocyst formation compared with the control group. Interestingly, an improvement in oolemma plasticity during manipulation and couplet fusion was evident, which moderated oocyte loss during these phases of the SCNT process. The inconsistent effectiveness of the SPOM system remains a quandary. The discrepancies between studies may be due to the different media used, as the cAMP modulators can interact with various media components, such as serum. Also, the studies often differ in the source of oocytes used and the embryo production methods applied. Importantly, in our study, the cAMP-elevating treatments yielded cloned embryos that gave rise to live foals, demonstrating that full developmental potential was not compromised by the SPOM protocol. Identifying the precise mechanisms through which cAMP-modulating agents promote the generation of equine oocytes with enhanced developmental competence remains an ongoing challenge. In this context, the study presented in Chapter 2 examined the effectiveness of the so called SPOM system, and, under the conditions used, the pre-IVM treatments did not enhance the developmental competence of equine oocytes. This was regardless of the treatment duration used; 18 h, as in the previous equine study by Metcalf et al (Metcalf et al., 2020), or 4 h, similar to that used in numerous cattle studies (Leal et al., 2022).

In Chapter 4, the effects of adding nicotinic acid (NA) to pre-IVM and IVM media on equine oocyte quality were investigated. Nicotinic acid (NA), a biologically active form of niacin, contributes to intracellular NAD^+ biosynthesis primarily through the Preiss–

Handler pathway. In parallel, NAD^+ can also be synthesized via the salvage pathway, in which nicotinamide (NAM) is recycled to NAD^+ through nicotinamide phosphoribosyltransferase (NAMPT), and via the de novo pathway from tryptophan through the kynurenine pathway. Together, these interconnected routes ensure the maintenance of cellular NAD^+ pools necessary for metabolic homeostasis and redox balance (Chini et al., 2021; Ryu et al., 2018).

NAD^+ plays a central role in multiple cellular processes, including mitochondrial energy metabolism, oxidative stress regulation, DNA repair, and cell survival. Beyond its metabolic function, NA exhibits antioxidant, anti-inflammatory, and lipid-modifying properties, which have been associated with improved cellular performance and gamete quality (Bertoldo et al., 2020; Kafi et al., 2019). Disruption of NAD^+ biosynthesis has been linked to severe developmental consequences, as congenital malformations have been reported in NAD^+-deficient humans and animal models. In this context, Shi et al. (2017) demonstrated that niacin supplementation during gestation prevents malformations in NAD^+-deficient mice, underscoring the critical requirement for adequate NAD^+ availability during early development.

At the oocyte level, NAD^+ functions as a key metabolic regulator by serving as a substrate for NAD^+-dependent enzymes, including sirtuins and poly(ADP-ribose) polymerases (PARPs). These enzymes are directly involved in chromatin remodeling, maintenance of genomic integrity, mitochondrial function, and regulation of meiotic progression, all of which are essential for successful oocyte maturation and the acquisition of developmental competence (Covarrubias et al., 2021; Pollard et al., 2022b). Collectively, these pathways provide a strong biological framework supporting the investigation of NA

supplementation as a strategy to modulate NAD^+ metabolism and improve oocyte quality and subsequent embryonic development.

Consequently, there has been a surge of research activity over recent years studying the impacts of NAD+-precursor supplementation to enhance oocyte quality and fertility, particularly in females of advanced reproductive age (Almubarak et al., 2021; Bertoldo et al., 2020; Miao et al., 2020; Wu et al., 2019). However, there is limited information regarding the effects of NAD+ precursors on oocyte quality in mares. In this study, the effects of NA supplementation during pre-IVM and IVM were examined in three experiments. In the first experiment, using oocytes obtained from slaughterhouse ovaries, pre-IVM medium contained different concentrations of NA (0, 50, and 200 μM) to determine the optimal concentration. While there were no differences between the groups in the rates of subsequent nuclear maturation, couplet fusion, and embryonic cleavage, the NA-treated groups developed to the blastocyst stage at higher rates compared with the control group (control: 19.9%; 50 μM NA: 27.1%; 200 μM NA: 32.9%). As the greatest improvement was observed in the 200 μM NA group, this NA concentration was used in the second experiment, which evaluated the effect of NA addition to pre-IVM medium in OPU-harvested oocytes. Disappointingly, no significant differences were found between the NA-treated and control groups in any of the embryo production parameters assessed. In the third experiment, OPU-derived oocytes exposed to 200 μM NA during the pre-IVM phase were subsequently treated with or without 200 μM NA during the IVM phase. Again, no significant differences were found between the NA-treated and control groups in the rates of nuclear maturation, couplet fusion, and embryonic cleavage. However, of the embryos that cleaved, the incidence of blastocyst formation was vastly improved by the NA treatment (control: 31.3%; 200 μM NA: 53.4%). These results provide compelling evidence that supplementing pre-IVM and

IVM media with NA has a positive impact on equine oocyte developmental potential. Additionally, the beneficial effects of NA supplementation during pre-IVM were clearly apparent in abattoir-derived oocytes, but not in OPU-derived oocytes, highlighting the disparate competencies of these two oocyte populations. The precise action of NA in stimulating improvements to cytoplasmic factors that support embryonic development and/or the reprogramming of donor nuclei remains to be determined. Overall, the study presented in this chapter successfully achieved its objective of determining the effect of NA supplementation on the developmental capacity of equine oocytes for cloned embryo production. These findings represent an exciting advance in the quest to improve equine embryo in vitro production.

5.2 Conclusions

In conclusion, the findings of this thesis expand our understanding of the influence of oocyte origin on the capacity of equine oocytes to form viable embryos and healthy cloned foals. As hypothesized, and consistent with the findings of previous studies (Choi et al., 2013; Lee et al., 2015), oocytes obtained from live mares exhibited far greater developmental potential compared to those retrieved post-mortem. To date, this study is the first to demonstrate that the production of cloned foals is vastly more efficient in terms of healthy offspring born, when the oocytes are collected by OPU from well maintained and reproductively healthy mares. These findings expose the deficiencies of abattoir-sourced oocytes and endorse the use of OPU to provide a sustainable source of high-quality oocytes, particularly in a context where access to slaughterhouse ovaries is becoming increasingly restricted in many countries, including Australia. This study also highlighted the importance of vitrification as a tool to ensure pregnancy success. Equine

embryo vitrification has advanced to the point where the post-warming survival rate of early stage blastocysts approaches 100% (Choi & Hinrichs, 2017). Our results show that vitrification does not compromise the in vivo viability of SCNT-produced blastocysts, consistent with the findings of an equine study in which the embryos were produced by OPU-ICSI (Claes & Stout, 2022). In fact, vitrified-warmed blastocyst transfers achieved better pregnancy rates than fresh blastocyst transfers, probably because the former were transferred at the optimal time into carefully selected recipient mares.

Next, the use of the SPOM system, in which cAMP modulators were applied during pre-IVM for short and long durations, was found to exert no benefit on cloned embryo generation. Although previous studies in several species, including horses, have shown the SPOM protocol to be effective in increasing intra-oocyte levels of cAMP and preventing the precocious resumption of meiosis (Leal et al., 2022; Metcalf et al., 2020), the results presented here suggest there was no improvement in the synchrony of nuclear and cytoplasmic maturation. Given the many differences between studies, such as the media formulations, serum use, and embryo production methods, it is difficult to postulate what contributed to the inconsistent results. Hence, the effectiveness of the SPOM system will remain contentious until the interactions of the cAMP modulators with the possible contributing factors are assessed in greater detail.

The improvement in oocyte quality achieved through the addition of nicotinic acid (NA) to pre-IVM and IVM media probably represents the most important research contribution of this thesis. By successfully enhancing equine oocyte developmental competence, which greatly increased the efficiency of cloned embryo production, the central objective of this thesis was accomplished. In the context of cloned embryo production in horses,

the blastocyst formation rates obtained from NA-treated oocytes are among the highest ever reported, regardless of the type of nuclear donor cell used (Olivera et al., 2016; Olivera et al., 2018). To fully understand the effects of NA supplementation on the acquisition of oocyte developmental competence, detailed assessments of oocyte properties must be carried out. Studies in murine and porcine oocytes have revealed that NAD+-elevating agents reduce the frequency of meiotic spindle defects (Pollard et al., 2022a; Wu et al., 2019), which have been implicated in the age-related decline in equine oocyte quality (Rizzo et al., 2019). Therefore, investigating the effects of supplementing media with NA on meiotic spindle assembly in equine oocytes is a priority, especially in those collected from older mares. Additionally, the influence of elevating intra-oocyte levels of NAD+ on the activities of NAD+-dependent enzymes, such as sirtuins and PARPs, should be explored. The use of NAD+-elevating treatments in oocyte IVM systems is gaining increasing attention, and the remarkable findings reported here, the first involving this strategy in equine oocytes, will further stimulate research in this area.

5.3 Future studies

The findings presented in this thesis contribute significantly to our understanding of fundamental aspects of oocyte quality in mares. In the first study, the impacts of oocyte origin on embryonic development were highlighted, with oocytes obtained from live mares exhibiting much greater developmental potential than oocytes recovered from post-mortem collected ovaries. There are several possible reasons why the oocytes from slaughterhouse ovaries are compromised so severely, including the age and reproductive status of the mares from which the ovaries were obtained, deterioration of the oocytes prior to removal from the follicles, and sub-optimal oocyte searching conditions. While

such studies would be difficult to implement, and access to slaughterhouse ovaries is often very limited, identifying the contributing factor(s) would provide useful insights that may lead to this source of oocytes having improved quality.

It is important to highlight that in all the studies the developmental potential of oocytes was assessed through the production of embryos by nuclear transfer. Therefore, the effects observed throughout the studies may involve the donor nucleus reprogramming process in cloned embryos. It is essential to determine whether similar effects on embryo development and in vivo viability are observed when other embryo production techniques such as intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) are employed. Applying the treatments examined in this thesis to ICSI and IVF embryo production systems would clarify their impact on oocyte cytoplasmic maturation and provide a more comprehensive understanding of their effectiveness.

A major limitation of the studies involved the constraints placed upon cloned embryo production by the commercial breeding operation. As the purpose of embryo production was to obtain cloned foals for different clients, each embryo production run used a different cell line to provide the donor nuclei. Also, only adult fibroblast cells, derived from skin biopsies, were used in the studies of Chapters 2 and 3, whereas mesenchymal stem cells, derived from sternal bone marrow, as well as adult fibroblast cells, were used in the Chapter 4 study. It is well established that the donor cell type affects the efficiency of cloned embryo production due to differences in nuclear plasticity (Olivera et al., 2018). Even different cell lines from the same tissue type exhibit differences in their ability to undergo nuclear reprogramming (Liu et al., 2013). To clarify, in all the thesis studies, the same cell line was used for all the experimental groups within each replicate. While some

effects of cell line were observed, as would be expected, analyses showed there were no interaction effects between treatments and cell lines. In other words, the treatments performed consistently across all the cell lines used. Also, as all morphologically normal blastocysts produced were transferred to recipient mares, or vitrified for later transfer, detailed assessment of blastocyst qualities was not possible. Future studies that are not impeded by commercial imperatives could assess blastocyst properties that are associated with viability, such as cell number and the distribution of inner cell mass and trophectoderm cells (Gardner & Schoolcraft, 1999). Alternatively, the developmental kinetics of individual embryos could be monitored non-destructively throughout culture using a time-lapse video system, such as those deployed in human fertility clinics.

Given the improvements achieved with NA supplementation, a follow-up embryo transfer study is being carried out. The blastocysts resulting from the NA experiments were all vitrified, and a number of them have since been transferred to recipient mares to assess their in vivo development. Owing to the long gestation interval in horses, sufficient data from monitoring the pregnancies through to foaling could not be obtained prior to thesis submission. Clearly, the results of this ongoing study will provide important evidence of NA treatment efficacy. As mentioned above, the use of ICSI and IVF procedures for embryo production will be critical to confirm that the NA treatment improves the quality of equine oocytes. Our NA findings will encourage further investigations into the addition of NA, and other NAD+-elevating agents, to the media used for oocyte IVM in horses and other species. Finally, the oral supplementation of NAD+ precursors has been proposed as a strategy to improve oocyte quality in vivo, particularly in females of advanced reproductive age (Pollard et al., 2022a; Pollard et al., 2021a). Studies that

explore the impact of NAD+ precursors on aged oocytes will be essential to support this proposition.

5.4 Closing remarks

Equine reproduction has historically lagged behind other domestic animal industries, like cattle and swine, due to the unique biological limitations of horses and the lack of genetic selection for fertility. The contributions to knowledge presented in this thesis will inform the refinement of advanced equine breeding programs that incorporate embryo in vitro production. The findings highlight that the quality of oocytes retrieved from live mares by OPU is far superior to that of abattoir-derived oocytes when used to produce cloned foals. Furthermore, the observed increase in cloned embryo production efficiency following nicotinic acid supplementation during pre-IVM and IVM supports the thesis hypothesis. Intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) techniques have gained increasing relevance in the equine reproduction industry in recent years. Ensuring the oocytes used for embryo production are of the highest quality possible will reduce the number of oocytes needed to achieve a successful pregnancy. This will ultimately benefit the welfare of mares by reducing the number of procedures performed, and potentially improve the health of the foals born.

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APPENDIX

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Cloning horses by somatic cell nuclear transfer: Effects of oocyte source on development to foaling

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ABSTRACT

The cloning of horses is a commercial reality, yet the availability of oocytes for cloned embryo production remains a major limitation. Immature oocytes collected from abattoir-sourced ovaries or from live mares by ovum pick-up (OPU) have both been used to generate cloned foals. However, the reported cloning efficiencies are difficult to compare due to the different somatic cell nuclear transfer (SCNT) techniques and conditions used. The objective of this retrospective study was to compare the in vitro and in vivo development of equine SCNT embryos produced using oocytes recovered from abattoir-sourced ovaries and from live mares by OPU. A total of 1,128 oocytes were obtained, of which 668 were abattoir-derived and 460 were OPU-derived. The methods used for in vitro maturation and SCNT were identical for both oocyte groups, and the embryos were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham medium supplemented with 10% fetal calf serum. Embryo development in vitro was assessed, and Day 7 blastocysts were transferred to recipient mares. The embryos were transferred fresh when possible, and a cohort of vitrified-thawed OPU-derived blastocysts was also transferred. Pregnancy outcomes were recorded at Days 14, 42 and 90 of gestation and at foaling. The rates of cleavage (68.7 ± 3.9% vs 62.4 ± 4.7%) and development to the blastocyst stage (34.6 ± 3.3% vs 25.6 ± 2.0%) were superior for OPU-derived embryos compared with abattoir-derived embryos ($P < 0.05$). Following transfer of Day 7 blastocysts to a total of 77 recipient mares, the pregnancy rates at Days 14 and 42 of gestation were 37.7% and 27.3%, respectively. Beyond Day 42, the percentages of recipient mares that still had a viable conceptus at Day 90 (84.6% vs 37.5%) and gave birth to a healthy foal (61.5% vs 12.5%) were greater for the OPU group compared with the abattoir group ($P < 0.05$). Surprisingly, more favourable pregnancy outcomes were achieved when blastocysts were vitrified for later transfer, probably because the uterine receptivity of the recipient mares was more ideal. A total of 12 cloned foals were born, 9 of which were viable. Given the differences observed between the two oocyte groups, the use of OPU-harvested oocytes for generating cloned foals is clearly advantageous. Continued research is essential to better understand the oocyte deficiencies and increase the efficiency of equine cloning.

1. Introduction

Twenty years ago, the first horse foal cloned from somatic cells was born [1]. The possibilities that somatic cell nuclear transfer (SCNT) presented for disseminating, perpetuating, and salvaging the genetics of rare and valuable horses were immediately obvious. The cloning of exceptional geldings would facilitate the use of the resulting male foals as breeders once sexually mature. Likewise, SCNT would allow the replication of champion mares that have no breeding opportunities during their most fertile years due to demanding competition schedules. Further, the genetic reconstitution of unbred individuals from biopsied tissue would now be possible following unexpected or accidental death. Despite the SCNT inefficiencies and associated challenges particular to equids, early studies showed that most cloned foals developed normally, and horse clone production was soon commercialised [2]. Many hundreds of horse clones have now been produced around the world, and the SCNT procedures have been refined to the stage

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where they can be readily applied in equine clinical practice [3,4].

While the equine cloning achievements to date are remarkable, the efficiency of SCNT remains low, with losses at each step of cloned embryo production and throughout gestation following embryo transfer. Hence, as large numbers of oocytes are needed to produce a cloned foal, a major constraint of equine SCNT is the supply of oocytes [5]. One source of immature oocytes that has been used widely by researchers to produce foals by SCNT and intracytoplasmic sperm injection (ICSI) involves the post-mortem recovery of ovaries from slaughtered mares [1,6–11]. Extensively flushing and scraping the antral follicles of ovaries collected post-mortem has been reported to yield a mean of 14 oocytes per mare [12], though typically about 4 cumulus-oocyte complexes (COCs) are recovered per abattoir-sourced ovary [8]. A regular and reliable supply of post-mortem ovaries is often difficult to gain access to, and horse abattoirs are scarce in most countries. In Australia, small numbers of mares are euthanized sporadically, and the importation of abattoir-derived oocytes is not a viable option due to biosecurity issues [13]. Furthermore, in some countries, like the United States, the slaughter of horses is banned [14].

