LIPID METABOLISM IN IN VITRO PRODUCED EMBRYOS AND EPIBLAST-DERIVED STEM CELLS

Gizem Guven Ates

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School of Biosciences

Sutton Bonington Campus

Loughborough Leicestershire LE12 5RD

To my family.

This thesis is dedicated to my parents and my sister. I am grateful to you for always supporting and guiding me on this long journey, for always making me feel your love and most importantly for making me who I am.

Love you!

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Declaration

I hereby declare that this thesis represents my own work which has been done for the degree of PhD at University of Nottingham and has not been submitted for any other degree or award. Other sources of information and assistance given to me during the preparation of this thesis have been acknowledged.

Gizem Guven Ates

General Introduction

Infertility is a disease of the reproductive system defined as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (PierreZegers-Hochschild et al., 2009). Between 9-15% couples will have infertility problems during their lifetimes and for these infertile people, the percentage searching for medical assistance is around 56% in more developed countries and 51% in less developed countries (Boivin et al., 2007). To treat infertility, Assisted Reproductive Technologies (ARTs) were developed beginning with artificial insemination (Jouannet, 2009). Today, ART also consists of in-vitro fertilization (IVF), intracytoplasmic sperm injection and cryopreservation of germ cells (i.e., sperm, oocyte and embryos) (Sejbaek et al., 2013).

On the contrary, infertility is not common in cattle. However still ART is applied in these animals to increase food production, increase the birth of healthy offspring and store the genetic materials for future. On one hand, while high productive animals calve around eight or nine times during their lifetime, it is possible to take a few times more offspring than they will get through in their lives in a year by ART. On the other hand, ART plays a commercially important role while the import-export. Intercontinental transport of live animals cost more than transport of frozen embryos (Mapletoft and Hasler, 2005). The other fact, the ART gives us a chance to create new breeds as a productive race and expedite the genetic improvement (Wu and Zan, 2012).

The human in vitro embryo production (IVP) protocols normally commence with *in vivo* maturation which takes place within the ovarian follicle during gonadotropin treatment. This is because IVM is not successful enough in human ART to encourage widespread uptake (Anckaert et al., 2012). In contrast, IVM of immature oocytes is routine practice prior to fertilization in cattle IVP programs. Recently there has been a trend towards transfer of Day 5/Day 6 blastocysts instead of Day 2 cleaved zygotes in human ART (Glujovsky et al., 2016), with many cycles embracing single transfer of frozen/thawed blastocysts (Tiegs et al., 2019). This change has been developed as a result of undesirable consequences of transferred cleaved

zygotes such as low implantation and birth rate and much cumulative pregnancy.

Limitations of human IVF research such as ethics and costs have led scientists to undertake animal studies. Mouse embryos have been used extensively to understand biochemical and physiology regulations of the human embryos. However, bovine and human preimplantation embryos have many similarities with respect to biochemical, paternal and maternal regulatory processes (Menezo and Herubel, 2002). Besides, bovine and human embryo development processes are completed almost at the same time. (Figure 1).

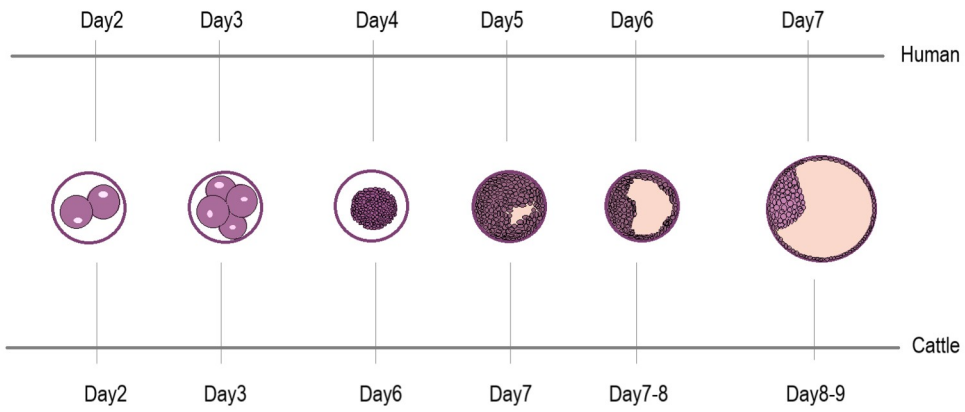


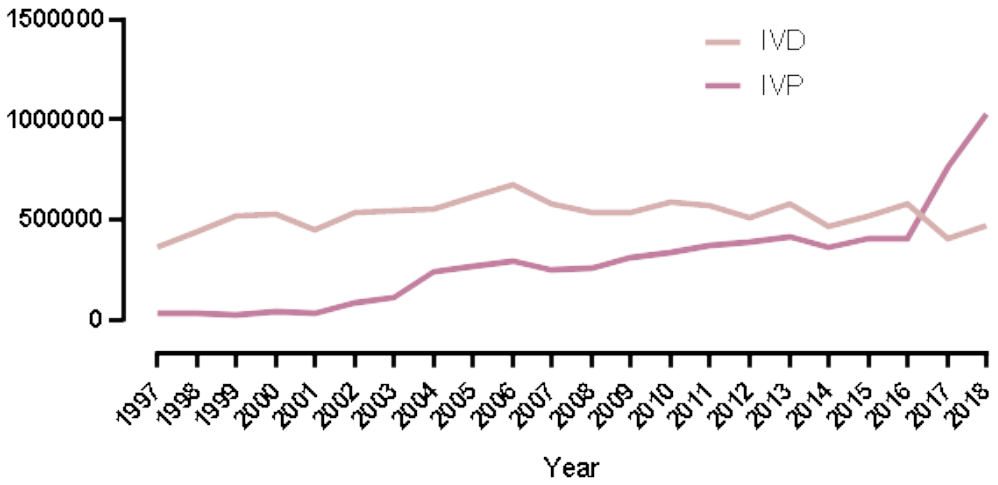
Figure 1 Comparison of embryo development stages in cattle and humans. Embryos reach the 2- and 3-cell stage around the same time. However, while human embryos form a morula around Day 4, this stage is seen on Day 5/6 in cattle. Thereafter, bovine embryo stages occur typically 1 to 2 later than human embryo stages. Credit artwork: Gizem Guven Ates

The number of cattle embryos transferred are steadily increased year on year as a consequence of improvements in bovine IVP (Figure 2A and B). The underlying reason of this development is comprehension of the metabolism of oocytes and embryos, thus determining the needs of cells viability culture. For instance, the human fluid composition of the oviduct where the zygote stays until formation of morula, demonstrates pyruvate and lactate levels significantly higher than uterus where the pre-implanted blastocyst is located. This contrasts with glucose concentrations which is over five times higher in the uterus than the oviduct and until this statement was understood required

pyruvate and glucose were not placed enough in the culture system (Leese et al., 2007).

Successful production of embryo also depends on maternal and paternal factors such as age, environmental pollution and parenteral nutrition. Nowadays numerous studies have been done especially about parenteral nutrition (Sharma et al., 2020). The scientists associated parenteral nutrition with blastocyst quality, DNA methylation, metabolism of germ cells and embryos and even offspring disease risk over their lifetime. Attention was drawn to over- and undernutrition can affect sperm and seminal plasma in male (Sinclair and Watkins, 2013), metabolites in follicular environment (Robker et al., 2009), ovulation rate and embryo viability (Gonzalez-Añover et al., 2011) during preconception period.

A.



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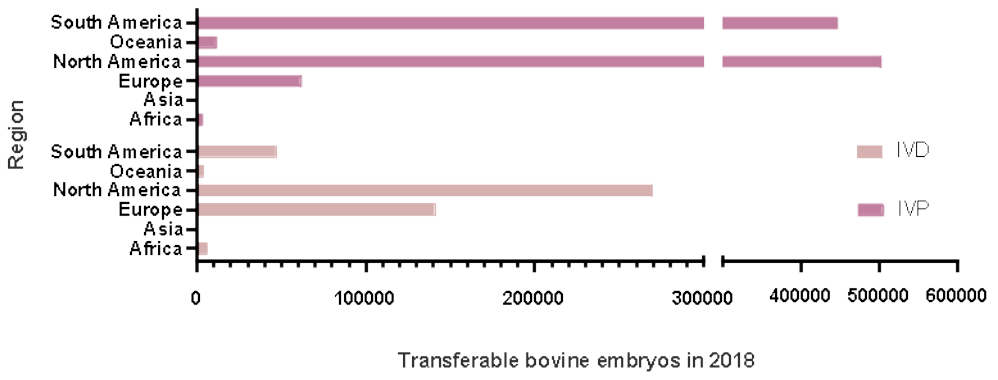

Figure 1 Number of cattle embryos transferred by year over the past 20 years (A) (IETS, 2016) and by geographical region in 2018 (B) (IETS, 2019). IVD = in vivo derived; IVP = in vitro produced

Culture environment during in vitro maturation (IVM), fertilisation (IVF and culture (IVC) is a major limitation in the success of contemporary systems of both human and bovine IVP. An understanding of gamete/embryo metabolism is key to the successful development of these systems. There are major requirements for energy associated with biosynthetic processes and proliferation in these different cell types. Energy is provided by the breakdown of carbohydrates, proteins and lipids. Well-studied sources of energy relate to carbohydrates, such as pyruvate, glucose and lactate (Gray et al., 2014), and amino acids (Hemmings et al., 2012). The importance of lipid metabolism, however, has become increasingly appreciated in recent years (Leese, 2015). Oocytes tend to accumulate fatty acids during the terminal stages of oocyte growth prior to IVM (Sturmey et al., 2009). Fatty acid oxidation (FAO) was shown to be indispensable for oocyte meiotic maturation and developmental competence in mice (Dunning et al., 2010) although oocyte lipid content in mice is relatively low compared to that of farm animal species such as cattle and pigs (McEvoy et al., 2000). Despite differences in oocyte lipid quantity, inhibition of FAO with the carnitine palmitoyl transferase 1 (CPT1) activity inhibitor etomoxir during IVM delayed the completion of meiotic maturation in murine, bovine and porcine oocytes (Paczkowski et al., 2014). In contrast, increasing FAO by supplementation of culture media with lipid metabolism modulators such as L-carnitine, resveratrol, conjugated linoleic acid and forskolin significantly improve oocyte competence in terms of fertilization and embryo development in mice (Dunning et al., 2011) and cows (Sutton-Mcdowall et al., 2012). On the other hand, lipids are key structural components of cell membranes and form complexes with glycose and/or proteins (Muro et al., 2014). In addition, lipids are precursors of steroids which have important roles in cell signalling (Kusumi et al., 2012).

With the foregoing discussion in mind, the aim of this thesis was to improve our understanding of lipid metabolism, and fatty acid oxidation in particular, during IVM of cattle oocytes, and to investigate the long-term consequences for embryo development and viability. The ultimate goal was to generate

improved systems for cattle IVP that would lead to better pregnancy outcomes following embryo transfer.

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Abbreviations

ALA	Alpha linolenic acid
ALC	Acetyl L-carnitine
AMPK	Monophosphate-activated protein kinase
ART	Assisted reproductive technique
ATP	Adenine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine mono phosphate
CAT	Catalyse
CAT	Carnitine acyl translocase
CC	Cumulus cells
CLA	Conjugated linoleic acid
COC	Cumulus oocyte complex
CPT	Carnitine palmitoyl transferase
CrAT	Carnitine-O-acetyltransferase
DFR	Dominant follicle removal
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxynucleic acid
EB	Embryoid body
EpiSC	Epiblast-derived stem cells
ER	Endoplasmic reticulum
ESC	Embryonic stem cell
ETS	Electron transport system
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Fatty acid oxidation

FASN	Fatty acid synthase
FCS	Fetal calf serum
FF	Follicular fluid
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
g	G force
g	gram
GC	Granulosa cell
GF	Growth factor
GPX	Glutathione peroxidase
GSH	Glutathione
GS-MS	Gas chromatography–mass spectrometry
GVBD	Germinal vesicle breakdown
HPLC	High pressure liquid chromatography
HSL	Hormone sensitive lipase
IAI	Inter alpha inhibitor
ICM	Inner cell mass
IETS	International Embryo Transfer society
IGF-1	Insulin like growth factor 1
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
LA	Linoleic acid
LC	L-carnitine
LD	Lipid droplets

LDL	Low density lipoprotein
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
Log	Logarithm
LOS	Large offspring syndrome
LPL	Lipoprotein lipase
MII	Metaphase 2
MEF	Mouse embryonic fibroblast
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mmol	Millimole
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acid
mtDNA	Mitochondrial DNA
NEB	Negative energy balance
OPU	Ovum pick up
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin and streptomycin
PFA	Paraformaldehyde
PKA	Protein kinase A
PPAR	Peroxisome proliferator activated receptor
PT	Pre-treatment
PUFA	Polyunsaturated fatty acid

PVP	Polyvinylpyrrolidone
RC	Reactive control
RNA	Ribonucleic acid
RNASeq	RNA Sequence
ROS	Reactive oxygen species
RT	Reverse transcription
SC	Stem cell
SCD	Stearoyl-CoA desaturase
SEM	Standard error means
SIRT	Sirtuin
SOD	Superoxide dismutase
SOF	Synthetic oviductal fluid
SREBF	Sterol regulatory element binding transcription factor
StAR	Steroidogenic acute regulatory protein
TAG	Triglyceride
TCA	Tricarboxylic acid cycle
TE	Trophectoderm
TGF-β	Transforming growth factor- β
VLDL	Very low-density lipoproteins
ZP	Zona pellucida
µg	Micrograms
µl	Microlitre
µm	Micrometre
µmol	Micromole

Chapter 1 Literature Review

1.1 Introduction

Successful embryo production depends on various factors such as genetic and epigenetic mechanisms operating within germ cells moderated by the environment, parenteral nutrition and sperm ability to fertilize; all of which are critical to promote embryo development following fertilization. A number of studies have sought to improve oocyte quality through establishing a better understanding of metabolism which is required for oocyte and preimplantation embryo viability (Scott et al., 2018). Fully grown oocytes from antral-stage follicles have extensive stocks of lipids, amino acids, pyruvate, mRNA and considerable numbers of mitochondria (Brown et al., 2017). Meiosis resumes prior to ovulation and nuclear maturation transits to metaphase II (MII) in readiness for fertilization. This is an energy demanding process and requires ATP from extra and intra-cellular compartments. The transition from oogenesis to post-fertilization zygote is critically dependent on energy metabolism. Cumulus oocyte complexes (COCs) and preimplantation embryos use intracellular metabolic substrates, namely pyruvate, fatty acids and glutamine, for ATP production (Bradley and Swann, 2019), all largely within mitochondria.

COCs prefer to use glucose as an initial energy source during maturation, which is metabolised in glycolysis, the pentose phosphate pathway, the hexosamine biosynthesis pathway and the polyol pathway to provide pyruvate for ATP production through oxidation (Sutton-McDowall et al., 2010). In the generation of ATP, pyruvate enters the tricarboxylic acid (TCA) cycle within the mitochondrion to generate acetyl-CoA. Further metabolism releases H+ via the electron transport system (ETS), which is captured as energy in the bonds of ATP. Oocytes have a low ability to uptake glucose via transporters, therefore they are dependent on the cooperation of cumulus cells via gap junctions (Gu et al., 2015). Cumulus cells generate pyruvate for oocytes via glycolysis during maturation (Sutton et al., 2003). From zygote formation until the morula stage, glucose metabolism and pyruvate uptake increase gradually. However, from the morula to blastocyst stage, hexokinase activity

and oxygen consumption increase and glucose uptake supresses pyruvate uptake (Chason et al., 2011).

In oocytes, amino acids are used for protein synthesis, serve as an intracellular buffer, act as organic osmolytes as well as participating in energy production (Van Winkle, 2001). Indeed, glutamine is one of the mayor energy sources required to support oocyte development. Glutaminolysis occurs in the cytoplasm of oocytes generating citrate within the TCA cycle, leading in turn to the production of ATP.

In recent years it has become increasingly apparent that lipid metabolism is key for the provision of energy during the pre-implantation stages of embryo development (McKeegan and Sturmey, 2011). Energy from fatty acids is derived via β-oxidation within the mitochondrion. Carnitine palmitoyl transferases (CPT1 and CPT2) (Dunning and Robker, 2012b) and their cofactor, carnitine, are critical for free FA transport into the mitochondria to undergo fatty acid oxidation (FAO) and thus to generate ATP (Bonnefont et al., 2004). During preimplantation development, carbohydrates, proteins and lipids are utilized as a source of energy by blastomeres. When they are compared, lipids provide the most energy and are used as a primary energy source (Gómez et al., 2001, Gomez et al., 2002). For instance, oxidation of palmitate can generate 106 ATP molecules, however oxidation of glucose can generate only 30 ATP molecules (Dunning et al., 2014b).

At the physiological level, some stimulants such as epinephrine and growth hormone increase intra-cellular hormone sensitive lipase levels by G-couple proteins. As a result of this stimulation, triacylglycerols are converted to free fatty acids and glycerol. These free fatty acids are activated by acyl-CoA then enter the mitochondria for β-oxidation by attaching to carnitine. Based on these processes, L-carnitine has been used in many studies as a regulator of lipid metabolism in bovine (Jiang et al., 2020, Jiang et al., 2019), porcine (Lowe et al., 2017, Yazaki et al., 2013), mouse (Khanmohammadi et al., 2016, Zare et al., 2015) and sheep (Saraiva et al., 2018) embryos. In addition to L-carnitine, conjugated linoleic acid (CLA) (Castañeda-Gutiérrez et al., 2007b), resveratrol (Martínez-Ibarra et al., 2018, Wang et al., 2014b) and acetyl-L-carnitine (ALC) (Xu et al., 2018b) have all been used as regulators of lipid metabolism. In the various studies related to lipid metabolism only a few have assessed the effects of antioxidants. Naturally, following β-oxidation, TCA and

ETS free radicals are generated. Up to a certain limit, free radicals assist metabolic processes within the cell. However if they exceed certain physiological limits, they have a detrimental effect on cell fate (Phaniendra et al., 2015).

With the foregoing discussion in mind, the aim of the following review is to determine the importance of lipid metabolism in germ cells and throughout preimplantation embryo development. Specifically, the effects of fatty acids on oocyte maturation, fertilization, early embryo development, cryopreservation and comparative effects of fatty acids on *in vivo* and *in vitro* processes are described. It then, briefly reports on current studies concerning assisted reproduction mainly in cattle but extended to include other mammalian species. Ultimately, an overarching working hypothesis is presented that sets the context for the work reported in subsequent chapters.

1.1.1 General aspects of fatty acid synthesis and oxidation in cellular metabolism

All mammalian cells derive fatty acids from exogenous sources or via endogenous synthesis. These fatty acids are involved in energy metabolism, synthesis of membrane components and generation of signalling molecules (Kastaniotis et al., 2017). When glucose stocks become limited in the body, most cells, except neurons, use fatty acids to generate energy. Additionally, fatty acids are converted into ketone bodies within the liver and these are used for energy in all cell types, including the brain (Houten and Wanders, 2010). Unused fatty acids are stored in cells as triglycerides within lipid droplets (LD). Their abundance varies with cell types. For instance, in *S. cerevisiae* (budding yeast) LD can be detected easily. LDs are found most distinctively during the inactive phase of *S. cerevisiae* and metabolism of LDs during the exponential phase is coordinated with an increased requirement for phospholipids needed for cell division (Walther and Jr., 2012). The variable abundance of LD within oocytes of different species leads to differences in cellular darkness when viewed by light microscopy (Ferguson and Leese, 1999). Lipid content of oocytes within the same species can increase with the use of, for example, serum supplements *in vitro* (Auclair et al., 2013).

The lipid composition of different cell types can differ markedly, suggesting that lipids are needed for distinct functions (Klose et al., 2013). While in cattle,

pig and sheep immature oocytes, palmitic, stearic and oleic acids are the most abundant (McEvoy et al., 2000), blastocysts cultured under serum free conditions have high levels of myristic, palmitic and steric acid (Sata et al., 1999). Presently the extensive roles of fatty acids within mammalian cells are not fully understood. However, it is known that lipids are important for oocyte maturation and subsequent embryo development (Figure 1.1).

For example, when α-linolenic acid (C18:3n-3) (one of the essential polyunsaturated fatty acids (PUFAs)) was added to bovine maturation media, oocyte maturation and subsequent blastocyst development was improved. The proportion of oocytes at metaphase II (MII) and phosphorylation of mitogen-activated protein kinase (MAPK) were both increased (Marei et al., 2009a). Similar observations have been reported in pig (Ji-Eun et al., 2017) and mouse studies (Truong et al., 2016). In contrast when the most abundance fatty acid in the bovine follicular fluid (linoleic acid (C18:2n-6)) was added to maturation media the proportion of bovine oocytes matured was reduced (Marei et al., 2010a).

Another abundant unsaturated fatty acid in female reproductive fluids is oleic acid (C18:1n-9) (Tsujii et al., 2001). Inclusion of this fatty acid in culture increases fatty acid storage, *in vitro* maturation (IVM), steroidogenesis and subsequent embryo development in a dose-dependent manner. For instance, while supplementation of oleic acid up to 250 μM leads to formation of smaller lipid droplets, 500 μM oleic acid leads to an increase in size and number of LD and impaired subsequent embryo development (Aardema et al., 2011, Fayezi et al., 2018b, Shaaker et al., 2012, Yenuganti et al., 2016).

Arachidonic acid (C20:4n-6) is a precursor for prostaglandin synthesis. PUFAs have additional essential roles in membrane structure, cell signalling pathways (when the G-protein coupled receptors are activated) and can affect sensitivity to oxidative damage (Khajeh et al., 2017).

Palmitic acid (C16:0) is a saturated fatty acid used for essential ATP generation via β-oxidation. However an overabundance of this fatty acid can lead to endoplasmic reticulum (ER) stress (Sutton-McDowall et al., 2016), mitochondrial dysfunction (Itami et al., 2018) and, consequently, impaired embryo development (Wu et al., 2012).

The third most abundant fatty acid in the bovine follicular fluid is stearic acid (C18:0) (Sata et al., 1999) which is also saturated Mirabi et al. (2017) found

that high-levels of saturated fatty acids in general, especially palmitic and stearic acid, have a detrimental effect on oocyte maturation, embryo implantation and development in humans.

As referred to earlier fatty acids are also important as a component of complex molecules. They contribute to cell membranes in the form of phospholipids (PL). Fluidity, permeability and stability of these membranes are altered by chain length, position and the number of double bonds (Bond et al., 2016). Therefore, these molecules are especially important for blastocyst formation.

Collectively, these findings highlight the importance of fatty acids and indicate that their proportions can be altered by environment during IVM and subsequent embryo development.

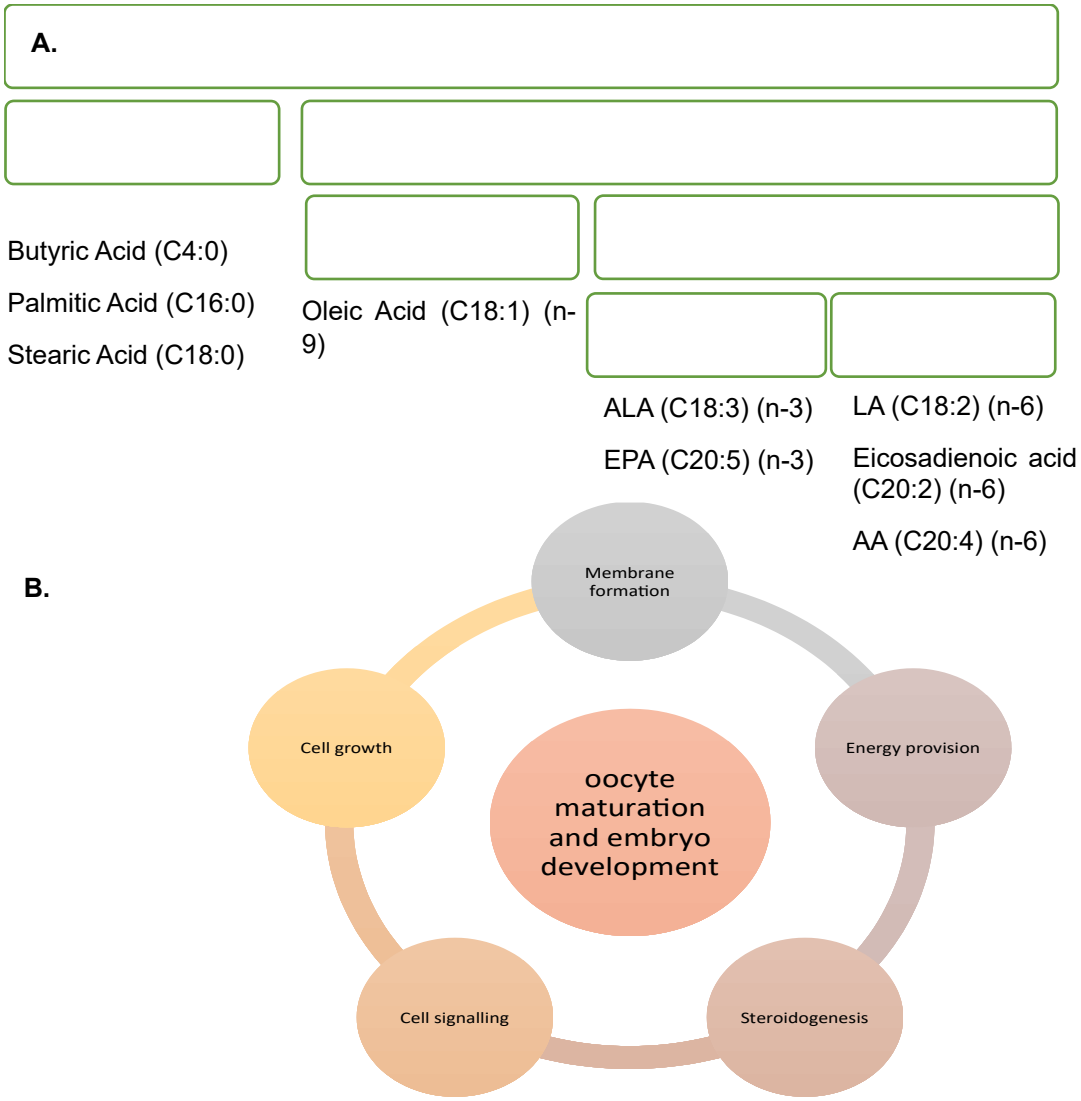


Figure 1.2 Fatty acid classification according to presence and number of double bonds within their carbon chain, with examples given (A), together with the effect of

fatty acids on oocyte maturation and embryo development via membrane formation, energy provision, cell growth and signalling, and steroidogenesis (B). ALA; alpha-linolenic acid, EPA; eicosapentaenoic acid, LA; linoleic acid, AA; arachidonic acid.

1.1.2 Fatty Acid Synthesis and Storage

In the body, fatty acids can be obtained from three sources: (i) exogenous fatty acids which are taken up by cells from blood, (ii) endogenous fatty acids which can be synthesised from acetyl-CoA, or (iii) fatty acids liberated from sources such as phospholipids and triglycerides within the cell by hydrolysis (Longo et al., 2016).

Oocytes are surrounded by cumulus cells (CCs) during antral follicle growth and fertilization. Cumulus cells have various roles such as preventing polyspermy, storing lipids in lipid droplets (LD), metabolism of glucose to pyruvate and providing ATP and free fatty acids for oocyte metabolism (Sanchez-Lazo et al., 2014a). These metabolites and additional signalling factors are transported via trans-zonal projections from cumulus cells to the oolemma. Once fatty acids gain access to the oocyte or blastocyst, they are converted to triglycerides for storage or modified in the synthesis of biologic molecules such as hormones, membranes and signalling factors. Also, cumulus cells can undergo *de novo* lipogenesis from acetyl-CoA derived from β-oxidation as well as conversion of citrate derived from pyruvate oxidation. However, acetyl-CoA cannot pass through the mitochondrial membrane, therefore it needs to attach to carnitine or convert to citrate in the matrix of mitochondria. When acetyl-CoA is released to the cytoplasm in the form of citrate or acetyl-carnitine, it is required to be reformed as acetyl-CoA under the control of ATP citrate lyse (ACLY) or carnitine-O-acetyltransferase (CrAT) enzymes (Adeva-Andany et al., 2019).