Immature oocytes have also been collected from live mares by transvaginal ultrasound-guided follicle aspiration, or ovum pick-up (OPU) to produce cloned foals [15–17]. The use of oocytes from the same maternal line as the nucleus donor animal avoids the presence of heterogenous mitochondrial DNA in the foal [15]. Although OPU is costly and technically challenging in mares, and there is some risk associated with the procedure, this oocyte source is increasingly being used for the production of ICSI embryos [18]. With the various refinements made to the equine OPU technique [19], experienced practitioners can now recover a mean of 9–14 oocytes per mare following aspiration of antral follicles 6–30 mm in diameter [20–22]. Mare age, breed and season have been found to influence the recovery of oocytes by OPU [20,23].

Following the collection and transportation of immature oocytes to the laboratory, current equine in vitro maturation (IVM) systems achieve nuclear maturation rates of around 60% for both abattoir- and OPU-derived oocytes [21,24]. Whilst there are few comparative studies, and the sample sizes are often small, the accumulating evidence suggests that OPU-derived embryos have superior in vitro and in vivo developmental potential compared with abattoir-derived embryos [8,17,25]. To the best of our knowledge, only one equine SCNT study has directly compared the developmental competence of both types of oocytes, and in that study, there was no assessment of in vitro development because the embryos were transferred to recipient mares immediately after couplet activation [17].

Blastocysts are commonly cryopreserved in equine embryo transfer programs when recipient mares are unavailable [26], and commercially acceptable pregnancy and foaling rates have been achieved following the transfer of cryopreserved equine embryos produced by ICSI [27,28]. In relation to the transfer of cryopreserved equine blastocysts produced by SCNT, Galli et al. [8] observed no difference in pregnancy rates between fresh and frozen embryos, but there is a paucity of literature on the effects of vitrification on the full-term viability of SCNT equine embryos.

The objective of this retrospective study was to compare the efficiency of equine SCNT using immature oocytes retrieved from live mares by OPU and from abattoir-sourced ovaries. The rates of oocyte maturation, couplet fusion, embryonic cleavage, and blastocyst formation were assessed. Following the transfer of Day 7 blastocysts to recipient mares, pregnancies and the birth of cloned foals were evaluated. As a proportion of the OPU-derived blastocysts were vitrified prior to transfer, the effect of vitrification on pregnancy outcomes was also analysed.

2. Materials and methods

2.1. Mares

The study was performed in the 2020 and 2021 breeding seasons (September to March in the southern hemisphere) at the Catalina Equine Reproduction Centre (North Richmond, NSW Australia). A total of 35 standardbred mares, aged 3–15 years, were used as oocyte donors and embryo transfer recipients. An additional 13 standardbred mares, of a similar age range, were used as embryo transfer recipients only. All procedures were carried out with informed consent from the owners and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [29], the NSW Animal Research Act (1985), the NSW Animal Research Regulations (2010), and other relevant legislation.

2.2. Chemicals and media

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Australia). Hepes-buffered Synthetic Oviductal Fluid (H–SOF) [30] was used for procedures performed outside of the CO_2 incubator. Unless otherwise stated, H–SOF contained 10% foetal calf serum (FCS; AU-FBS/PG; Cellsera, Rutherford, NSW, Australia). A 1:1 mix of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; D8437) and Medium 199 (M3769) supplemented with 10% FCS was used to transport and hold oocytes prior to in vitro maturation.

2.3. Collection of immature oocytes by ovum pick up (OPU)

The OPU procedure was performed as described previously [31]. The day before OPU, mares were scanned transrectally using an ultrasound machine (Mindray M9; Mindray, Shenzhen, China) equipped with a 5–8 MHz linear-array transducer (6LE5Vs) to determine the number and size of the follicles present on the ovaries. Only mares that had at least 15 follicles, with the largest follicle <25 mm in diameter, were scheduled for OPU. In a total of 20 sessions, with one or two sessions carried out in any single week, 64 donor mare retrievals were performed (mean of 3.2 retrievals per session). Some of the mares underwent OPU on multiple occasions with a minimum of two weeks between sessions.

In preparation for OPU, mares were sedated using a combination of detomidine hydrochloride (4 mg iv) and butorphanol tartrate (6 mg iv). Immature oocytes were collected by transvaginal ultrasound guided aspiration of follicles 5–30 mm in diameter using a 12G double-lumen needle attached to a vacuum pump. After aspirating the follicular fluid, each follicle was flushed 10 times with 0.5–5 mL (depending on follicle size) of embryo flushing medium (BoviFlush; Minitube Australia Pty. Ltd., Smythesdale, VIC, Australia) supplemented with sodium heparin (5 IU/mL, Pharma, Denmark) and pre-warmed to 37 °C. The follicular fluid and lavage medium were collected in 500 mL flasks kept at 37 °C. The collected fluids were poured through a sterile embryo collection filter (EmCon filter; Immuno Systems Inc., Spring Valley, WI, USA) immediately after the end of the OPU procedure, and the residual fluid and follicular material were then rinsed into a sterile Petri dish. Subsequently, immature oocytes were identified using a stereomicroscope, washed three times with H–SOF and transferred to a cryovial filled with transport medium at 20–22 °C. The cryovial was tightly capped and the oocytes were kept in a polystyrene container at 20–22 °C overnight (18–24 h) prior to in vitro maturation (IVM).

2.4. Collection of immature oocytes from ovaries harvested post-mortem

Equine ovaries were obtained from an abattoir and processed within 2 h of slaughter. Cumulus-oocyte complexes (COC) were recovered from follicles <30 mm in diameter by aspirating with an 18G needle and extensively scraping the follicle walls with a bone curette. The follicular material obtained was transferred to a 100 mm Petri dish containing H–SOF at 37 °C. Only COCs that had at least two complete cumulus layers were selected for IVM because immature equine oocytes with incomplete cumulus or only the corona radiata have limited developmental potential [32]. The COCs selected for IVM were washed three times with H–SOF and transferred to a cryovial filled with transport medium at 20–22 °C. The cryovial was tightly capped and the oocytes were transported to the laboratory in a polystyrene container at 20–22 °C overnight (18–24 h) prior to IVM.

2.5. In vitro maturation (IVM)

Immature oocytes retrieved from both groups (abattoir- and OPU-derived) were washed 3 times with H–SOF, and then transferred to maturation medium and incubated for 18–24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The maturation medium consisted of DMEM/F-12 medium supplemented with 0.1 IU/mL follicle-stimulating hormone and 0.1 IU/mL luteinizing hormone (Menopur, Ferring Pharmaceuticals, Copenhagen, Denmark), 50 ng/mL epidermal growth factor (EGF; E9644), 1 mM sodium pyruvate, insulin-transferrin-sodium selenium mixture (I1884), and 10% FCS.

2.6. Somatic cell nuclear transfer (SCNT)

A total of 14 different fibroblast cell lines were derived from the subcutaneous tissue of 14 adult horses (12 females and 2 males). Two of the fibroblast cell lines were purchased from Avantea (Cremona, Italy). To culture the fibroblast cells, DMEM/F-12 medium supplemented with 1 mM glutamine, 0.2 mM pyruvate, 10 ng/mL EGF and 10% FCS was used. Briefly, after plating and initial expansion, cells were passaged twice, frozen at −80 °C in culture medium containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Upon thawing, the donor cells were cultured for at least two days to ensure confluence-induced cell cycle arrest for a minimum of 24 h prior to use. Immediately before SCNT, the donor cells were harvested by trypsinization, washed and suspended in H–SOF containing 2% FCS, and kept at room temperature (RT) until use (approximately 10 min).

Following IVM, cumulus cells were removed from the oocytes by gentle pipetting in H–SOF supplemented with 1 mg/mL hyaluronidase. Denuded oocytes and donor cells were transferred to a droplet of H–SOF covered with mineral oil. The polar body and the metaphase plate of each mature oocyte were aspirated using an enucleation pipette attached to a Piezo drill (PMAS-CT150; Prime Tech Ltd., Ibaraki, Japan), which assisted penetration of the zona pellucida with speed and intensity set to 7 and 8, respectively. The donor cells were then loaded into an injection pipette and a single cell was deposited within the perivitelline space of each cytoplast. The couplets were held for 1 h in H–SOF at 38.5 °C, before being transferred to fusion medium on a fusion chamber slide between electrodes 0.5 mm apart. The fusion medium consisted of 3.0 M D-sorbitol, 0.05 mM $CaCl_2$, 0.10 mM $MgCl_2$ and 0.05% (w/v) fatty acid-free bovine serum albumin (BSA; 700-102P; Gemini Bio-Products, West Sacramento, CA, USA). A direct current (DC) pulse of 2.2 kV/cm strength and 15 μs duration was immediately applied to the couplets using the Voltain™ EP-1 system (CryoLogic, Mulgrave, VIC, Australia). The pulsed couplets were washed 3 times in H–SOF, transferred to embryo culture medium, and incubated for 2 h. The embryo culture medium consisted of DMEM/F-12 medium supplemented with 10% FCS. Activation was carried out by exposing the fused couplets to 5 μM ionomycin (I0634) for 5 min in H–SOF. After several washes in H–SOF, the fused and activated couplets were treated with 1 mM 6-dimethylaminopurine and 5 μg/mL cycloheximide in embryo culture medium for 4 h. Finally, the cloned embryos produced were washed several times, transferred to 10 μL droplets of embryo culture medium (maximum of 7 embryos in each droplet; volume >1.4 μL per embryo), and incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C. The cloned embryos were transferred to fresh droplets of culture medium every 2 days. Embryonic cleavage was assessed at the first change of culture medium and blastocyst development was assessed on Day 7 of in vitro culture, using well described morphological features to identify blastocyst formation [33]. For the fresh embryo transfers, Day 7 blastocysts were transferred to fresh embryo culture media (equilibrated and warmed to 38.5 °C) and loaded immediately in about 50 μL of the embryo culture medium into 0.25 mL straws, which were plugged and transported at 38.5 °C in a portable incubator to the site of embryo transfer (within 30 min).

2.7. Blastocyst vitrification and thawing

Blastocysts were vitrified using the Cryotop method according to the manufacturer's instructions (Vitrification Kit VT601-TOP; Kitazato Corporation, Shizuoka, Japan). In brief, embryos were transferred to the top of a 300 μL droplet of equilibration solution (ES) at RT for up to 15 min until a cycle of shrinkage (dehydration) and re-expansion (ES infiltration) was observed. This took 12–15 min, depending on the initial quality and size of the blastocyst. Equilibrated embryos with a minimum volume of ES were then transferred to the top of a 300 μL droplet of vitrification solution 1 (VS1) at RT for 30 s, during which the embryos were displaced three times within the VS1 droplet to completely wash out ES. Embryos with a minimum volume of VS1 were then transferred to a 300 μL droplet of VS2 for another 30 s with twice stirring and displacing of embryos within VS2 until complete dehydration was observed. As soon as the embryos were placed onto the thin polypropylene strip of the Cryotop, the excess VS2 around the embryos was removed and the device was immediately submerged vertically into liquid nitrogen. For thawing (Thawing Kit VT602-KIT, Kitazato Corporation), the Cryotop was immersed directly into 1 mL of pre-warmed (37 °C) thawing solution (TS) for 1 min. Thawed embryos in TS were gently deposited at the bottom of a 300 μL droplet of dilution solution (DS) for a gradual displacement of TS to DS for 3 min at RT. Then embryos with a 2 mm column of DS in the pipette were gently deposited at the bottom of a 300 μL droplet of WS1 for gradual displacement of DS to WS1 for 5 min at RT. Embryos with a minimal volume of WS1 were washed by twice submerging in WS2 for 1 min. Finally, the embryos were deposited in embryo culture medium (equilibrated and warmed to 38.5 °C) and loaded immediately in about 50 μL of the embryo culture medium into 0.25 mL straws, which were plugged and transported at 38.5 °C in a portable incubator to the site of embryo transfer (within 30 min).

2.8. Embryo transfer (ET)

Mares that had been used as oocyte donors were allowed to recover for approximately 2 months before being used as recipients. Once in oestrus, the recipient mares were scanned daily by transrectal ultrasound to determine the day of ovulation, observing the disappearance of the preovulatory follicle and the appearance

of the corpus luteum (CL). Day 7 blastocysts were transferred transcervically to recipients on Day 5 post-ovulation. One day before ET, potential recipients were examined by transrectal ultrasound to confirm the absence of endometrial oedema and excessive intrauterine fluid accumulation, and the presence of adequate uterine tone, a tight cervix, and at least one CL of expected echogenicity. If considered suitable for ET, the mares were sedated with detomidine hydrochloride (4 mg iv) and the procedure was performed. Following the procedure, mares were administered long-acting progesterone (1.5 g im; P4-300; Botupharma, Botucatu, Brazil) and every 7 days thereafter until Day 100 of gestation. The mares were examined by transrectal ultrasonography on Days 14 (Day 9 after ET), 42 and 90 of gestation to determine their pregnancy status. Additional scans were subsequently performed to monitor the progress of ongoing pregnancies.

The numbers of embryos transferred per recipient (multiple vs single) and the type of embryos transferred (fresh only vs fresh and vitrified-thawed) differed between the groups. In most of the replicates that utilised abattoir-derived oocytes, the number of blastocysts produced was much greater than the number of recipients suitable on the day of fresh ET. Based on previous reports [8,11,17], multiple (up to four) fresh abattoir-derived blastocysts were transferred to each mare. In this way, all abattoir-derived blastocysts were transferred fresh (89 blastocysts transferred to 29 recipients). In some of the replicates that utilised OPU-derived oocytes, the number of blastocysts produced was greater than the number of recipients suitable on the day of fresh ET. Those mares that were suitable on the day of fresh ET received a single OPU-derived blastocyst. The remaining OPU-derived blastocysts were vitrified and stored in liquid nitrogen as described in subsection 2.7. Later, when a recipient mare was at the appropriate stage for ET, a single vitrified OPU-derived blastocyst was thawed and transferred. In this way, all OPU-derived blastocysts were transferred singly (48 blastocysts transferred to 48 recipients).

2.9. Statistical analyses

Data were analysed using the Genstat statistical software package (18th edition; VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The embryo in vitro production data (oocytes matured, couplets fused, embryos cleaved, and blastocysts formed) were subjected to logistic regression analysis with oocyte source and cell line as factors. The pregnancy and foaling data were analysed using Fisher's exact test to evaluate the null hypothesis that there is no difference between the abattoir and OPU groups beyond Day 42 of gestation. Kendall's rank correlation was used to test the similarities in the ordering of the in vitro and in vivo data for the different donor cell lines (the probability of tau was not adjusted for ties). A P value of less than 0.05 designated a significant difference.

3. Results

3.1. In vitro production of cloned embryos

The effects of oocyte source on the in vitro production and development of cloned embryos are shown in Table 1. For the abattoir-derived oocytes, a total of 668 oocytes were collected in the 15 replicates (mean of 44.5 oocytes per replicate). For the OPU-derived oocytes, a total of 460 oocytes were collected from a total of 64 donor mare retrievals (mean of 7.2 oocytes per retrieval) in the 20 replicates (mean of 23.0 oocytes per replicate). A greater proportion of the abattoir-derived oocytes matured to the metaphase II stage by the end of IVM compared with the OPU-derived oocytes (61.6 ± 3.1% vs 49.9 ± 2.6%; $P < 0.001$). The OPU-derived couplets tended to fuse at a higher rate than the abattoir-derived couplets, but the difference was not significant (91.0 ± 2.5% vs 82.1 ± 4.9%; $P = 0.069$). The cleavage and blastocyst formation rates of the OPU-derived embryos were both superior to those of the abattoir-derived embryos (68.7 ± 3.9% vs 62.4 ± 4.7% and 34.6 ± 3.3% vs 25.6 ± 2.0%, respectively; $P < 0.05$). Using abattoir-derived oocytes, blastocysts were produced in every replicate (mean of 6.2 blastocysts per replicate). Using OPU-derived oocytes, blastocysts were produced in 18 of the 20 replicates (mean of 3.5 blastocysts per replicate).

3.2. Pregnancies and foals from cloned embryos

The results of pregnancy diagnosis and foaling following the transfer of cloned embryos to recipient mares are shown in Fig. 1. The pregnancy rates at Day 14 of gestation for the abattoir and OPU groups were 41.4% (12/29) and 35.4% (17/48), respectively. In three of the mares of the abattoir group that were diagnosed as pregnant at Day 14, two embryonic vesicles were detected. One of the embryonic vesicles in each of these mares was subsequently ablated to maximize the likelihood of the remaining conceptus surviving. Therefore, of the 89 abattoir-derived blastocysts transferred, 15 were viable at Day 14. Calculated per transferred embryo, the Day 14 viability rates of abattoir- and OPU-derived blastocysts were 16.9% (15/89) and 35.4% (17/48), respectively.

The pregnancy rates at Day 42 of gestation for the abattoir and OPU groups were 27.6% (8/29) and 27.1% (13/48), respectively. When considering the post-attachment viability of conceptuses (beyond Day 42 of gestation), a smaller proportion of mares remained pregnant at Day 90 in the abattoir group compared with the OPU group (3/8 vs 11/13; $P = 0.037$). Moreover, the proportion of viable foals born was lower in the abattoir group compared with the OPU group (1/8 vs 8/13; $P = 0.049$). Of the 3 foals born in the abattoir group, one had a markedly enlarged umbilicus and died, and one had severe angular limb deformities and died. Of the 9 foals born in the OPU group, one had severe craniofacial malformations and was euthanized, and one was declared healthy after successful treatment of minor forelimb and umbilical cord problems. All the other foals were born normal and healthy.

3.3. Vitrified-thawed vs fresh cloned embryos

When cloned embryos were produced there was often insufficient suitable recipient mares to transfer all Day 7 blastocysts as single fresh embryos. Therefore, the remaining Day 7 blastocysts were vitrified, stored, and transferred later as single vitrified-

Table 1
The effect of oocyte source on the rates of oocyte maturation, couplet fusion, embryonic cleavage, and blastocyst formation.