De novo fatty acid synthesis in the cytoplasm commences with the conversion of acetyl-CoA to malonyl-CoA under the control of acetyl-CoA carboxylase (ACAC) which is regulated by sterol regulatory element binding transcription factor 1 (SREBF1) (Eberlé et al., 2004) (Figure 1.2). Under the control of fatty acid synthase (FASN), ketoacyl-, 3-hydroxyacyl, trasn-2-enoyl and acyl-CoA are formed respectively from malonyl-CoA. This process is divided into four

parts namely, in turn, condensation, reduction, dehydration and reduction. At the end of the pathway butyric acid is formed. However, if the cycle repeats six times, palmitate is formed.

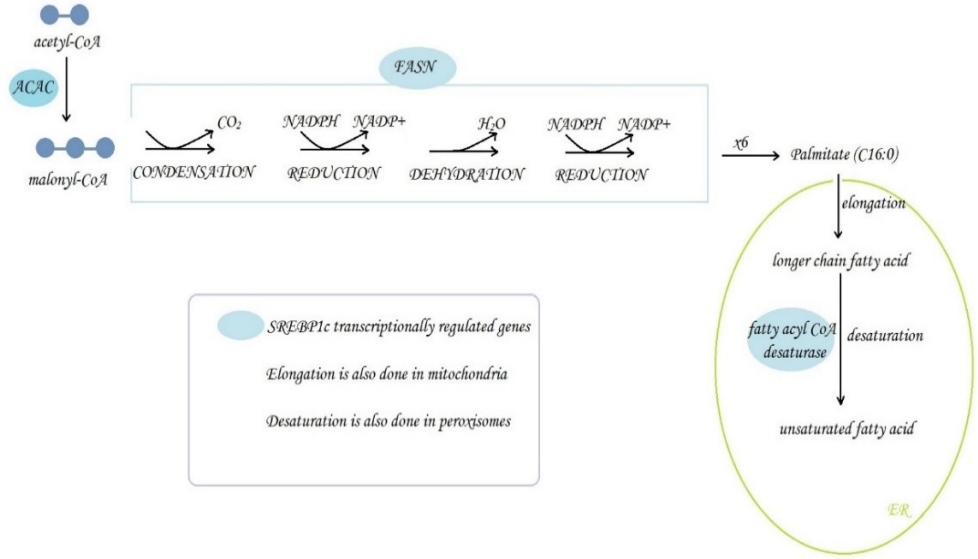


Figure 1.3 Palmitate formation starts with conversion of acetyl-CoA into malonyl-CoA under the control of acetyl-CoA carboxylase (ACAC). Following condensation, reduction I, dehydration and reduction II, palmitate is formed, and these successive reactions are controlled by fatty acid synthase (FASN). As a result of elongation (it is also done in mitochondria) longer fatty acid and following desaturation (also in peroxisomes) unsaturated fatty acid is generated in endoplasmic reticulum (ER). Credit artwork: Gizem Guven Ates

According to the type and length of the fatty acid, sequential reactions occur in specific organelles. When triglycerides (TAG) are required, free fatty acids are transferred to the ER to be turned to LDs which are storage form of neutral lipids in the cells (Bradley and Swann, 2019). Complex molecules (i.e., lipoproteins and glycolipids) are formed in the golgi that also cooperates with the ER to generate different classes of fatty acids (i.e., saturated, mono- and poly-unsaturated) by operating elongation, saturation and desaturation pathways.

1.1.3 Mechanism of Lipid Degradation and Oxidation

Stored triglycerides within LDs are hydrolysed by lipoprotein lipase (LPL) under basal and stimulated conditions by hormone sensitive lipase (HSL) under the influence of adrenalin, noradrenaline, glucagon and

adrenocorticotropic hormone (Houten and Wanders, 2010). Hormones interact with cell surface proteins, such as G-protein coupled receptors linked to cyclic adenosine mono phosphate (cAMP). Thereafter, protein kinase A (PKA) is activated. It is also known as cAMP-dependent protein kinase. This enzyme catalyses the phosphorylation reaction of perilipin which is the surface protein of LDs (Moro and Lafontan, 2012). Phosphorylated perilipins allow LPL and/or HSL to transfer from the cytoplasm into lipid droplets (Sztalryd and Brasaemle, 2017). Triacylglycerols are broken down under the control of enzymes into diacylglycerol, monoacylglycerol and fatty acids and glycerol, respectively. At the end of these reactions, three fatty acids and glycerol are formed. The other lipid degradation process is lipophagy. Phagophore is formed in the ER and attached to microtubule associated proteins (LC3 proteins). This complex then envelopes a portion of large LDs or all small LDs to form a lipo-autophagosome. With the merging of lysosomal acid lipase in the lysosome, fatty acids are formed in the lipo-autophagosome. These are then released to the cytoplasm (Braun et al., 2018).

When fatty acids are released into the cytoplasm, they are activated in a reaction catalysed by fatty acyl-CoA synthetase in the outer mitochondrial membrane to form fatty acyl-CoA. Fatty acyl-CoA compounds formed in the outer mitochondrial membrane cannot pass through the inner mitochondrial membrane; they require carnitine to be taken into the mitochondrial matrix. Initially L-carnitine groups play a crucial role in transport of fatty acid esters (fatty acyl-CoA) across the mitochondrial membrane (Figure 1.3). Carnitine palmitoyltransferase-1 (CPT1) is involved in the esterification process. It connects the acyl groups in acyl coenzyme A to fatty acids for uptake into the mitochondria (Adeva-Andany et al., 2019). Acyl-carnitine then passes through the inner mitochondrial membrane by the action of the carnitine-acyl carnitine translocase (CAT) which is located in the inner mitochondrial membrane. When Acyl-carnitine passes through the inner membrane, carnitine is transported out by CAT. In other words, CAT acts as a carnitine exchanger in the mitochondrial inner membrane (Adeva-Andany et al., 2017). Then, acyl-carnitine reacts with the CoA group in the presence of carnitine palmitoyltransferase-2 (CPT-2). Following that reaction, fatty acyl-CoA will be in the mitochondria and ready for β-oxidation. The β-oxidation pathway of fatty acyl-CoA esters consist of four sequential enzymatic reactions, which are oxidation (dehydrogenation 1), hydration, oxidation (dehydrogenation 2) and

thiolysis. When the cycle is complete, acetyl-CoA is extracted from the carboxyl end of the fatty acid. In addition, 1 mole of FADH2 and 1 mole of NADH, together with five moles ATP, are obtained by oxidative phosphorylation. B-oxidation continues until 1 mole acetyl- CoA remains. Newly synthesised acetyl-CoA enters the tricarboxylic acid cycle (TCA) where the majority of ATP production occurs (Schönfeld and Wojtczak, 2016).

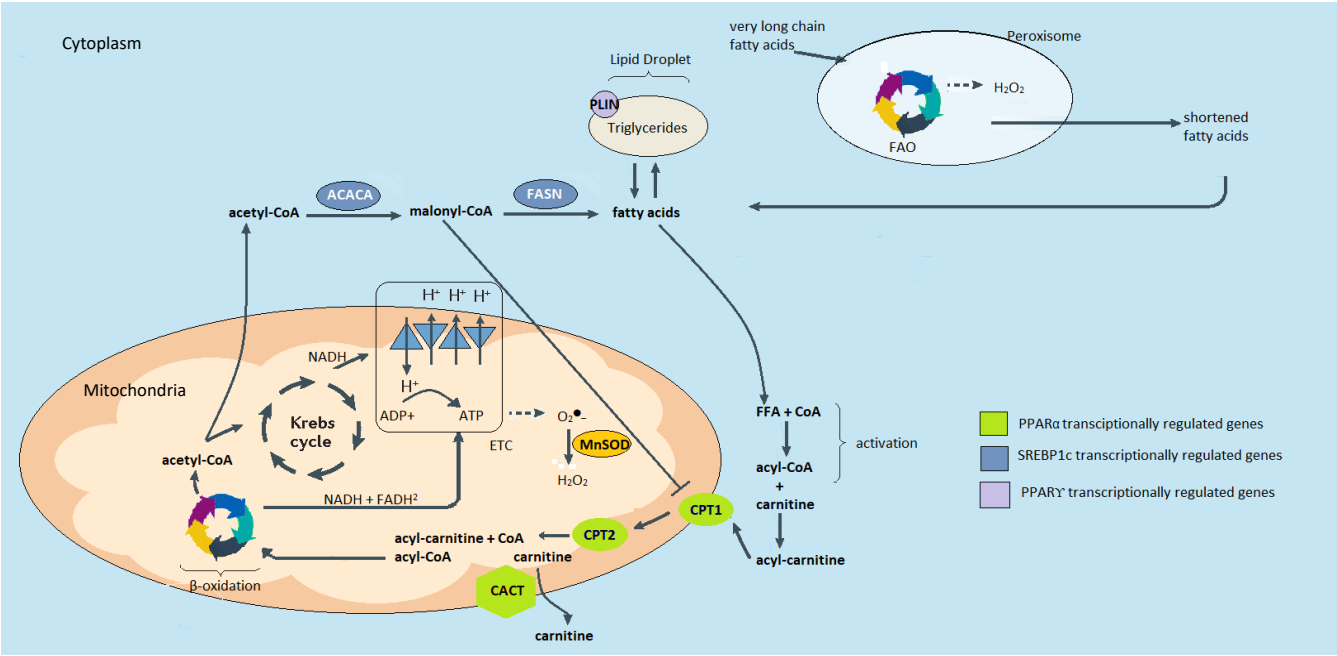


Figure 1.4 β-oxidation and the tricarboxylic acid (TCA) cycle. Free fatty acids are activated by CoA leading to the production of acyl-CoA in the cytoplasm. In β-oxidation, acyl-CoA attaches to carnitine to form acyl-carnitine, which is catalysed by carnitine palmitoyl transferase 1 (CPT1). This enzyme is located in the outer membrane of the mitochondrion. Upon uptake of acyl-carnitine into the matrix of mitochondria, -CoA is attached and carnitine is released under the control of CPT2, which is on the inner membrane of mitochondria. Acyl-CoA undergoes β-oxidation. At the end of each cycle, it loses 2C, in the form of acetyl-CoA, and generates ATP. Acetyl-CoA then enters the TCA cycle & ETS to generate more ATP or is converted to Malonyl-CoA to synthase fatty acids. Credit artwork: Gizem Guven Ates

1.2 Fatty acid metabolism within the ovarian follicle and during IVM

The follicle-enclosed oocyte is in intimate contact with surrounding somatic cells, namely, cumulus, granulosa and theca cells. These participate in bi-directional exchange of nutrients, growth factors and cell-signalling molecules. Oocyte development commences with differentiation of primordial germ cells which occurs during early fetal development (Collado-Fernandez et al., 2012). Following puberty, antral follicles are formed in a series of waves over several days. During antral follicle formation, granulosa cells (GC) differentiate into cumulus cells (CC), which surround the oocyte and mural granulosa cells, which forms the follicle wall (Wigglesworth et al., 2015). During antral follicle development, glycogen, fatty acids, proteins and mRNAs accumulate in the ooplasm of the growing oocyte (RG Sturmey, 2009). Furthermore, specific cytoplasmic organelles such as the ER, golgi, LDs, ribosomes and mitochondria increase and are redistributed within the cytoplasm (Fair et al., 1997, Picton et al., 1998).

1.2.1 Endocrine and paracrine regulation of ovarian-follicle physiology

As a consequence of this, oocyte and follicular somatic cell signalling occurs under the influence of gonadotropins. During the follicular phase, oocyte-secreted paracrine factors, growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and fibroblast growth factor (FGF), regulate proliferation and differentiation of granulosa cells (McNatty et al., 2005, McNatty et al., 2003, Roy et al., 2018). Following release of these transforming growth factor-β (TGF-β) family members, granulosa and theca cells produce their own paracrine factors such as kit ligand, activin, inhibin, leukemia inhibitory factor (Stoecklein et al., 2021) and anti-Mullerian hormone; all of which co-operate with the oocyte during antral development up to the point of ovulation (Sugiura and Eppig, 2005).

During the antral stage, these processes are regulated by gonadotropins. When follicle stimulating hormone (FSH) is released, it triggers uptake of theca cell derived-androgens which are converted into oestrogens catalysed by aromatase or, alternatively, *de novo* cholesterol synthesis (which is formed

from acetyl-CoA and catalysed by HMG-CoA reductase within the ER) to obtain oestrogen within granulosa cells (Singh et al., 2013). Meanwhile, following the FSH surge, protein kinase B (PKB/also known as AKT) increases within bovine granulosa cells. AKT is essential for aromatase activation (Zeleznik et al., 2003). Moreover, insulin like growth factor 1 (IGF-1) enhances the effect of FSH on these processes within the GC (Beg et al., 2001, Zhou et al., 2013).

During the ovulatory phase, luteinizing hormone (LH) triggers uptake of cholesterol, in the form of low-density lipoprotein (LDL), from circulation by theca cells. Thereafter, to synthesise pregnenolone (P5), cholesterol is transported into the mitochondria, and this part is the rate-limiting step of steroidogenesis. For transportation to occur, cholesterol requires to attach to steroidogenic acute regulatory protein (StAR) (Zhang et al., 2015a). In the mitochondrial matrix, cholesterol is converted to P5 under the control of cholesterol-side chain cleavage (P450scc), which is encoded by CYP11A1 (Strauss et al., 2014) (Figure 1.4).

1.2.2 Fatty acids and ovarian follicle physiology

Over the last few decades, the relation between nutrition and fertility has been extensively studied (Leroy et al., 2008). In dairy cows, this relationship is critical around conception, early gestation and lactation, because these physiological states are associated with negative energy balance (NEB) which reduces fertility (Esposito et al., 2014). A contributing factor to reduced fertility is impaired oocyte quality which affect their ability to support embryo development (Leroy et al., 2014). Changes in follicular fluid (FF) fatty-acid composition can alter the fate of oocytes and subsequent embryo development. Van Hoeck et al. (2014) indicated that during NEB in dairy cows, NEFAs in FF increase and that this can lead to lipotoxicity and reduced oocyte quality. It follows that high-levels of saturated-fatty acids in FF can lead to lower fertility in lactating cows than in heifers (Bender et al., 2010). In another study, an association between FFA and triglycerides in human FF and ER stress was reported and this was linked to impaired oocyte maturation (Yang et al., 2012). Increasing the amount of fatty acids in FF due to aging in humans also decreases oocyte quality (Cordeiro et al., 2018). All these studies indicate that overaccumulation of FAs reduce oocyte quality and

subsequent embryo development. It may also partially explain lower embryo development form abattoir derived bovine oocytes compared to OPU oocytes, as the former are derived from animals that are generally fatter (Rizos et al., 2002). Changes in cytoplasmic lipid content of oocytes and FF during follicular development, together with the lipid profile of blastocysts have been investigated (Annes et al., 2019). According to this study, when the oocytes were obtained from large follicle (> 8 mm), lipid contents of oocytes and blastocyst were the highest, and the follicular fluid from large follicle contains lower lipid compared to follicular fluid obtained from small follicle. Moreover, cleavage and blastocyst rate was higher in the large compared to the small follicle (≤ 2 mm) group. Additionally, the effect of PUFAs (especially n-3) on cultured theca and granulosa cells, and *in vitro* embryo development has been investigated in sheep (Hughes et al., 2010). Omega-3 PUFAs increased progesterone (P4) production by theca cells, associated with an increase in STAR expression. During in vitro embryo culture, albumin appears to be the primary vehicle responsible for cellular uptake of these FAs.

The beneficial effect of enhanced P4 secretion on early embryo developmental competence has been demonstrated in the cow (McNamara et al., 2003). Diets enriched with two n-6 PUFAs (namely LA and ALA) impaired progesterone production in lactating dairy cow (Robinson et al., 2002). On the other hand, other two other PUFAs, eicosadienoic (n-6) and docosahexaenoic acids (n-3) did not alter P4 secretion in lactating cows (Mattos et al., 2002). The difference of dietary n-3 and n-6 PUFA on P4 production was compared and data indicated that P4 concentration in FF was higher in n-3 than n-6 supplemented cows.

1.2.3 Fatty acids and oocyte maturation

The lipid composition of IVM culture media is as important as in follicular fluid. Therefore, several studies have sought to understand the effect of lipid metabolism during oocyte maturation *in vitro*. In general, fetal calf serum (FBS/FCS) and/or bovine serum albumin (BSA) are added as sources of fatty acids during IVM (Ferguson and Leese, 1999, Del Collado et al., 2015, Santiquet et al., 2017, Canel et al., 2018), as these components have been found to improve the proportion of oocytes that complete maturation (Sanbuissho and Threlfall, 1990). Furthermore, serum provides growth

factors, cytokines and metabolites which can increase intracellular lipids by mediating fatty acid uptake and/or triglyceride biosynthesis (Dunning et al., 2014b).

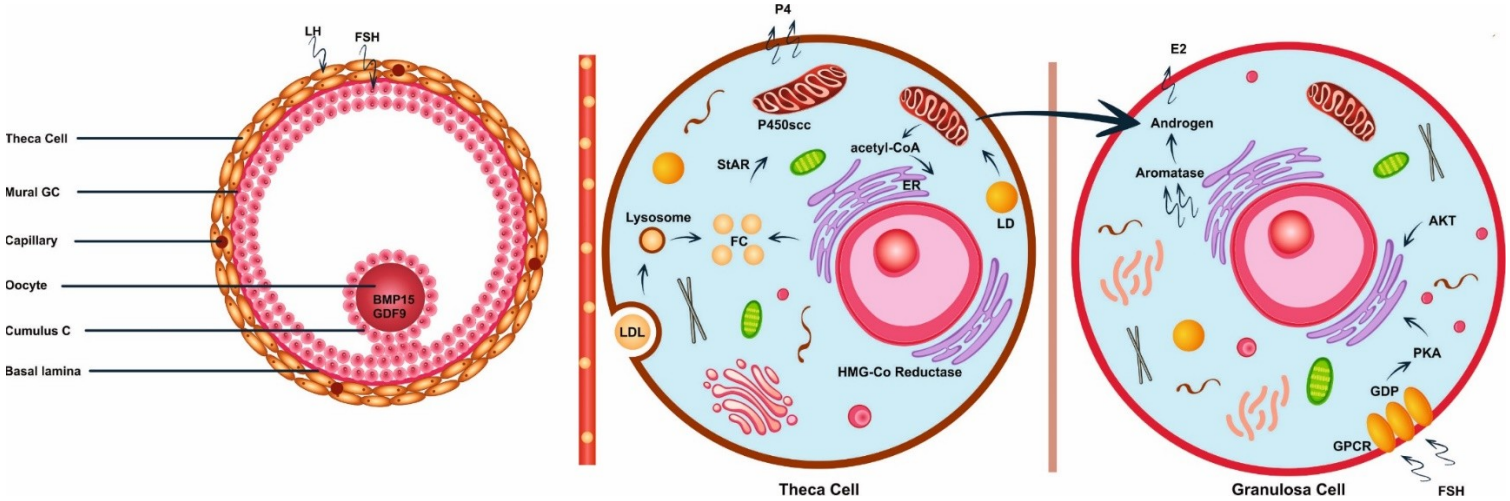


Figure 1.5 Depiction of key physiological prosses influenced by fatty acids within the ovarian follicle. Production of progesterone (P4) within theca cells and oestrogen (E2) within granulosa cells are illustrated. Low density lipoproteins (LDLs) enter theca cells from capillary circulation following invagination by cell membranes (endosome). Thereafter, endosome complexes with vesicles form golgi and lysosome are formed. LDLs are broken down to free cholesterol within the lysosomes and released to the cytoplasm. FC is transported into the mitochondria via steroidogenic acute regulatory protein (STAR) and converted into androgens or P4 by cytochrome 450scc. FC can also be formed from acetyl-CoA by HMG-Co reductase located in endoplasmic reticulum (ER). Androgen is transported to granulosa cells under the influence of FSH. FSH attaches to its G-protein-coupled receptor and the activated receptor converts GDP to cAMP which activates PKA. Increased levels of PKA as well as AKT direct the ER to release aromatase which is required for the conversion of androgen to E2. Thereafter, E2 is transported to follicular fluid and general circulation. Credit artwork: Gizem Guven Ates

It is now quite well established that β-oxidation is important for oocyte maturation. For example, the addition of etomoxir (an inhibitor of β-oxidation) in mouse maturation medium inhibited the progression of oocytes to MII (Dunning et al., 2010). Additionally, there is evidence that total triglyceride content is decreased during oocyte maturation in the pig (Sturmey and Leese, 2003), and cow (Ferguson and Leese, 1999); indicative of β-oxidation. In addition, lipase enzymatic activity increase in bovine COCs during IVM (Cetica et al., 2002). Fatty acid oxidation was inhibited by etomoxir during oocyte maturation and expression of related genes in COCs, together with nuclear maturation, examined in three distinct species (i.e., mouse, cow and pig) (Paczkowski et al., 2013). Results confirmed the importance of fatty acid oxidation, particularly in the mouse. This might be result of various lipid contents in oocytes of different species (Figure 1.5).

FCS supplements added to IVM media increases lipid accumulation in COCs, and it is believed to impair embryo development in vitro (Del Collado et al., 2017). Hence, there is an interest in the inclusion of lipid modulators or removal of FCS from media during IVM to prevent the overaccumulation of lipid and increase embryo development. For instance, the effect of follicular fluid containing maturation medium on oocyte maturation was examined in the absence of FCS and found that it increased oocyte developmental competence in sheep (Tian et al., 2019).

Lipid metabolism is controlled by a number of transcription factors such as sterol regulatory element binding proteins (SREBPs) and peroxisome proliferator activated receptor (PPAR) in COCs. Another key regulator enzyme, activated protein kinase AMPK, is associated with lipid metabolism as well as glucose metabolism and meiosis resumption (Tomek and Smiljakovic, 2005). AMPK inhibits acetyl-CoA carboxylase (ACAC) to prevent lipid accumulation. Consequently, acetyl-CoA cannot be converted to Malonyl-CoA. On the other hand, since Malonyl-CoA is not formed, CPT1 cannot be inhibited (Long and Zierath, 2006).

Cumulus cells are essential for oocyte maturation. They can determine oocyte developmental competence by cell-signalling, supporting cytoplasmic maturation by transporting metabolites, and promote fertilisation by releasing signals to sperm (Tanghe et al., 2002). Moreover, they play a key role in lipid

metabolism of oocytes (Auclair et al., 2013). These authors found that denuded oocytes have less lipid than oocytes enclosed with cumulus cells. This might be primarily due to CC lipid utilisation, as CCs are the first cell type which closely interacts with media (Aardema et al., 2013), and inhibiting lipid metabolism in CCs has a greater detrimental effect when compared to inhibiting oocyte lipid metabolism (Sanchez-Lazo et al., 2014b).

Additionally, lower levels of fatty acid synthetase (FAS) protein were found in denuded oocytes. Cumulus cells transport fatty acids, by the solute carrier protein family (SLC) and fatty acid translocase (CD36), to the oocyte during the last six hours of bovine oocyte maturation (Sanchez-Lazo et al., 2014a). Thereafter, FAs are transported to the ER or mitochondria by fatty acid cytosolic transporters (FABPs). During maturation, the increased expression of PLIN2 and CPT1A indicate that β-oxidation is required for completion of meiosis (Downs et al., 2009). On the other hand, during germinal vesicle break down (GVBD) (within the first 6 h of maturation), the expression of SREBF1 increases followed 18 hours later by ACAC and FASN (Sanchez-Lazo et al., 2014a). In the pig, supplemented stearic acid in maturation medium increased ACACA, PLIN, FASN and stearoyl-CoA desaturase (SCD) expression in cumulus cells but not in oocytes (Pawlak et al., 2020). It has been found that elongation of fatty acid (elongation of very long chain fatty acids (ELOVL)) during IVM is increased in the presence of serum; this is in contrast to what happens *in vivo* (Del Collado et al., 2017).

Consequently, follicular somatic cells use fatty acids to generate the steroid hormones progesterone and oestrogen, which are required for follicular growth, ovulation and to maintain pregnancy. On the other hand, all mammalian oocytes require fatty acid oxidation to promote oocyte maturation.

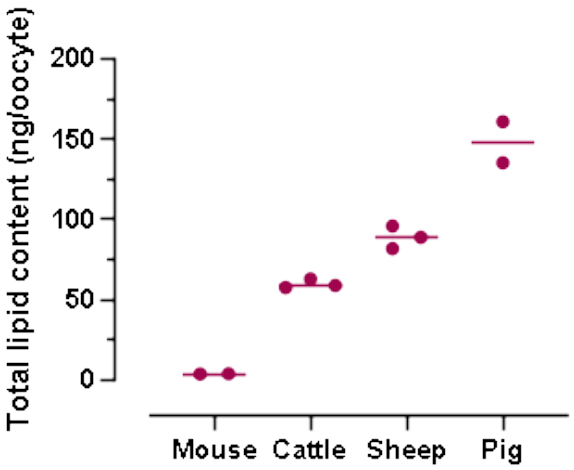


Figure 1.6 Lipid content of oocytes from the mouse, cow, sheep and pig. Each point corresponds to an average value derived from McEvoy et al. (2000), Sturmey and Leese (2003), Loewenstein and Cohen (1964), Ferguson and Leese (1999), Kim et al. (2001) and Coull et al. (1998).

1.3 Fatty acid metabolism in the pre-implantation embryo

The importance of fatty acid metabolism in determining fertility in cattle has been understood for a number of years (Mattos et al., 2000). The effect of dietary FAs on embryo development, however, depends on body condition score (BCS). Whilst dietary FAs reduced blastocyst yields in low BCS heifers, blastocyst yields were not altered in moderate BCS heifers (Adamiak et al., 2006). Fatty acid oxidation during early post-fertilization development is represented by a decrease in triglyceride content in all species studied (Ferguson and Leese, 1999, Wonnacott et al., 2010, Dunning et al., 2010). The importance of FA oxidation in subsequent embryo development was demonstrated following supplementation of a CPT inhibitor in both IVM and IVC media. Blastocyst formation and cell numbers were both reduced in the presence of this inhibitor (Ferguson and Leese, 2006). Moreover, genes related with lipid metabolism (*ACSL3*, *ELOVL5* and *ELOVL6*) were upregulated during this early stage (up to the morula), however their expression decreased throughout the blastocyst stage (Sudano et al., 2016).

During embryo development, while the number of LDs decrease (Ordoñez-Leon et al., 2014) their size increases (Bradley et al., 2016). On the other hand, during embryo development, cell numbers increase, and this means that FA requirement for membrane structures also increases. Consequently, the proportion of PUFAs increases parallel with embryo development (Wonnacott et al., 2010).

It is known that the lipid profile of early embryos is altered by oocyte lipid metabolism (Del Collado et al., 2015). Enhanced β-oxidation with regulators such as L-carnitine, resveratrol and melatonin can increase embryo development in the pig (Lowe et al., 2017), cow (Sutton-Mcdowall et al., 2012) and mouse (Dunning et al., 2010). Moreover, stimulation of β-oxidation with L-carnitine during IVC increases the proportion of blastocysts, total cell number, mitochondrial activity and cryotolerance, and decreases the number of apoptotic cells and lipid droplets in cattle (Ghanem et al., 2014).

Although, lipid metabolism has a significant role in energy metabolism, cell proliferation and membrane formation during embryo development, excessive amounts of lipid can enhance oxidative stress and impair embryo development (Cagnone and Sirard, 2014).

1.4 Attempts to regulate fatty acid metabolism during mammalian IVP

There are several transcription factors that can be categorised based on their function such as cell differentiation, proliferation and metabolism. The factors responsible for lipid metabolism are peroxisome proliferator-activated receptor (PPAR) (Desvergne and Wahli, 1999) and sterol regulatory element-binding protein (SREBP) family (Shimano and Sato, 2017); however their subtypes differ between cell types.

Three forms of PPARs are known, namely, PPARα, γ and β. A number of biological molecules such as PUFAs, saturated FAs, very low density lipoproteins (VLDLs) and leukotriene B4 regulate PPAR activity, serving as endogenous PPAR ligands and some of them are specific to only one PPAR subtype (Komar, 2005). Moreover, there are numerous synthetic ligands such as non-steroidal anti-inflammatory drugs (NSAIDs), thiazolidinediones (TZDs) and troglitazones that also regulate these factors (Vélez et al., 2013). A possible reason for differences in ligand specific receptors lies in the variation in binding domain structure of each PPAR (Xu et al., 2001). Induced-PPARs attach to specific region of DNA, termed retinoic acid receptor (RXR) (Feige et al., 2005), therefore regulate gene expression.

The PPAR agonist, rosiglitazone causes an increase oocyte membrane potential and expression of FAO related genes during oocyte maturation in mice (Dunning et al., 2014a). PPARα regulates FA uptake, FA binding within

the cytoplasm and activation of FAs into acyl-CoA esters. Moreover, they are involved in the degradation acyl-CoA via FA oxidation pathways, and ketone body synthesis (Yoon, 2009). Briefly, PPARγ regulates follicular development, ovulation, oocyte maturation, function of the corpus luteum and implantation of embryos by regulating related gene expression (Froment et al., 2006). It has been shown that the addition of specific PPARβ agonists increase the proportion of hatching blastocysts, total cell number in blastocysts and ATP production via β-oxidation (Guo et al., 2017).During early embryo development PPARβ agonists prevent embryo loss by inhibiting endometrial PGF2α synthesis (MacLaren et al., 2006).

The second lipid regulating transcript factor is SREBP consisting of two subtypes which are SREBP1 and SREBP2. SREBP1 has two further subtypes, namely SREBP1a and SREBP1c. Each SREBP is synthesized as inactive precursors and attach to the ER membrane (Brown and Goldstein, 1997). When cholesterol is required within the cell, SREBP cleavage-activating protein (SCAP) mediates transportation of inactive SREBP from the ER to the golgi where SREBP is formed via dividing by site-1 protease (S1P) and site-2 protease (S2P) (Horton et al., 2002). Thereafter, active SREBP attaches to specific DNA sites which are called sterol regulatory elements (SREs), and these regulate lipid metabolism genes. While SREBP1c is responsible for stimulating lipogenic genes, SREB2 controls cholestrogenic genes, and SREBP1a stimulates both lipogenic and cholestrogenic genes (Shimano and Sato, 2017) (Figure 1.6).

It has been suggested that PUFAs repress SREBPs synthesis thereby altering cholesterol production (Tabor et al., 1998). It was found that mRNA levels of *SREBP1*, *SCD1* and *FADS2* (genes involved in fatty acid synthesis) decrease in bovine embryos when cultured with the PUFAs (docosahexaenoic, linoleic and α-linolenic acid) (Al Darwich et al., 2010). Additionally, dietary conjugated linoleic acid (CLA) reduced *SREBP1c* within non-lactating cows in adipose tissue (Urrutia et al., 2017).

Addition of HMG-CoA inhibitor in human granulosa cell culture increased *SREBP1a* and *SREBP2* expression and decreased mRNA expression of LH receptor (Li et al., 2019). This suggests that cholesterol is required for facilitating the actions of LH on granulosa cells. Moreover, SREBP1a and SREP2 were directly activated as a result of decreased cholesterol level.

Supplementation of insulin during bovine IVM resulted in increased levels of *SREBP1* in Day 8 blastocysts (Laskowski et al., 2018).

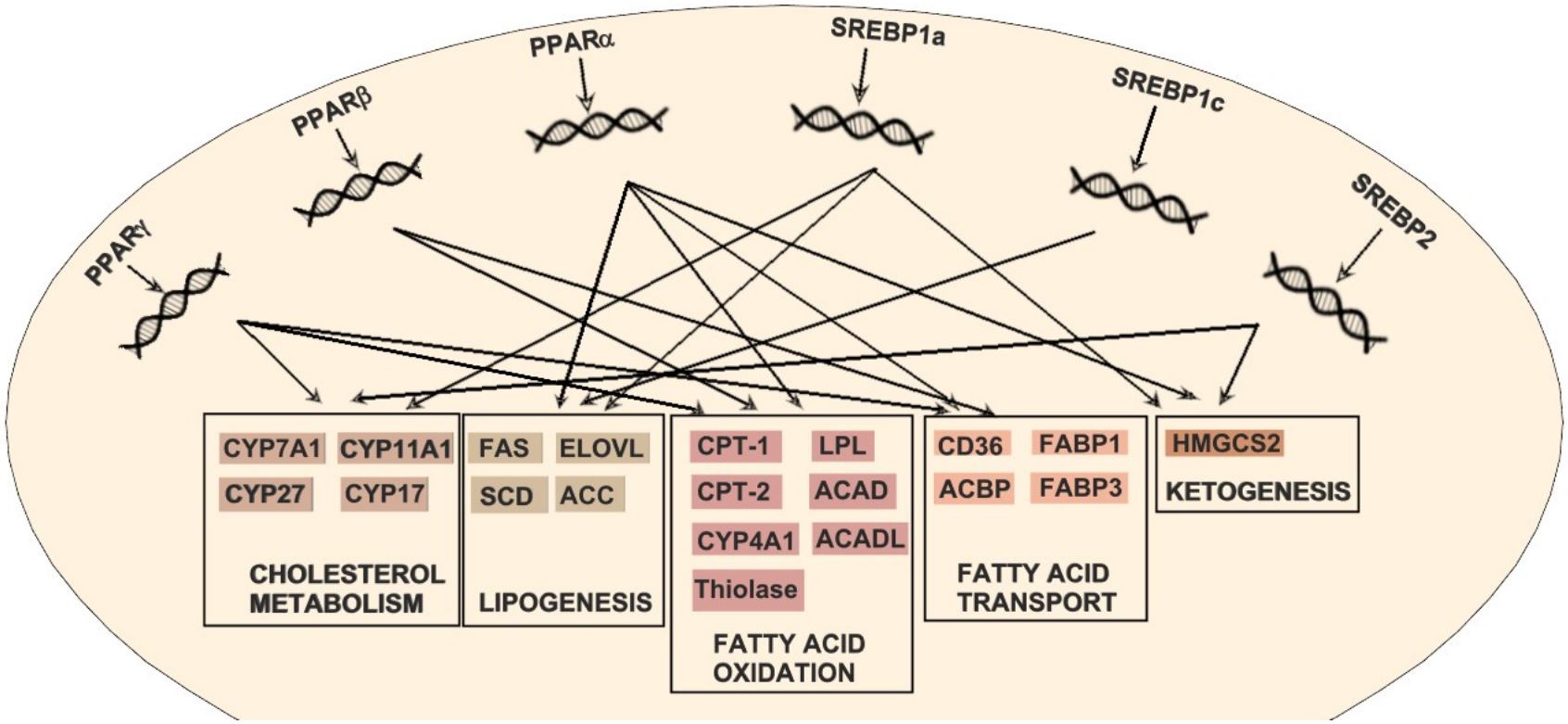


Figure 1.7 Genes which are involved in lipid metabolism and their regulators, transcription factors. Credit artwork: Umut Ates

1.5 Lipid Regulators

When *in vivo* matured oocytes are used for IVP then blastocyst yields are higher than is the case with *in vitro* matured oocytes (Khalil et al., 2014). There are several factors that may affect embryo development during IVM. One of the factors is lipid accumulation by reason of serum supplementation. Serum is used during IVM as it contains numerous factors and generally improves success rate of embryo development (Khurana and Niemann, 2000a). Additionally, oocyte maturation in the absence of serum leads to impaired blastocysts yields and increased number of apoptotic cells (Korhonen et al., 2010). Furthermore, supplementation of high-level serum or bovine serum albumin (BSA) during IVC caused large offspring syndrome in cattle and sheep (Lazzari et al., 2002). Therefore, several studies have been undertaken to protect oocytes from increased lipid accumulation and to improve the quality and proportion of blastocysts. In this regard, various combinations of lipid regulators and antioxidants have been included in the diet, IVM, IVF and/or IVC media with a view to mitigating the detrimental effects of serum and to improve blastocyst cryopreservation.

1.5.1 Carnitine

All cells require carnitine to carry long-chain fatty acids into the mitochondrion where oxidation enzymes are located. Carnitine has several isomers and one of them, levocarnitine (L-carnitine) is biologically active (Agarwal et al., 2018). L-carnitine has beneficial effects on fertility. It can increase motility and chromatin quality of testicular sperm (Aliabadi et al., 2012). L-carnitine also plays a major role on sperm maturation, ATP provision and viability by preventing reactive oxygen species (ROS) (Dokmeci, 2005).

Oocyte quality moderately correlates with metabolism. Therefore, fertilization rate and blastocyst development increased when β-oxidation was enhanced with 1 mM L-carnitine during follicle culture (from preantral to antral stages) for 12 days in the mouse (Dunning et al., 2011). Additionally, L-carnitine treatment increased oocyte maturation within early antral follicles by

increasing mitochondrial activity (Hashimoto, 2009). L-carnitine has also had a beneficial effect on ovulation and pregnancy rate in women with clomiphene-resistant polycystic ovary syndrome (Ismail et al., 2014).

Treatment with L-carnitine during culture increases FAO, oocyte maturation and subsequent embryo development in a dose-dependent manner. In a camel study, 0.5mg/ml L-carnitine supplementation during maturation improved the proportion of matured oocytes and cleaved embryos (Fathi and El-Shahat, 2017). The same amount of L-carnitine led to an increase in the proportion of matured oocytes, blastocyst formation, glutathione (GSH) concentration, reduced ROS levels and apoptosis in porcine blastocysts (Wu et al., 2011). Maturation and subsequent embryo development increased when bovine oocytes were treated with 0.3 and 0.6mg/ml L-carnitine (Phongnimitr et al., 2013). In the same study a higher dose (1.2mg/ml) was determined to be detrimental. Supplementation of L-carnitine during sheep oocyte IVM regulates apoptosis, acting as an antiapoptotic agent, and induces proliferative genes during embryo development (Mishra et al., 2016a).

When bovine embryos were cultured in 5 mM L-carnitine supplemented medium in the absence of carbohydrates for four days the proportion of morula stage embryos and ATP increased (Sutton-Mcdowall et al., 2012). In other work, while 0.5 mg/ml L-carnitine treatment during IVC increased zona pellucida (ZP) thickness, cell number within the ICM and the proportion of hatching mouse blastocysts, 4 mg/ml inhibited embryo development and impaired blastocysts quality (Khanmohammadi et al., 2016).

Farm animal cryopreservation is still limited because frozen-thawed oocytes lead to impaired embryo development (Hwang and Hochi, 2014). Several explanations exist which suggest that the large size of bovine oocytes, high volume-surface ratio, plasma membrane and ooplasm lipid content all limit cryopreservation (Saragusty and Arav, 2011). L-carnitine has been used to reduce lipid content of embryos. 3 mM L-carnitine improves cryotolerance when supplemented in bovine (Zolini et al., 2019) and pig (Lowe et al., 2017) culture media. During IVC, 2.5 mM L-carnitine supplementation improved re-expansion rates after thawing (Held-Hoelker et al., 2017).

Studies which are mentioned above indicate that mM concentrations of L-carnitine during IVM increases the proportion of matured oocytes and ATP production whilst reducing ROS. Additionally, supplementation of L-carnitine

during IVC increases cell number of blastocysts and cryotolerance of embryos. Interestingly, the levels of L-carnitine used in *in vitro* studies are higher than those observed *in vivo*. Robker et al. (2009) analysed L-carnitine levels in human follicular fluid and carnitine levels ranged between 2-37 μM.

1.5.2 Conjugated Linoleic Acid (CLA)

Conjugated linoleic acid is a positional and geometric isomer of linoleic acid with conjugated double bonds, biosynthesized by rumen microorganisms. It is known that CLA reduces fatty acid uptake and, LPL activity in adipocytes (Pariza et al., 2001), and reduces SCD activity in hepatocytes (Choi et al., 2001).

Within the CLA group, cis-9, trans-11 (c9, t11) and trans-10, cis-12 (t10, c12) have reported effects on reproductive processes. Studies investigating the effects of CLA on reproductive processes have been quite general and often assess the effects of dietary supplementation. While t10, c12 decreased milk fat, c9, t11 has been found to be more abundant in milk (Bauman and Lock, 2007). Dietary CLA can reduce negative energy balance, possibly by decreasing milk fat. However, supplementation of less than 10 g/d CLA did not alter energy balance (Castañeda-Gutiérrez et al., 2005). On the other hand, in the same study, dietary CLA induced early ovulation during the postpartum period and increased pregnancy rate. Supplementation of PUFAs in the diet may have a synergistic effect by reducing embryo losses via suppression uterine prostaglandin-F2α (PGF2α) (Mattos et al., 2000). Concordantly, CLA can inhibit prostaglandin synthesis by acting on PPARs in kidney cells (Belury et al., 2002). However, while the effect of CLA on uterine PGF2α has not been observed following short-term feeding, increased circulating levels of IGF-1 has been reported (Castañeda-Gutiérrez et al., 2007a). In a long-term dietary CLA study, the expression of FASN, and SREBP1 was not differed, however lipid composition was altered in bovine FF (González-Serrano et al., 2015). Addition of CLA dissolved in DMSO (100 μM final concentration) in bovine maturation medium did not alter cumulus cell expansion, nuclear maturation or the proportion of cleaved embryos and blastocyst formation (Khalil et al., 2014). However, in the same study treatment with CLA during IVM increased total cell numbers in blastocysts. In a separate IVM study, CLA once again failed to have any effect on oocyte maturation, however, CLA improved

embryo quality morphologically. Moreover, fatty acid composition of matured oocytes and surrounding cumulus cells was reduced in the presence of CLA (Lapa et al., 2011). Furthermore, in the same study CLA treatment increased both palmitic and myristic acid and decreased linoleic and arachidonic acid in oocytes, and additionally decreased linoleic acid in cumulus cells. Decreased linoleic and arachidonic acid suggests that these fatty acids were utilized during IVM. Interestingly, in a porcine study, 50 μM CLA supplementation during IVM significantly increased the proportion of matured oocytes, cleaved embryos, blastocyst formation and total cell numbers in blastocysts (Jia et al., 2014). In the same study, cyclooxygenase (COX2) and mitogen activated protein kinase (p-MAPK3) expression, and glutathione concentrations, were all increased in the treatment group.

In a large-scale experiment, different concentrations (15, 25, 50, 100 μM) of CLAs (c9, t12 and t10, c12) were supplemented during bovine IVM and IVC separately (Absalon-Medina et al., 2014). Supplementation of CLA during IVM did not alter oocyte maturation and subsequent embryo development. Only the low dose of t10, c12 increased the proportion of cleaved embryos. However, the beneficial effect was not observed at the blastocyst stage. Treatment with isomers t10, c12 during the last 36 h of culture decreased expression of fatty acid desaturase 2 (FADS2) in embryos.

Addition of 100 μM t10, c12 CLA isomer in culture media decreased lipid accumulation in blastocysts, however it did not alter embryo development (Pereira et al., 2007). CLA did not alter cryotolerance of embryos, however fatty acid synthase (FASN) expression was decreased in D7/D8 blastocysts.

1.5.3 Resveratrol

Resveratrol is naturally found in grapes, red wine, peanuts and various medicinal plants. It is a far-reaching chemical, having cardioprotective, anticancer, antiapoptotic, anti-inflammatory and antioxidant effects (Gambini et al., 2015). The effects of resveratrol on reproductive processes have been investigated (Ortega and Duleba, 2015). For example, granulosa cells harvested from rat ovaries were cultured in the presence of 100 μM resveratrol. Sirtuin (SIRT1), STAR and P450 aromatase expression were all increased as a result of treatment (Morita et al., 2012). These data suggest that resveratrol has an additive effect on producing steroid hormones and

subsequently follicular development, also preventing granulosa cell apoptosis by increasing the activation of SIRT enzyme (Zhao et al., 2014). It has been reported that supplementation of 0.1-5μM resveratrol during IVM increases glutathione peroxidase (GPX) concentration within the oocyte, reduces ROS levels and improves embryo development in the pig (Kwak et al., 2012), cow (Wang et al., 2014a) and goat (Mukherjee et al., 2014).

Treatment with 0.5 μM resveratrol during IVC increased formation and total cell numbers of porcine blastocysts, and reduced expression of BCL-2 and caspase-3 genes (related to apoptosis); however higher concentrations (1 and 25 μM) impaired embryo development (Lee et al., 2010). DMSO is widely used for antioxidant dilution in *in vitro* studies. However, 0.125-1.07% DMSO and ethanol were found to be cytotoxic by impairing differentiation (Adler et al., 2006). Based on this information, 0.5-10 μM resveratrol-cyclodextrin complex was used for bovine IVM or IVC (Torres et al., 2018). This study found that the proportion of matured oocytes and embryo development were not altered. However, the expression of various apoptotic genes and SOD1 were reduced and the expression of lipolytic genes (*PLIN* and *CYP1B1* (*P450)*) increased. Additionally, these authors suggested that the higher concentration of resveratrol-cyclodextrin complex did not impair embryo development. Therefore, impaired embryo development in the other studies may be due to higher doses of DMSO.

1.5.4 Melatonin

Melatonin is secreted mostly from the pineal gland but recently it was found to be secreted from the retina, extra-orbital lacrimal gland, skin and ovary (Acuña-Castroviejo et al., 2014). The amino acid, tryptophan, is a precursor for melatonin synthesis. Initially it needs to be hydroxylated to 5-hydroxytryptophan which is then decarboxylated to form serotonin. Thereafter, serotonin is acetylated with acetyl-CoA to N-acetylserotonin (AANAT) to produce melatonin (Hickman et al., 1999). AANAT activity is the rate limiting factor for melatonin synthesis (Klein and Weller, 1970).

Melatonin can affect the female reproductive system (Tamura et al., 2009). It regulates circadian rhythms and can influence insulin resistance (Cerezo et al., 2016). It protects the immune system, has an anti-inflammatory effect and scavenges free radicals (Rodrigues-Cunha et al., 2016). Melatonin has been

detected in the follicular fluid of humans (Nakamura et al., 2003), pigs (Shi et al., 2009) and cattle (Coelho et al., 2013). The expressions of melatonin receptors (MT1 and MT2) were identified in cumulus cells (CC), granulosa cells and oocytes in cattle (Tian et al., 2014), goat (Saeedabadi et al., 2018), pig (Kang et al., 2009), sheep (Tian et al., 2017) and mice (Lee et al., 2001).

Based on these observations various IVP studies have been undertaken to understand the effects of melatonin on oocytes and on embryo development. He et al. (2018) observed that supplementation of melatonin during IVM increased the proportion of oocytes that reached MII. It also increased lipid droplet accumulation but decreased mitochondrial (mtDNA) and ROS levels in porcine oocytes. In contrast, a separate study observed that melatonin increased β-oxidation, mitochondrial biogenesis and lipogenesis by upregulating related genes (Jin et al., 2017). The difference can arise due to presence of serum in maturation media in the first study. Rodrigues-Cunha et al. (2016) suggested that melatonin has an antioxidant effect by upregulating *SOD2* expression when supplemented in IVM medium. During IVC, and in high concentrations (20%) of O2, melatonin increased bovine blastocysts formation. This effect was thought to arise due to melatonin protecting blastocysts from oxidative stress, thereby, decreasing apoptosis (Papis et al., 2007). Interestingly, melatonin activated AMPK, PPAR-α and CPT1 which are associated with lipolysis. Meanwhile, downregulated expression of lipogenic genes such as *ACACA*, *SREBP-1c* and *FAS*, have been reported in the presence, but not absence of oleic acid in HepG2 cells (Mi et al., 2018).

1.5.5 Forskolin

Forskolin is a herbal diterpene and activator of cAMP (Alasbahi and Melzig, 2012). Within the ovarian follicle, until the LH surge, mural granulosa cell-derived natriuretic peptide type C (NPPC) stimulates production of cyclic guanosine monophosphate (cGMP) in cumulus cells via natriuretic receptor 2 (NPR2) (Zhang et al., 2010). Increased cGMP is transmitted to the oocyte through gap junctions and inhibits phosphodiesterase 3A (PDE3A) activity, therefore avoiding hydrolysis of cAMP (Tripathi et al., 2010). Unhydrolyzed cAMP levels increase protein kinase A (PKA) activity which inhibits maturation promoting factor (MPF) activity. Inactivated MPF leads to maintenance of meiotic arrest (Pan and Li, 2019). When COCs are aspirated from antral

follicles for IVM, the effect of mural granulosa cells on meiotic arrest disappears, therefore meiosis resumes automatically (Razza et al., 2019). On the other hand, isolated COCs from small antral follicles (1-3mm) leads to a delay of cytoplasmic maturation (Wu et al., 2006). Thus, it has been proposed that delay of nuclear maturation provides more time for cytoplasmic maturation and accumulation of mRNA, nutrients and redistribution of organelles that are required to complete oocyte maturation, fertilization and embryo development (Bilodeau-Goeseels, 2012).

To understand these changes on lipid metabolism of oocytes and blastocysts in cattle, pre-matured COCs were treated with forskolin before maturation then membrane lipid profile and lipid content of blastocyst and oocyte were examined (Razza et al., 2018). Embryo development and lipid content of treated pre-matured COCs were not altered. Following maturation, lipid content of oocytes was increased however lipids were reduced in blastocysts. In contrast, treatment of pre-matured COCs with forskolin increased embryo development and glutathione (GSH) levels as gap junctions remained open, and H_2O_2 levels were reduced (Li et al., 2016). Moreover, forskolin treatment during pre-maturation COC culture increased cryoresistance of matured porcine oocytes (Fu et al., 2011).

1.6 Antioxidants

Reactive oxygen species (ROS), namely, superoxide anions (O_2^-), hydrogen peroxides (H_2O_2) and hydroxyl radicals ($^\bullet OH$) are generated as a result of metabolic reactions (Agarwal et al., 2005a). Superoxide is formed from the addition of an election on the oxygen molecule which might be formed as a result of ETC. Hydrogen peroxide is a short-lived molecule and formed from superoxide and hydrogen. The reaction is catalysed by superoxide dismutase (SOD). However, H_2O_2 can cross cell membranes easily and be converted into $^\bullet OH$ in the presence of ferrous ions. Hydroxyl radicals are more toxic and reactive than the others and therefore can cause prevalent injury to cells. While superoxide causes ischemia, $^\bullet OH$ causes cytotoxicity and both inhibit glycolysis, oxidative phosphorylation and ATP depletion. Nevertheless, at physiological levels they regulate cell proliferation, growth and differentiation by affecting cell signalling pathway (Sauer et al., 2001, Poli et al., 2004).

Under optimal conditions there is a balance between ROS and antioxidants. However, when conditions change, ROS levels exceed those of antioxidants and can damage cells. As a consequence of this imbalance, oxidative stress arises. An idea has been put forward that oxidative stress impairs follicular oocytes and follicular somatic cells by altering mitochondrial functions *in vivo* (Cerezo et al., 2016) and *in vitro* (Combelles et al., 2009). Reproductive events *in vivo* occur under low oxygen levels (Mastroianni and Jones, 1965) and higher oxygen (20%) levels trigger DNA damage in bovine embryos and impair embryo development (Takahashi et al., 2000, Pabon et al., 1989).

In vitro manipulations such as culture conditions, centrifugation, pH and osmolarity can cause oxidative stress in oocytes, sperm and embryos, and impair embryo development (Agarwal et al., 2014). For this reason, antioxidants and/or free radical scavengers are added to medium to improve oocyte and embryo quality. In these systems there are two types of compound used in the defence of cells against ROS and free radicals; namely antioxidants and ROS scavengers. They differ slightly in their modes of action. While antioxidants (Figure 1.7) supress oxidation to prevent the formation of ROS, ROS scavengers react with ROS and free radicals directly.

Superoxide dismutase forms the first line of defence against ROS. Superoxide anions are converted to hydrogen peroxidase and oxygen, and this reaction is catalysed by superoxide dismutase. Thereafter, hydrogen peroxide formed is removed from the medium by catalase or glutathione peroxidase. In mammals, there are three forms of SOD, Cu-SOD contains copper and occupies the cytosol, manganese superoxide dismutase (Mn-SOD) is to be found in mitochondria, and extracellular dismutase (EC-SOD) is found in extracellular liquids and the surface of cells. It is synthesized by fibroblasts, glia and endothelial cells (Nozik-Grayck et al., 2005).

Catalyse (CAT) consists of four protein subunits and each one contains nicotinamide adenine dinucleotide phosphate (NADPH). It is located widely in the peroxisome and is found in mitochondria and in the endoplasmic reticulum in smaller amounts. It catalyses the conversion of H_2O_2 into H_2O and O_2.

Glutathione peroxidase (GPX) exists in the cytoplasm and has a similar role to that of CAT. It consists of four protein subunits, and each subunit contains selenium (Se). If Se is located in the active part of GPX, it is called active GPX (Se-GPX) which mainly reacts with H_2O_2. The other form of GPX does not

contain Se in the active part, and it metabolises organic hydroperoxides. During metabolic reactions involving GPX, glutathione (GSH) acts as a donor of hydrogen as well as playing a role in cell proliferation during embryo development (Gardiner and Reed, 1994). Therefore, while H_2O_2 and hydroperoxides are reduced, GSH is oxidized which is reversable. Cysteine is a rate-limiting factor of GSH synthesis (de Matos and Furnus, 2000). Moreover, supplementation of cysteine during IVM increases GSH level and the proportion of cleaved embryos and blastocysts in cattle (Furnus et al., 2008).

Glutathione reductase is a flavoprotein enzyme and contains flavin adenine dinucleotide. It catalyses the conversion of oxidised glutathione back to GSH by transferring one electron of NADPH to the disulphide bond of oxidised glutathione. Therefore, NADPH is required to avoid depredation of free radicals (Zare et al., 2015).

There have been several studies undertaken to evaluate the effects of antioxidants on oocyte maturation, embryo development and even fertilisation. Addition of quercetin (2 μM), cysteamine (100 μM), carnitine (0.5 mg/ml), vitamin C (50 μg/ml) or resveratrol (2 μM) separately during IVM, did not alter oocyte maturation. However, treatment decreased ROS levels and increased blastocyst formation and total cell number of blastocysts (apart from quercetin) in cattle (Sovernigo et al., 2017). Supplementation of cysteine and cysteamine, and/or CAT, during bovine IVM increased GSH levels and cytoplasmic maturation. However, this did not alter embryo development (Rocha-Frigoni et al., 2016).

In contrast, supplementation of combined antioxidants, namely 10 μM Acetyl-L-Carnitine (ALC), 10 μM N-Acetyl-L-Cysteine (NALC), 5 μM α-Lipoic Acid (ALA) in IVF and/or culture medium under 20% oxygen tension, increased total cell number in blastocysts, as well as cleavage and blastocyst rates in mouse IVP (Truong and Gardner, 2017). LC supplementation in mouse culture medium increased embryo development and decreased DNA damage (Abdelrazik et al., 2009).

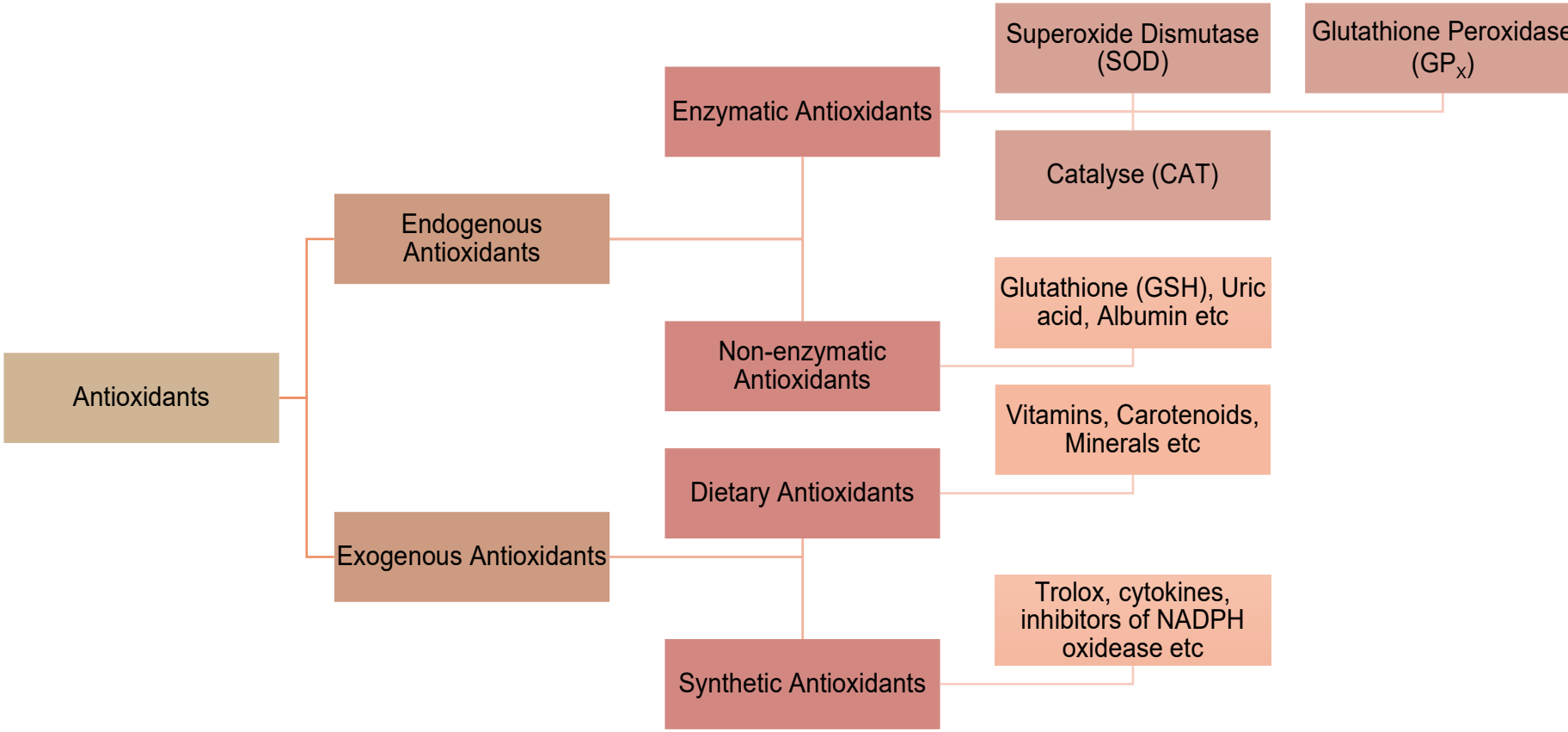


Figure 1.8 Classification of antioxidants.