Oocyte source	Oocytes n	Maturation (n)	Fusion (n)	Cleavage[a] (n)	Blastocysts[a] (n)
Abattoir	668	61.6 ± 3.1%[a] (416)	82.1 ± 4.9% (353)	62.4 ± 4.7%[a] (221)	25.6 ± 2.0%[a] (93)
OPU	460	49.9 ± 2.6%[b] (218)	91.0 ± 2.5% (197)	68.7 ± 3.9%[b] (141)	34.6 ± 3.3%[b] (70)

Percentage values are presented as the mean ± SEM.
[a,b]Within columns, values labelled with different letters are significantly different ($P < 0.05$).
[a] Cleavage and blastocyst formation rates were calculated from couplets fused.

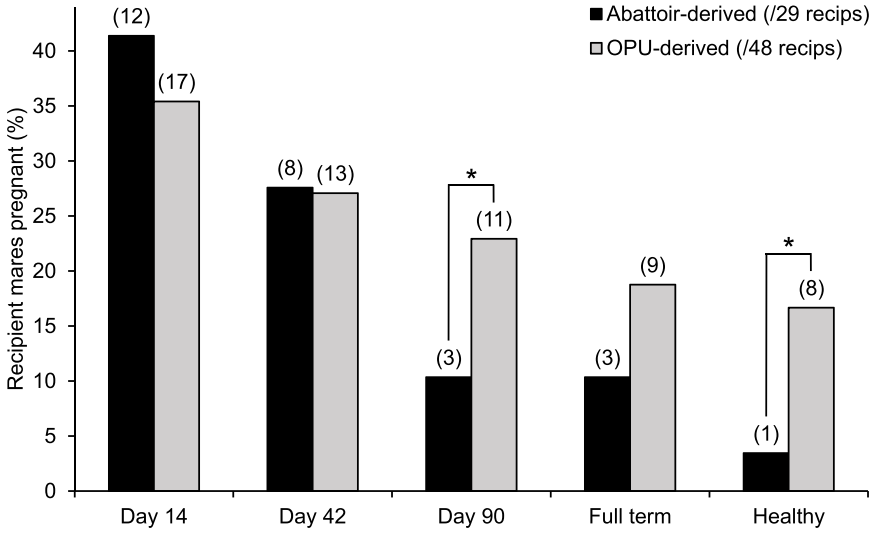

Fig. 1. The percentages of recipient mares that were diagnosed as pregnant at Days 14, 42 and 90 of gestation, carried the pregnancy full term, and gave birth to a healthy foal following transfer of cloned embryos. Day 7 abattoir- and OPU-derived blastocysts were transferred to 29 and 48 recipient mares, respectively. The numbers in parentheses above each bar indicate the number of recipient mares positive for that parameter. The asterisk brackets indicate that conceptus and foal viability beyond Day 42 of gestation is significantly different between groups ($P < 0.05$).

thawed embryos. Of the 48 OPU-derived blastocysts transferred singly to recipient mares, 34 were transferred as fresh embryos, and 14 were transferred as vitrified-thawed embryos. Of the nine donor cell lines used to produce blastocysts for the fresh vs vitrified-thawed comparison, seven yielded blastocysts that were transferred both fresh and vitrified-thawed, and two yielded blastocysts that were transferred fresh only. The results of pregnancy diagnosis and foaling following the transfer of fresh and vitrified-thawed cloned embryos to recipient mares are shown in Table 2. The proportions of mares detected as pregnant following transfer of vitrified-thawed blastocysts were greater than those of mares detected as pregnant following transfer of fresh blastocysts at Day 14 (57.1% vs 26.5%; $P = 0.036$) and Day 90 (42.9% vs 14.7%; $P = 0.039$). The proportions of mares detected as pregnant at Day 42, carried the pregnancy full term, and gave birth to a healthy foal did not differ between the two groups of blastocysts ($P > 0.05$).

3.4. *In vitro and in vivo development from different fibroblast cell lines*

A total of 14 different fibroblast cell lines were used to provide the donor nuclei for cloned embryo production. Three of the cell lines were used to produce embryos from both abattoir- and OPU-derived oocytes. Four of the cell lines were used to produce embryos from abattoir-derived oocytes only. Seven of the cell lines were used to produce embryos from OPU-derived oocytes only. The distributions of couplet fusion, embryonic cleavage, and blastocyst formation rates obtained for the cell lines are shown in Fig. 2. The mean couplet fusion rate was 89.8% and there was no effect of cell line on the rate achieved ($P > 0.05$). However, the cell line used to provide the donor nuclei affected the rates of embryonic cleavage and blastocyst formation ($P < 0.05$). The mean cleavage rate was 65.2% and the mean blastocyst formation rate was 31.2%.

The pregnancy outcomes obtained for the different fibroblast cell lines following transfer of the cloned blastocysts to recipient mares are shown in Table 3. Blastocysts obtained from one cell line that was used in only one SCNT replicate were not transferred to recipient mares. Therefore, this cell line was excluded from the analysis of pregnancy outcomes. Blastocysts produced from 11 of the 13 cell lines formed embryonic vesicles that were detected at the Day 14 pregnancy scan. The two cell lines from which no embryonic vesicles developed were only used once or twice to produce cloned embryos. Of the 11 cell lines from which embryonic vesicles developed, 8 resulted in conceptuses at Day 90, a critical milestone of pregnancy, when attachment is complete. While some post-attachment losses were observed, all 8 cell lines from which Day 90 conceptuses developed resulted in the birth of a foal. Only one of the cell lines that resulted in full term development did not produce a viable foal. Hence, healthy foals were obtained from 7 of the 13 cell lines used.

Further analysis showed there was a correlation between the rates of embryo development in vitro and the rates of foaling (Fig. 3). The cell line ranking of healthy foals born from the number of embryo transfers performed was similar to the cell line ranking of cleavage and blastocyst formation rates ($P < 0.05$). Despite low cleavage and blastocyst formation rates (41.3% and 12.5%, respectively), one of the donor cell lines resulted in the birth of a healthy foal following transfer of two OPU-derived blastocysts. This

Table 2
The percentages of recipient mares that were diagnosed as pregnant at Days 14, 42 and 90 of gestation, carried the pregnancy full term, and gave birth to a healthy foal following transfer of fresh and vitrified-thawed cloned embryos.

Embryo group	Embryos n	Day 14 (n)	Day 42 (n)	Day 90 (n)	Full term (n)	Healthy (n)
Fresh	34	26.5%[a] (9)	20.6% (7)	14.7%[a] (5)	11.8% (4)	11.8% (4)
Vitrified-thawed	14	57.1%[b] (8)	42.9% (6)	42.9%[b] (6)	35.7% (5)	28.6% (4)

Percentages are the number of positive recipients divided by the total number of recipients (a single embryo was transferred to each).
[a,b]Within columns, values labelled with different letters are significantly different ($P < 0.05$).

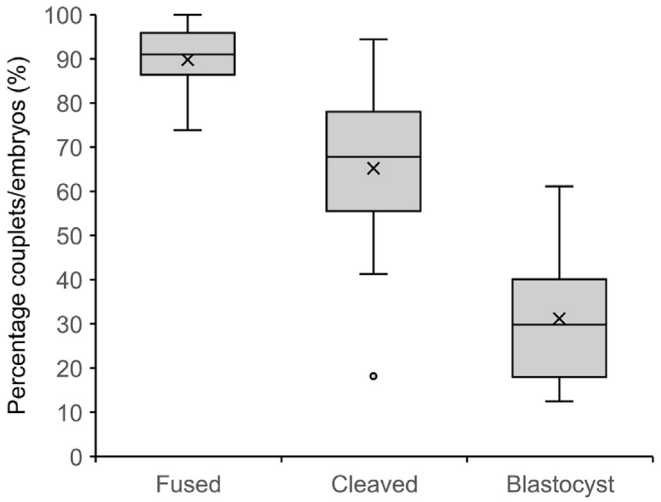

Fig. 2. Box plots of the percentages of couplets that fused, and cloned embryos that cleaved and developed to the blastocyst stage, showing the distributions due to the fibroblast cell lines used to provide the donor nuclei. The mean values are indicated by crosses and an outlier is marked by a point. While the fusion rate was not affected by the cell line used ($P > 0.05$), the cleavage and blastocyst formation rates were ($P < 0.05$).

apparent outlier was not excluded from the analysis (Fig. 3). The other cell lines that resulted in the births of healthy foals had cleavage rates greater than 65% and blastocyst formation rates greater than 35%.

4. Discussion

The results of this retrospective somatic cell nuclear transfer (SCNT) study, carried out over two breeding seasons at a commercial stud in Australia, reveal insights into several factors that influence the efficiency of equine cloning. A total of 77 embryo transfers were performed from 35 replicates in which cloned embryos were produced using abattoir- or OPU-derived oocytes and 14 different nuclear donor cell lines. A total of 12 cloned foals were born, of which 9 were healthy and 3 were not viable. Firstly, the sources of oocytes used to provide the recipient cytoplasts differed greatly in their capacity to support positive pregnancy outcomes. Secondly, vitrification of OPU-derived Day 7 blastocysts, a necessary additional procedure that addresses the unavoidable shortage of suitable recipient mares on the day of single fresh ET, did not reduce their potential to generate healthy foals. Thirdly, the fibroblast cell line used to provide the donor nuclei affected the efficiency of cloned embryo production, which was reflected in the pregnancy outcomes achieved.

Surprisingly, a greater proportion of abattoir-derived oocytes matured to the metaphase II stage compared with OPU-derived oocytes. Considerably more oocytes were recovered from abattoir-sourced ovaries in each replicate, and approximately 15% were eliminated because they had few cumulus or only the corona radiata, which has been associated with poor developmental competence [32]. In contrast, no oocytes harvested from live mares by OPU were discarded because their number was very limited. This finding suggests that the oocytes of the OPU group with few cumulus or only the corona radiata had substantially reduced meiotic competence.

With Day 7 blastocysts produced in nearly every replicate, we found the rates of cleavage and blastocyst formation were higher for OPU-derived embryos than for abattoir-derived embryos. To the best of our knowledge, this is the first such reported comparison of equine cloned embryo development in vitro. A previous study by Lee et al. [17] compared the in vivo development of equine cloned embryos produced using abattoir- and OPU-derived oocytes, but in vitro development was not assessed because the embryos were transferred to recipient mares immediately after couplet activation. In other equine SCNT studies that produced embryos from oocytes of either source, the reported rates of cleavage and blastocyst formation vary greatly. The differences in equine cloned embryo in vitro development between studies is likely due to the many differences in the SCNT technique and treatments, the nuclear donor cells, and the conditions for oocyte maturation and embryo culture. When used to produce equine embryos by intracytoplasmic sperm injection (ICSI), OPU-derived oocytes supported higher rates of development to the blastocyst stage compared with abattoir-derived oocytes [25].

Following transfer of Day 7 blastocysts, 37.7% of the recipient mares were diagnosed as pregnant at Day 14 of gestation, and a single viable conceptus was maintained in 27.3% of the recipient mares at Day 42 of gestation. Pregnancy loss beyond Day 42, when conceptus attachment is established [34], was greater at Day 90 for the abattoir group than for the OPU group. Abnormal placental

Table 3
Summary of the pregnancy outcomes obtained for the different fibroblast cell lines used to provide the donor nuclei.

Cell line	SCNT reps[a]	Oocyte source	Recipient mares n	Recipient mares positive n[b]				
				Day 14	Day 42	Day 90	Full term	Healthy
#01	2	Abattoir	5	2	1	1	1	1
	3	OPU	9	3	2	0		
#02	1	Abattoir	2	0				
	4	OPU	9	3	3	3	2	2
#03	5	Abattoir	10	5	5	2	2	0
#04	4	OPU	10	5	3	3	2	2[c]
#05	4	Abattoir	8	2	1	0		
#06	1	OPU	6	1	1	1	1	1[c]
#07	1	OPU	5	2	1	1	1	1[c]
#08	1	OPU	5	2	2	2	2	1
#09	2	Abattoir	2	2	0			
#10	2	OPU	2	1	1	1	1	1
#11	2	OPU	2	0				
#12	1	Abattoir	1	1	1	0		
#13	1	Abattoir	1	0				

[a] Number of replicates cloned embryos were produced.
[b] Number of recipient mares diagnosed as pregnant at Days 14, 42 and 90 of gestation, carried the pregnancy full term, and gave birth to a healthy foal.
[c] Healthy foals developed from vitrified-thawed blastocysts.

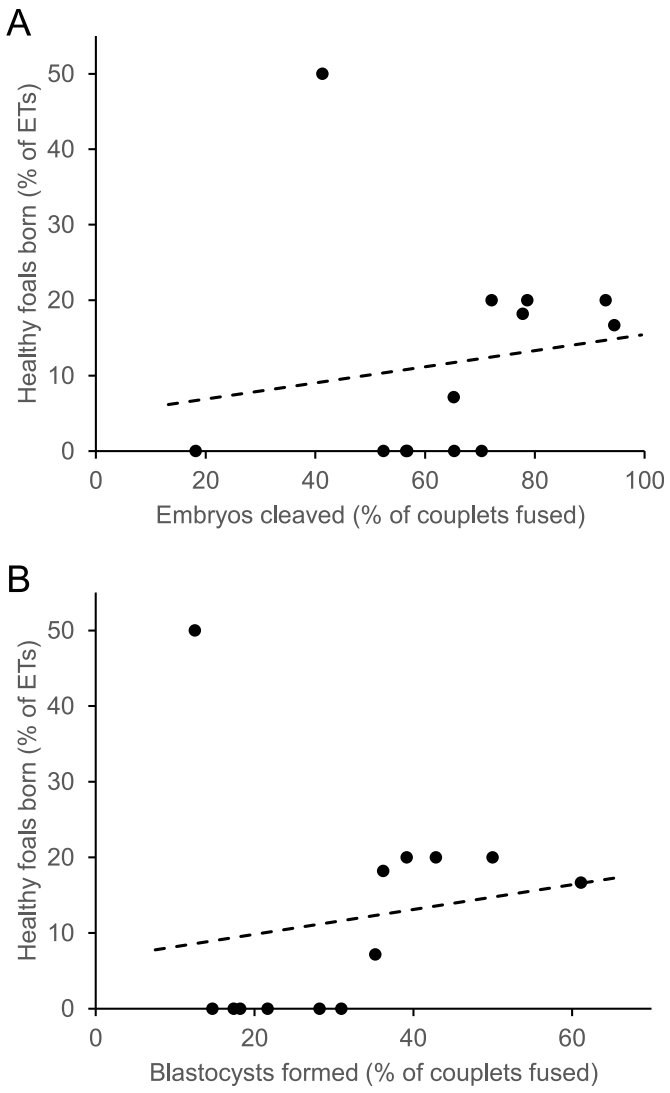

Fig. 3. Scatter plots showing the relationship between the percentage of embryo transfers resulting in the birth of a healthy cloned foal and the in vitro embryo development results for each donor cell line. The linear trendline is indicated by the dashed line. Kendall's rank correlation revealed that the healthy foaling rate was ranked in a similar order to the rates of (A) cleavage ($P = 0.038$) and (B) blastocyst formation ($P = 0.021$).

development is a common cause of pregnancy loss in SCNT conceptuses [35]. In mares, equine chorionic gonadotrophin (eCG) secreted by the endometrial cups promotes the formation of accessory CL from around Day 40 to elevate progesterone levels [34,36]. However, given that exogenous progesterone was administered, pregnancy loss due to progesterone insufficiency seems unlikely. The difference between groups was also reflected in the viability of foals, with 3.4% and 16.7% of embryo transfers resulting in the birth of a healthy foal in the abattoir and OPU groups, respectively. To date, equine SCNT studies have mostly utilised abattoir-derived oocytes for cloned embryo production. The pregnancy and foaling rates for abattoir-derived embryos reported here compare favourably with those reported previously [1,6–11,17]. Relatively few studies have used OPU-derived oocytes for SCNT and obtained live foals after ET [15–17]. In the comparison by Lee et al. [17], one foal was obtained in the in vivo (OPU) group from 26 fused couplets transferred to 13 recipients, and no foals were obtained in the in vitro (abattoir) group from 42 fused couplets transferred to 11 recipients.

Of the three non-viable foals born, one had a markedly enlarged umbilicus, one had severe angular limb deformities, and one had severe craniofacial malformations. Another foal had minor forelimb and umbilical cord problems that were resolved. Developmental abnormalities are frequently observed in foals generated by SCNT [11,36–40]. In a detailed assessment of 14 cloned foals born alive by Johnson et al. [38], seven had an umbilical cord abnormality, and eight had a limb abnormality. Craniofacial malformations are a commonly observed equine congenital defect [41]. It is interesting to note that the cloned foals assessed in the Johnson et al. [38] study were generated using abattoir-derived oocytes [6,9,10,38]. Our results indicate that using oocytes collected from live mares reduced the incidence of cloning-associated abnormalities, referred to as "cloned offspring syndrome" [42]. Recently, a similar improvement in live cloned offspring efficiency was reported in dromedary camels, although the oocytes obtained by OPU were matured in vivo following gonadotrophin stimulation [43]. Studies in cattle and buffalo have also demonstrated the superior developmental competence of OPU-derived oocytes compared with abattoir-derived oocytes [44–47]. Given the high incidence of abnormalities seen in the cloned offspring of livestock species, particularly in cloned calves, we recommend the use of oocytes collected by OPU for SCNT embryo production.