1.7 Other factors

Sirtuins (SIRTs) are a class of proteins that participate in several important cellular processes such as genomic stability, DNA repair, transcriptional silencing and lipid metabolism (Qiang et al., 2011). SIRT1 is expressed in oocytes and granulosa cells at various stages of follicular development and, with SIRT3, protects oocytes and granulosa cells against oxidative stress within the aging ovary (Tatone et al., 2015) and obese mouse ovary by regulating *SOD2* activity (Zhang et al., 2015b). Moreover, SIRTs have an important effect on steroidogenesis during luteinization and SIRT1 helps to reduce granulosa cell apoptosis (Zhao et al., 2014).

Apart from biological molecules, enzymes and chemicals, other metabolic factors play a significant role in regulating lipid metabolism. Recently, the effect of glucose metabolism on lipid metabolism was evaluated in porcine oocytes. Maturation was performed with/without pyruvate and lactate. Glucose metabolism was found to affect the distribution of lipid droplets (Lowe et al., 2018). The balance between glucose and lipid metabolism is important in oocyte metabolism and maturation, and in the production of ATP. The balance between different components of metabolism is regulated by transcription factors. Treatment of bovine oocytes with PPARβ agonist, whilst decreasing *CPT1* expression, increased phosphoenolpyruvate carboxylase (PEPCK) which is related with glycogenesis (Idrees et al., 2019).

1.8 Stems cells: Models of development that extend beyond blastocysts

The first embryonic stem cells were derived from mouse embryos in 1981 (Evans and Kaufman, 1981). This advanced technique opened up new opportunities such as genetic modifications, drug trials and regenerative therapy (Leventhal et al., 2012). Stem cells are classified from their origin and/or their ability to differentiate (Figure 1.8). In this classification method, embryonic stem cells are identified as being pluripotent. Embryonic stem cells are classified based on the stage of the blastocyst from which they are derived; naïve or primed ESC. Naïve ESCs are derived from the pre-

implantation embryo and primed (also called EpiSC = epiblast stem cell) are derived from post-implantation embryos (Nichols and Smith, 2009). EpiSC can be derived from pre-implantation embryos (Bogliotti et al., 2018). Both ESC types (i.e., naïve and primed) are pluripotent. However due to different blastocyst stages their signaling pathways change and thus naive ESCs are not capable to generate primordial germ-like cells which are precursors of sperm and oocyte, unless they are transitioned into epiblast-like cells (EpiLCs) (Hayashi et al., 2011). Naïve stem cell derivation has been successfully applied in mouse and human embryos (Gafni et al., 2013, Weinberger et al., 2016). However, it was still challenging in the cow and pig (Blomberg and Telugu, 2012). On the other hand, EpiSC has been derived from advanced blastocysts from pig (Park et al., 2018), cattle (Bogliotti et al., 2018), mice and humans (Kinoshita et al., 2021a).

To be classified as pluripotent, under specific conditions stem cells must differentiate into different cell types which represent the three germ layers. To observe their differentiation, a teratoma test has been used for many years (Liu et al., 2020). Additionally, some *in vitro* techniques were developed such as embryoid body (EB) formation or direct differentiation into different cell types which represent individual germ layers.

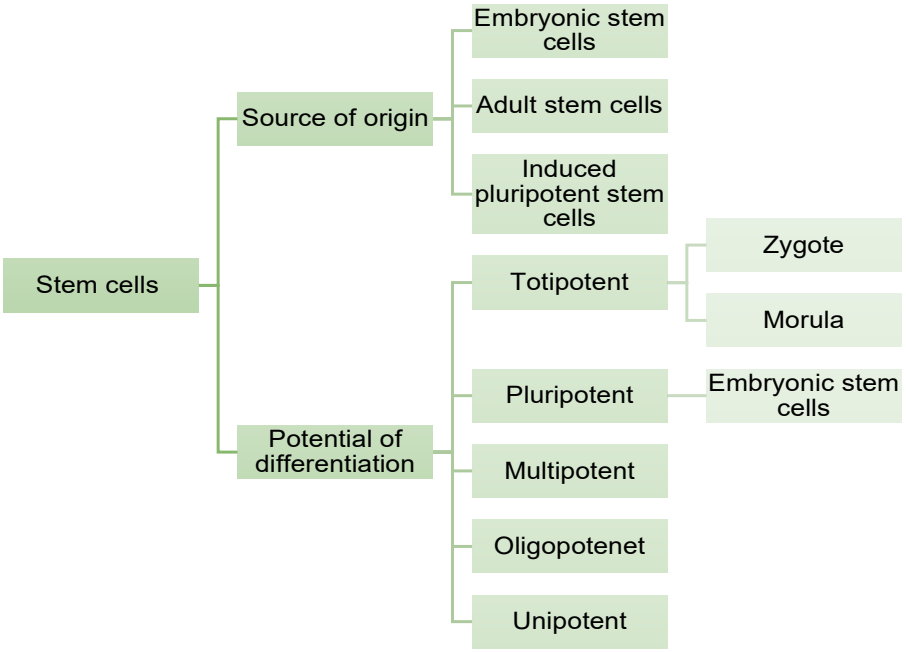


Figure 1.9 Stem cell classification based on their origin and according to their differentiation potential. Totipotent; differentiation into three germ layers and extra-embryonic tissues, pluripotent; into three germ layers, multipotent; into multiple

specialized cell types, oligopotent; into 2 or more cell types, unipotent; into only one cell type (Kolios and Moodley, 2013).

1.8.1 Prospect of using ESCs as a model

Understanding early embryo development in cattle is important to prevent pregnancy failure and improve calving; and when using bovine cells as a model for human reproduction. Unfortunately, *in vitro* embryo production is limited to development up to 8 days after fertilization due to culture system incapacity. To observe further development of embryos embryo transfer (ET) is performed, and embryos recovered and assessed at various intervals (van Leeuwen et al., 2015). However, such approaches are expensive and subject animals to invasive procedures. Therefore, new *in vitro* techniques are being developed to observe peri- (Machado et al., 2013) and gastrulating embryo development (Moris et al., 2020).

To optimize the present techniques for peri-gastrulating embryo culture, ESC culture media (N2B27) was used. Typically bovine (Ramos-Ibeas et al., 2020) and ovine embryos (Ramos-Ibeas et al., 2022) are cultured in SOF media until D8, then cultured in N2B27 media up to D15. Under such conditions 55-60% blastocysts develop further, and the hypoblast migrates under the trophectoderm lineage.

Moreover, it was noted that ESC allows us to compare embryo development without ET. In mice, ESCs which were derived from embryos generated from old and young donors and pluripotency and aneuploidy evaluated (Khurana et al., 2022). Additionally, in the same study differentiation of ESCs was used to observe the characteristic features of blastocysts to evaluate signaling in organoids.

Recently, gastrulated embryos were successfully generated from human and mouse ESCs, and it was observed that *in vitro* model development accurately represents the natural gastrulating embryo. Moreover, this technique allows us to observe germ layers differentiation and cell migration over the 72 h (Moris et al., 2020).

1.9 Development of Working Hypothesis and Specific Aims

In vitro produced embryos exhibit lower implantation rates compared to *in vivo* derived embryos (Mahdavinezhad et al., 2019). To increase the yield of embryos, undefined media components such as serum and/or BSA have been used for many years. Due to variations in embryo production efficiency and batch differences in undefined media components, the desire to develop defined culture conditions has become important in recent years. Additionally, serum supplementation during *in vitro* culture is linked to fetal developmental anomalies leading to the large offspring syndrome (LOS) (Young et al., 1998). Moreover, it was observed that serum causes lipid droplet accumulation, and this impairs cryopreservation (Abe et al., 2002) which is important for embryo transport and storage.

For oocytes/embryos from a number of species, lipid metabolism regulators have been incorporated in culture where serum is added in order to avoid lipid accumulation (see Section 1.6). One of the most frequently used regulators is L-carnitine (LC). It has been observed that, whilst addition of L-carnitine in oocyte maturation media decreased lipid accumulation in embryos, LC supplementation during culture or freezing media improved cryotolerance in blastocysts (Section 1.6.1). Moreover, it is known that LC affects antioxidation, therefore increasing blastocyst cell number by reducing apoptosis caused by ROS. Therefore, this thesis focuses on lipid metabolism in bovine oocytes and blastocysts by adding LC during IVM and/or IVC to improve blastocyst quality and yields from oocytes recovered from pubertal heifers. Additionally, this thesis aimed to improve culture systems by removing undefined components. Moreover, it is well known that most pregnancy losses occur due to early embryo death (Diskin et al., 2006). However, the reasons remain unclear because, with the current culture systems it is possible to observe embryo development only up to D8-9. On the other hand, new *in vitro* techniques were developed such as tunnel and ESC differentiation. In the last few years ESCs became more important as they allow us to generate gastruloids which represent gastrulating embryos. They have not been generated from bEpiSC thus far, and this so thesis also aimed to develop a model of the bovine gastrulating embryo.

The acetylated form of LC (ALC) is an essential source of acetyl moieties and the easiest form to be taken into cell, even being able to cross the blood-brain

barrier (Inano et al., 2003). Moreover, various LC isoforms were identified in follicular fluid in the μM range which is much lower than that used in the vast majority of studies. Chapter 2, therefore, focused on the effects of ALC supplementation during maturation and culture at more physiological levels on embryo development and quality. The aim of this chapter was to improve embryo development by increasing antioxidation. Specifically, the aim of this chapter was to determine the effect of physiological doses of ALC supplementation during IVM and IVC on molecular changes during maturation and embryo development during culture.

As previously described, due to the effect of undefined chemicals such as serum and BSA on impaired embryo development and offspring, Chapter 3 sought to remove serum and BSA from maturation media and replace them with defined components. Specifically, this study sought to replace serum and BSA in culture with a mixture of growth factors, L-carnitine, fatty acids and other selected components of serum identified with reference to previously published data. Success of these alterations were determined by molecular changes in cumulus cells, and measures of embryo development and quality.

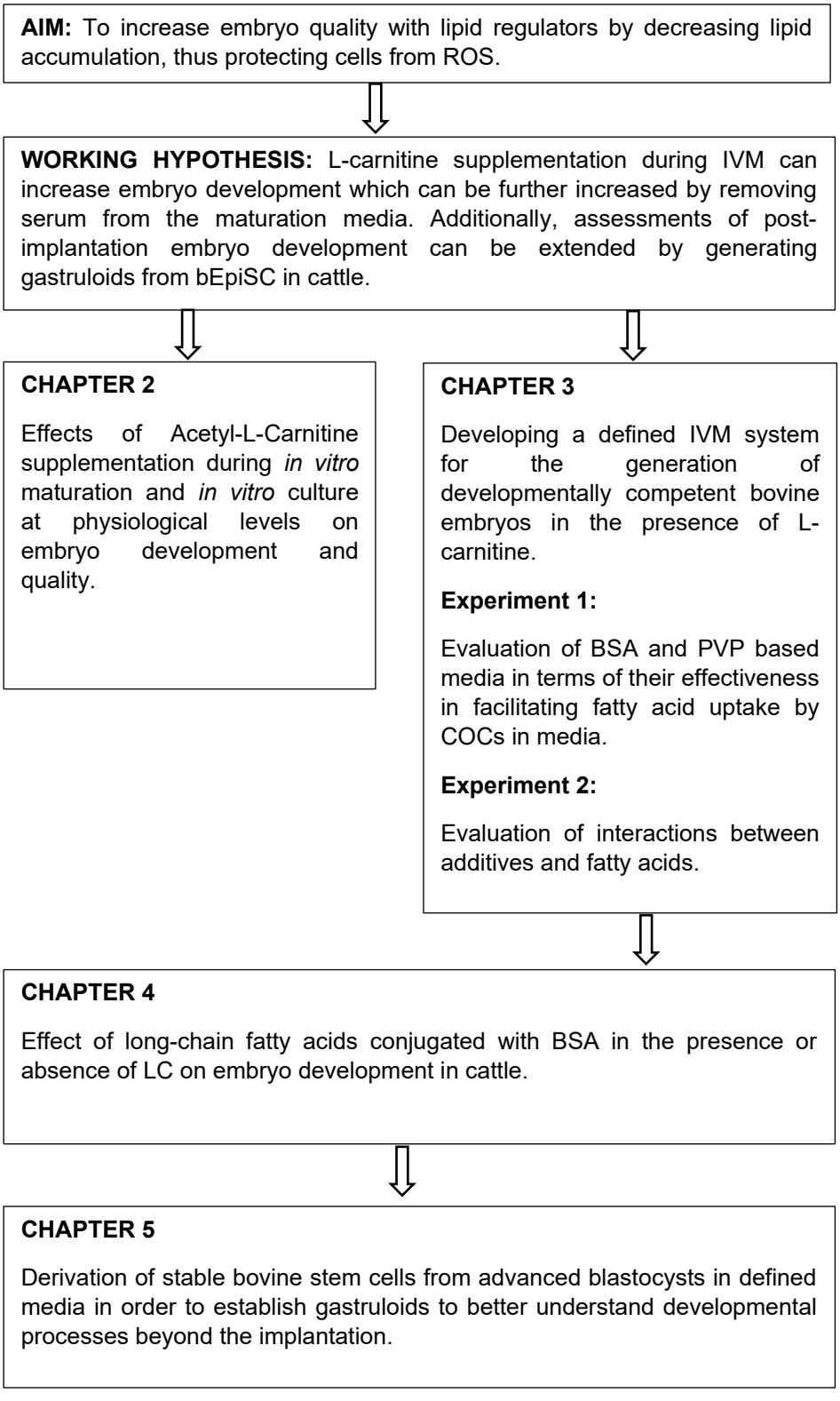
Albumin (BSA) is known as a lipid transporter and the primary source of fatty acid uptake by embryos (Hughes et al., 2010). Fatty acid-free forms of BSA are available on the market presenting the opportunity to add specific fatty acids to culture media. Additionally, to the best of our knowledge, the effect of L-carnitine in the presence of specific mixtures of fatty acids has never been investigated. Therefore, in addition to assessing the effects of a specific mixture of growth factors, Chapter 4 sought to assess the effects of LC in the presence of specific fatty acids conjugated to fatty acid free BSA. It was hypothesized that LC improves embryo development, ATP production and protects mitochondria from the ROS. As there is a delicate balance between fatty acid and glucose metabolism in cells (Lowe et al., 2018). This chapter also sought to identify the effects of changes in glucose metabolism in the presence or absence of LC on various markers of embryo development.

Following the bovine oocyte fertilization (D0), zygotes undergo mitotic cell divisions and by D6 morula is formed. From this point of development differentiation occurs and trophectoderm (TE) and inner cell mass (ICM) are formed. Blastocysts expand and hatch by D9 and two different linages are appeared within the ICM; epiblast and hypoblast. Up to D9 embryo

development can be monitored and the developmental process is investigated *in vitro*. By D11 and D17 blastocysts undergo elongation, by D18-D20 embryo attaches to the uterus and by D29 placentation occurs *in vivo*. As previously mentioned (see Section 1.9.1), ESCs allows us to understand the mechanism of further development of embryos. Therefore, Chapter 5 focuses on stem cell derivation from the advanced blastocysts (expanded, hatching and hatched blastocysts) which are matured and cultured in serum-free media as also stem cell culture media is chemically defined. In recent years, embryonic stem cells (ESC) became more important as their ability to form gastruloids which represents gastrulating embryos. This opportunity gives us a chance to observe changes that naturally occur in gastrulating embryos following the implantation.

In summary, this thesis hypothesized that embryo quality and yield can be increased with lipid regulators by decreasing lipid accumulation, preserving cells from ROS. At the same time this thesis aimed to improve defined culture media. Moreover, new *in vitro* technique was developed using bEpiSC. Finally, in Chapter 6, the findings of each experiment were discussed, and remaining gaps in knowledge identified to direct future research endeavors.

1.10 Thesis Structure Diagram



Chapter 2 Effect of Acetyl-L Carnitine supplementation during in vitro maturation and culture on bovine embryo development

2.1 Introduction

Carnitine is a carrier protein which is synthesized from lysine and methionine endogenously or digestion in gastro-intestinal track as exogenously (Rebouche, 2004a). It is taken up by cells from the extracellular compartment via carnitine carrier proteins such as OCTN2. Carnitine has two optic isomers, L-carnitine and D-carnitine. While L-carnitine has a vital role for β-oxidation, D-carnitine reduces uptake of L-carnitine into cells (Gross and Henderson, 1984), inhibits CPT activation (Vashistha and Bhushan, 2015), and therefore, inhibits β-oxidation. Carnitine locates in the cytoplasm within cells and mediates the uptake of activated long-chain fatty acids into the matrix of mitochondria under the control of carnitine palmitoyl transferase I (CPT I), which is in the outer membrane, and CPT II in the inner membrane. Following transport, carnitine is released into the cytoplasm by translocase which is located in the mitochondrial membrane. The other role of carnitine is to transfer acetyl-CoA, which is derived from peroxisome β-oxidation, into the mitochondria to participate in the Krebs cycle (Vaz and Wanders, 2002). Every molecule of NADH and FADH2 formed in the TCA steps is transferred to the electron transport system (ETS). Following the ETS free radicals are formed, and antioxidants are synthesized against them.

L-carnitine comes in several forms in animal cells. The acetylated form is an essential source of acetyl moieties and the easiest form to transfer, being able to cross the blood-brain barrier. Therefore, acetyl-L-carnitine (ALC) has to date been the most popular form for neurological studies. In sperm and macrophages it has been speculated that acetyl-L-carnitine is a storage form of energy (Lee Carter et al., 1995). Liu et al. (2004a) found that both LC and ALC can increase ATP. However, only ALC decreased oxidative damage in the brains of old rats.

A number of studies have been undertaken in embryos of different species where the effects of L-carnitine in contrast to acetyl-L carnitine, was

investigated. In a few studies, ALC was incorporated into media at the same concentration as L-carnitine. During IVM 2.5mM ALC increased blastocyst development rate, blastocyst cell number and decreased concentrations of ROS (Xu et al., 2018a). According to Xu et al. (2019), ALC supplemented to IVM media increased the proportion of inseminated oocytes that cleaved and blastocyst formation from vitrified oocytes. On the other hand, in humans physiological concentrations of L-carnitine in follicular fluid ranged from 0.6 µmol/L to 45.8 µmol/L in pregnant and non-pregnant women (Montjean et al., 2012). In a more physiological study, treatment with 10 µM Acetyl-L-Carnitine, 10 µM N-Acetyl-L-Cysteine and 5 µM α-Lipoic Acid during IVF and IVC enhanced embryo development and reduced concentration of ROS (Truong and Gardner, 2017). More specific information about the effects of ALC has come from Loots et al. (2004), whereby ALC prevented formation of hydroxyl free radicals which has harmful effects including cytotoxicity (Hiraishi et al., 1991), inhibition of glycolysis and oxidative phosphorylation and intracellular ATP depletion (Wu et al., 1996).

To the best of our knowledge, the overall effects and benefits of ALC is not yet clear, and there is no information about the cumulative effects of ALC on embryo development. Furthermore, the optimal level of inclusion of ALC in IVM and IVC media has yet to be established. Therefore, the current study sought to establish the effects of ALC on *in vitro* embryo production, and so focussed on the cumulative effects of different concentrations of ALC (from the micromolar to millimolar range) during IVP on blastocyst development, and biochemical and molecular markers of metabolism.

2.2 Material and Method

2.2.1 Experimental Design

This experiment consist of adding the following concentrations of ALC to IVM and IVC: 0, 0.01, 0.1 and 1.0 mM. The experiment was replicated nine times. Embryo development to the Day 8 blastocyst stage was morphological assessed in nine replicates, the first 5 replicates were spared for immunostaining to examine total cell number of blastocysts and the last 4 replicates were used for gene expression analysis. In an additional four

replicates, gene expression analysis was undertaken separately in cumulus cells and oocytes both before and following IVM.

2.2.2 Chemicals and medium

All media chemicals were ordered from Sigma Aldrich (Dorset, England) and all the media were prepared in the lab, pH and osmolality (mOsm) checked and then filtered for sterilization. Details of media composition are provided in Appendix I.

2.2.3 Oocyte collection and in vitro embryo production (IVP)

Abattoir-derived oocytes were transported to the laboratory in phosphate buffer saline (PBS) at 35˚C. Cumulus-oocyte complexes (COCs) were aspirated from 2-8 mm follicles using a 10 ml syringe. Grade I and II which have translucent and homogenous ooplasm and they are surrounding completely (at least three cell layers) by compact cumulus cells (Boni et al., 2002) COCs were transferred to 4-well plates containing 30 oocytes per well in 400µl maturation medium with different concentrations of acetyl L-carnitine; 0mM, 0.01mM, 0.1mM, 1mM (Appendix I.6). Maturation was undertaken in a humidified environment under 5% CO_2 at 38.8ºC for 22 h.

Frozen-bull semen from a single sire was thawed in a 37˚C water bath for 1 minute, washed in sperm-TALP and centrifuged 2,000 x g for 2 min. Pelleted sperm then underwent a swim-up procedure at 38ºC for 90 min (Magdanz et al., 2019). Incubated sperm was centrifuged again at 2,000 x g for 20 min. Pellet was resuspended in remaining Sperm TALP medium. Once mixed, 5 µl of sperm suspension was taken and added to 95 µl of water to establish sperm concentration. Water-sperm suspension was mixed well and 10 µl of sperm suspension was added into each of Nauber chamber. Sperm was counted in 5 squares and multiply by 50,000 x the dilution factor (in this case 20) to give concentration of sperm/ml. Matured oocytes were placed into pre-warmed 50 µl drops of HEPES TALP medium then washed in a 200µl fertilization drop under mineral oil (Sage Oil for Tissue culture, Cat No ART-4008-5PA). At the end of this process, they were transferred in 50 µl drops of fertilization media.

Sperm concentration was adjusted to 26 x 10^6 sperm/ml then added to fertilization drops for syngamy. The plate was replaced in a CO2 incubator (5% CO2, 38.8ºC – 39ºC and maximum humidity) over night.

After fertilisation, presumptive zygotes were transferred into eppendorf tubes containing 0.5 ml of synthetic oviduct fluid holding medium and vortexed for 1 minute to denude them from attached sperm and remaining cumulus cells. They were then placed into 4-well plates containing 400 µl of SOF culture medium with 30 presumptive zygotes per well and incubated at 5% CO_2, 5% O^2 at 38.8ºC – 39ºC and maximum humidity for eight days. The number of cleaved zygotes and their developmental stages were recorded 36-48 h post-fertilization according to Stringfellow D (2010), media were replenished at this time, and again on Day 5.

2.2.4 Day 8 blastocyst assessments: staining with DAPI

On D8 blastocysts were transferred into SOF holding medium (enriched with 3mg/ml BSA-FAF), separated into hatched and non-hatched groups within treatment. Hatched groups were washed with 50 µl drops of PBS three times. Non-hatched groups were washed following pronase treatment (10 µg/ml, 50 µl drop) until zona pellucida (ZP) looks jelly to remove their ZP. ZP-free blastocysts were transferred to 300 µl 4% paraformaldehyde (PFA) for 1hr at room temperature, then washed again three times in 1% BSA-PBS drops. After fixation, they were washed again with fresh PBS-PVP then incubated in 300 µl triton for another 1h. Following the permeabilization, for staining, 3 µl drop histology mounting medium (DAPI) was warmed on the microscope slides. Blastocysts were placed into drops covered by circular lamellae, then nail polish was applied around the cover glass for immobilization.

Fluorescence microscope (Leica) filters were set at blue for DAPI. Image J software (NIH Image, Bethesda, MD) was used to capture images.

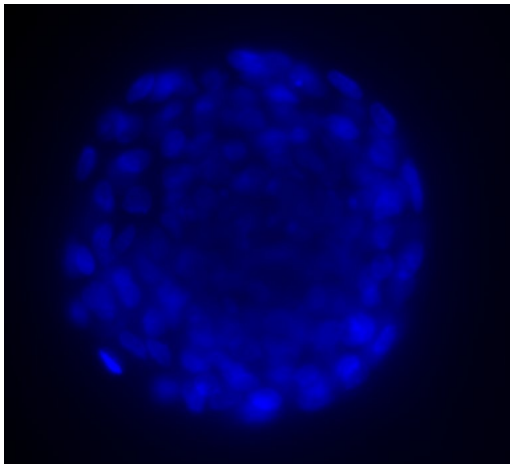


Figure 2. 1 Blue colour indicates total cells. The picture was taken under 20 × zoom lens. Cell counts conducted using Image J software (NIH Image, Bethesda, MD).

2.2.5 Transcript expression in cumulus cells, oocytes and blastocysts

To provide an insight into key events occurring in lipid metabolism, the following target genes were identified for quantitative real-time PCR (qPCR): *ACACA, SLC22A5, CPT1, SOD2, CAT, GPX1, FASN, PLIN3* and *PRKAA1* (Table 1).

Both before and following IVM, 20 COCs per treatment were transferred into eppendorf tubes containing 200 µl 0.1% PVP-PBS solution and vortexed for 10 min. Suspensions containing oocytes and cumulus cells (CC) were transferred to 35 mm dishes to collect denuded oocytes which were individually stored in 5µl 0.1% PVP-PBS at -80ºC following washing in 50 µl 0.1% PVP-PBS drops. The leftover solutions containing CCs were transferred into fresh eppendorf tubes for centrifugation (at 12,100 g for 10 min) to obtain CC pellets. Pellets were also stored at -80ºC. On Day 8, after assessing embryo development, blastocyst were washed within 50 µl 0.1% PVP-PBS drops and stored at -80ºC.

Primers were designed on Primer Blast programme. Based on the given accession numbers of primers (from PubMed Nucleotide), primers were selected that gave rise to 120-150 bp PCR products containing exon-exon junctions. Prepared primers were purchased from Eurofins Genomics (Wolverhampton, UK).

Table 2. 1 List of primers with their sequences which were used for qPCR analysis. FP, forward primer, RP, reverse primer.

Gene	Encoded Protein	Accession Number	Primer Sequence		Product Length (bp)	PCR Efficienc (%)
		Fatty acid synthesis and storage				
ACACA	Acetyl-CoA Carboxylase α	NM_174224	FP	CTGAACCAGCACTCCCGATT	145	96.5
			RP	CAGAGAGCGTGTCTGAGCTG		
FASN	Fatty Acid synthase	NM_001012669.1	FP	CACTCCATCCTCGCTCTC	181	94.6
			RP	GCCTGTCATCATCTGTCACC		
PLIN3	Perilipin 3	NM_001077046	FP	CGTTTCCACCCTCGCTTTTG	127	116.1
			RP	CCTGGTCACCACACTCAGTT		
		Oxidative stress				
SOD2	Superoxide dismutase 2 [Mn]	NM_201527.2	FP	TTGCTGGAAGCCATCAAACGTGAC	135	96.9
			RP	AATCTGTAAGCGTCCCTGCTCCTT		
GPX1	Glutathione Peroxidase 1	NM_174076.3	FP	CATCGACATCGAGCCTGACA	112	92.1
			RP	GGCAAAATCCCTGGAGAGCA		
CAT	Catalase	NM_001035386.2	FP	AGGAGAAACCTAAGAACGCAGT	135	93.9
			RP	CATGCTCTGCTTCACAAGCG		
		Beta- oxidation				
CPT1A	Carnitine Palmitoyl Transferase 1A	NM_001304989	FP	TTCGCGATGGACTTGCTGTA	149	105.2
			RP	CAGAGCGGAATCGTAGACCC		
Gene	**Encoded protein**	**Accession Number**	**Primer Sequence**		**Product Length (bp)**	**PCR Efficienc (%)**
		Membrane Transporter Proteins				
SLC22A5	Solute Carrier Family 22 Member 5	NM_001046502	FP	CTCAGACAGGTTTGGTCGGA	136	106.2
			RP	GATCTGGCCCATGCCTACAA		

		Transcription Factors				
PRKAA1	AMP-activated catalytic subunit alpha 1	XM_024981363.1	FP	GAGCTTGCCAAAGGAATGAT	158	104.4
			RP	TAGATTCGCGCACATCTCAA		
		Reference Genes				
HMBS	Hydroxymethylbilane Synthase	NM_001046207.1	FP	CCCGACACCGGAGGACATT	102	95.4
			RP	TGGGGTGTCTTCTTCCGCTAT		
B2M	Beta-2-Microglobulin	NM_173893	FP	ATCCAGCGTCCTCCAAAGATTC	132	121.5
			RP	CTCCCCATTCTTCAGCAAATCG		
H2A	H2A Histone Family	NM_174809.2	FP	GGAATTCGAAATGGCTGG	149	90.8
			RP	CACGTCCATGGCTAGTCG		
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_001034034	FP	GTGGCGCCAAGAGGGTCA	115	92.9
			RP	CAGGAGGCATTGCTGACAATC		
RPL19	Ribosomal Protein L19	NM_001040516	FP	AGCGGATCCTCATGGAACAT	72	96.0
			RP	CCTGGTCCGCCAGAAGCT		

2.2.5.1 RNA Extraction from CC and quantification

The RNeasy Mini Kit (cat. Nos. 74104 and 74106, QIAGEN Group, Manchester/UK) was used and the protocol which is provided by company was followed.

RLT buffer was mixed with β-mercaptoethanol (β-ME) as described in the Quick-Start Protocol. The ratio of these two chemicals was 10 µl β-ME to 1ml RLT Buffer (1:100). 600 µl from this solution was added to each sample. Homogeneity was induced by pipetting using an insulin syringe and 19G needle. Then 70% ethanol was added to the RLT- β-ME solution (600 µl). From this mixture, 600 µl was added to the column which was placed in the collection tube and centrifuged at the max speed (12,000 x g) for 30s. During this process RNA is bound to the column and all the liquid collected was discarded. Samples were then transferred to fresh 2 ml tubes. 700 µl RW1 buffer was added to the column and spun for 30s at max speed – liquid collected was discarded. 500µl RPE buffer was added and spun twice, 30s and 2 min at max speed, respectively. Membranes were dried by centrifugation at full speed for 1 min. Columns were placed into eppendorf tubes and 40 µl RNA-free water added directly to the column membrane and spun at full speed for 75 min.

For RNA quantification, Qubit RNA HS Assay Kits (Cat Nos Q32852, Q32855, Thermo Fisher, UK) were used and the given protocol followed.

Qubit RNA HS reagent was diluted (1:200) with Qubit RNA HS Buffer in a plastic container to prepare Qubit working solution. For standard 190 µl, for samples 199 µl Qubit working solution were added into the tubes. 10 µl of each Qubit standard was added to the appropriate tube (into 190 µl), then mixed by vortexing for 2-3 s. 1 µl of each sample was added to each tube which contained 199 µl working solution. This was then vortexed. Final volumes of each tubes including samples were 200 µl. Concentrations of extracted RNA was determined by nanodrop spectrometer (ND1000, Nanodrop, Wilmington, USA) and illustrated in appendix.

2.2.5.2 RNA extraction from oocytes and blastocysts

Dynabeads mRNA Direct Kit (Cat:610-11, Thermo Fisher, UK) were used for RNA extraction from blastocysts and oocytes. Following kit instructions, samples were lysed in 150 µl lysis buffer for 15 min at room temperature. Meanwhile required volume of beads was transferred to the magnet and, after a few seconds a pellet was formed, and the supernatant was removed. Pellets were washed with a similar volume of lysis buffer and transferred to the magnet again. Washing procedure was repeated two times before resuspending with the same volume of lysis buffer. 40 µl pre-washed beads were added to lysed oocytes and blastocysts and incubated for 10 min at room temperature in rotation machine. Samples were spun down and placed in magnet. Supernatant was removed and samples were washed with 100 µl buffer A. After each washing, samples were placed in magnet and repeated 3 times. Then, samples were washed with 100 µl buffer B same as buffer A but repeated 2 times. Following the last wash with buffer B, pellet was resuspended with 10 µl PCR water and incubated at 65ºC for 2 min. Samples were transferred in magnet for the extracted RNA collection.

2.2.5.3 cDNA synthesis and PCR amplification

cDNA was produced from extracted RNA by using QuantiTect reverse transcription kit (Qiagen Ltd., West Sussex, UK) following manufacturers guidelines. 120ng total RNA in a maximum volume of 12µl was reverse transcribed to single stranded cDNA. mRNA, 2 µl gDNA Wipeout buffer and, according to mRNA volume, RNase-free water was added to complete final volume to 14 µl. This master mix has been incubated for 2 min at 42 ºC. After genomic DNA elimination reaction, 1 µl was kept for -RT and the rest was added to the master mix for RT reaction. 1 µl Quantiscript reverse transcriptase, 4 µl RT buffer, 1 µl RT primer mix and 1 µl RNase-free water was mixed. This 20 µl master mix was incubated for 30 min at 42ºC and 3 min at 95ºC to generate cDNA. Amplification of the cDNA was carried out using QuantiNova SYBR Green PCR Master mix and the reaction was run in Bio-Rad CFX Maestro. The master mix was prepared for amplification based on the manufacturer guidelines (10 µl SYBR green, 1 µl forward primer, 1 µl reverse primer, 6 µl RNase-free water and 2 µl cDNA (20 µl in total per

reaction)). Reaction started with initial heating step to activate enzymes for 3 min at 95ºC. The samples were then PCR amplified for 39 cycles each consisting of 5 seconds denaturation at 95°C, 10 sec annealing at 60°C. Following amplification, a melt curve was recorded for 5 sec at 65 °C to 95 °C.

2.2.5.4 Sequencing

Primer verification was confirmed by sequencing. 2% agarose/TAE buffer with 5 µl/ml ethanol bromide was prepared. Teeth of comb were covered by type as holes prepared for marker (100 bp), reactive control (RC) and samples. When the comb was placed in the plate, the gel was poured and left to dry. Meanwhile, from the PCR plate which was run before and stored at -20˚C, two groups which were containing high concentration of mRNA of each gene was collected and mixed with 8 µl loading dye (5 :1). Following vortexing and centrifugation, they were loaded onto the gel and electrophorese were run. 110V was applied for 20 min and the gel cut under UV and placed into measuring tubes and their weight were recorded. Zymoclean gen DNA recovery kit (Cat No: D4007) was used to prepare samples for sequencing. Following company instructions, gel melted with three-fold of gel weight ADB solution, washed and DNA collected via EB buffer from the matrix of column by spinning. Forward and reverse primers were separately diluted in 1:100 from stock or diluted in 1:10 working solutions which were already diluted (1:10).

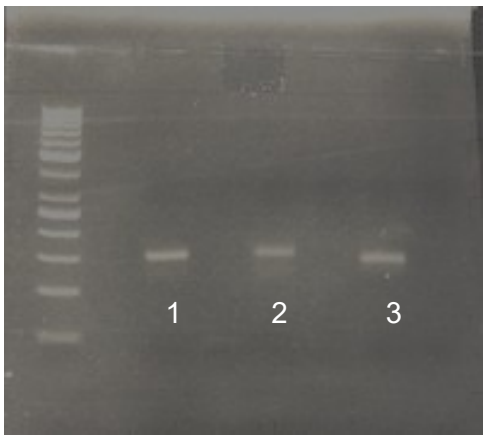


Figure 2. 1 Gel electrophoresis. Single bands, left to right, represent *ACACA* (1), *CPT1A* (2) and *SLC22A5* (3), respectively. The marker was 100 bp.

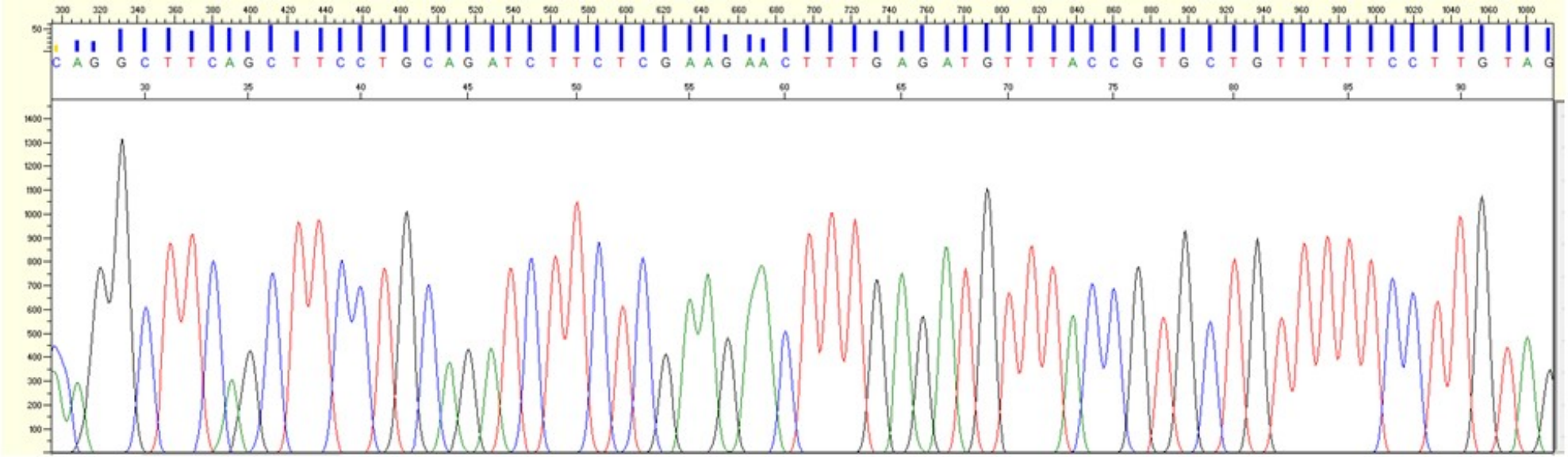


Figure 2. 2 Representative sequencing result for *SLC22A5*. As the result indicates the picks are clear and baseline 'noise' are quite minimal which is an indicator of primer quality.

2.2.5.5 Optimisation of gene expression and selection of housekeeping genes (HKGs)

2.2.5.5.1 Cumulus Cells

Different concentrations of RNA were tested and optimised as 4 ng per reaction. To identify the most reliable genes in this treatment, all genes were run together and GeNorm analysis (St-Pierre et al., 2017) (Appendix II) and Bio-Rad gene study performed (Figure 2.4).

For the standard curve, 35 ng RNA per reaction was determined as a highest concentration of standard curve and serial diluted in 1:5 concentrations. In further studies to avoid having to generate standard curve each time, a calibrator also was performed in this project. 4 ng RNA per reaction was standardized and applied triplicate. Dilutions for standard curves and replicate of samples were run in duplicate.

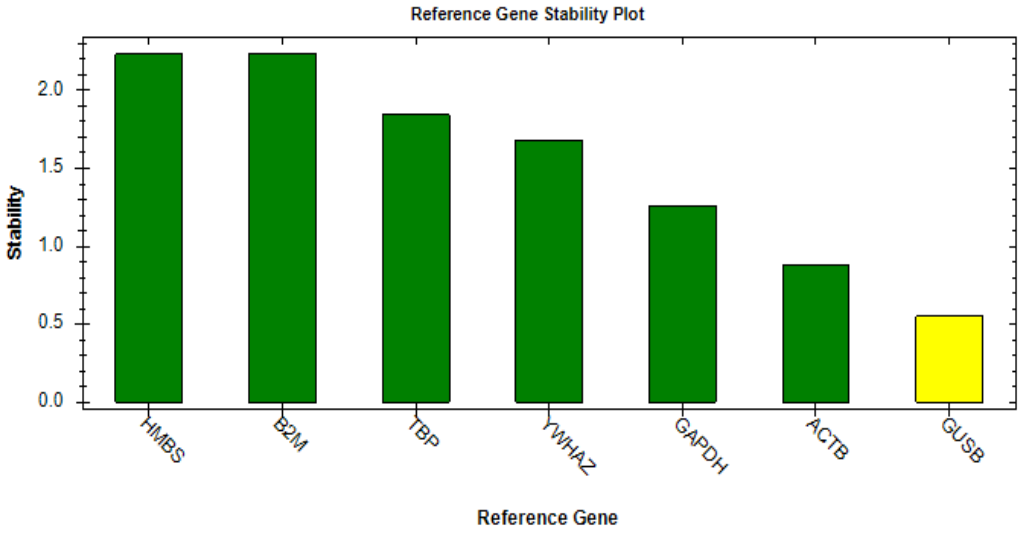


Figure 2. 3 The most stable genes were determined as *HMBS* and *B2M* for CC by Bio-Rad system.

2.2.5.5.2 Blastocysts and Oocytes

Before running the real samples, 2 blastocysts and oocytes per gene, 3 per the highest concentration of standard curve were tested. Neat was diluted 1:5 concentrations to obtain 5 serial dilution. Each reaction was duplicated to control for pipetting error. For HKGs selection, Bio-Rad reference gene stability program was used (Figure 2.5, 2.6), based on the M value 2 HKGs were selected for real samples.

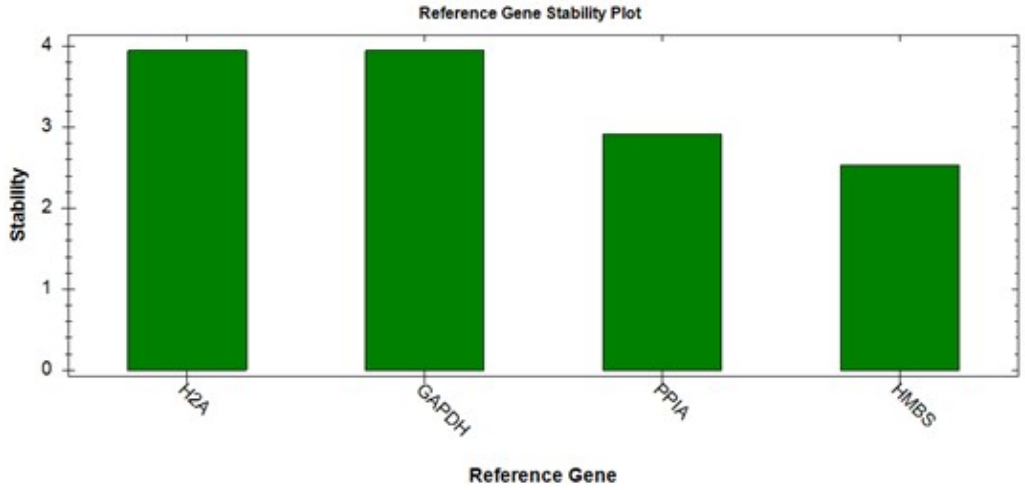


Figure 2. 4 Reference gene selection results for blastocysts obtained by Bio-Rad. As the *H2A* and *GAPDH* exhibited higher stability compared to others, these two genes were selected for HKGs.



Figure 2. 5 Reference gene stability result for oocytes indicated that the most stable genes are *RPL19* and *GADPH* and these genes were selected for HKGs.

2.2.6 mtDNA copy number

Pedersen et al. (2016) has provided a protocol and it was modified for Sybr green. For standard curve preparation, *ND4* qPCR product was run on the gel in electrophoresis. After the run, gel was cut in the dark room under the UV light to get rid of the gel which did not involve the product, in other words only bands were collected by cutting. Following the cutting the gel, Zymoclean gen DNA recovery kit (Cat No: D4007) was used for purification. The DNA concentration of samples was measured by Qubit HS dsDNA assay kit (Cat: Q32851, Thermo Fisher, UK). The working solution was diluted with reagent 1:200 and split in 3 tubes (2 standard and 1 sample), 190 µl was required for both standards and 199 µl for sample. Standards (0 and 10) and 1 µl sample were added appropriate tubes. Tubes were vortexed and incubated in dark at room temperature during 2 min, then measured by spectrometer (DeNovix DS-11VS, Thermo Fisher,UK).

Based on the DNA concentration, standard was diluted with 5ng/ul tRNA to obtain 6 points, 5-fold serial dilution from $4x10^{-7}$ to $1.28x\ 10^{-10}$.

Oocytes were lysed with lysing buffer and 4ul was diluted with 5ng/ml tRNA in 1:7 ratio to reduce the density of lysate buffer. From dilutions, 4ul was taken for preparing master-mix to run qPCR.

$$Number\ of\ copies = \frac{Amount\ (ng) \times 6.022\ \times 10^{23}}{Length\ (bp)\ \times 1 \times 10^{9}\ \times 660}$$

Figure 2. 6 mtDNA copy number was calculated based on this formula. Amount of DNA was multiplied by Avogadro's constant then divided by length of DNA template, conversion factor to convert bp to ng and average mass of 1bp of dsDNA.

2.2.7 Statistical Analysis

Embryo production data were analysed using Generalised Linear Models within GenStat (GenStat 19th Edition, 2018, VSN International Ltd, Hemel Hempstead, UK) assuming binomial errors and with logit link functions. Terms fitted to this model were replicate and dose of ALC. Data are presented as means ± Standard Error of Means (SEM). F distribution (F pr.) was used for p value.

2.3 Results

2.3.1 Embryo Development

There was no effect of ALC dose on proportion cleaved of inseminated (Figure 2.8A), Day 2 zygotes of cleaved (Figure 2.8B), blastocysts of inseminated (Figure 2.8C) and blastocysts of cleaved (Figure 2.8D).

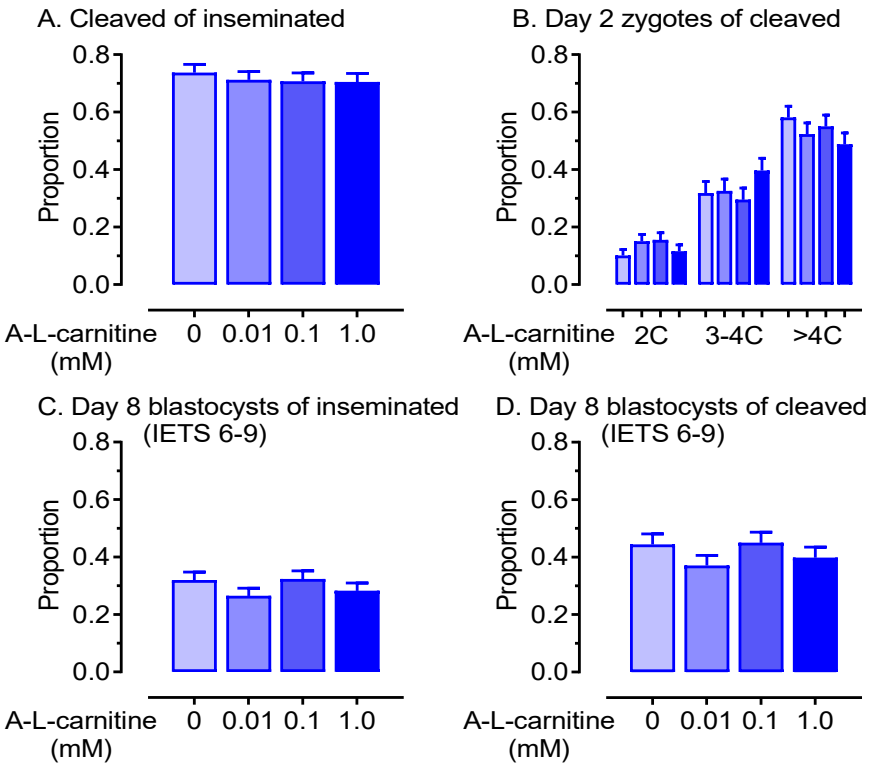


Figure 2. 7 No effect of dose (0.0, 0.01, 0.1 and 1.0 mM) of acetyl-L-carnitine (A-L-carnitine) was observed on *in vitro* embryo development; the proportion of inseminated oocytes (n= 30) that cleaved by Day 2 (A), Day 2 zygotes were categorised as 2, 3-4 and >4cells (B). Blastocyst stage was graded according to the recognised by the International Embryo Transfer society (IETS): stage 6 = expanding, 7 = expanded, 8 = hatching and 9 = hatched blastocysts. Proportion of inseminated oocytes reached blastocyst stage (C) and proportion of embryos reached the blastocyst stage by Day 8 (D).

On the other hand, by Day 8 there was an indication (not statistically significant) that blastocyst stage varied between treatment groups. While the proportion of expanded blastocysts appeared highest in the 0.01 mM ALC group (Figure 2.9A), the proportion hatching blastocysts appeared highest in 0.1mM ALC group (Figure 2.9B), and hatched blastocysts was highest in the control group (Figure 2.9C).

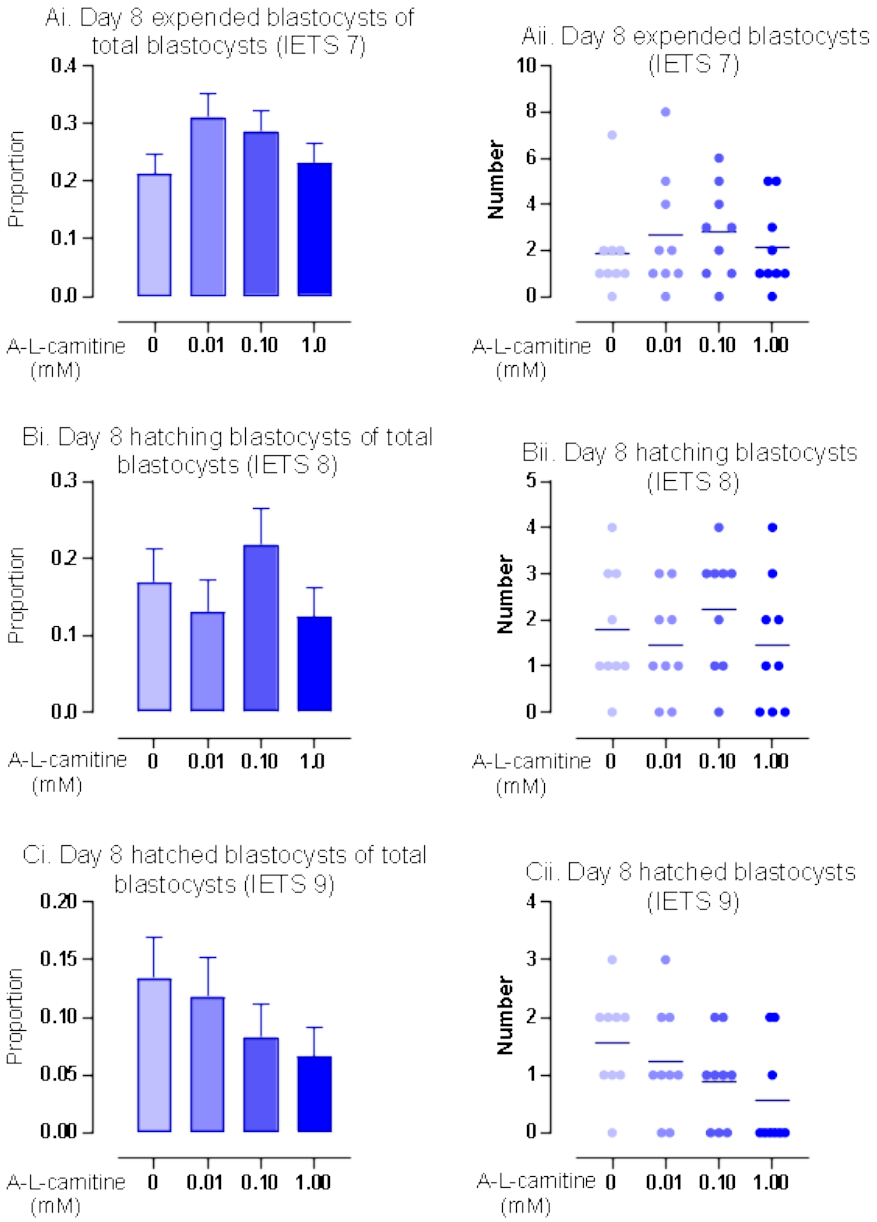


Figure 2. 8 No effect of dose (0.0, 0.01, 0.1 and 1.0 mM) of acetyl-L-carnitine (A-L-carnitine) was observed on *in vitro* embryo development Day 8 blastocysts; blastocyst stage was graded according to the recognised by the International Embryo Transfer society (IETS): stage 6 = expanding, 7 = expanded, 8 = hatching and 9 = hatched blastocysts. The proportion of D 8 expended (IETS 7) (Ai), hatching (IETS 8) (Bi) and hatched blastocysts (IETS 9) (Ci) in total blastocysts were not affected by treatment. Scatter plots data of expended (Aii), hatching (Bii) and hatched (Cii) blastocysts indicates distribution of blastocysts number with median from each replicate.

2.3.2 Total cells of Day 8 Blastocysts

Day 8 blastocysts cell numbers decreased as ALC concentration increased (Figure 2.9).

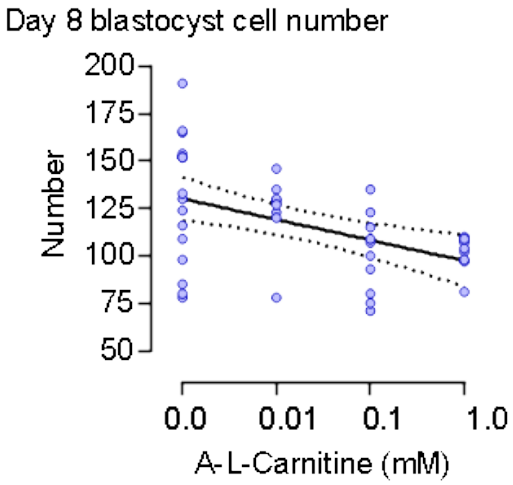


Figure 2. 9 Relationship between dose of acetyl-L-carnitine (A-L-Carnitine) and blastocyst cell number. Preliminary analyses indicated that cell number decreased (P= 0.011) with dose of A-L-Carnitine. (0mM; 130.3 ± 6.6, 0.01mM; 123.5 ± 9.1, 0.1mM; 100.8 ± 7.3, 1mM; 101.8 ± 7.8)

2.3.3 Gene Expression Analysis

2.3.3.1 In Cumulus Cells

Significant difference was found between pre-matured and matured group in terms of *CAT* gene abundance (Figure 2.11.Ai). Moreover, statistical analyses of control versus all three different concentrations was performed and significantly decreasing (p=0.044) was observed in ALC groups (Figure 2.11.Aii). Additionally, Whilst *SOD2* expression was getting lower in matured group compared to premature (Figure 2.11.Bi), with ALC treatment decreasing level prevented significantly (p= 0.03) (Figure 2.11.Bii). In *GPX1* expression, significant decrease was observed over the maturation (Figure 2.11.Ci), however there was not any treatment effect between matured groups (Figure 2.11.Cii). Raw data scatter plots of gene expression in CC are placed in Appendix V.1.