The pregnancy loss and compromised neonatal health associated with equine cloning have been attributed to defective epigenetic reprogramming that results in aberrant gene expression [2]. A recent transcriptomic analysis of equine placentas identified 1,651 differentially regulated genes between control artificial insemination (AI) pregnancies and cloned pregnancies that yielded non-viable foals; pathway analysis indicated that angiogenesis was disrupted in the cloned placentas [48]. Our results suggest that the epigenetic reprogramming ability of oocytes from slaughtered mares is inherently worse than that of oocytes harvested from live mares. Populations of slaughtered horses usually include a greater proportion of old and sub-fertile mares, so the quality of oocytes in the abattoir group would be expected to be poorer [49]. Oocytes from mares of advanced maternal age have been found to have impaired metabolic activity and a compromised ability to align chromosomes compared with oocytes from young mares [50–52]. Therefore, aging-induced depletion of oocyte factors involved in spindle formation, which is accompanied by aneuploidy and developmental defects in bovine and murine SCNT embryos [53–55], may have contributed to the greater incidence of pregnancy loss observed in the abattoir group. Additionally, the phase of the oestrous cycle was unknown at the time of slaughter, whereas the wave of developing follicles was closely monitored in preparation for OPU. Therefore, differences in follicular wave development between the two groups of donor mares very likely influenced oocyte quality [56].

Not all OPU-derived blastocysts were transferred fresh because there were not enough recipient mares at the desired stage (Day 5 post-ovulation) on the day of fresh ET. Hence, a proportion of blastocysts were vitrified and then thawed and transferred when recipient mares attained the desired stage. Unexpectedly, the pregnancy outcomes achieved for the vitrified-thawed blastocysts (four healthy foals from 14 transfers) were more favourable than for the fresh blastocysts (four healthy foals from 34 transfers). This finding highlights the importance of a synchronous embryo-uterine interaction and suggests that some of the mares used for the fresh ETs did not have optimal uterine receptivity. Previously, Galli et al. [8] observed no differences in pregnancy and foaling rates between fresh and frozen cloned embryos. The clear advantage of transferring a vitrified-thawed embryo is that the transfer can be delayed until a mare is deemed to be at the "perfect" stage.

Cuervo-Arango et al. [57] found that the number of days after ovulation and the number of CL at ET influenced the likelihood of pregnancy in mares following transfer of in vitro produced (IVP) embryos. The retrospective analysis of frozen ICSI ET cycles showed that the optimal recipient mare stage for transfer of Day 7–8 IVP blastocysts was Day 4 post-ovulation [57]. Interestingly, the rate of ongoing pregnancies was lower in mares with two CL on Day 5 post-ovulation compared to mares with one CL on Day 5 post-ovulation [57]. Therefore, the number of CL that the recipient mares had on Day 5 post-ovulation may have influenced the pregnancy rates obtained in the present study. Recently, excessive heat conditions on the day of and/or in the week after ET, a relevant issue in the context of global warming [58], has been associated with early embryonic loss in horses [59]. While the results of prospective cohort studies are needed to support the previous findings, transferring equine SCNT embryos on Day 4 post-ovulation, and scheduling to avoid extreme heat on the day of and in the week after ET, is proposed to improve pregnancy rates.

Fourteen nuclear donor cell lines were used to produce the cloned embryos in this study, and blastocysts from 13 of these cell lines were transferred to recipient mares. All the fibroblast cell lines were generated from biopsied skin tissue using the same procedure and were treated in the same way to prepare the donor cells for embryo reconstruction. Even though the same protocols were used, the cell line influenced the rates of cleavage and development to the blastocyst stage, presumably because of different epigenetic characteristics that affected nuclear reprogramming. It is well established that the reprogramming plasticity of donor cells, even those derived from the same tissue, can vary significantly [11,60,61]. In a horse cloning study that compared the use of adult fibroblast cells and bone marrow-mesenchymal stem cells (BM-MSC) as nuclear donors, preimplantation embryo development and foal viability were superior from BM-MSC [62], reinforcing the assertion that less differentiated donor cells increase cloning efficiency. However, acquiring BM-MSC from donor animals involves an invasive procedure that comes with some risk of complications [63], such that horse owners prefer the collection of cells from a superficial skin biopsy. An alternative strategy to improve the developmental potential of SCNT embryos is to apply treatments that overcome the epigenetic reprogramming barriers [64]. The findings of recent pig cloning studies suggest that dual inhibition of DNA and histone methyltransferases may be the most effective approach to improve SCNT efficiency [65,66].

Pregnancies were established from 11 of the cell lines and healthy cloned foals were obtained from 7 of these, demonstrating that most gained totipotency following SCNT reprogramming in at least a proportion of the embryos produced. Interestingly, as the cleavage and blastocyst formation rates achieved for a cell line increased, so too did the percentage of embryo transfers that resulted in the birth of a healthy foal. Apart from one outlier, the cell lines that yielded a healthy foal also produced blastocysts at rates greater than 35% (of couplets fused). This relationship may be attributed to the capacity of the cell line to be reprogrammed to a totipotent state. Alternatively, this finding may reflect that the generation of cloned offspring is just “a numbers game”. Put simply, the more Day 7 blastocysts produced and transferred, the more likely a foaling will be. For the cell lines that produced a healthy foal, the foaling rate ranged from 7.1% (1 foal from 14 transfers) to 50.0% (1 foal from 2 transfers), with a mean of 21.7%, which equates to about 5 embryo transfers for each healthy foal born. Based on our in vitro and in vivo development results, we believe a useful strategy to increase the likelihood of foaling is to first determine the chosen cell line's capacity to form Day 7 blastocysts, and to only perform ET, with or without blastocyst vitrification, if the blastocyst rate achieved is greater than 35% using OPU-derived oocytes, or greater than 25% using abattoir-derived oocytes.

While the results show a clear difference in cloning efficiency between abattoir- and OPU-derived oocytes, some limitations of this retrospective study should be acknowledged. Firstly, the production of cloned embryos in each replicate involved the use of either abattoir- or OPU-derived oocytes. Ideally, oocytes from both sources should be utilised in each replicate to account for any variation between the separate days of embryo production. Such an immense undertaking was simply not possible due to the logistical constraints. Secondly, the glaring procedural difference between the groups was the number of blastocysts transferred to each recipient mare; abattoir-derived blastocysts were transferred as multiples and OPU-derived blastocysts were transferred singly. As the number of blastocysts produced using abattoir-derived oocytes (mean of 6.2 blastocysts per replicate) exceeded the number of recipients suitable on the day of fresh ET, the decision was made to transfer the embryos of the abattoir group as multiples based on the results of previous equine SCNT studies [8]. Galli et al. [8] transferred a total of 118 SCNT embryos to 46 mares (up to four embryos per recipient) and obtained 13 pregnancies with one embryonic vesicle each. Given that mare factors contribute to pregnancy loss, the embryonic vesicle development results of the present study should be viewed with some caution. Finally, commercial constraints prevented the use of all the donor cell lines in both oocyte groups. To investigate the effects of oocyte source on in vitro and in vivo development it would be ideal to use only a single donor cell line.

5. Conclusions

In conclusion, here we report that conventional equine SCNT procedures were successfully applied in a commercial horse breeding operation to generate 12 cloned foals using abattoir- and OPU-derived oocytes. Although the quality of oocytes would be expected to be poorer from slaughtered mares compared with live mares monitored for OPU, the results provide a measure of the difference in SCNT reprogramming efficiency between the two oocyte sources. Despite the considerable resourcing, expense and effort involved, we find the efficiency gains achieved with OPU-harvested oocytes make this source of oocytes undeniably preferrable for producing healthy cloned foals. Vitrification had no detrimental impact on the developmental potential of OPU-derived Day 7 blastocysts, and enabled embryo transfers to be performed when the uterine receptivity of the recipient mares was more ideal. Healthy cloned foals were obtained from seven different adult fibroblast cell lines used to provide the donor nuclei, demonstrating the reliability of the SCNT method used. These findings will inform strategies to further improve the efficiency of SCNT programs and help direct the focus of future cloning research efforts. Clearly, further studies are needed to better understand the oocyte maturational requirements that facilitate complete epigenetic reprogramming. As the birth of live offspring is the only definitive measure of embryo viability, the generation of cloned foals provides an invaluable model for addressing fundamental questions in reproductive and developmental biology.

CRediT authorship contribution statement

Jenin V. Cortez: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Kylie Hardwicke:** Methodology, Investigation, Data curation. **Juan Cuervo-Arango:** Methodology, Investigation, Writing – review & editing. **Christopher G. Grupen:** Conceptualization, Formal analysis, Writing – original draft, Visualization, Supervision.

Declaration of competing interest

None.

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animals

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Article

Effect of Pre-IVM Duration with cAMP Modulators on the Production of Cloned Equine Embryos and Foals

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Simple Summary

The efficiency of equine embryo in vitro production is mainly limited by the capacity of current in vitro maturation (IVM) systems to support the acquisition of oocyte developmental competence. Oocyte quality may be improved by better coordinating nuclear and cytoplasmic maturation using pre-IVM treatments that modulate cAMP levels. The aim of this study was to evaluate the effect of pre-IVM treatment with cAMP modulators for short and long durations on equine oocyte quality. Following maturation with or without the pre-IVM treatments, the oocytes were used to produce cloned embryos and early development was assessed. Additionally, cohorts of blastocysts were transferred to recipient mares and the resulting pregnancies were monitored. The in vitro development of embryos did not differ significantly between the groups, though blastocyst formation tended to be inferior when the oocytes were subjected to the short pre-IVM. The in vivo development of transferred blastocysts was not adversely impacted by the pre-IVM treatments, with pregnancies established and foals born in all groups. While the use of cAMP modulators in this biphasic IVM system supported successful outcomes, it did not enhance the production of cloned equine embryos and foals.

Abstract

The asynchrony of cytoplasmic and nuclear maturation in cumulus–oocyte complexes (COCs) due to prematurely declining concentrations of cyclic adenosine monophosphate (cAMP) has been shown to result in reduced oocyte developmental competence. The objective of this study was to evaluate the effect of pre-IVM treatment with cAMP modulators for different durations on the developmental potential of equine oocytes used for cloned embryo production. Collected COCs were transferred to cryovials filled with transport medium at 20–22 °C. Within the cryovials, the COCs were either untreated (Control) for 18 h or treated with 50 µM forskolin and 100 µM 3-isobutyl-1-methylxanthine for the first 4 h (Pre-IVM 4 h) or the entire 18 h (Pre-IVM 18 h). Oocytes were then transferred to maturation medium and incubated for a further 22–24 h at 38.5 °C in 5% CO_2 in air. Somatic cell nuclear transfer embryos were then produced using the meiotically mature oocytes and donor cells from six different fibroblast cell lines. The rates of maturation and embryo development did not differ significantly between the groups, though blastocyst formation tended to be inferior in the Pre-IVM 4 h group compared with the Control group ($p = 0.06$). Of 67 blastocysts produced, 23 were transferred to recipient mares on Day 4 or 5 post-ovulation. Regarding the pregnancy outcomes, no significant differences were found between the groups, and four viable foals were born, each derived from a different donor cell line. The findings expand on those from previous evaluations of this biphasic IVM

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system, and indicate that the cAMP-modulating treatments exert limited effects under the pre-IVM conditions used here.

Keywords: in vitro maturation (IVM); biphasic IVM; simulated physiological oocyte maturation (SPOM); somatic cell nuclear transfer (SCNT); blastocyst; embryo transfer; horse

1. Introduction

In horses, assisted reproductive technologies (ARTs) are constantly being refined to enhance or extend the reproductive potential of valuable animals. While oocyte in vitro maturation (IVM) can successfully generate viable embryos after transfer [1], a major focus in recent years has been on improving oocyte IVM systems. This is particularly important when oocytes are used to produce cloned embryos, as the inefficiencies inherent in the somatic cell nuclear transfer (SCNT) procedure necessitate a large number of oocytes as starting material [2]. In vivo, oocyte maturation is regulated by signals from the somatic compartment of the follicles, including granulosa and cumulus cells, which coordinate the acquisition of developmental competence, such that the oocyte cytoplasmic components correctly direct the intricate events following fertilization and oocyte activation [3,4].

The development of IVM for equine embryo production has been slower compared to other domestic animals due to lower success rates and the constraints in obtaining immature oocytes [4]. Asynchrony of nuclear and cytoplasmic maturation results when immature oocytes are removed from antral follicles, contributing to the poor developmental potential of IVM oocytes [5]. A commonly used maturational synchronizing strategy is to simulate the conditions that maintain oocyte meiotic arrest at prophase I stage by elevating the intra-oocyte concentrations of cAMP, a key regulator of meiotic progression [6,7]. Transient exposure of oocytes to cAMP-elevating agents inhibits the degradation of germinal vesicles through the activation of protein kinase A and prevents the spontaneous resumption of meiosis, thereby reducing the asynchrony of cytoplasmic and nuclear maturation [8,9].

The cAMP modulating treatments used in the so called Simulated Physiological Oocyte Maturation (SPOM) system have been shown to exert beneficial effects on the in vitro production of embryos in several species, including cattle [10–12], goats [13], horses [14], and mice [10,15]. However, the effectiveness of the SPOM system remains contentious, owing to the inconsistent results achieved by different research groups [16]. This pre-IVM approach uses a combination of intra-oocyte cAMP modulators, specifically forskolin (FSK), which activates adenylate cyclase and increases cAMP synthesis, and isobutyl-1-methylxanthine (IBMX), which acts as a phosphodiesterase (PDE) inhibitor and prevents cAMP catabolism. The combined activities stimulate greater elevation of cAMP levels than either activity alone and supposedly maintain oocyte meiotic arrest in a way that more closely mimics that in an in vivo environment [10]. Interestingly, the duration of the SPOM pre-IVM incubation varies considerably between studies, with an overnight duration being used successfully in horses [14], and shorter durations (2–6 h) being beneficial in cattle [11,12]. Overnight pre-IVM treatment of equine oocytes is logistically convenient, as this coincides with the period typically needed to transport them from the site of collection to the laboratory for subsequent maturation and embryo production.

The objective of this study was to evaluate the effectiveness of the pre-IVM treatment (FSK and IBMX combined) applied for different durations (4 and 18 h) on the developmental competence of equine oocytes recovered from slaughterhouse-sourced ovaries. After IVM (with or without the pre-IVM treatments), matured oocytes were used to produce cloned embryos by SCNT, and the rates of maturation, couplet fusion, embryonic cleavage, and

blastocyst formation were assessed. Following the transfer of blastocysts to recipient mares, pregnancy outcomes were also monitored.

2. Materials and Methods

2.1. Mares

This study was conducted at the Catalina Equine Reproduction Centre located in North Richmond, NSW, Australia. A total of 23 standardbred mares, ranging in age from 3 to 15 years, served as recipients for embryo transfer. All procedures were performed with the informed consent from the animals' owners and in compliance with the NSW Animal Research Act (1985), which incorporates the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [17].

2.2. Chemicals and Media

Unless specified otherwise, all chemicals and reagents were obtained from Sigma-Aldrich (Bayswater, VIC, Australia). Hepes-buffered Synthetic Oviductal Fluid (H-SOF) [18] supplemented with 10% fetal calf serum (FCS; AU-FBS/PG; Cellsera, Rutherford, NSW, Australia) was used when procedures were conducted outside of the CO_2 incubator. The transport medium used to hold oocytes prior to IVM (including the pre-IVM treatments) was a 1:1 mixture of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; D8437) and Medium 199 (M3769) supplemented with 10% FCS. The oocyte maturation medium and the couplet fusion medium were prepared as previously described [19].

2.3. Collection of Immature Oocytes

Mare ovaries were obtained at a slaughterhouse and processed within 2 h. The process of cumulus–oocyte complex (COC) recovery has been described in detail previously [19]. Briefly, using an 18G needle, antral follicles < 30 mm in diameter were aspirated, and, using a bone curette, the follicle walls were scraped extensively. Following transfer of follicular material to H-SOF warmed at 37 °C within a 100 mm Petri dish, COCs were selected and washed three times with H-SOF. A total of 715 COCs were collected in 6 replicates (mean of 119.2 COCs per replicate; range of 82 to 146). In each replicate, the COCs were allocated to one of three pre-IVM treatment groups such that each group was transferred to a separate 1.5 mL cryovial containing transport medium at 20–22 °C. The cryovials were securely capped and kept within a polystyrene container at 20–22 °C for overnight transport to the laboratory and before IVM. Due to the logistics of SCNT processing after oocyte IVM, the allocation of COCs to the cryovials of transport medium, the transfer of COCs to IVM medium 18 h later, and the processing of oocytes for SCNT were staggered in the same order to achieve consistent timing. The three cryovials contained either (i) non-supplemented transport medium for the entire 18 h duration (Control), (ii) transport medium supplemented with 50 μM forskolin (FSK) and 100 μM 3-isobutyl-1-methylxanthine (IBMX) for the first 4 h and then non-supplemented transport medium for the next 14 h (Pre-IVM 4 h), and (iii) transport medium supplemented with FSK and IBMX for the entire 18 h duration (Pre-IVM 18 h). The concentrations of FSK and IBMX were selected based on the findings of Metcalf and co-workers [14,20].

2.4. Oocyte In Vitro Maturation (IVM)

At the end of the 18 h transport and holding period, the COCs of each group were washed with H-SOF three times and placed into wells of 4-well dishes (144444; Nunc, Roskilde, Denmark) containing 500 μL of pre-equilibrated maturation medium (20–50 COCs

per well). The COCs were incubated for 22–24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.5. Somatic Cell Nuclear Transfer (SCNT)

A total of 6 different fibroblast cell lines were derived from the subcutaneous tissue of 6 adult horses (one male warmblood, and two male and three female polo ponies). A different cell line (denoted as #01 to #06) was used in each of the 6 replicates. DMEM/F-12 medium supplemented with 1 mM glutamine, 0.2 mM pyruvate, 10 ng/mL EGF, and 10% FCS was used to culture the fibroblast cells. After plating and initial expansion, the cells were subcultured twice before freezing at −80 °C in culture medium supplemented with 10% dimethyl sulfoxide (DMSO) and storing in liquid nitrogen. After thawing the cells, they were cultured for at least two days. To arrest the cell cycle, the cells were grown to confluence at least 24 h before use as donor cells. About 10 min prior to use for SCNT, a suspension of the donor cells was prepared at room temperature (RT) by trypsinizing, washing, and transferring them to H-SOF supplemented with 2% FCS. Fresh donor cells were prepared at the start of the SCNT procedure for each group of oocytes.