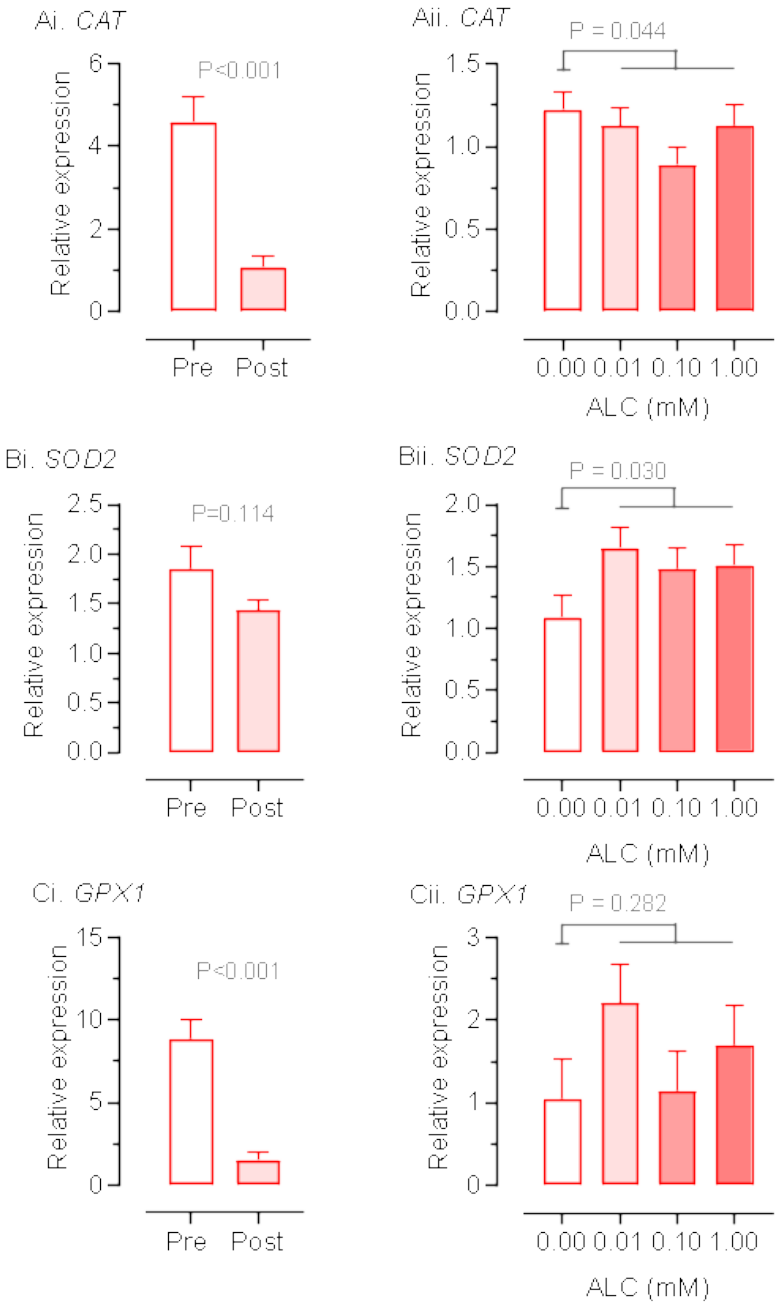


Figure 2. 10 Antioxidative gene expression in cumulus cells; *CAT* (Ai), *SOD2* (Bi) and *GPX1* (Ci) were analysed in CC during the maturation. Following the maturation when the COCs were treated with 0, 0.01, 0.1 and 1 mM ALC; *CAT* (Aii), *SOD2* (Bii) and *GPX1* (Cii) expressions were evaluated between control and all three treatment groups. Pre: pre-maturation, Post: post-maturation.

The abundance of *PRKAA1* decreased during the IVM (2.12.Di), however prevention of decreasing level was observed significantly (p= 0.045) with ALC treatments compared to control (2.12.Dii). In the treatment groups *FASN* was increased slightly when compared to control group however that was not

significant statistically (2.12.Eii). On the other hand, matured group has expressed less *FASN* than pre-matured group significantly (2.12.Ei). It was observed that abundance of *ACACA* was decreased during the maturation (2.12.Ci) and the treatment effect was found in *ACACA* expression with the lowest concentration of ALC (2.12.Fii).

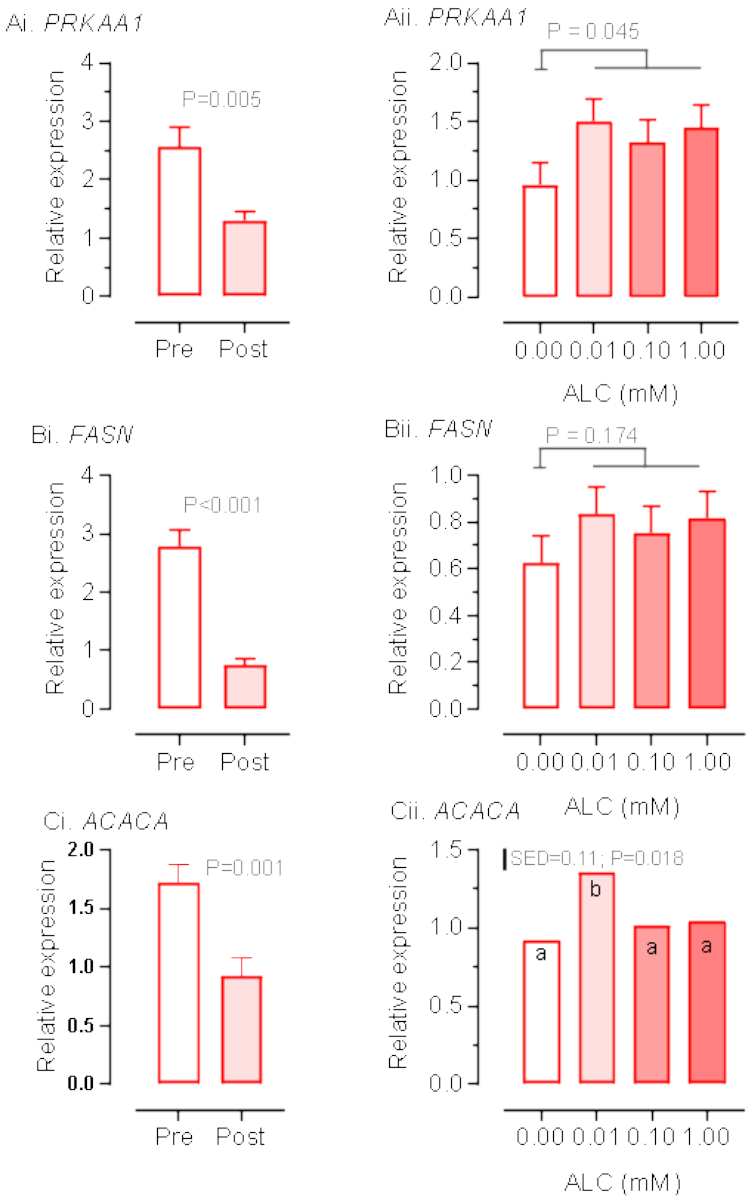


Figure 2. 11 Expression of genes involved in fatty acid synthesis; *PRKAA1* (Ai), *FASN* (Bi) and *ACACA* (Ci) were analysed in CC during the maturation. Following the maturation when the COCs were treated with 0, 0.01, 0.1 and 1 mM ALC; *PRKAA1* (Aii), *FASN* (Bii) and *ACACA* (SEM= 0.02) (Cii) expressions were evaluated between control and all three treatment groups. Pre: pre-maturation, Post: post-maturation.

No treatment effect was observed in term of *CPT1A* expression (2.13.Aii), only increase was observed in matured group compared to pre-matured (2.13.Ai). The comparison between pre- and matured group indicated that *SLC22A5* (2.13.Bi) abundance decreased however no treatment effect was observed (2.13.Bii).

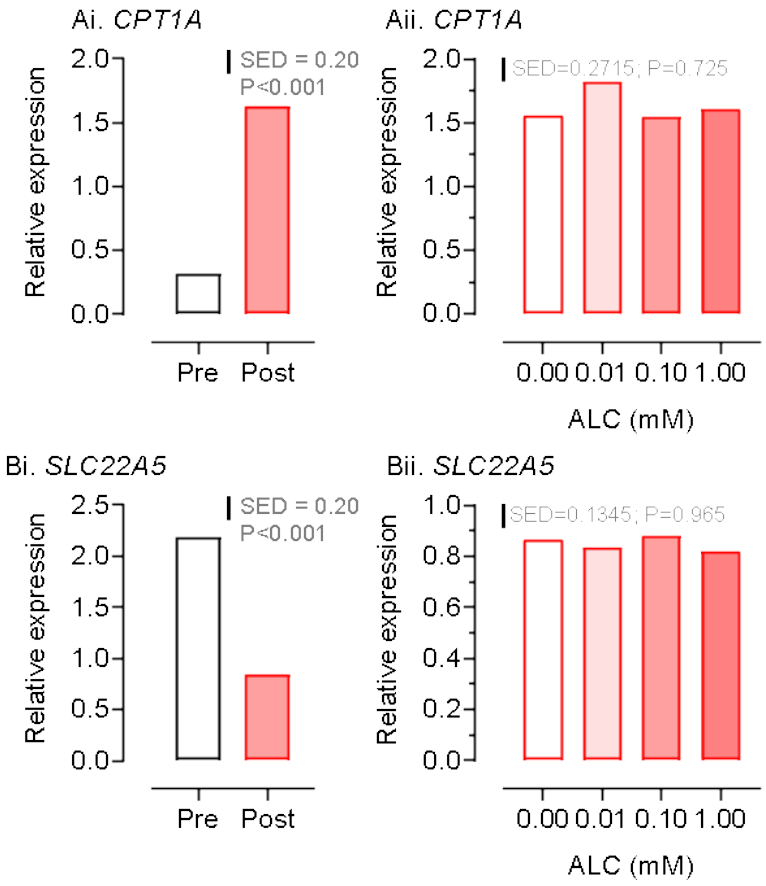


Figure 2. 12 Expression of genes involved in β-oxidation; *CPT1A* (Ai) and *SLC22A5* (Bi) in CC during the maturation. Following the maturation when the COCs were treated with 0, 0.01, 0.1 and 1 mM ALC; *CPT1A* (Aii) and *SLC22A5* (Bii) expressions were evaluated between control and all three treatment groups. Pre: pre-maturation, Post: post-maturation.

2.3.3.2 In Oocyte

There was not any significant difference between treatment groups in terms of *PRKAA1* expression following maturation (p= 0.207), however data showed that following maturation, *PRKAA1* expression was increased significantly when compared PM versus all matured groups (p=0.01) (A). *SOD2* abundance was increased in matured oocytes compare to pre-matured (p= 0.008) as well as *CAT* (p= 0.003), however the treatment effect could not have been found neither in *SOD2* expression (p= 0.163) nor in *CAT* expression (p= 0.178) (B, C). Data indicated that *GPX1* expression was increased during maturation, however that was not found as significant (p= 0.445). Additionally, there was not any treatment effect observed in *GPX1* expression (p= 0.381) (D). *FASN* expression was increased during maturation, but not significantly (p= 0.077). There was not any treatment effect on *FASN* (p= 0.775) and *PLIN3* (p= 0.475) as well as *PLIN3* abundance during maturation (p= 0.342) (E, F). Raw data scatter plots of gene expression in oocyte are placed in Appendix V.2.

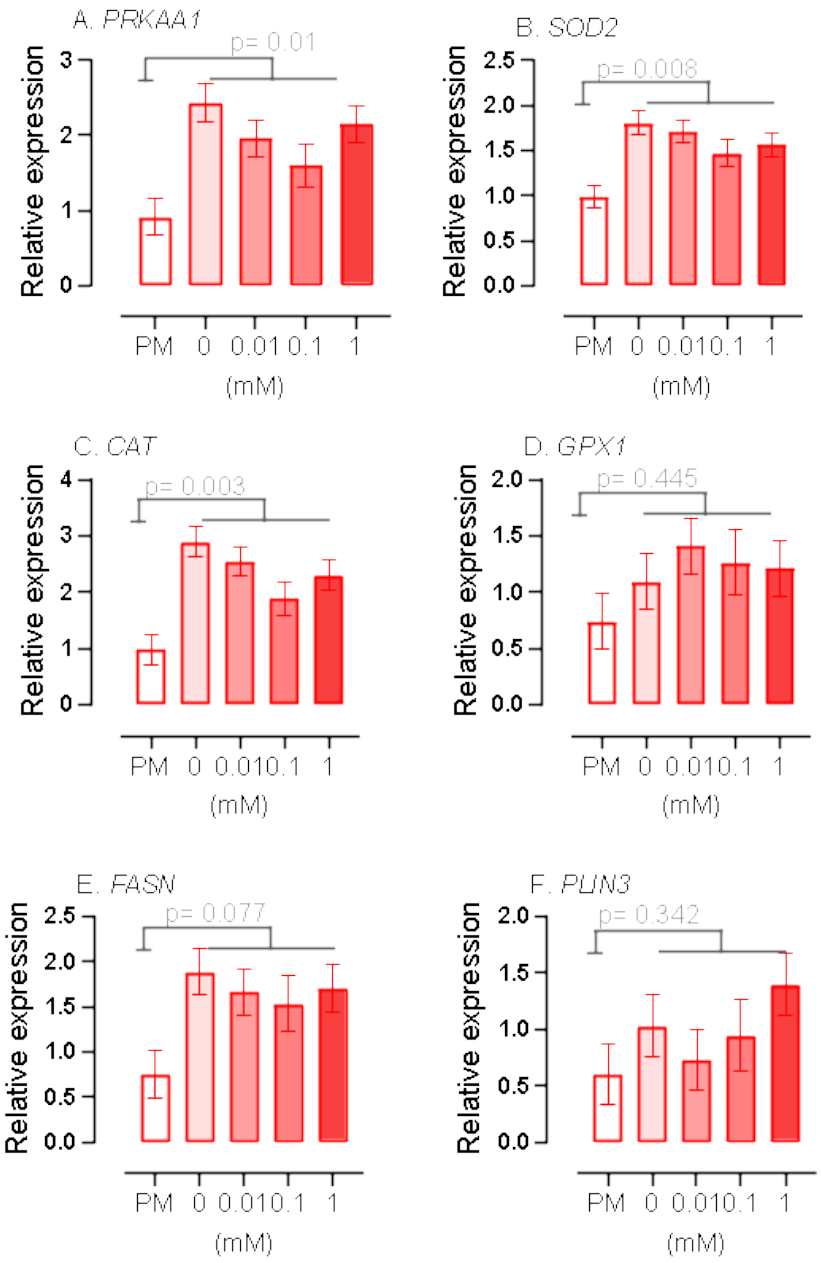


Figure 2. 13 *PRAA1* (A), *SOD2* (B), *CAT* (C), *GPX1* (D), *FASN* (E) and *PLIN3* (F) expression comparison between pre-matured (PM) and matured oocytes when the COCs were matured with 0, 0.01, 0.1 and 1 mM ALC.

2.3.3.3 In Blastocysts

Within the antioxidative genes, significant difference was observed only in *CAT* (p= 0.04). Data indicated that abundance of *SOD2* and *GPX1* was also increased treated with 0.1 ALC, however difference was not found as

significant. (A, C). *PLIN3* expression was increased presence of 0.1 ALC compared to the other groups (p= 0.04) (D). However, no difference was observed in terms of *FASN* expressions (p= 0.111) (E). Raw data scatter plots of gene expression in blastocysts are placed in Appendix V.3.

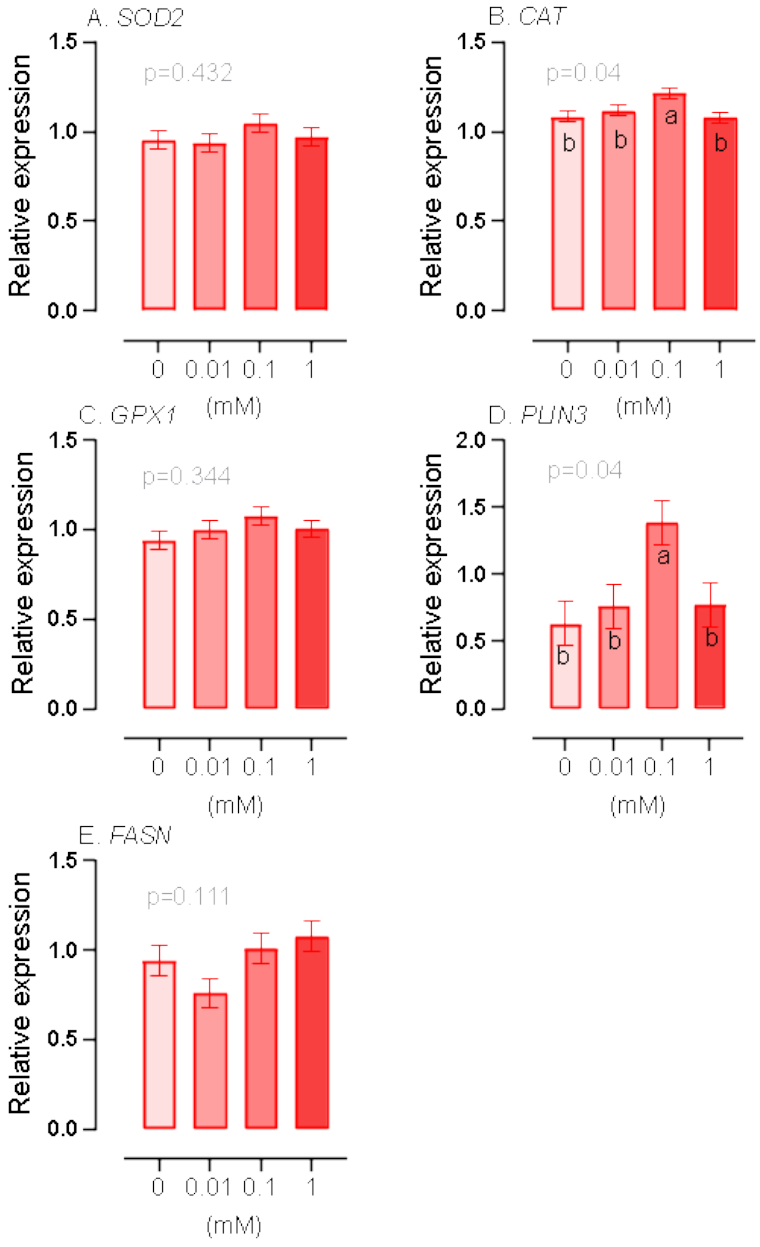


Figure 2. 14 *SOD2* (A), *CAT* (B), *GPX1* (C), *PLIN3* (D) and *FASN* (E) expression on D8 blastocysts following the zygotes were cultured with 0, 0.01, 0.1 and 1 mM ALC.

2.3.4 mtDNA copy number

During the maturation, mtDNA copy number was increased significantly when compared all matured groups to PM (p= 0.009) (Ai). Copy numbers were increased with ALC treatment, however the different was not significant (p= 0.084) (B). Log base 10 (Log_{10}) was used together with scatter plot to transform data to stabilize variation within the groups and account for curvature in a linear model.

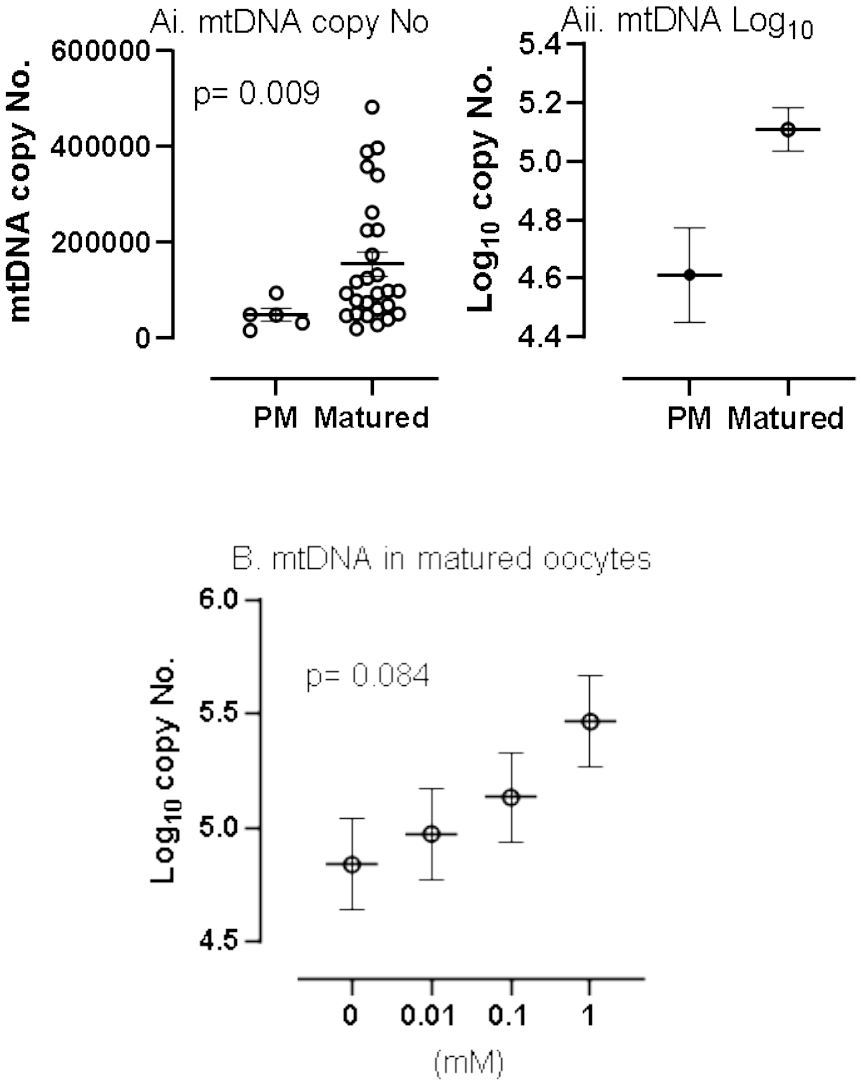


Figure 2. 15 Oocyte mtDNA copy number; mtDNA copy number (Ai) and log_{10} (Aii) of PM and matured oocytes and matured oocytes which were treated with 0, 0.01, 0.1 and 1 mM ALC (B).

2.4 Discussion

This series of experiments demonstrated the effect of supplementation of ALC during IVM and IVC on embryo development and molecular markers of function and competency. To the best of our knowledge, it is the first study to assess the cumulative effect of ALC during IVP (i.e., IVM and IVC) in cattle. No difference was observed in the proportion of cleaved zygotes following ALC treatment. Similarly, the proportion of D8 blastocysts of inseminated oocytes was unaltered, although there was evidence of decreasing cell number with increasing dose of ALC. Oocyte mtDNA copy number increased during IVM and also tended to increase with ALC dose. A cumulative effect of ALC was observed on blastocyst gene expression. Transcripts for *CAT* and *PLIN3* were both increased at the 0.1 mM dose of ALC, although the expression of other selected genes was unaltered.

There was evidence of active antioxidation pathways in cumulus cells during IVM. *CAT* expression was found in high abundance prior to IVM and significantly decreased during maturation. In ALC treated groups *CAT* expression was significantly lower than for the control group. In contrast, *SOD2* expression was increased in post-maturation cumulus cells exposed to ALC relative to the control group. In contrast to CC, transcript abundance for *PRKAA1*, *SOD2* and *CAT* in oocytes generally increased, however there was no apparent effect of ALC on these pathways.

Collectively, these findings indicated that ALC does not any affect the embryo development however it played a role on antioxidative pathway to protect the cells from oxidative stress.

2.4.1 Selected range of ALC doses and embryo development competence

Various carnitines have been investigated in numerous studies in different species. The doses of carnitines employed during IVM or IVP have varied between 1 to 10mM in a number of species including the camel (Fathi and El-Shahat, 2017), pig (Wu et al., 2011), mouse (Zare et al., 2015), sheep (Reader et al., 2015) and cattle (Xu et al., 2018a). Data indicate that blastocyst formation and total cell numbers are both improved with carnitines

supplementation, however, there is no information concerning pregnancy outcomes following embryo transfer. Carnitine levels in human follicular fluid range from 0.6 to 45.8 µmol/L (Montjean et al., 2012). Moreover, supplementation of 10 µM carnitine increased blastocysts formation, total cell numbers and decreased ROS formation in mouse (Truong and Gardner, 2017).

It has long been established that culturing gametes and fertilized oocytes with physiological levels of media constituents can enhance cytoplasmic and nuclear maturation (Krisher and Bavister, 1998) and support the developmental competence of offspring to adulthood (Eppig and O'Brien, 1998). The aim of the current study was to establish the optimal carnitine concentration within recognised physiological ranges. Therefore, carnitine supplementation was kept within the µM range as in FF. However, in current experiment, the data indicated that no different was observed in blastocysts formation and total cell number was reduced as ALC dose increased.

2.4.2 ALC as an antioxidant

Whilst L-carnitine is commonly used for preventing lipid accumulation and to assist in generating ATP by β-oxidation, ALC was found to be more associated with the antioxidative pathway in cells (REBOUCHE, 2004b). During oocyte maturation, lipid metabolism is essential for energy production to resume meiosis for preparing successful fertilization (Dunning and Robker, 2012a). Therefore, in oocytes maintaining the proper level of ROS is crucial during maturation to prevent oxidative damage (Dunning et al., 2010).

It is well documented that both carnitine forms offer a good defensive against ROS (Dokmeci, 2005). However Liu et al. (2004a) suggested that ALC is more effective in reducing ROS levels compared to LC. Additionally, in various studies gene expression data support the antioxidative effect of carnitines in different species. Carnitine supplementation in mM range during IVC increased *SOD2* expression in blastocysts in cattle (Ghanem et al., 2014) and sheep (Mishra et al., 2016b) and *SOD1* expression in pigs (Xu et al., 2020). In the current study, there was no significant difference between ALC and control groups in terms of *CAT*, *SOD2* and *GPX1* expression in oocytes. However, in CC, *CAT* and *SOD2* expression were both altered by ALC treatment. Whilst

SOD2 was upregulated by the treatment, *CAT* was found to be downregulated. It might be due to the specific effect of ALC on antioxidative pathways as it prevents hydroxyl free radicals and uric acid formation (Loots et al., 2004); suggesting that ALC works as an ROS scavenger. It may explain the lower expression of *CAT* in ALC treated groups. However, our data indicates that the cumulative effect of ALC in blastocysts did not alter *SOD2* expression and only *CAT* expression was upregulated with 0.1mM ALC group. In direct measurements, carnitine supplementation during IVM led to increased GSH level and reduced ROS levels in matured pig oocytes (You et al., 2012) as well as sheep oocytes (Mishra et al., 2016b). This indicates that ALC works as a non-enzymatic antioxidant.

2.4.3 ALC, mitochondrial function and fatty acid metabolism

Mitochondria are important not only for ATP production but also for ROS formation involved in cell signalling, regulating cell proliferation and apoptosis (Annesley and Fisher, 2019). Dysfunction of this organelle can affect fertility. Therefore, many studies have focused on improving mitochondrial function and protection of mitochondria from the oxidative damage in order to treat or prevent infertility. As with many of other antioxidants, it's been observed that ALC can improve mitochondrial function in brain tissues (Hota et al., 2012). As a direct effect on mitochondrial function, ALC provides acetyl groups and facilitates long chain fatty acid transport from the cytosol to mitochondria for β-oxidation (LIU et al., 2004b).

Lipid metabolism is partially regulated by AMPK (*PRKAA1*) activation. Increased AMP:ATP ratio triggers AMPK phosphorylation for ATP production, and activated AMPK regulates β-oxidation inhibiting fatty acid synthesis (Jeon, 2016). It was confirmed in our experiment that *PRKAA1* expression was upregulated with ALC treatment in CC. However, our results indicated that *CPT1A* expression was not altered and, moreover, *ACACA* expression was increased with 0.01mM ALC. This may have arisen because of the acetyl component of ALC, as increased concentrations of acetyl-Co and NADH inhibit the Kreps cycle (Akram, 2014). As a long-term consequence of ALC supplementation, *PLIN3* expression was changed with 0.1mM ALC in D8 blastocysts. PLIN3 is one of the lipid droplets (LDs) protein and it's degradation was observed during starvation (Kaushik and Cuervo, 2015). It

was observed that while LDs synthesis, *PLIN3* expression was increased (Nose et al., 2013). Additionally, mtDNA copy number tended to increase with ALC. This might be an indirect effect of ALC on mitochondria by protecting from oxidative stress (Musicco et al., 2009). Moreover, it is well known that ALC increases mitochondria biogenesis by stimulation PPAR-γ signalling (Pesce et al., 2012).