After IVM, the oocytes were denuded of cumulus cells by gentle pipetting with 1 mg/mL hyaluronidase in H-SOF. Each metaphase-II-stage oocyte was visualized using an inverted microscope equipped with Hoffman modulation contrast optics and enucleated using a micropipette attached to a Piezo drill (PMAS-CT150; Prime Tech Ltd., Ibaraki, Japan). Oocytes were considered to be successfully enucleated when the birefringence properties of the meiotic spindle were observed upon aspiration. Each resulting cytoplast then had a donor cell placed in the perivitelline space. A total of 308 couplets were constructed in 6 replicates (mean of 51.3 couplets per replicate; range of 33 to 65). The couplets were incubated for 1 h in H-SOF at 38 °C, before being transferred to fusion medium placed between the electrodes (0.5 mm apart) of a fusion chamber slide. A direct current (DC) pulse of 2.2 kV/cm strength and 15 µsec duration was applied to the couplets immediately, after which they were washed several times and then incubated for 2 h in embryo culture medium at 38.5 °C. Subsequently, the fused couplets were activated by exposing them to 5 µM ionomycin in H-SOF for precisely 5 min, washed several times in H-SOF, and incubated for 4 h in embryo culture medium containing 1 mM 6-dimethylaminopurine and 5 µg/mL cycloheximide. After further washes, the SCNT embryos were transferred to 10 µL droplets of embryo culture medium (maximum of 7 embryos in each droplet), and incubated in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C. The SCNT embryos were transferred every 2 days to droplets of fresh embryo culture medium [19]. At the first change of embryo culture medium, cleavage was assessed. Blastocyst development was evaluated at Days 7 and 8, using well-described morphological features to classify blastocyst quality [21].

2.6. Blastocyst Vitrification and Thawing

Blastocysts were vitrified using the Cryotop method following the manufacturer's directions (Kitazato BioPharma, Shizuoka, Japan). In summary, 300 µL of equilibration solution (ES) and vitrification solution 1 (VS1) were deposited in separate wells of a 4-well dish at RT. Embryos were transferred to the ES drop for 12–15 min and a cycle of contraction (dehydration) and re-expansion (ES infiltration) was observed. Then, the equilibrated embryos were transferred to VS1 for 1 min, after which each embryo was placed with a minimal amount of medium on the thin polypropylene strip of the Cryotop, and the device was immediately immersed vertically in liquid nitrogen. To thaw, the Cryotop was directly immersed in 1 mL of prewarmed (37 °C) thawing solution (TS) for 1 min. Embryos thawed in TS were gently placed at the bottom of a 300 µL drop of dilution solution (DS)

for a gradual shift from TS to DS over 3 min at RT. The embryos were drawn up into a pipette tip with a 2 mm column of DS and then carefully placed at the base of a 300 µL drop of warming solution 1 (WS1), for a gradual shift from DS to WS1 over 5 min at RT. The embryos were then washed twice for 1 min in warming solution 2 (WS2). Finally, the embryos were transferred to equilibrated and warmed (38.5 °C) embryo culture media.

2.7. Embryo Transfer (ET)

Non-surgical embryo transfers were performed in 23 standardbred mares as described previously [19]. For each embryo that was transferred, the size of the somatic cell nucleus donor animal was considered to select an appropriately sized recipient mare able to carry the pregnancy to term. Within 30 min of warming, blastocysts were transferred trans-cervically to recipients on Day 4 or 5 post-ovulation. Pregnancies were diagnosed by transrectal ultrasonography on Days 14 (Day 9 after ET), 45 and 90 of gestation. During pregnancy, fetal movements, placental quality, and heart rate were monitored. Additional scans were subsequently performed once a month to monitor the progress of ongoing pregnancies.

2.8. Statistical Analysis

Analyses of the data were performed using Genstat for Windows 22nd Edition statistical software (Version 22.1.0.195; VSN International, Hemel Hempstead, UK). The proportional SCNT embryo production data (oocytes matured, couplets fused, embryos cleaved, and blastocysts formed) were subjected to logistic regression analysis using the logit transformation, with treatment group and cell line as factors. When no significant differences were detected, the variance between groups was determined using ANOVA and Fisher's unprotected pairwise comparison. The pregnancy and foaling data were analyzed using chi-square tests to evaluate the null hypothesis that there was no difference between the groups. A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. In Vitro Development of SCNT Embryos

3.1.1. Effect of cAMP-Modulating Pre-IVM Treatments

The effects of the cAMP-modulating pre-IVM treatments on the in vitro production and development of SCNT embryos are shown in Figure 1. In the six replicates, a total of 266 oocytes were allocated to the Control group (mean of 44.3 oocytes per replicate), a total of 191 oocytes were allocated to the Pre-IVM 4 h group (mean of 31.8 oocytes per replicate), and a total of 258 oocytes were allocated to the Pre-IVM 18 h group (mean of 43.0 oocytes per replicate). The rates of maturation, couplet fusion, embryonic cleavage and overall cloning efficiency did not differ between the groups ($p > 0.05$). However, the rates of blastocyst formation, as proportions of fused couplets and cleaved embryos, tended to be lower in the Pre-IVM 4 h group than in the Control group ($p < 0.1$). The blastocyst rates of the Pre-IVM 18 h group did not differ from those of the Control and Pre-IVM 4 h groups ($p > 0.05$). Using untreated Control oocytes, blastocysts were produced in all six replicates (mean of 4.5 blastocysts per replicate). Conversely, blastocysts were produced in five of the six replicates using the Pre-IVM 18 h treated oocytes (mean of 4.5 blastocysts per replicate), and in four of the six replicates using the Pre-IVM 4 h treated oocytes (mean of 2.2 blastocysts per replicate).

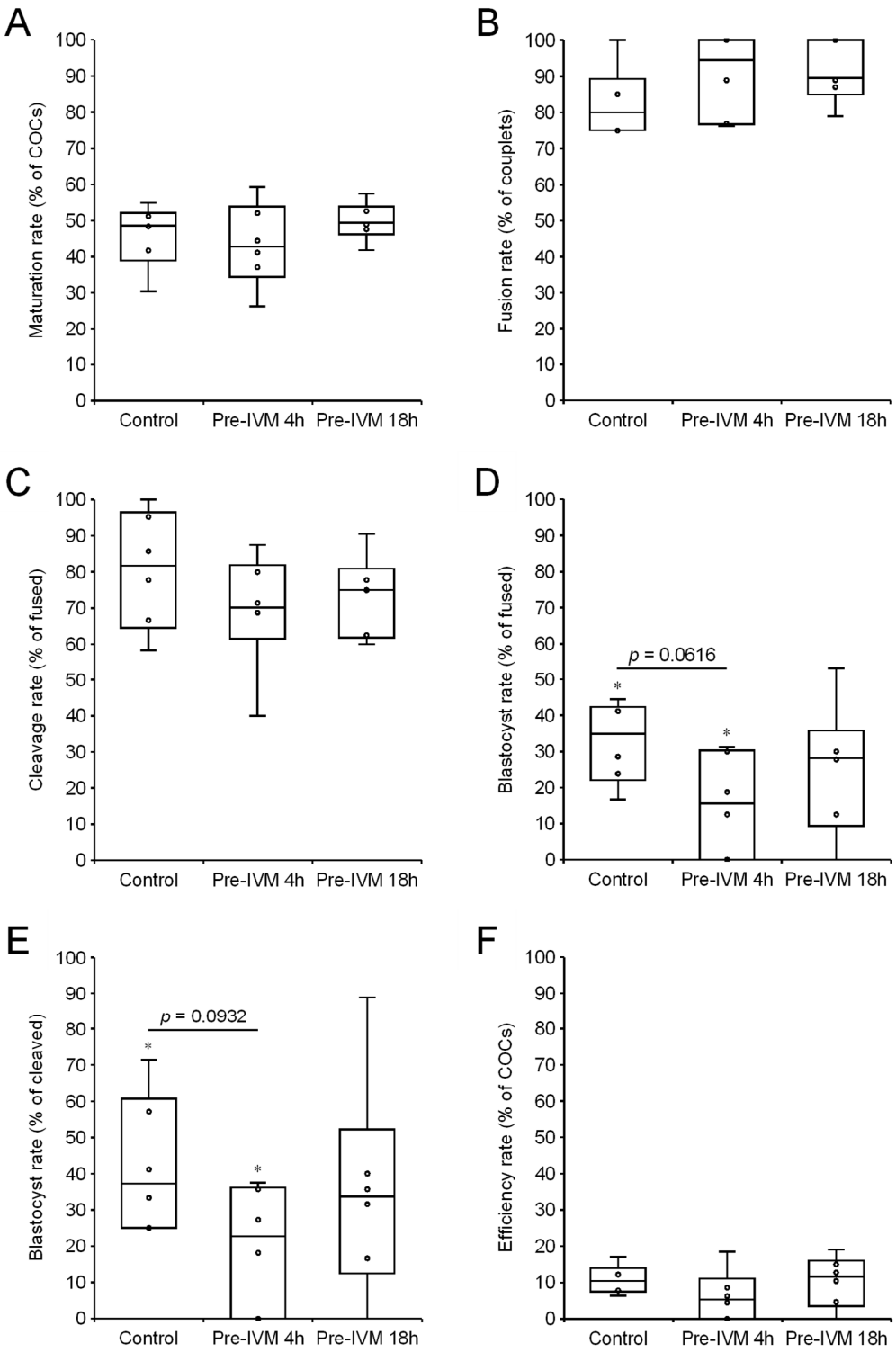

Figure 1. Effect of the cAMP-modulating pre-IVM treatments on SCNT embryo production (Control, Pre-IVM 4 h, and Pre-IVM 18 h groups). (**A**) The percentage of oocytes that matured after an additional 22–24 h of IVM, as determined by the presence of a first polar body. (**B**) The percentage of constructed couplets that fused. (**C**) The percentage of fused couplets that cleaved after 48 h of embryo culture. (**D**) The percentage of fused couplets that formed blastocysts. (**E**) The percentage of cleaved embryos that formed blastocysts. (**F**) The overall cloning efficiency rate, expressed as the percentage of cumulus–oocyte complexes (COCs) that resulted in blastocysts. Values are presented as the mean ± s.e.m. No significant differences were detected between the groups ($p > 0.05$). Values labeled with an asterisk tend to differ ($p < 0.1$).

3.1.2. Effect of Donor Cell Lines

A total of six different fibroblast cell lines, generated from separate skin biopsies, were used to provide the donor nuclei used to produce the SCNT embryos. Each cell line was used in all three experimental groups (Control, Pre-IVM 4 h, and Pre-IVM 18 h), such that a different cell line was used in each of the six replicates. There was no effect of donor cell line on the rates of couplet fusion, embryonic cleavage, or development to the blastocyst stage ($p > 0.05$; Figure 2). There was no significant interaction between treatment and cell line, and a mean of 10.8 blastocysts was produced for each cell line (range of 7 to 16).

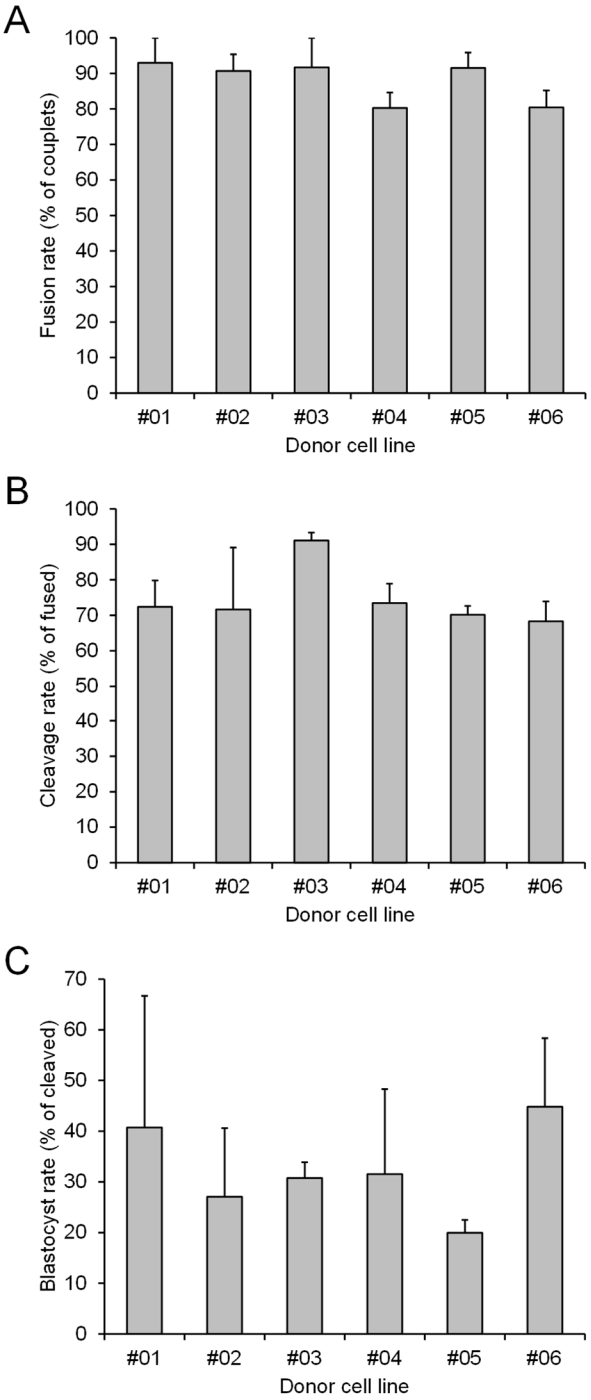

Figure 2. Effect of the donor cell lines (denoted as #01 to #06) on SCNT embryo production. (**A**) The percentage of constructed couplets that fused. (**B**) The percentage of fused couplets that cleaved. (**C**) The percentage of cleaved embryos that formed blastocysts. Values are presented as the mean ± s.e.m. No significant differences were detected between the donor cell lines ($p > 0.05$).

3.2. Pregnancy Outcomes After ET

3.2.1. Effect of cAMP-Modulating Pre-IVM Treatments

Of the 67 blastocysts produced and vitrified, a total of 23 blastocysts were warmed and transferred (4, 2, and 17 for the Control, Pre-IVM 4 h, and Pre-IVM 18 h groups, respectively). The results of pregnancy diagnosis after transfer of the SCNT embryos to recipient mares are shown in Table 1. As determined by ultrasonographic detection of an embryonic vesicle, 10 were viable on Day 14. Although the proportions of mares in which embryonic vesicles were detected appeared to vary between the groups, the Day 14 pregnancy rates did not differ significantly (25%, 50%, and 47% for the Control, Pre-IVM 4 h, and Pre-IVM 18 h groups, respectively; $p > 0.05$).

Table 1. Effect of cAMP-modulating pre-IVM treatments on pregnancy and foaling.

Group	Recipient Mares Total	Pregnant at Day 14 [1]	Pregnant at Day 45 [1]	Pregnant at Day 90	Mares Foaling
Control	4	1 (25.0%)	1 (25.0%)	1	1
Pre-IVM 4 h	2	1 (50.0%)	1 (50.0%)	1	1
Pre-IVM 18 h	17	8 (47.1%)	2 (11.8%)	2	2
Total	23	10 (43.5%)	4 (17.4%)	4	4

[1] Values in parentheses are the percentage pregnant of total recipient mares.

At Day 45 of pregnancy, the conceptuses of the Control and Pre-IVM 4 h groups remained viable, while pregnancy losses occurred in the Pre-IVM 18 h group, and two conceptuses remained viable. Beyond Day 45 of gestation, no pregnancy loss occurred in any of the groups. A total of four foals were born (one, one, and two in the Control, Pre-IVM 4 h, and Pre-IVM 18 h groups, respectively). Two of the foals were born normal and healthy, and the other two were declared healthy after successful treatment of minor forelimb and umbilical problems.

3.2.2. Effect of Donor Cell Lines

The pregnancy results obtained for the different cell lines are shown in Table 2. A total of 23 blastocysts produced from four of the six cell lines were transferred to 23 recipient mares, and 10 of the transferred blastocysts formed embryonic vesicles at Day 14 of pregnancy. All of the resulting Day 45 conceptuses completed full-term development, such that four foals were born, one derived from each of the four donor cell lines involved.

Table 2. Effect of the donor cell lines on pregnancy and foaling.

Donor Cell Line	Recipient Mares Total	Pregnant at Day 14 [1]	Pregnant at Day 45 [1]	Pregnant at Day 90	Mares Foaling
#01	10	3 (30.0%)	1 (10.0%)	1	1
#02	5	1 (20.0%)	1 (20.0%)	1	1
#03	4	3 (75.0%)	1 (25.0%)	1	1
#05	4	3 (75.0%)	1 (25.0%)	1	1

[1] Values in parentheses are the percentage pregnant of total recipient mares.

3.2.3. Effect of Embryo Grade

The effect of the quality of the transferred embryo (Grade 1 vs. Grade 2) on pregnancy outcome is shown in Table 3. A total of 16 Grade 1 blastocysts and 7 Grade 2 blastocysts were transferred to the recipient mares. While pregnancies were detected at Day 14 for both embryo grades, only Grade 1 blastocysts established ongoing pregnancies and generated viable births (Grade 1: 4/16; Grade 2: 0/7). However, the apparent difference was not statistically significant ($p > 0.05$).

Table 3. Effect of embryo grade on pregnancy and foaling.

Embryo Grade	Recipient Mares Total	Pregnant at Day 14 [1]	Pregnant at Day 45 [1]	Pregnant at Day 90	Mares Foaling
Grade 1	16	8 (50.0%)	4 (25.0%)	4	4
Grade 2	7	2 (28.6%)	0 (0.0%)	0	0

[1] Values in parentheses are the percentage pregnant of total recipient mares.

3.2.4. Effect of Recipient Mare's Day Post-Ovulation

The effect of the recipient mare's day post-ovulation (Day 4 vs. Day 5) at ET was also analyzed. As shown in Table 4, the transfer of embryos to recipients on Days 4 and 5 after ovulation resulted in similar Day 14 pregnancy rates. While more pregnancy losses occurred in the Day 5 post-ovulation group compared with the Day 4 post-ovulation group, the pregnancy rates at Days 45 and 90 and the foaling rates did not differ significantly (2/7 and 2/16, respectively).

Table 4. Effect of the recipient mare's day post-ovulation at embryo transfer on pregnancy and foaling.

Day Post-Ovulation	Recipient Mares Total	Pregnant at Day 14 [1]	Pregnant at Day 45 [1]	Pregnant at Day 90	Mares Foaling
Day 4	7	3 (42.9%)	2 (28.6%)	2	2
Day 5	16	7 (43.8%)	2 (12.5%)	2	2

[1] Values in parentheses are the percentage pregnant of total recipient mares.

4. Discussion

The results of this study show that the developmental competence of equine oocytes used to produce embryos by somatic cell nuclear transfer (SCNT) was not improved following pre-IVM treatment with the cAMP modulators forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), a previously described biphasic IVM system referred to as Simulated Physiological Oocyte Maturation (SPOM) [10,16]. A total of 715 oocytes were recovered from slaughterhouse-sourced ovaries in six replicates, and a total of 67 blastocysts were produced. While no significant differences between the treatments and Control groups were found, the blastocyst formation rates tended to be lower in the Pre-IVM 4 h group than in the Control group. Within the constraints of a commercial equine cloning program, 23 blastocysts were transferred to recipient mares, resulting in the birth of four healthy foals derived from four different donor cell lines. There were no significant differences in pregnancy outcomes between the treatments and Control groups, demonstrating for the first time that pre-IVM exposure of equine oocytes to FSK and IBMX does not prevent full-term development of SCNT embryos. These findings expand the evaluation of the SPOM system in equine oocytes.

The developmental competence of in vitro-matured oocytes is compromised due to the initiation of spontaneous meiotic resumption, which occurs in response to a decrease in the cAMP levels responsible for maintaining meiotic arrest [3,22]. Adequate levels of cAMP in oocytes are generated and maintained by granulosa cells, cumulus cells, and metabolites of the follicular compartment to ensure that meiosis resumes in an orchestrated manner [23]. By disrupting the oocyte–follicle connection, the intra-oocyte cAMP levels decrease, such that the resumption of meiosis occurs in a more rapid and uncontrolled manner [24]. In horses, the problem is exacerbated because the procedures used to recover oocytes via ovum pick up (OPU) or from slaughterhouse ovaries are more challenging than those used in other species [25,26]. Similar to the approaches described previously in horses and other species [16,20], the rationale for the pre-IVM treatments used here was to

maintain the intra-oocyte cAMP concentrations immediately after collection, thus avoiding spontaneous meiotic resumption.

Research carried out in equine oocytes using treatments to inhibit meiosis is limited. The study by Choi et al. [27] was one of the first to report the manipulation of meiotic resumption in this species using roscovitine, which is an analog of purine that specifically inhibits M-phase-promoting factor activity. The addition of roscovitine to pre-IVM medium for 16–18 h effectively maintained oocytes at the germinal vesicle stage, but their developmental competence after subsequent IVM and intracytoplasmic sperm injection (ICSI) was not enhanced [27]. Conversely, Metcalf et al. [14,20] found that the rates of maturation and blastocyst development were increased, compared with the Control, when equine oocytes were held overnight in medium with 50 μM FSK and 100 μM IBMX (i.e., the SPOM system) [14,20]. Using the same concentrations of FSK and IBMX as those used by Metcalf et al. [14], which were found to be optimal for equine oocytes in a pilot study [20], the results of the present study did not show any significant differences in the rates of maturation, couplet fusion, cleavage, and blastocyst formation between the pre-IVM and Control groups. The inconsistent results between the studies may be due to the different media used or the possibility that supplementation with serum may have interfered with the pre-IVM treatments [16]. Another major difference between the studies is that Metcalf et al. [14,20] produced ICSI embryos using oocytes harvested from live mares by OPU, whereas here we produced SCNT embryos using oocytes recovered from slaughterhouse-sourced ovaries. In a previous study we found that oocyte source (OPU-derived vs. abattoir-derived) significantly impacted the development of SCNT embryos to the blastocyst stage [19].

The SPOM system has been applied to embryo production in numerous species, including cows [10,11,28–31], mice [10,15,32], sheep [7,33], goats [13], horses [14], and cats [34]. The effectiveness of the SPOM treatment has not been consistent, with only 34.7% (8/23) of studies achieving an improvement in blastocyst production [16]. Further, in those studies conducted in cattle, only 25% (4/16) of them succeeded in improving blastocyst production [16]. Differences between studies in the composition of the pre-IVM base medium and the supplements used suggest there are complex interactions with factors in the media that influence the effectiveness of the cAMP modulators [16].

A major variable of the SPOM system is the duration of the FSK and IBMX treatment. In horses, oocytes are often collected at a site distant to the laboratory, such that transportation overnight, as was the case in this study, is necessary. Hence, for the Pre-IVM 18 h group, the oocytes were kept in transport medium supplemented with FSK and IBMX for the entire transportation period, whereas for the Pre-IVM 4 h group, the oocytes were first held in transport medium supplemented with FSK and IBMX for 4 h before being transferred and kept in transport media without the cAMP modulators for the remaining 14 h. The oocytes of the Control group were kept in transport medium without the cAMP modulators for 18 h. It is unclear why the Pre-IVM 4 h treated oocytes tended to have poorer developmental potential compared with the untreated Control oocytes. A possible explanation is that the additional handling and medium change had a negative influence. In the only other horse studies of the SPOM system, an overnight pre-IVM duration was used with some success [14,20], while in cattle studies of the SPOM system, using pre-IVM durations of 2 and 6 h achieved positive results [10,12,35,36]. Further studies are needed to determine whether an alternative pre-IVM duration may be optimal. A key feature of equine oocyte maturation is the ability to hold immature oocytes at room temperature for 18 h, which appears to promote chromatin condensation at the germinal vesicle stage without reducing developmental competence [37,38]. As the modulation of cAMP levels by the SPOM

system has been carried out at 37–38.5 °C in other species [10], an influence of the pre-IVM incubation temperature on the treatments cannot be ruled out.

The blastocysts produced were vitrified and then transferred into previously synchronized recipient mares. Due to the commercial imperative to obtain foals from particular donor cell lines, a similar number of blastocysts from each group could not be transferred. Pregnancy rates on Day 14 of gestation did not differ among the groups. Of the embryonic vesicles that developed, 40% (4/10) were viable conceptuses on Day 45 of gestation. Pregnancy loss and compromised neonatal health associated with equine cloning have been attributed to defective epigenetic reprogramming resulting in aberrant gene expression [39] and the reproductive status of recipients carrying the pregnancy [40]. There was no pregnancy loss after Day 45 and viable foals were born in each group, demonstrating that the pre-IVM treatment with cAMP modulators did not have an adverse effect on fetal development. Similarly, in mice, Albuz et al. [10] showed that viable offspring can be obtained following the transfer of embryos produced from oocytes subjected to the SPOM system.

The success of nuclear transfer depends on the ability of the donor cells to be reprogrammed to a totipotent state, guided by the reprogramming factors present in the recipient cytoplast [41]. The developmental plasticity of cells from different lines is an important factor that determines the capacity of embryos to develop and give rise to healthy offspring [42,43]. In this study, pregnancies were established, and healthy cloned foals were obtained from all the cell lines used to produce the transferred blastocysts, demonstrating that complete cellular reprogramming to a totipotent state was achieved. Overall, viable foals were obtained from 17.4% (4/23) of the blastocysts transferred, which equates to approximately six embryo transfers for each foal born. This foaling rate is comparable to that reported previously for SCNT embryos [19].

The relationship between embryonic morphological features and pregnancy outcomes was also evaluated. Day 7 blastocysts classified as being of Grade 1 or 2 quality, according to well-defined criteria [21], were transferred to recipient mares. The morphological properties of in vitro-produced (IVP) equine blastocysts have been associated with their speed of development, and embryos that develop faster are more likely to be classified as Grade 1 and have a greater chance of generating births [44–46]. In the present study, births were only obtained from Grade 1 embryos. Assessing the kinetics of embryonic development is now routine practice to predict the outcome of transferred embryos in domestic species and humans [47].

Regarding the recipient mare's day post-ovulation, similar pregnancy outcomes were achieved when ET was carried out 4 or 5 days after ovulation. In retrospective studies where many more ICSI-produced embryos were transferred to recipient mares on days 3–6 after ovulation, the best pregnancy rates were obtained when ET was performed on Day 4 [44,48], suggesting that the mare's uterine environment and stage of IVP embryo development are optimally synchronized on that day. The success of equine ET is influenced by multiple factors, including other recipient mare factors such as age and uterine tone [49,50]. Given the multitude of factors at play, the relatively small number of embryos transferred to recipient mares in the present study likely precluded the detection of any differences between groups.

5. Conclusions

In conclusion, the results show that pre-IVM treatment with cAMP modulators for 4 or 18 h did not enhance the quality of equine oocytes, as the rates of oocyte maturation, couplet fusion, embryonic cleavage, and development to the blastocyst stage did not differ significantly from those of the Control group. Indeed, the rates of blastocyst formation tended to be lower in the Pre-IVM 4 h group, compared with the Control group. Following

the transfer of blastocysts to recipient mares, four cloned foals were generated, including three from embryos produced using oocytes treated with the cAMP modulators, demonstrating for the first time that this treatment is compatible with full-term development in horses. Furthermore, the four cloned foals were derived from four different cell lines, demonstrating the reliability of the SCNT methods used. While effects on pregnancy outcomes due to the various factors analyzed (pre-IVM treatment, donor cell line, embryo grade, and recipient mare's day post-ovulation) were not detected, transferring greater numbers of SCNT embryos may reveal significant differences. Regardless, as the cAMP modulators exerted no beneficial effects under the pre-IVM conditions described here, the findings do not support the use of the so-called SPOM system for equine oocyte maturation, adding to the controversy in this area. Further studies are needed to evaluate the merit of biphasic IVM approaches that modulate cAMP levels during equine oocyte maturation.

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Institutional Review Board Statement: In the present study, all procedures conducted on the mares were part of routine breeding practices at a commercial stud, where the mares were privately owned and housed, and informed written consent had been obtained from their respective owners. Following consultation with the University of Sydney Animal Ethics Committee, separate institutional ethical approval was not required, provided the procedures adhered to relevant regulatory standards and were conducted by a registered equine veterinarian. All procedures were performed by Dr Kylie Hardwicke, Head Veterinarian at the stud and a co-author of this manuscript, who is a registered specialist in embryo transfer and advanced equine reproduction.

Informed Consent Statement: Written informed consent was obtained from the owners of the animals used in this study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Original Research Article

Nicotinic acid treatment improves the developmental potential of equine oocytes for cloned embryo production

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ABSTRACT

Nicotinic acid (NA) treatment during in vitro maturation (IVM) has been shown to elevate nicotinamide adenine dinucleotide (NAD^+) levels and improve oocyte developmental competence. Suboptimal equine oocyte IVM systems currently limit the efficiency of viable embryo in vitro production. This study evaluated NA supplementation during IVM for cloned equine embryo production, using oocytes from abattoir-sourced ovaries and live mares via ovum pick-up (OPU). Abattoir-derived oocytes (n = 694) were treated without or with 50 or 200 μM NA during the 18 h holding period (Pre-IVM). Next, OPU-derived oocytes (n = 147) received either no treatment or 200 μM NA during Pre-IVM. Additionally, 285 OPU-derived oocytes were treated with 200 μM NA during Pre-IVM and then either without or with 200 μM NA during the final 22–24 h of IVM. While different cell lines provided the donor nuclei, all experimental groups used the same cell line in each replicate. In abattoir-derived embryos, the Pre-IVM NA treatment increased blastocyst rates compared with the control (50 μM: 27.1 ± 1.4%; 200 μM: 32.9 ± 3.0%; control: 19.9 ± 1.7%; $P < 0.05$). In OPU-derived embryos, the Pre-IVM NA treatment had no effect ($P > 0.05$), but NA supplementation during IVM improved the blastocyst rate (53.4 ± 9.6% vs 31.3 ± 8.1%; $P < 0.05$). The rates of nuclear maturation, couplet fusion, and cleavage were not influenced by NA supplementation. These results show that NA treatment during Pre-IVM and IVM enhanced equine oocyte developmental potential. Further research is needed to clarify the underlying mechanisms and assess embryo viability post-transfer.

1. Introduction

The complete acquisition of oocyte developmental competence is essential for successful fertilization, the development of viable embryos, and the birth of healthy offspring [1]. Consequently, the in vitro production of embryos via somatic cell nuclear transfer (SCNT) or intracytoplasmic sperm injection (ICSI) is largely constrained by the effectiveness of the oocyte in vitro maturation (IVM) system used [2,3]. In horses, embryo in vitro production is further constrained, compared with other livestock species, by the limited access to slaughterhouse ovaries, which are typically from mares of advanced maternal age, and are approaching reproductive senescence [4,5]. Alternatively, equine oocytes can be collected from live mares by ovum pick-up (OPU), which effectively overcomes the lack of an efficient superovulation treatment [6,7]. Also, many mares are bred into their older years, when the quantity and quality of their oocytes are declining [8,9]. While the OPU technique has developed to the stage where highly trained practitioners can consistently harvest COCs, the numbers recovered can vary greatly between mares and collections, such that acceptable embryo yields may not be obtained from each oocyte retrieval [10,11]. Hence, a major focus of research efforts in this area is to improve the success of equine IVM systems to make the most of the precious few oocytes obtained.

Numerous factors have been identified that influence the acquisition of oocyte developmental competence, including those related to energy metabolism, cell survival, and DNA repair [12,13]. An essential cofactor implicated in many cellular processes during oocyte maturation is nicotinamide adenine dinucleotide (NAD^+) [14]. Recent studies carried out in murine oocytes have shown that treatments that promote the production of NAD^+ can enhance mitochondrial function, reduce DNA damage, suppress apoptosis, and improve oocyte developmental competence, especially in cases of maternal and oocyte aging [15–18]. These processes are largely driven by NAD^+-dependent enzymes, including sirtuins (SIRTs) and poly-ADP-ribose polymerases (PARPs) [14]. During meiosis, SIRT1 associates with spindle microtubules and

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facilitates cytoskeletal remodelling and epigenetic reprogramming, which are essential for establishing correct gene expression patterns in the resulting embryo [19,20]. Synthesis of NAD^+ can occur through the catalytic conversion of the amino acid tryptophan (Trp) via the *de novo* synthesis pathway, recycling of NAD^+ metabolites via the salvage pathway, and metabolism of nicotinic acid via the Preiss-Handler pathway [14].

As a precursor of NAD^+, nicotinic acid (NA), also known as niacin or vitamin B3, plays a crucial role in a myriad of cellular processes [21]. In addition, NA has antioxidative and cell membrane protection properties [22,23]. Supplementation of IVM medium with NA has been reported to increase the rate of nuclear maturation in bovine and porcine oocytes [24,25] and reduce the frequency of meiotic spindle defects in aged murine oocytes [18]. It is worth noting that aberrant nuclear maturation and chromosome segregation errors associated with impaired meiotic spindle assembly are hallmarks of the maternal age-related decline in equine oocyte quality [26]. To date, the effect of NA supplementation on the maturation of equine oocytes has not been reported.

Having successfully established a commercial equine breeding program that incorporates embryo in vitro production by SCNT, a major priority is to improve the effectiveness of the IVM system being used. The procedures and conditions applied in our laboratory to produce cloned equine embryos have been described previously [27,28], with embryos from OPU-derived oocytes found to have superior in vivo viability after transfer to recipient mares compared with embryos from abattoir-derived oocytes [27]. In the Australian context, access to ovaries from slaughtered mares is extremely limited, making the collection of oocytes from live mares by OPU highly preferable for commercial cloned embryo production. Additionally, refinement of the SCNT procedure has been informed by the finding that bone marrow mesenchymal stem cells (MSCs) are more efficiently reprogrammed compared with adult fibroblast cells (AFCs) [29], which has led to their use as the preferred nuclear donor cell type.

Therefore, the aim of this study was to determine the effect of supplementing the oocyte maturation medium with NA on the capacity of equine oocytes to support cloned embryo production. As equine oocyte IVM typically involves a prolonged transport period (18 h) prior to maturation at the laboratory (22–24 h), NA supplementation during the transport period was first examined. After determining the optimal NA dose using oocytes from abattoir-sourced ovaries, the effect of this NA treatment was then assessed using oocytes harvested from live mares by OPU. Lastly, the effect of supplementing the final IVM medium with NA was evaluated using OPU-derived oocytes. Following oocyte maturation, SCNT embryos were produced using either MSCs or AFCs to compare the in vitro development of the different treatment groups.