2.5 Conclusions

In conclusion, a modest cumulative effect of ALC within the μM range was observed but in the absence of differences in embryo development. However, a cumulative effect of ALC during IVP (IVM and IVC) was a decrease in total cell number in D8 blastocysts. Whilst antioxidative gene expressions in CC and blastocysts were altered by ALC treatment (i.e., *CAT* and *SOD2*), no treatment effect was observed in β-oxidative gene expression. For the further investigations, ROS levels need to be examined to identify the direct effect of ALC on antioxidative pathways. mtDNA copy number in oocytes was not altered by ALC treatment, however mitochondrial distribution and activity might have been altered and so this also needs to be examined in future studies. As *PLIN3* expression was increased in blastocysts as a result of the cumulative effects of ALC, future studies should assess the abundance lipid droplets to identify potential changes in fatty acid metabolism.

In general, results from the current series of experiments indicate that L-carnitine supplementation could potentially lead to improvements in embryo development by decreasing ROS formation and lipid accumulation. However, total cell numbers were decreased as ALC dose increased. As ALC treatment did not exhibit any major improvements in post-fertilisation developmental competence, the subsequent chapters of this thesis focused on the effects of L-carnitine during IVP.

Chapter 3 Effect of modified maturation medium on bovine *in vitro* embryo production (IVP)

3.1 Introduction

Embryo development following *in vitro* maturation of oocytes is lower than that for *in vivo* matured oocytes. One school of thought is that this relates to impaired cytoplasmic maturation during ovarian stimulation and/or *in vitro* maturation (Mao et al., 2014). Commonly, maturation media is supplemented with serum in order to improve oocyte nuclear and cytoplasmic maturation and subsequent embryo development (Thompson, 2000). Serum contains growth factors, chelate heavy metal cations, serves as an osmolyte and contains fatty acids (Gardner, 2011). As a result of serum supplementation during bovine oocyte *in vitro* maturation, the proportion of blastocysts is increased compared to non-supplemented PVA and PVP based formulations (Mingoti et al., 2011).

However, embryos never normally come into direct contact with serum *in vivo* (Moore et al., 2007). Additionally, serum supplementation causes variable results due to the undefined components it contains and has the additional risk of possible contamination with viruses and bacteria (Moore et al., 2007). Moreover, during embryo culture, serum increases fatty acid content of Day 7 blastocysts in cattle (Reis et al., 2003). It is known that serum supplementation induces accumulation of lipid droplets in IVP embryos and impairs cryotolerance of embryos (Abe et al., 2002). Bovine blastocysts cultured in the presence of serum also have more chromosomal abnormalities (Lonergan et al., 2004). Additionally, the presence of serum and somatic cells in culture media can lead to the Large Offspring Syndrome (LOS) (Young et al., 1998, Hill, 2014).

To avoid issues related with serum, defined culture media have been proposed. There are several commercial products that can be used as serum replacers; however, there are still differences between companies as well as between batches for such components, leading to variable results in terms of embryo development (Duque et al., 2003, Sagirkaya et al., 2007).

To standardize culture systems, undefined constituents are usually replaced by synthetic macromolecules, such as polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP). PVP is the more commonly used polymer in medicine because of its solubility and low cytotoxicity, being more supportive of embryo development than PVA (Wu et al., 2020, Lopes and Felisberti, 2003). In addition, synthetic macromolecules play a role in preventing the loss of oocytes and embryos due to adhesion to the surface of culture dishes or pipettes (Chung et al., 2007). The addition of PVP in bovine IVM media can lead to improvements in embryo development and blastocyst cell number (Chung et al., 2007).

Growth factors added to culture media have been in the focus of attention in recent years. Three important growth factors in serum are transforming growth factor- β (TGF-β), IGF-1 and FGF-2. Physiologically, TGF-β and FGF-2 are responsible for ovarian follicle development (Knight and Glister, 2003), and recently, FGF-2 added to IVM media was found to promote oocyte maturation (Du et al., 2021). It is known that IGF-1 also plays a role in promoting meiosis (Conti et al., 2002). Whilst the physiological level of IGF1 in follicular fluid has been reported to vary between 70-350 ng/ml, in maturation media as little as 10 ng/ml has been reported to produce an effect (Yuan et al., 2017).

Insulin concentrations in serum and follicular fluid range between 0.1 and 2 ng/ml depending on diet and energy status in cattle (Landau et al., 2000, Garnsworthy et al., 2009). Up to 5 ng/ml insulin has been shown to have a beneficial effect on bovine oocyte maturation (Stefanello et al., 2006). However, combination studies indicate that IGF1 activity may be reduced when insulin is present at higher concentrations (>10 ng/ml) (Entingh-Pearsall and Kahn, 2004). Apart from insulin, two other important components of biological fluids are transferrin and selenium which each promote oocyte maturation. Selenium levels have been reported to vary between 70-135 ng/ml in bovine follicular fluid (Xiong et al., 2018); and selenium has been shown to improve oocyte maturation at levels between 10 ng-2 µg/ml during IVM (Xiong et al., 2018, Lizarraga et al., 2020). Transferrin has been detected at between 0.9-2.8 mg/ml in human follicular fluid, and it is synthesized by granulosa cells and has a receptor on oocytes (Briggs et al., 1999). It has been reported that, in the presence of transferrin, cytoplasmic maturation is increased, and polyspermy reduced in pig (Jeong et al., 2008).

Serum-free IVM results in reduced cumulus mucification and intracellular cohesion, due to the absence of Inter-α-trypsin-Inhibitor protein (IAIP) (Chen et al., 1992). The IAIP complex is a ligand for hyaluronic acid (HA) to form link proteins which stabilises the extracellular matrix (ECM) (Salustri et al., 2019). Addition of IAIP (at 1 µg/ml) to oocyte maturation media has been shown to restore protein linking of HA in the ECM (Sun et al., 2002).

Additionally, to protect COCs from oxidative stress, different types of antioxidants have been studied. Glutathione (GSH) is the major non-enzymatic antioxidant but requires the presence of glutamate, glycine and cysteine for synthesis (Furnus et al., 2008). It cannot be taken up by cells as whole glutathione but must be broken down into amino acids. Therefore, IVM studies have focused on increasing the availability of GSH components. Cysteine is unstable in culture and is oxidized to cystine which makes it unavailable for the cellular uptake (Ishii and Bannai, 1985). Therefore, it needs to be mixed with active transport catalysts such as s β-mercaptoethanol (Curnow et al., 2008). As the other required substrate, addition of glycine in media also increases GSH production (Li et al., 2018). Whilst GSH acts a scavenger for hydrogen peroxidase (OOH), ascorbic acid (AA) has a role for detoxification of hydrogen peroxide (H_2O_2) (Arrigoni and De Tullio, 2002). The beneficial effect of AA in oocyte maturation and embryo development has been demonstrated in pigs (Tatemoto et al., 2001) and cattle (Córdova et al., 2010).

With the foregoing discussion in mind, this chapter focuses on developing a defined IVM system for the generation of developmentally competent bovine embryos. In the following series of experiments various factors, including growth factors, antioxidants and fatty acids, were added to basal maturation media containing PVP in a modular fashion in order to replace serum and albumin. In this chapter OPU was undertaken and oocytes were also derived from dominant follicle removal (DFR) for use in pilot studies to compare the effects of enriched media with albumin vs PVP so the effectiveness of fatty acid inclusion in media and uptake by COCs could be assessed. Fatty acid analysis of spent media was undertaken, and embryo development assessed to identify the effects of fatty acid inclusion on embryo development. Finally, RNA extracted from cumulus cells following IVM was sequenced to gain an insight into transcriptional changes related to the experimental treatments.

3.2 Pilot experiments to assess the inclusion of growth factors and fatty acids in PVP and BSA supplemented TCM199 IVM media

This section describes two experiments where oocytes were collected from cycles of DFR. DFR was undertaken seven times in total. The first three cycles generated oocytes for the first pilot experiment and the last four cycles generated oocytes the second experiment.

In the first experiment (Expt. 3.1A), BSA and PVP were assessed in terms of their effectiveness in facilitating fatty acid uptake by COCs in media that contained a cocktail of growth factors (GFs) and Inter-α-trypsin-Inhibitor (Table 3.1). Specifically, three treatment groups were examined (Table 3.2). The first two treatment groups incorporated the GF mixture with IAI alone (GF-IAI) or with added fatty acids (GF-IAI-FA). Both were incorporated into PVP-based TCM199 media (Table 3.1). The third treatment group involved adding the same fatty acid mixture to BSA-based TCM199 media containing GFs and IAI. The rationale for this experiment was that the inclusion of FAs to the mixture may enhance the effects of GFs and IAI in promoting oocyte maturation and subsequent embryo development. The BSA treatment was included to assess the effectiveness of FA uptake between BSA and PVP. This experiment was replicated three times.

The second experiment (Expt. 3.2) was based on emerging findings from Expt. 3.1A (discussed later). Only PVP-based media was used and, in the third treatment, GFs and IAI were excluded, and FAs added as the sole supplement to TCM199-based media (Table 3.2).

3.2.1 Material and Methods

Animal procedures were undertaken under the auspices of the Animals (Scientific Procedures) Act, 1986 (Project Licence: *PDBF3E539*). Associated protocols complied with the ARRIVE guidelines and were reviewed by the University of Nottingham Animal Welfare and Ethical Review Board (AWERB).

3.2.1.1 Experimental design and details of treatment groups

Based on published levels contained in serum, and previously assessed in bovine culture media, combinations of selected growth factors, antioxidants/antioxidant precursors and key fatty acids found in follicular fluid

were incorporated in IVM media (Table 3.1). Additionally, all treatment groups contain 2.5 mM L-carnitine.

Table 3. 1 Ingredients added to in vitro maturation media, for the details of media preparation see Appendix I.7.

Treatments	Ingredients	Amount	Reference
Growth Factor Cocktail (GFs)	IGF1	10 ng/ml	(Stefanello et al., 2006, Yuan et al., 2017)
	FGF2	0.5 ng/ml	(Zhang and Ealy, 2012)
	Insulin	2 ng/ml	(Laskowski et al., 2016)
	Selenium	10 ng/ml	(Xiong et al., 2018)
	Transferrin	10 µg/ml	(Briggs et al., 1999)
	Ascorbic Acid	40 µg/ml	(Tatemoto et al., 2001)
	L-cysteine	90 µg/ml	(Deleuze and Goudet, 2010)
	Beta-mercaptoethanol	0.1mM	
	Glycine	450 µg/ml	(Li et al., 2018)
IAI	Inter-α-Inhibitor	1 µg/ml	(Sun et al., 2002)
Fatty Acids mixture (FAs)	Palmitic	50 µM	(Van Hoeck et al., 2011)
	Stearic	25 µM	
	Oleic	75 µM	
	Linoleic	40 µM	(Marei et al., 2010a)
	A-Linolenic	40 µM	(Marei et al., 2009a)

Experiment 3.1A consisted of three treatments that assessed the effects adding FAs to PVP based TCM199 media containing IAI and was replicated three times (Table 3.2). Experiment 3.1B also consisted of three treatments but here TCM199 media was supplemented with PVP only. The addition of FAs to this media was compared in the presence or absence of GFs and IAI (Table 3.2).

Table 3. 2 Experimental Design for the two exploratory experiments

Experiment 3.1A	GF	IαI	FA
GF-IAI (PVP)	+	+	-
GF-IAI-FA (PVP)	+	+	+
GF-IAI-FA (BSA)	-	-	+

Experiment 3.1B	GF	IαI	FA
GF-IAI (PVP)	+	+	-
GF-IAI-FA (PVP)	+	+	+
FA (PVP)	-	-	+

3.2.1.2 Follicle Aspiration and IVP

Ten sexually mature 12-15-month-old Holstein-Friesian heifers were used in the experiment. Follicle aspiration was performed by trained veterinarian from a commercial company (Paragon Veterinary Group, UK). Aspiration of follicles >4 mm in diameter (dominant follicle removal; DFR) was performed in a procedure's room which was maintained at between 28 and 33 ºC. The DFR procedure was described in previously (Tutt et al., 2021). Briefly, U=under epidural anaesthesia, COCs were collected into 5ml of Tyrode's lactate (TL) based aspiration media using a Cook Medical vacuum pump with a 7.5 MHz ultrasound scanner (Exapad, IMV Imaging, Glasgow, UK) with the aspiration pressure set at 70 mmHg. An 18G needle and 1.4 m of 1.4 mm (I.D.) silicone tubing were used for aspiration and COC collection.

Aspirated samples were filtered with a heated (~37°C) filter and transferred to 100 mm petri dishes on a heated stage (~38°C) for COC recovery. They were graded 1-4 based on cumulus-cell compactness and homogeneity of oocyte ooplasm (Khurana and Niemann, 2000b). Grade 1-3 COCs were matured in HEPES buffered TCM199 media supplemented with 0.2 mM pyruvate, 50 µg/ml gentamicin, 5 µg/ml FSH, 0.5 µg/ml LH (Calier/ Pluset), 1 µg/ml E2 and/or 0.6% growth factors (depending on treatment; Table 3.2), insulin-selenium-transferrin and antioxidant mixture and/or 1 µg/ml inter alpha inhibitor (IAI) (again depending on treatment; Table 3.2) and 2.5mM L-

carnitine. A fatty acid mixture (palmitic 50 µM, stearic 25 µM, oleic 75 µM, α-linolenic 25 µM and linoleic 25 µM) was added to these media, and 0.4% BSA or 0.4% PVP used as macromolecules (again depending on treatment; Table 3.2). All media were prepared in duplicate; i.e., those containing COCs and those without. Maturation occurred in a screw top cryovial (Thermo Fisher Scientific Inc. Loughborough, UK) at atmospheric CO_2 at 38.5°C, for 23-24 h.

Following maturation, surplus cumulus cells (trimmed from COCs) were collected from 35mm dishes containing heated HEPES buffer TCM199 media on a heated stage (~38°C) by pipetting. These cells were destined for gene expression analyses. Media/spent media was collected for FA analyses. Remaining COCs were transferred to 50 µl fertilization drops (5 oocytes/drop) under oil for fertilization.

Fertilization media was composed of modified TL media with 0.6% (w/v) fatty acid free BSA, 1.5 µg/ml heparin, 0.2 mM sodium pyruvate, PHE (0.08 mM penicillamine, 0.04 mM hypo taurine, 10 mM epinephrine) and 50 µg/ml gentamicin (Tutt et al., 2021). Frozen semen from a single sire was thawed in a hot water bath and prepared by centrifugation through a 45%/90% BoviPure (Nidacon, Mölndal, Sweden) gradient. Following 10 min centrifugation at 900g, the pellet was centrifuged in 600 µl gradient medium to wash for another 5 min at 900g. 120 µl sperm pellet transferred into fresh eppendorf tubes and placed into a hot box during the period required to calculate sperm concentration as described in 2.2.3. 2 µl of the sperm-gradient mixture was added to each drop to obtain a final concentration of 70,000 sperm per drop. Fertilization dishes containing oocytes and sperm were incubated for 18-21 h in a humidified environment of 5% CO_2 in air at 38.5°C.

Zygote denudation was performed by vortexing for 1 min. Putative zygotes were then cultured in modified SOF (mSOF) based serial culture drops (10 zygotes per 10 µl drop) as described previously (Khan et al., 2016) in a humidified environment under oil at 5% CO_2, 5% O_2 and 38.5°C. Cleavage was assessed on Day 2 (30 h following transfer) and embryos classified according to cell number (i.e., 1, 2-3, and >4 cells). Embryos were transferred approximately 42 h later (Day 4) to 10 µl drops of the second culture media. Progression to the morula stage was assessed 48 h later (Day 6), and embryos this time transferred to 20 µl drops of the third culture media. Embryos were assessed again 48 h later (Day 8) for stage and quality in

accordance with the International Embryo Transfer Society (IETS) guidelines for bovine embryo assessment (Baillargeon et al., 2001).

3.2.1.3 Fatty Acid Analysis of Maturation Media

Following the maturation, spent media and COCs were transferred to 35 mm dish to evaluate COCs. After COCs were transferred to fertilisation media leftover maturation media was centrifuged at 12,100 g for 1 min to make sure CCs were collected at the bottom of eppendorf tube. Spent media was collected carefully without disturbing the pellet, then they were frozen. To avoid effect of incubation process, same amount media was incubated without COCs, and these were frozen directly following the incubation. Frozen media samples (in Eppendorf tubes) were thawed at room temperature, then centrifuged at 12,100 g for 5 sec to ensure that no thawed media was trapped in the lid. Meanwhile, 6 µl of 100 µg/ml C15:0 in ethanol (internal standard (IS)) was added to each Pyrex tube. From each sample, 150 µl medium was transferred to Pyrex tubes containing the IS. After adding 100 µl LC-MS grade water, each sample was mixed for 30 s. Following addition of 1 ml of chloroform: methanol (2:1 v/v) using a glass syringe, samples were vortexed for 2 min and spun at 3000 g for 10 min at room temperature. The bottom layer was then collected and transferred to high performance liquid chromatography (HPLC) crimp top vials before being dried under nitrogen. 300 µl of 0.5 ml methanolic hydrochloric acid (HCl) was added using a positive dispenser and purged with nitrogen, then the tubes sealed for vortexing for 1 min. Following 2h incubation at 70°C, samples were left for 10 min to cool. After adding 1ml of hexane, each vial was mixed for 1 min before letting them sit at room temperature until the two layers formed, after nearly 1h. The upper phase was then transferred to fresh HPLC screw top vials containing 10 µl of methylated C19 standard at 100 µg/ml and dried under nitrogen. Resuspended dried lipids with 100 µl hexane were transferred to 150 µl inserts which was placed in HPLC vials. For the fatty acid methyl ester (FAME) standard, 10 µl of methylated C19 standard (50 µg/ml) was added into HPLC screw top vials. Following evaporation, 100 µl diluted FAME mix was added from each concentration. Following preparation, all samples were run in gas chromatography-mass spectrometry (GC-MS) (Appendix III).

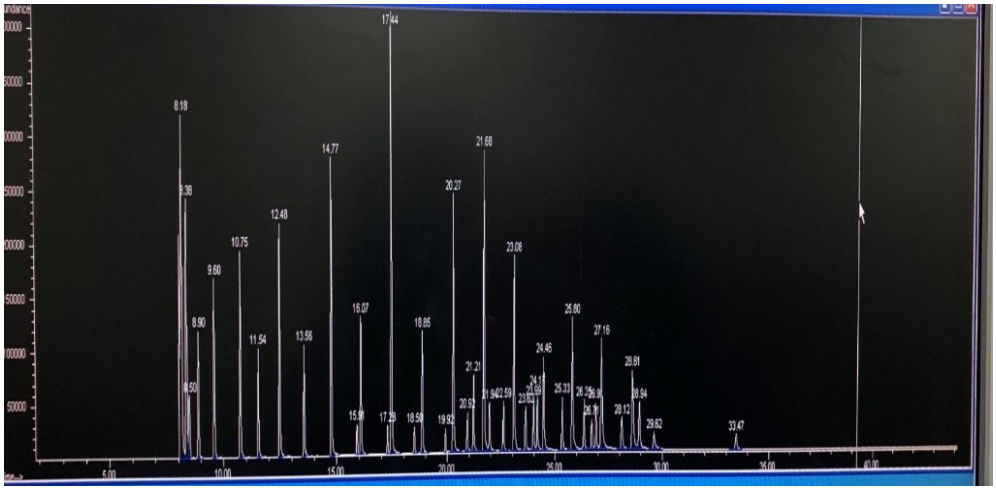


Figure 3. 1 Representative output of fatty acid analysis indicated the clear and sharp peaks. Each peaks indicates the presence of specific ions which represent fatty acids.

3.2.1.4 Statistical Analysis

Embryo production data were analysed using Generalised Linear Models within GenStat (GenStat 19th Edition, 2018, VSN International Ltd, Hemel Hempstead, UK) assuming binomial errors and with logit link functions. Terms fitted to these models were DFR-cycle and IVM-treatment. Data are presented as means ± Standard Error of Means (SEM). Total blastocyst-cell numbers were analysed using similar models but assuming position distribution. Finally, FA concentrations in spent media were analysed using general linear regression with the same terms added to this model. Data are also presented as means ± Standard Error of Means (SEM).

3.2.2 Results

3.2.2.1 Experiment 1A

3.2.2.1.1 Embryo Development

No significant treatment difference was observed in the proportion of putative zygotes that had cleaved (Fig. 3.2A). However, FA supplementation to PVP media in the presence of GF and IAI increased (P = 0.038) the proportion of embryos with more than 4 cells on Day2 (Fig 3.2B). However, the same was not true in the presence of BSA. There was an indication that the proportion Day 8 blastocysts of inseminated (P = 0.161) and cleaved (P = 0.122) was increased in the presence of FA (particularly in the presence of PVP). The

proportion advanced (IETS Stage 7-9) of Day 8 blastocysts was unaffected by treatment.

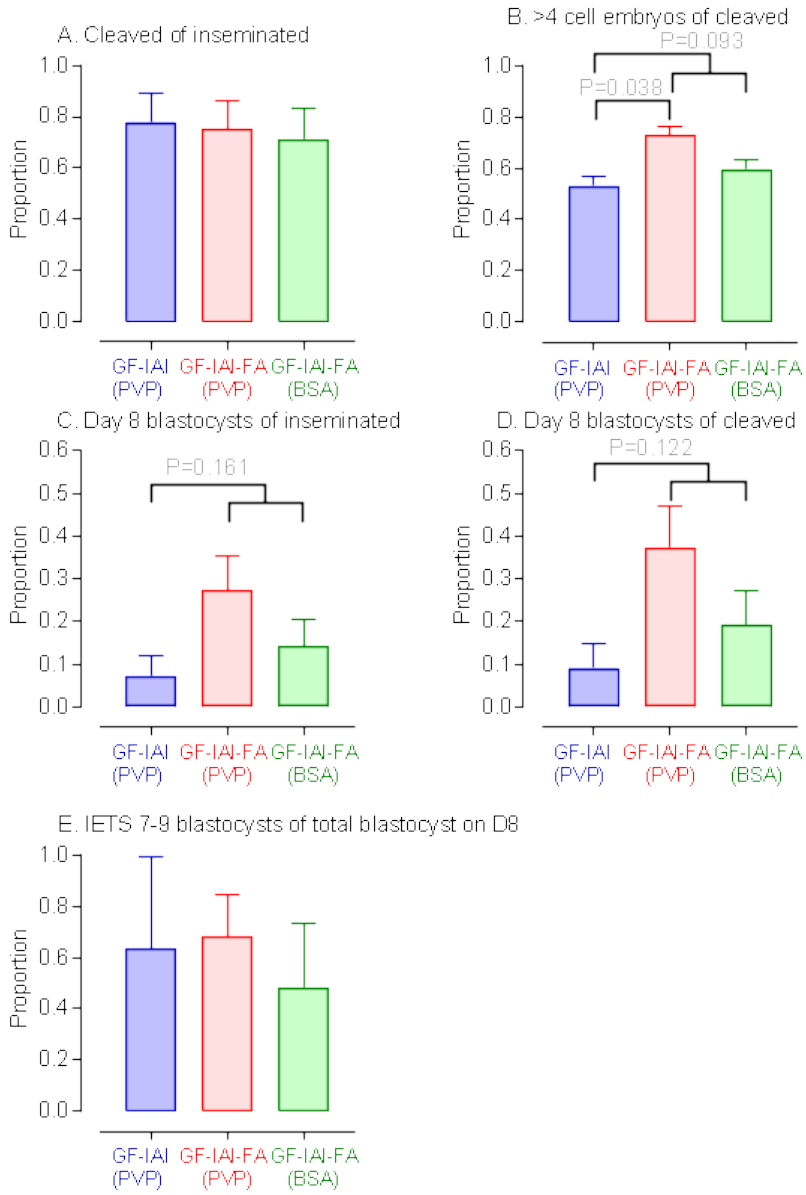


Figure 3. 2 Embryo development assessed morphologically on Day 2 and Day 8 based on the IETS scale (Robertson and Nelson, 2010) . Treatment groups are GF-IAI; growth factor cocktail and IAI, GF-IAI-FA (PVP); growth factor cocktail, IAI and fatty acid mixture in the presence of PVP, GF-IAI-FA (BSA); growth factor cocktail, IAI and fatty acid mixture in the presence of BSA. No difference was observed on cleavage (A), fatty acids increased the proportion of >4-cells embryos in the presence of PVP (B), no effect of fatty acids on D8 embryos (C and D), as well as proportion of transferable embryos (E).

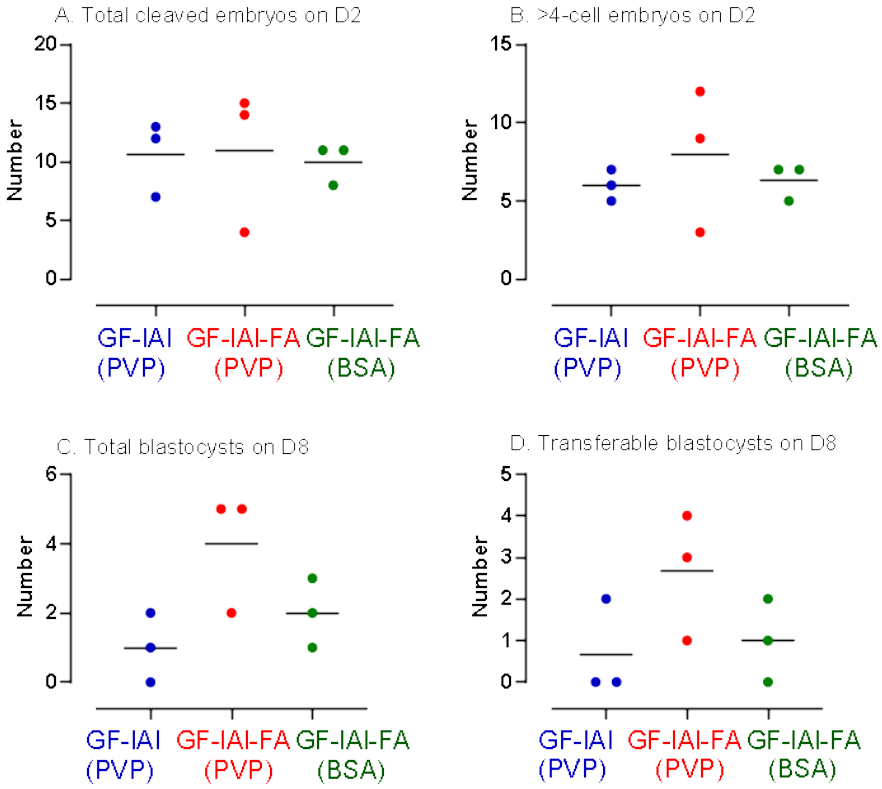


Figure 3.3 Scatter plots data of total cleaved embryos (A), and more advanced embryos on D2 (B), total blastocysts (C), and transferable blastocysts on D8 (D) indicate distribution of blastocysts number with median from each replicate.

3.2.2.1.2 Fatty Acid uptake in maturation media

Incorporation of FAs into maturation media was greater ($P<0.001$) for BSA than PVP supplemented media (Fig. 3.4A). Indeed, that measured for BSA supplemented media was close to the predicted amount added for each FA, whereas it was orders of magnitude lower for PVP indicating that little FA was incorporated into PVP supplemented media. In contrast, the proportion uptake of FAs was similar for both treatments (Fig. 3.4B). There was an indication that, proportionally, uptake of palmitic (C16:0) and stearic (C18:0) acids was greater for PVP, whilst uptake of linoleic acid (C18:2) was greater ($P<0.05$) for BSA, supplemented media.

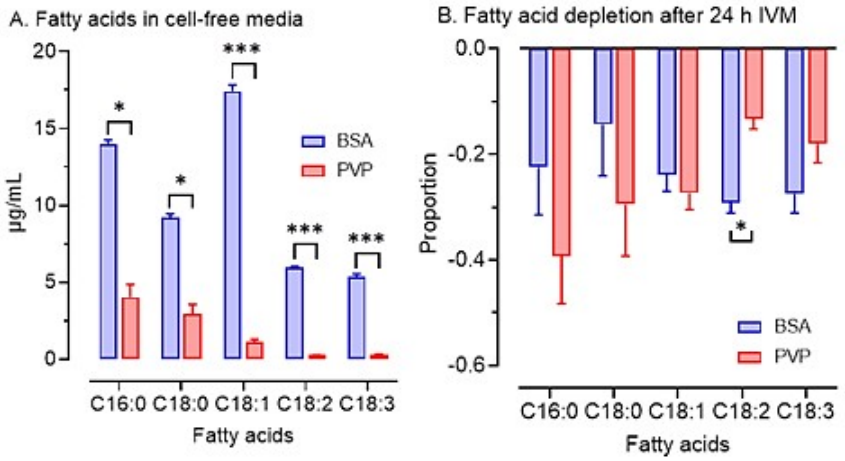


Figure 3. 4 Fatty acid analysis data were obtained by GC-MS. FAs were measured in stock and spent media then depletion was calculated.