2. Materials and methods

2.1. Mares

The study was performed at the Catalina Equine Reproduction Centre (North Richmond, NSW, Australia). A total of 23 standardbred mares, aged 3 to 15 years, were used as oocyte donors. All procedures were carried out with informed consent from the owners of the animals and in accordance with the NSW Animal Research Act (1985), which incorporates the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [30].

2.2. Chemicals and media

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Australia). Hepes-buffered Synthetic Oviductal Fluid (H-SOF) [31] supplemented with 10% fetal calf serum (FCS; AU-FBS/PG; Cellsera, Rutherford, NSW, Australia) was used for procedures performed outside of the CO_2 incubator. The medium used to transport and hold oocytes prior to IVM consisted of a 1:1 mix of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; D8437) and Medium 199 (M3769) supplemented with 10% FCS. A stock solution of 50 mM NA (N4126) was prepared by dissolving NA powder in ultra-pure water and 20 μL aliquots were stored at −20 °C until use. The oocyte maturation medium and the couplet fusion medium were prepared as described previously [27].

2.3. Retrieval of immature oocytes from ovaries collected post-mortem

Equine ovaries were obtained and processed at a slaughterhouse. The recovery of cumulus-oocyte complexes (COCs) from abattoir-sourced ovaries was performed as described previously [27]. Briefly, antral follicles <30 mm in diameter were aspirated with an 18G needle and the follicle walls were scraped extensively with a bone curette. Following transfer to a 100 mm Petri dish containing H-SOF at 37 °C to facilitate searching, selected COCs were washed three times with H-SOF. In each replicate, ovaries were collected within a 2 h period and processed over an additional 2 h period, such that the maximum time from slaughter to transferring the retrieved oocytes to vials of transport medium was approximately 4.5 h. A total of 694 COCs were collected in 6 replicates (Experiment 1). In each replicate, the COCs were randomly allocated to one of three vials filled with transport medium at 20–22 °C. In Experiment 1, the vials contained transport medium that was either unsupplemented or supplemented with 50 or 200 μM nicotinic acid (NA). These concentrations of NA were selected based on the findings of Pollard and co-workers [25]. The vials were tightly capped and the COCs were transported to the laboratory in a polystyrene container at 20–22 °C overnight, for IVM 18 h after COC recovery.

2.4. Retrieval of immature oocytes by ovum pick-up (OPU)

The OPU procedure was performed as described previously [27]. The day before OPU, mares were scanned transrectally using an ultrasound machine (Mindray M9; Mindray, Shenzhen, China) equipped with a 5–8 MHz linear-array transducer (6LE5Vs) to determine the number and size of the follicles present on the ovaries. Only mares that had at least 15 follicles, with the largest follicle <25 mm in diameter, were scheduled for OPU. In a total of 15 sessions, OPU was performed once a week on 2 or 3 scheduled mares. Some of the mares underwent OPU on multiple occasions with a minimum of two weeks between sessions.

In preparation for OPU, initial sedation consisted of 6 mg butorphanol tartrate i.v. (Butorgesic; 10 mg/mL; Troy Animal Health, Glendenning, NSW, Australia) and 4 mg detomidine hydrochloride i.v. (Sedator; 10 mg/mL; Randlab Australia, Revesby, NSW, Australia), administered in the stall regardless of body weight. Mares were then moved to stocks, where the rectum was emptied and the perineum cleansed with 2% chlorhexidine solution and water. The vaginal vestibule was scrubbed using sterile saline-soaked cotton wool. A sterile 22 Fr Foley catheter was inserted to empty the bladder and remained in place throughout the OPU procedure. After placement of a sterile IV catheter, mares received pre-medication consisting of 1.1 mg/kg flunixin meglumine i.v. (Ilium Flunixil, 50 mg/mL, Troy Animal Health), 22 mg/kg procaine benzylpenicillin i.m. (Benacillin, 300 mg/mL, Troy Animal Health), and 6.6 mg/kg gentamicin sulphate i.v. (Ilium Gentam, 100 mg/mL, Troy Animal Health). Approximately 5 min before commencing OPU, mares were given 0.1 mg/kg butylscopolamine bromide i.v. (Buscopan Compositum, Boehringer Ingelheim, NSW, Australia), with additional doses administered as required to reduce rectal straining. Immediately before starting OPU, a second sedation bolus (4 mg butorphanol, 3 mg detomidine) was administered via the IV catheter. Additional 2 mg detomidine boluses were administered as needed to maintain adequate sedation.

Immature oocytes were harvested by transvaginal ultra-sound guided aspiration of follicles 5 to 30 mm in diameter using a 12G double-lumen needle attached to a vacuum pump. After aspirating the follicular fluid, each follicle was flushed 10 times with 0.5–5 mL

(depending on follicle size) of embryo flushing medium (BoviFlush; Minitube Australia Pty. Ltd., Smythesdale, VIC, Australia) supplemented with sodium heparin (2.5 IU/mL, Pharma, Denmark) and pre-warmed to 37 °C. The follicular fluid and lavage medium were collected in 500 mL flasks kept at 37 °C. The collected fluids were poured through a sterile embryo collection filter (EmCon filter; Immuno Systems Inc., Spring Valley, WI, USA) immediately after the end of the OPU procedure, and the residual fluid and follicular material were then rinsed into a sterile Petri dish. Subsequently, COCs were identified using a stereomicroscope, washed three times with H-SOF, and transferred to vials filled with transport medium at 20–22 °C. Depending on the experimental design, the vials contained transport medium that was either unsupplemented or supplemented with 200 μM NA. The vials were tightly capped and the oocytes were kept overnight in a polystyrene container at 20–22 °C, for IVM 18 h after COC recovery.

For each mare, the duration of the OPU procedure was 15 to 25 min. Following OPU, mares were moved to a box stall and monitored until fully recovered, remaining under observation for at least 48 h. During the two days after OPU, a daily physical examination was performed, and mares received flunixin meglumine 1.1 mg/kg i.v. SID, procaine benzylpenicillin 22 mg/kg i.m. BID, and gentamicin sulphate 6.6 mg/kg i.v. SID. If no abnormalities were detected, mares returned to pasture at the beginning of the third day after OPU.

2.5. In vitro maturation (IVM)

At the end of the 18 h transport and holding period (Pre-IVM), the COCs of each group were washed 3 times with H-SOF, and then transferred to wells containing 500 μL of pre-equilibrated maturation medium (maximum of 20–50 COCs per well). The oocyte maturation medium consisted of DMEM/F-12 medium supplemented with 0.1 IU/mL follicle-stimulating hormone and 0.1 IU/mL luteinizing hormone (Menopur, Ferring Pharmaceuticals, Copenhagen, Denmark), 50 ng/mL epidermal growth factor (EGF; E9644), 1 mM sodium pyruvate, insulin-transferrin-sodium selenium mixture (ITS; I1884), and 10% FCS. Depending on the experimental design, the maturation medium was either unsupplemented or supplemented with 200 μM NA. The COCs were incubated for 22–24 h at 38 °C in a humidified atmosphere of 5% CO_2 in air.

2.6. Nuclear donor cell culture

A total of 17 different cell lines were derived from the tissues of 17 adult horses, 5 male and 12 female. For the establishment of primary adult fibroblast cell (AFC) lines, seven skin biopsies were obtained and processed as previously described [32]. Mesenchymal stem cells (MSCs) were isolated from ten sternal bone marrow aspirates following the protocol described by Sellon [33]. Briefly, horses were intravenously sedated with 0.01 mg/kg detomidine hydrochloride (10 mg/mL; Randlab Australia) and 0.01 mg/kg butorphanol tartrate (10 mg/mL; Troy Animal Health). The region between the 4th and 5th sternebrae was identified by ultrasonography, clipped, and surgically prepared. Approximately 6 mL of lignocaine hydrochloride (20 mg/mL; Troy Animal Health) was infiltrated through the subcutis, muscle layers, and into the periosteum to provide local anesthesia. A stab incision was made using a #10 surgical blade and ~20 mL of bone marrow was aspirated using a 13-gauge 5 cm Jamshidi aspiration needle (BD; Becton Dickinson, NJ, USA) under negative pressure. Aspirates were collected into sodium citrate-containing vacutainers and transported to the laboratory at 5 °C. At the laboratory, the samples were centrifuged at 1000×*g* for 3 min, treated to selectively lyse erythrocytes [34], and washed twice using centrifugation under the same conditions (1000×*g* for 3 min). The resulting cell pellet was resuspended with 5 mL of cell culture medium and the cell suspension was seeded in culture flasks. Both AFCs and MSCs were maintained in medium consisting of DMEM/F-12 medium supplemented with 1 mM glutamine, 0.2 mM pyruvate, 10 ng/mL EGF, and 10% FCS, and cultured in a humidified atmosphere of 5% CO_2 in air at 38.5 °C. After 4–7 days, adherent cells were subcultured and expanded until reaching optimal confluency for cryopreservation in aliquots of culture medium containing 10% dimethyl sulfoxide (DMSO) [27]. All MSCs had a fibroblastic, spindle-like morphology characteristic of MSCs [35], with the expanded cell populations appearing homogeneous in all cases.

2.7. Somatic cell nuclear transfer (SCNT)

A different donor cell line was used in each of the six replicates of Experiments 1 and 2 (one cell line was used once in both experiments). In Experiment 3, seven different donor cell lines were used in the nine replicates, such that two of the cell lines were used twice (one of these had also been used once in Experiment 1). After thawing, the cells were cultured for no less than two days and grown to confluence to induce cell cycle arrest at least 24 h before use as donor cells for SCNT. Immediately before SCNT, the cells were trypsinized, washed, and suspended in H-SOF containing 2% FCS, and kept at room temperature (RT) until use (approximately 10 min).

The cumulus cells were removed from the oocytes after IVM by gentle pipetting in H-SOF supplemented with 1 mg/mL hyaluronidase. The micromanipulation process involved the removal of the polar body and the metaphase plate from each oocyte using an enucleation pipette attached to a Piezo drill (PMAS-CT150; Prime Tech Ltd., Ibaraki, Japan), and placement of a donor cell in the perivitelline space of each cytoplast. The couplets were held for 1 h in H-SOF at 38 °C, before being transferred to fusion medium, which contained 3.0 M D-sorbitol, 0.05 mM $CaCl_2$, 0.10 mM $MgCl_2$ and 0.05% (w/v) fatty acid-free bovine serum albumin (BSA; 700-102P; Gemini Bio-Products, West Sacramento, CA, USA), on a fusion chamber slide between electrodes 0.5 mm apart. A direct current (DC) pulse of 2.2 kV/cm strength and 15 μsec duration was immediately applied to the couplets. The pulsed couplets were washed 3 times in H-SOF, transferred to embryo culture medium, which consisted of DMEM/F-12 medium supplemented with 10% FCS, and incubated for 2 h. Activation was carried out by exposing the fused couplets to 5 μM ionomycin in H-SOF for 5 min. After several washes in H-SOF, the fused and activated couplets were treated with 1 mM 6-dimethylaminopurine and 5 μg/mL cycloheximide in embryo culture medium for 4 h. Finally, the reconstructed embryos were washed several times, transferred to droplets of culture medium, and incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C. The SCNT embryos were transferred to fresh culture medium every 2 days. Embryonic cleavage was assessed at the first change of culture medium and blastocyst development was assessed on Days 7 and 8 of in vitro culture, using well-described morphological criteria to identify key developmental features [36].

2.8. Experimental design

In Experiment 1, in which abattoir-derived oocytes were used, the recovered COCs were randomly allocated to tubes containing transport medium that was either unsupplemented (Control) or supplemented with 50 or 200 μM nicotinic acid (NA). After the 18 h transport and holding period (Pre-IVM), the COCs of the three groups were transferred to wells of IVM medium (containing no NA supplement). In Experiments 2 and 3, OPU-derived oocytes were used. In Experiment 2, the recovered COCs were randomly allocated to tubes containing transport medium that was either unsupplemented (Control) or supplemented with 200 μM NA. After the 18 h transport and holding period (Pre-IVM), the COCs of the two groups were transferred to wells of IVM medium (containing no NA supplement). In Experiment 3, all the recovered COCs were transferred to tubes containing transport medium supplemented with 200 μM NA. After the 18 h transport and holding period (Pre-IVM), the COCs were randomly allocated to wells of IVM medium that was either unsupplemented (Control) or supplemented with 200 μM NA. Following

the IVM period in all three experiments, the meiotically mature oocytes of each group were used to produce SCNT embryos. Experiments 1, 2, and 3 were replicated 6, 6, and 9 times, respectively.

2.9. Statistical analysis

Data were analyzed using the Genstat statistical software package (18th edition; VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The embryo in vitro production data (oocytes matured, couplets fused, embryos cleaved, and blastocysts formed) were subjected to logistic regression analysis with treatment group, donor cell line, and donor cell type as factors. A P value of less than 0.05 designated a significant difference.

3. Results

3.1. Experiment 1: The effect of nicotinic acid supplementation during Pre-IVM on abattoir-derived oocytes

A total of 694 oocytes were collected from abattoir-sourced ovaries in six replicates (mean of 115.7 oocytes per replicate). As shown in Table 1, the oocyte maturation rates did not differ significantly among the groups (control: 52.6%; 50 μM NA: 58.8%; 200 μM NA: 58.6%; $P > 0.05$). From the 391 MII-stage oocytes obtained, a total of 300 SCNT donor cell-ooplast couplets were constructed. The rates of couplet fusion and embryonic cleavage did not differ significantly among the groups ($P > 0.05$; Table 1). Treatment with 200 μM NA during Pre-IVM increased the proportion of fused couplets that formed blastocysts (32.9% vs 19.9%; $P < 0.05$), and the proportion of cleaved embryos that formed blastocysts (50.0% vs 30.7%; $P < 0.05$), compared with the control group (Table 1). Of fused couplets, the blastocyst formation rate of the 50 μM NA group was also superior to that of the control group (27.1% vs 19.9%; $P < 0.05$; Table 1). Blastocysts were produced in all six replicates in the control, 50 μM NA, and 200 μM NA groups (mean of 3.0, 3.3, and 5.6 blastocysts per replicate, respectively).

Of the cell lines used to provide the donor nuclei for SCNT in Experiment 1, three were AFC lines (#1.3, #1.4, #1.6), and three were MSC lines (#1.1, #1.2, #1.5). No significant interaction between cell line and treatment was found. As shown in Fig. 1, couplets constructed using two of the cell lines (#1.3 and #1.4) achieved a 100% fusion rate, which was significantly greater than that attained by couplets constructed using the other four cell lines (range of 82.4% to 86.4%). There was no effect of cell line on the rates of cleavage and blastocyst formation ($P > 0.05$; Fig. 1). The fusion rate was greater for AFC-derived couplets than for MSC-derived couplets (94.8 ± 3.0% vs 84.6 ± 2.3%; $P < 0.05$), while the cleavage (70.1 ± 3.0% vs 63.1 ± 3.5%) and blastocyst formation (27.9 ± 3.3% vs 25.3 ± 1.3%) rates did not differ due to donor cell type ($P > 0.05$).

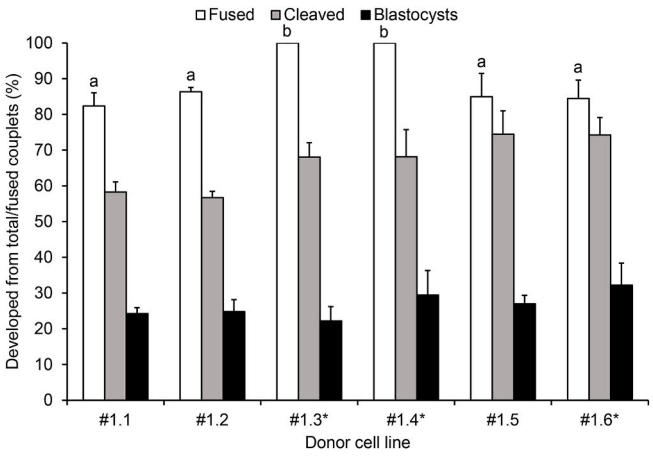

Fig. 1. The effect of donor cell line (#1.1–#1.6) on the development of SCNT embryos in Experiment 1. Combining the data from the three experimental groups for each cell line, the values are expressed as the percentages (mean ± s.e.m.) of couplets that fused (from total couplets constructed; white bars), embryos that cleaved (from fused couplets; grey bars), and blastocysts that formed (from fused couplets; black bars). Donor cell lines that are labelled with an asterisk are adult fibroblast cells, and those that are unlabelled are mesenchymal stem cells. Bars without a common letter differ significantly ($P < 0.05$).

3.2. Experiment 2: The effect of nicotinic acid supplementation during Pre-IVM on OPU-derived oocytes

A total of 147 oocytes were collected by OPU in six replicates (mean of 24.5 oocytes per replicate). As shown in Table 2, the oocyte maturation rates did not differ significantly between the groups (Control: 63.9%; 200 μM NA: 54.9%; $P > 0.05$). From the 88 MII-stage oocytes obtained, a total of 81 SCNT donor cell-ooplast couplets were constructed. The rates of couplet fusion, embryonic cleavage, and blastocyst formation did not differ significantly between the groups ($P > 0.05$; Table 2). Blastocysts were produced in all six replicates in the control and 200 μM NA groups (mean of 2.2 and 2.3 blastocysts per replicate, respectively).

Of the cell lines used to provide the donor nuclei for SCNT, one was an AFC line (#2.2), and five were MSC lines (#2.1, #2.3, #2.4, #2.5, #2.6). No significant interaction between cell line and treatment was found. There was no effect of cell line on the rates of fusion, cleavage, and blastocyst formation ($P > 0.05$; Fig. 2). Also, the rates of fusion (100 ± 0% vs 85.3 ± 5.0%), cleavage (64.4 ± 24.4% vs 80.2 ± 4.2%), and blastocyst formation (47.8 ± 7.8% vs 39.7 ± 5.9%) did not differ due to donor cell type ($P > 0.05$).

Table 1
Effect of nicotinic acid (NA) supplementation during Pre-IVM of abattoir-derived oocytes on the in vitro production of cloned embryos. Percentage values are expressed as the mean ± s.e.m.

	Control	50 μM NA	200 μM NA
Total COCs	245	189	260
Oocytes matured/COCs (%)	129/245 (52.6 ± 4.4)	111/189 (58.8 ± 4.4)	151/260 (58.6 ± 3.4)
Total couplets constructed	104	82	114
Couplets fused/constructed (%)	91/104 (87.8 ± 4.5)	74/82 (90.8 ± 3.5)	103/114 (90.5 ± 4.0)
Embryos cleaved/fused (%)	58/91 (65.1 ± 2.0)	50/74 (67.9 ± 4.4)	69/103 (66.9 ± 6.0)
Blastocysts formed/fused (%)	18/91 (19.9 ± 1.7)[a]	20/74 (27.1 ± 1.4)[b]	34/103 (32.9 ± 3.0)[b]
Blastocysts formed/cleaved (%)	18/58 (30.7 ± 2.6)[a]	20/50 (40.1 ± 1.0)[ab]	34/69 (50.0 ± 4.1)[b]

[a,b]Within rows, values without a common letter differ significantly (P < 0.05).

Table 2
Effect of nicotinic acid (NA) supplementation during Pre-IVM of OPU-harvested oocytes on the in vitro production of cloned embryos. Percentage values are expressed as the mean ± s.e.m.

	Control	200 μM NA
Total COCs	73	74
Oocytes matured/COCs (%)	46/73 (63.9 ± 6.4)	42/74 (54.9 ± 5.6)
Total couplets constructed	42	39
Couplets fused/constructed (%)	34/42 (83.8 ± 7.3)	37/39 (91.7 ± 5.3)
Embryos cleaved/fused (%)	28/34 (83.3 ± 4.2)	27/37 (71.9 ± 8.7)
Blastocysts formed/fused (%)	13/34 (39.0 ± 5.8)	14/37 (43.0 ± 8.7)
Blastocysts formed/cleaved (%)	13/28 (46.5 ± 6.3)	14/27 (62.8 ± 13.3)

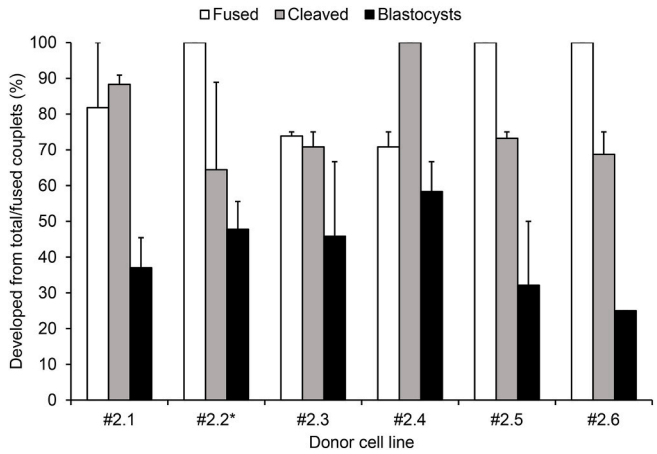

Fig. 2. The effect of donor cell line (#2.1–#2.6) on the development of SCNT embryos in Experiment 2. Combining the data from the two experimental groups for each cell line, the values are expressed as the percentages (mean ± s.e.m.) of couplets that fused (from total couplets constructed; white bars), embryos that cleaved (from fused couplets; grey bars), and blastocysts that formed (from fused couplets; black bars). Donor cell lines that are labelled with an asterisk are adult fibroblast cells, and those that are unlabelled are mesenchymal stem cells. There were no significant differences among the cell lines.

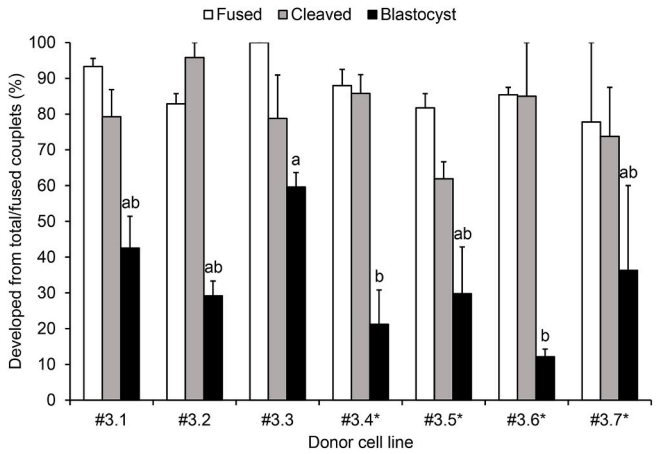

Fig. 3. The effect of donor cell line (#3.1–#3.7) on the development of SCNT embryos in Experiment 3. Combining the data from the two experimental groups for each cell line, the values are expressed as the percentages (mean ± s.e.m.) of couplets that fused (from total couplets constructed; white bars), embryos that cleaved (from fused couplets; grey bars), and blastocysts that formed (from fused couplets; black bars). Donor cell lines that are labelled with an asterisk are adult fibroblast cells, and those that are unlabelled are mesenchymal stem cells. Bars without a common letter differ significantly ($P < 0.05$).

3.3. Experiment 3: The effect of nicotinic acid supplementation during IVM on OPU-derived oocytes

A total of 285 oocytes were collected by OPU in nine replicates (mean of 31.7 oocytes per replicate). As shown in Table 3, the oocyte maturation rates did not differ significantly between the groups (Control: 71.3%; 200 μM NA: 71.5%; $P > 0.05$). From the 200 MII-stage oocytes obtained, a total of 177 SCNT donor cell-ooplast couplets were constructed. The rates of couplet fusion and embryonic cleavage did not differ significantly between the groups ($P > 0.05$; Table 3). Treatment with 200 μM NA during IVM increased the proportion of fused couplets that formed blastocysts (41.0% vs 24.5%; $P < 0.05$), and the proportion of cleaved embryos that formed blastocysts (53.4% vs 31.3%; $P < 0.05$), compared with the control group (Table 3). Blastocysts were produced in all nine replicates in the control and 200 μM NA groups (mean of 2.6 and 3.3 blastocysts per replicate, respectively).

Of the seven cell lines used to provide the donor nuclei for SCNT, four were AFC lines (#3.4, #3.5, #3.6, #3.7), and three were MSC lines (#3.1, #3.2, #3.3). No significant interaction between cell line and treatment was found. There was no effect of cell line on the rates of couplet fusion and embryonic cleavage ($P > 0.05$; Fig. 3). Also, the rates of fusion (92.4 ± 2.6% vs 84.2 ± 4.0%) and cleavage (83.3 ± 5.1% vs 78.4 ± 4.8%) did not differ due to donor cell type ($P > 0.05$). However, the rates of blastocyst formation differed significantly due to the donor cell line used and the donor cell type. Couplets constructed using cell line #3.3 formed blastocysts at a greater rate than those constructed using cell lines #3.4 and #3.6 ($P < 0.05$; Fig. 3). Moreover, the blastocyst formation rate was greater for MSC-derived couplets than for AFC-derived couplets (43.5 ± 5.9% vs 24.1 ± 6.0%; $P < 0.05$).

Table 3
Effect of nicotinic acid (NA) supplementation during IVM of OPU-harvested oocytes on the in vitro production of cloned embryos. Percentage values are expressed as the mean ± s.e.m.

	Control	200 μM NA
Total COCs	146	139
Oocytes matured/COCs (%)	102/146 (71.3 ± 3.2)	98/139 (71.6 ± 3.1)
Total couplets constructed	93	84
Couplets fused/constructed (%)	82/93 (89.0 ± 2.9)	73/84 (86.6 ± 4.5)
Embryos cleaved/fused (%)	66/82 (79.6 ± 3.4)	59/73 (81.6 ± 6.2)
Blastocysts formed/fused (%)	21/82 (24.5 ± 6.0)[a]	30/73 (41.0 ± 6.5)[b]
Blastocysts formed/cleaved (%)	21/66 (31.3 ± 8.1)[a]	30/59 (53.4 ± 9.6)[b]

[a,b]Within rows, values without a common letter differ significantly (P < 0.05).

4. Discussion

The results of this SCNT study show that the developmental potential of equine oocytes matured in vitro can be significantly enhanced by supplementing the medium with nicotinic acid (NA). Treatment with NA during the 18 h transport and holding period (Pre-IVM) improved the developmental competence of abattoir-derived oocytes, markedly increasing the proportion of fused couplets that developed to the blastocyst stage. Likewise, treatment with NA during the 22-24 h IVM incubation enhanced the developmental competence of oocytes harvested from live mares by OPU, again, greatly increasing the proportion of fused couplets that developed to the blastocyst stage. Through the consistent production of blastocysts in a commercial equine cloning program, the beneficial effect of NA supplementation on oocyte developmental competence was found to be independent of the donor cell line and the type of cell used to provide the donor nuclei. These findings suggest that the provision of NA during Pre-IVM and/or IVM helps to meet an important metabolic requirement of equine cumulus-oocyte complexes (COCs), thereby increasing the efficiency of embryo in vitro production.

Nicotinic acid is a precursor of NAD^+, which is a key molecule involved in fundamental cellular processes such as energy production, apoptosis regulation, and DNA repair [21,37]. Moreover, the activities of NAD^+-consuming enzymes, including sirtuins (SIRTs) and poly-ADP-ribose polymerases (PARPs), are essential for oocyte maturation [14,38]. Additionally, NA possesses antioxidant properties and functions as a lipid modulator [39], positioning it as a promising candidate treatment to improve oocyte quality. Recently, reduced intra-oocyte NAD^+ levels have been linked with the age-related decline in female fertility [15,16,18]. In female mice of advanced age, treatments that elevated NAD^+ levels were found to improve oocyte developmental competence and pregnancy outcomes [15,16,18]. Furthermore, supplementing IVM medium with NA has been found to enhance nuclear and cytoplasmic properties of oocytes in pigs and cattle [24,25,40,41].

Nuclear and cytoplasmic maturation of oocytes must proceed in a coordinated manner to ensure successful fertilization and subsequent

embryonic development [1,2]. In equine oocytes, the time required to complete maturation, characterized by the extrusion of the first polar body, is directly related to the ability of the oocyte to support optimal embryonic development [42]. A unique feature of equine oocyte IVM systems is a commonly implemented 18 h (overnight) holding period at 20–22 °C (Pre-IVM), which facilitates the transport of COCs from the site of collection to the laboratory where IVM is then carried out [6,28,43]. In the present study, treatment with NA during the Pre-IVM phase (Experiments 1 and 2) did not influence the subsequent rates of maturation to the MII-stage. Similarly, the nuclear maturation rate of oocytes treated with 200 μM NA during IVM were comparable to that of the control group (Experiment 3). Similar results have been reported in studies with porcine oocytes, where no significant increases in nuclear maturation rates were observed following treatment with NA [40,41]. In contrast, the addition of NA at a higher concentration (400 μM) during IVM increased nuclear maturation rates in bovine oocytes [24]. The inconsistent nuclear maturation results observed among studies may be due to the different concentrations of NA used.

Regarding the acquisition of oocyte developmental competence, or cytoplasmic maturation, the Pre-IVM NA treatments (50 and 200 μM) enhanced the capacity of abattoir-derived oocytes to develop to the blastocyst stage following SCNT embryo production (Experiment 1). As the benefits to blastocyst production were more apparent at the higher NA dose in Experiment 1, the 200 μM concentration was used to assess the effects of NA on OPU-derived oocytes in Experiments 2 and 3. Interestingly, the Pre-IVM NA treatment did not enhance the capacity of OPU-derived oocytes to form blastocysts (Experiment 2), whereas the IVM NA treatment did (Experiment 3), compared with the untreated controls. The contrasting effect of NA during the Pre-IVM period on the differently sourced oocytes suggests that the cytoplasmic deficiencies of abattoir-derived oocytes differ from those of OPU-derived oocytes. This finding is not surprising, given the previously reported effects of oocyte source on the development of equine cloned embryos [27]. The intrinsic quality of oocytes would be expected to be poorer from slaughtered mares than from live mares, because slaughtered mares are often older and less fertile, and their oestrous cycle phase is unknown, compared with live mares monitored for OPU [5,9,44]. Further, abattoir-derived oocytes have compromised meiotic competence due to post-mortem changes [45], and their developmental potential declines as the interval between ovary excision and oocyte retrieval increases [2,46]. The variation among the experiments in overall cleavage rates (Experiment 1: 66.6%; Experiment 2: 77.6%; Experiment 3: 80.6%) further highlights the effect of oocyte source on cloned embryo production. Nevertheless, the oocytes from live mares benefited from the NA treatment when it was applied during the IVM period (Experiment 3). The effect of NA supplementation during IVM on abattoir-derived oocytes warrants additional investigation. Finally, it should be noted that the NA treatments had no effect on the rates of couplet fusion and development to the early cleavage stages in any of the experiments.

These findings are consistent with those from studies in pigs, cattle, and mice, where supplementation of IVM media with NA has been shown to improve the developmental competence of oocytes [18,25,40,41]. In porcine oocytes from small antral follicles, Pollard et al. (2021) also found that the addition of 200 μM NA to IVM medium increased the blastocyst formation rate, without affecting the cleavage rate, compared with the control [25]. The conversion of NA to NAD^+ is thought to promote normal chromosome segregation and spindle assembly, because inhibition of the Preiss-Handler pathway during IVM increased the incidence of aberrant metaphase-II spindles in porcine oocytes [41]. Also, NA treatment elevated the NAD^+ content and reduced the frequency of spindle defects in oocytes from old mice [18]. At a higher concentration (600 μM), NA-enhanced embryo development was associated with higher levels of glutathione (GSH), lower levels of reactive oxygen species (ROS), and lower lipid droplet content, within porcine oocytes [40]. More detailed assessments of equine oocytes are needed to determine the effects of NA treatments during Pre-IVM and IVM on GSH and ROS levels, lipid droplet content, and spindle morphology.

Given that the Pre-IVM NA treatment alone improved the developmental competence of abattoir-derived oocytes, but not OPU-derived oocytes, further investigation is needed to elucidate the specific deficiencies of these oocyte groups. As already mentioned, oocytes from abattoir-sourced ovaries are compromised due to post-mortem changes, and slaughtered mares are often older and less fertile than mares selected for OPU [5]. Studies in mice have demonstrated that NAD^+ levels are lower in oocytes from females of advanced age, and that intra-oocyte NAD^+ levels can be restored by supplementing NAD^+ precursors in vivo, thereby improving fertility [15,16]. Interestingly, studies in mares have shown that oral administration of NA increases the concentrations of several NAD^+ precursors and metabolites in plasma and follicular fluid [47,48]. Our in vitro findings support the proposal that supplementing the diet with NAD^+-elevating compounds may be a useful strategy to improve oocyte quality and pregnancy outcomes in mares, especially those of advanced maternal age [14]. Moreover, our findings have important implications for the in vitro production of equine embryos using other techniques, such as intra-cytoplasmic sperm injection (ICSI) and conventional in vitro fertilization (IVF). An embryo transfer trial is currently in progress to evaluate the efficacy and safety of the NA treatment during Pre-IVM and IVM on conceptus development and foal health.

In the present study, a total of 17 different cell lines were used to provide the donor nuclei for cloned embryo production. Of these, 7 were AFC lines derived from skin biopsies, and 10 were MSC lines derived from aspirated sternal bone marrow. It is well known that the efficiency of SCNT embryo production is influenced by the donor cell type [49] and can vary considerably between donor cell lines derived from the same tissue [50]. Ideally, in a study examining the effects of an oocyte treatment on cloned embryo development, the same donor cell line would be used in all replicates. However, the use of different donor cell lines was unavoidable, due to the commercial imperative of the horse breeding enterprise. Importantly, the same donor cell line was used for all experimental groups in each replicate, and no interactions were detected between treatment and cell line, indicating that the reported effects of the NA treatments were independent of the donor cell line used. An effect of cell type on blastocyst formation was detected in Experiment 3 (replicated 9 times), but not in Experiments 1 and 2 (relicated 6 times each). A previous equine SCNT study has shown that the development of bone marrow MSC-derived embryos is superior to that of AFC-derived embryos [29]. In the present study, an inability to consistently detect significant differences due to the donor cell type used can be attributed to a lack of statistical power. Notably, blastocysts were produced in all experimental groups in every replicate, demonstrating the reliability and robustness of the SCNT procedures used.

5. Conclusion

These results demonstrate that exposure to 200 μM NA during the Pre-IVM and IVM periods significantly enhanced the developmental competence of equine oocytes. A substantial improvement to blastocyst production was achieved regardless of whether the oocytes were collected from abattoir-sourced ovaries or from live mares by OPU. This is the first study to evaluate the effect of NA supplementation on equine oocytes, and our results align with those from studies in other species, highlighting the positive impact of NAD^+-elevating treatments on the acquisition of oocyte developmental competence. These findings pave the way for more detailed investigations into the molecular mechanisms involved during the maturation of oocytes and inform future refinements to IVM systems that are key to improving the efficiencies and outcomes of assisted reproductive technologies in clinical practice.

CRediT authorship contribution statement

Jenin V. Cortez: Writing – original draft, Visualization,

Methodology, Investigation, Formal analysis, Data curation. **Danton Cervi:** Methodology, Investigation. **Agustin J. Ruiz:** Writing – review & editing, Methodology, Investigation. **Christopher G. Grupen:** Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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