3.2.2.1.3 Total Cell number

There was no effect of treatment on total cell number of Day 8 blastocysts (Figure 3.5).

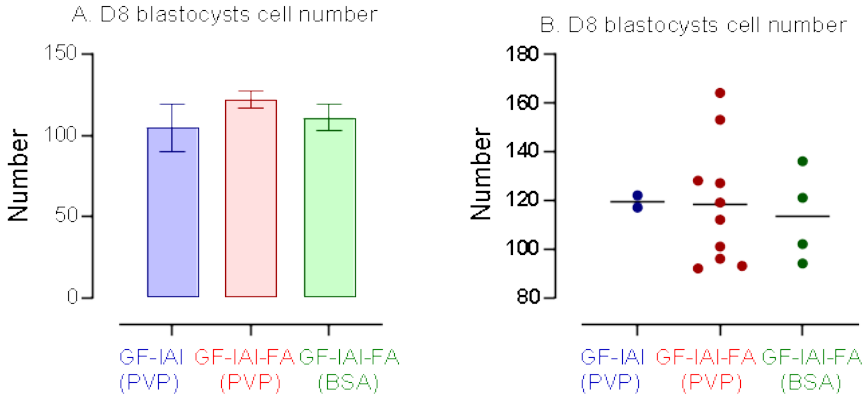


Figure 3. 5 Total cell numbers on D8 blastocysts (A) were observed by staining with DAPI. Treatment groups are GF-IAI; growth factor cocktail and IAI, GF-IAI-FA (PVP); growth factor cocktail, IAI and fatty acid mixture in the presence of PVP, GF-IAI-FA (BSA); growth factor cocktail, IAI and fatty acid mixture in the presence of BSA. Scatter plots data (B) indicates distribution of blastocysts cell number with median from each replicate.

3.2.2.2 Experiment 1B

3.2.2.2.1 Embryo Development

There was no effect of treatment on the proportion cleaved of inseminated by Day 2 (Fig. 3.6A). Similarly, the proportion of advanced (>4 cells) embryos on Day 2 was unaltered by treatment (Fig. 3.6B). However, there was an indication that the proportion Day 8 blastocysts of inseminated (P = 0.122; Fig. 3.6C) and cleaved (P = 0.139; Fig. 3.6D) was increased following the inclusion of FAs. Nevertheless, the proportion advanced (IETS Stages 7-9) and therefore transferrable Day 8 blastocysts was unaltered by treatment.

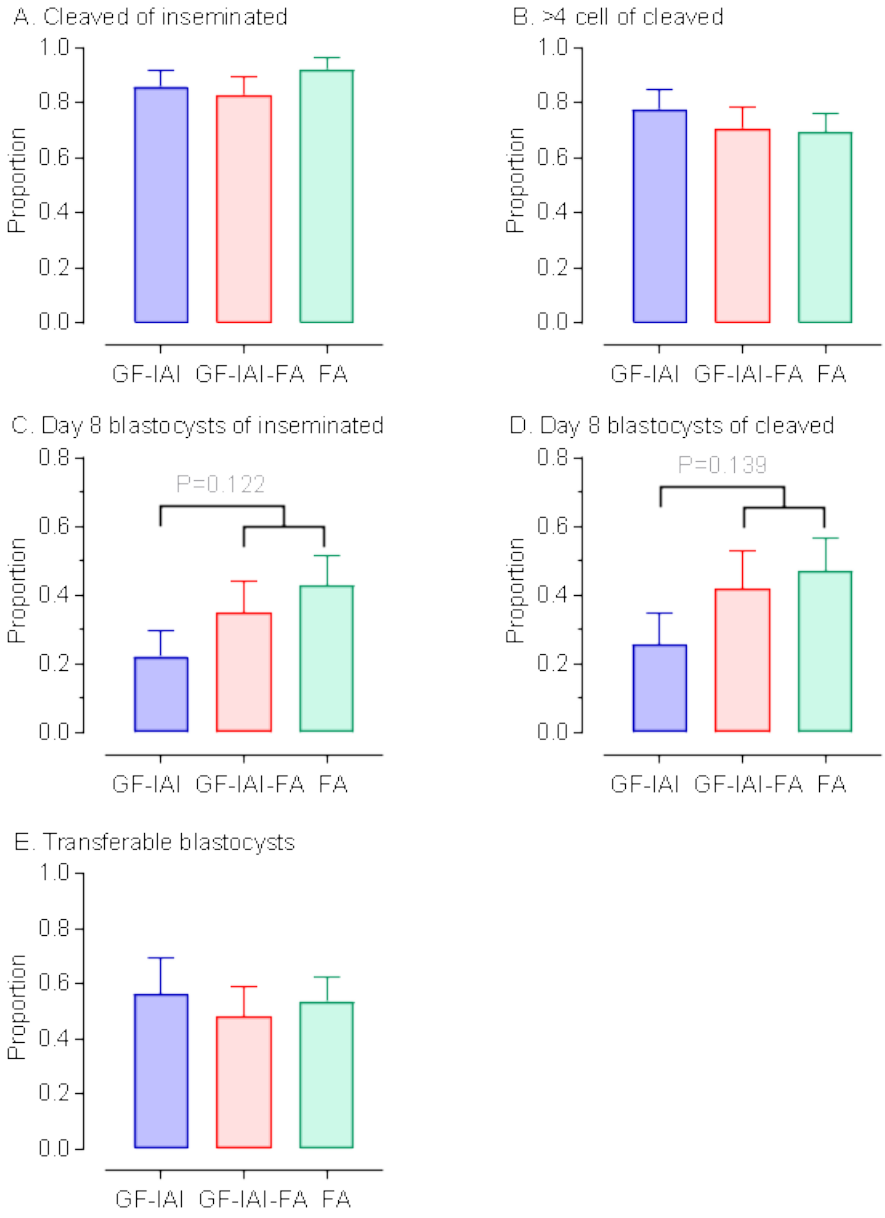


Figure 3. 6 Embryo development assessed morphologically on Day 2 and Day 8 based on the IETS scale. Treatment groups are GF-IAI; growth factor cocktail and IAI, GF-IAI-FA (PVP); growth factor cocktail, IAI and fatty acid mixture in the presence of PVP, GF-IAI-FA (BSA); growth factor cocktail, IAI and fatty acid mixture in the presence of BSA. No difference was observed neither on cleavage (A) nor proportion of advanced embryos (B) on D2. Fatty acids have no effect on the proportion of blastocysts (C and D). And no treatment effect was observed on proportion of transferable blastocysts (E).

3.2.2.2.2 Fatty acid uptake during the IVM

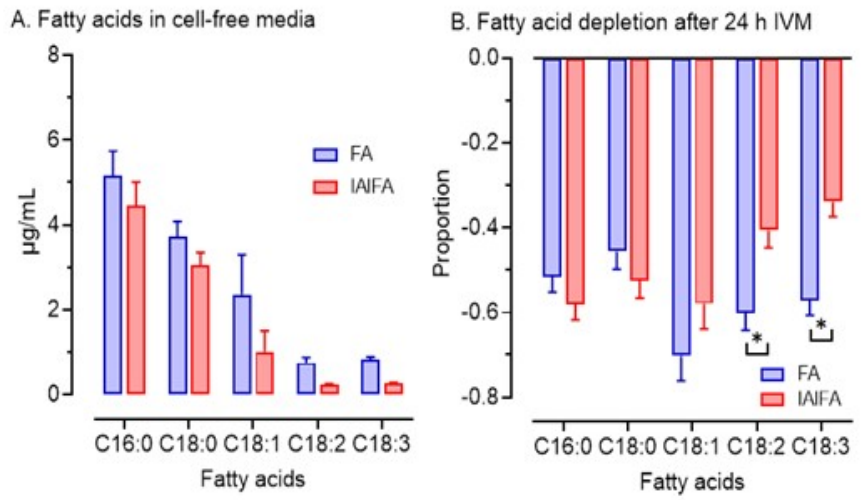


Figure 3. 7 Fatty acid analysis by GC-MS. Fatty acids were measured in stock and spent media (A), then depletion was calculated (B). FA = Fatty acid treatment group, IAIFA = GF-IAI-FA treatment group.

Incorporation of FAs into PVP supplemented maturation media was compared for both the GF-IAI-FA and FA treatment groups and found to be similar (Fig. 3.7A). However, there was evidence ($P<0.05$) that uptake of linoleic (C18:2n-6) and alpha-linolenic (C18:3n-3) acids were greater for the FA than the GF-IAI-FA treatment group (Fig. 3.7B).

3.2.2.2.3 Total Cell Number

There was no effect of treatment on total cell number of Day 8 blastocysts (Fig. 3.8).

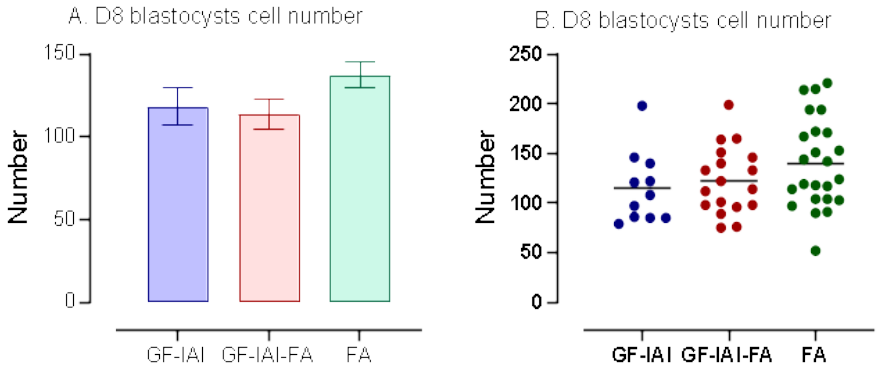


Figure 3. 8 Total cell number of Day 8 blastocysts (A) observed by staining. Scatter plots data (B) indicates distribution of blastocysts number with median from each replicate.

3.2.2.3 DFR embryo development data from total 7 replicates

As two treatment groups (i.e., GF-IAI and GF-IAI-FA) were common across both experiments, and as these two experiments involved seven sequential cycles of DFR using the same donors, so it was possible to combine these two sets of data into a single analysis.

Although the inclusion of FAs to PVP media containing GFs and IAI did not affect the proportions cleaved of inseminated (Fig. 3.9A) and >4cells of cleaved (Fig 3.9B), there were strong indications that it did improve Day 8 blastocysts of inseminated (P = 0.097; Fig 3.9C) and of cleaved (P = 0.056; Fig. 3.9D). However, the proportion advanced (IETS Stages 7-9), and hence potentially transferrable, blastocysts of total Day 8 blastocysts was unaffected by FA inclusion in IVM media (Fig. 3.9E).

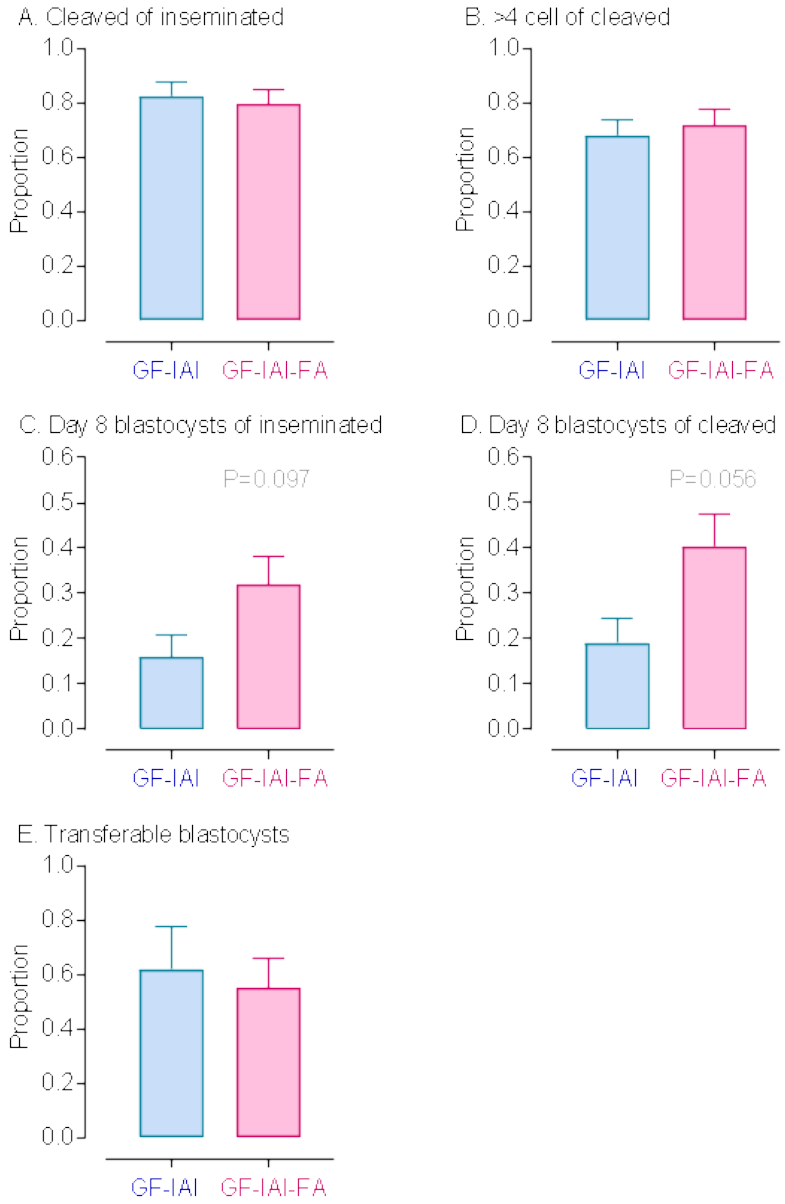


Figure 3. 9 Embryo development assessed morphologically on Day 2 and Day 8 based on the IETS scale. Data derived from seven replicated cycles of DFR pooled from Experiments 1A and 1B. Fatty acids have no effect on cleavage (A) or proportion of advance embryos on D2 (B). No treatment effect was observed on blastocysts of inseminated (C), however blastocysts of cleaved (D) indicated that FA supplementation increased embryo development by supporting cleaved embryos to reach blastocysts on D8. However, no effect of FA was observed on proportion of transferable embryos (E).

3.3 Experiment 2: Effect of growth factors and fatty acids during IVM on IVP embryo development

Based on the outcome of the two pilot experiments, IAI was excluded from IVM media in the current experiment, which was a factorial arrangement investigating the effects of presence or absence of the GF cocktail and presence or absence of FAs in PVP-based IVM media (Table 3.3). A fifth treatment group included serum in IVM media which served as a positive control.

3.3.1 Materials and Method

3.3.1.1 Experimental Design

The current experiment extended beyond that of Experiments 1A and B and used the same batch of ten donor heifers with oocytes recovered from five sequential cycles of OPU. At the end of each cycle, donors were rotated between treatments; with oocytes donated to each treatment from two donors during each cycle (Table 3.3).

Table 3. 3 The factorial arrangement of Experiment 2, indicating the number of oocyte donors for each of the five treatment groups and for each of the five cycles of OPU. Note, donors were rotated between treatments at the end of each cycle.

Growth factors (GF)	**-**	**-**	**+**	**+**	**Serum**
Fatty acids (FA)	**-**	**+**	**-**	**+**	
Cycle 1	2	2	2	2	2
Cycle 2	2	2	2	2	2
Cycle 3	2	2	2	2	2
Cycle 4	2	2	2	2	2
Cycle 5	2	2	2	2	2

3.3.1.2 Follicle Aspiration and IVP

Stimulated cycles of transvaginal OPU (ovum pick-up) were performed as described previously (Tutt et al., 2021), Briefly, these cycles commenced with an intravaginal progesterone (P4)-releasing device (CIDR Vaginal Delivery System, 1.38g, Zoetis UK Ltd, Leatherhead, UK) inserted and a GnRH analogue (Acegon, Zoetis UK Ltd, Leatherhead, UK) administered (2 ml/per animal) intramuscularly. Six and seven days later prostaglandin ($PGF_{2\alpha}$) analogue Cloprostenol (Estrumate, MSD Animal Health, UK) was administered (2 ml/per animal; i.m), and the CIDR was changed and DFR was performed at that time. Two days later, follicle stimulating hormone (FSH; Folltropin, 70IU dose per injection, Vetoquinol UK Ltd, Towcester, UK) was given at intervals of 12 hours for three days. The first stimulated cycle of OPU was performed approximately 38–42 h following the final FSH injection.

The OPU and IVP procedures, and IVP endpoints were as described for Experiments 1A and B. However, in the current experiment, IVM was performed in 2 ml of media instead of 20 µl media/oocyte as in the two previous experiments.

3.3.1.3 RNASeq: Library preparation, sequencing an informatics

Following the 24 h maturation, cumulus cells were trimmed by mouth pipetting and washed with 0.1%PVP-PBS solution. Centrifugation was at 13,793 g for 10 minutes at 4 °C in a cold room. Snap freezing of pellets was performed, and they were stored at -80 °C.

On the day of assay, PicoPure RNA isolation kit (Cat no: KIT0204 Thermo Fisher,UK) was used for RNA isolation. The method followed manufacturer's instructions. 100µl extraction buffer was added to each sample tube and pelleted cells were re-suspended by gentle pipetting, then incubated at 42°C for 30 min. Meanwhile, 250 µl of conditioning buffer was added to each column and left at room temperature for 5 min then centrifuged at 16,000 g for 1 minute to complete the pre-condition process for columns. Incubated cells were then centrifuged at 3,000 g for 2 min. Liquid was transferred to fresh tubes without touching the pellet as the pellet contains RNA-free cells and

debris and 100 µl of 70% EtOH was added to each and gently mixed by pipette. Thereafter, the cell extracts were transferred to columns and centrifuged at 100 g for 2 min to bind RNA to the column. After binding, samples were quickly spun and 100 µl of wash buffer 1 was added, then spun down at 8,000 g for 1 min. At this point, each column was treated with DNAse to purify RNA from any contamination. For each sample 10 µl DNAse was diluted with 70 µl RDD buffer and 80 µl mixture was added to each column, then incubated 15 min. Following incubation, each sample was washed twice with 100 µl wash 2 buffer and, after each wash, centrifuged for one minute at 8,000 g and 16,000 g respectively. Columns were transferred to 0.5 ml microcentrifuge tubes which was provided by the kit. 15 µl elution buffer was then added to each column matrix directly and incubated for 1 min at room temperature. Samples were centrifuged first at 1,000 g for 1 min to distribute elution buffer, then centrifuged again at 16000 g for a min to elute.

Library preparation, sequencing and bioinformatics were undertaken at QMC. Accordingly, indexed libraries were generated from total RNA using the QuantSeq 3' mRNA-Seq library preparation kit for Illumina (FWD) (Lexogen; 015.96), thereafter sequenced on the Illumina NextSeq 500, using a NextSeq 500 High Output v2.5 75 cycle kit (Illumina; 20024906), to generate approximately 5 million 75bp single-end reads per sample. To pre-analyse the results, BlueBee platform was used. Thereafter, mapping of reads were recorded and counted by using FeatureCounts 1.6.0 version. Principal component analysis (Alessandra Corallo Nicacio2 et al.) and DESeq analysis were performed. As a gene ontology analyser GOSeq was performed for GO Enrichment analysis.

3.3.1.4 ATP assay

On Day 8, after embryo development was recorded, the zona pellucida (ZP) was removed by incubating in 50 µl pronase until zona disappeared. Blastocysts were then washed and stored in 25 µl 0.1%PVP-PBS individually. Based on the previous tests for optimization, eight different spots (5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 and 0 pmol/blastocyst) were determined as standards. On the day of assay, according to manufacturer instructions, 50 µl somatic cell releasing agent. After vortexing, the samples were incubated on ice for 5 min. One volume of assay mix stock was diluted with 24 volumes of assay mix dilution buffer. From that mixture 100 µl was added into each tube.

After vortexing, each plate was loaded and before running, incubated at room temperature in the dark for 5 min. Plate was run by MicroLumat Plus LB 96 V (Berthold Technologies, Germany) and the data obtained on WinGlow software.

3.3.1.5 Statistical analyses

Embryo production data were analysed using Generalised Linear Mixed Models within GenStat (GenStat 19th Edition, 2018, VSN International Ltd, Hemel Hempstead, UK) assuming binomial errors and with logit link functions. Terms fitted to the fixed model were OPU-cycle, growth factors, fatty acids and interactions between these terms. Serum was separately contrasted to serum-free (PVP) in these analyses. Donor was included as a random effect in these models. Data are presented as means ± Standard Error of Means (SEM). ATP generation in Day 8 blastocysts were analysed using similar Mixed Linear Models but assumed normal errors. Data are also presented as means ± Standard Error of Means (SEM).

3.3.2 Results

3.3.2.1 Embryo Development

There was no apparent effect of treatment on the proportion of oocytes that cleaved following insemination, nor on embryo stage at Day 2 following insemination (Table 3.4). Similarly, the proportion of embryos that developed to Day 6 and embryo stage at Day 6 was unaffected by IVM media composition.

Table 3. 4 Embryo development at Day 2 and Day 6.

Growth Factors (GF)	-	-	+	+	**Serum (S)**	Significance (P)			
Fatty Acids (FA)	-	+	-	+		S#	GF	FA	GFxFA
No. replicated cycles	5	5	5	5	5				
Day 2 cleavage-stage embryos									
Inseminated per cycle, n	23.5±3.34	19.4±3.45	21.7±3.38	17.5±3.28	23.0±3.19	-	-	-	-
Cleaved of inseminated	0.895±0.0337	0.880±0.0417	0.950±0.0251	0.875±0.044	0.861±0.0364	-	-	-	-
Proportion 2 cell	0.032±0.0156	0.034±0.0217	0.051±0.0213	0.066±0.0301	0.042±0.0172	-	-	-	-
Proportion 3-4 cell	0.195±0.0388	0.180±0.0492	0.154±0.0381	0.116±0.0419	0.140±0.0334	-	-	-	-
Proportion 5-6 cell	0.284±0.0341	0.269±0.0412	0.190±0.0309	0.262±0.0426	0.226±0.0316	-	-	-	-
Proportion >6 cell	0.489±0.0602	0.518±0.0767	0.613±0.0625	0.557±0.0799	0.591±0.0590	-	-	-	-
Day 6 embryos									
Proportion on inseminated	0.652±0.0588	0.737±0.0670	0.682±0.0622	0.613±0.0769	0.706±0.0542	-	-	-	-
Proportion of cleaved	0.729±0.0582	0.839±0.0615	0.717±0.0629	0.697±0.0791	0.819±0.0498	-	-	-	-
>12 cells of Day 6	0.241±0.0570	0.217±0.0679	0.211±0.0603	0.292±0.0872	0.209±0.0806	-	-	-	-
Morula of Day 6	0.524±0.0595	0.595±0.0708	0.524±0.0645	0.532±0.0852	0.548±0.0536	-	-	-	-
Blastocysts of Day 6	0.234±0.0555	0.187±0.0631	0.267±0.0612	0.168±0.0778	0.245±0.0557	-	-	-	-

S#: Contrast – Serum vs PVP based media treatment. No significant difference observed up to Day 6 after insemination.

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In contrast to developmental results up to Day 6 following insemination, there were significant treatment effects on the proportion and developmental stage of blastocysts on Day 8 (Fig. 3.10). In general, embryo development was greater for oocytes matured in the presence than the absence of serum during IVM. Furthermore, the addition of GFs to PVP media reduced ($P<0.05$) the proportion of Day 8 blastocysts of inseminated (Fig. 3.10A) and cleaved (Fig. 3.10B), as well as the proportion of Day 6 embryos that developed to Day 8 blastocysts (Fig. 3.10C). There were no interactions between GFs and FAs. Although, as a main effect, the inclusion of FAs was not significant in itself, the addition of FAs to GF media appeared to be additive; that is, the lowest yields of blastocysts were observed when FAs were combined with GFs.

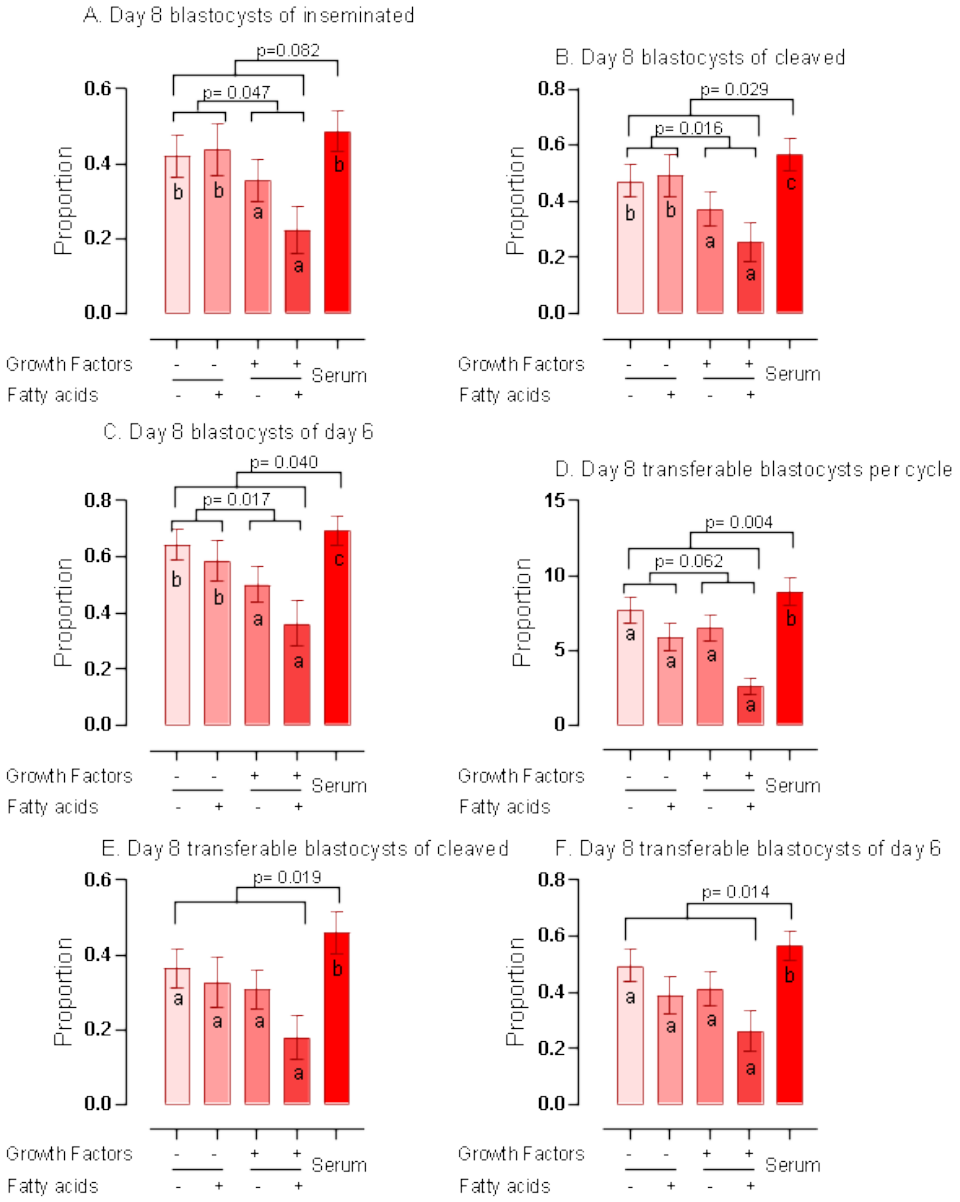


Figure 3. 10 Day 8 blastocyst development following oocyte maturation in PVP based media supplemented with combinations of growth factors and fatty acids; '+' indicates the supplement exists in the media and '-' is for absence of supplements. Maturation in the presence of serum served as a positive control. Letters indicate statistically similarities and differences between treatment groups. Transferrable blastocysts refers to IETS Stages 7, 8 and 9. Whilst effect of GF was identified D8 blastocysts of inseminated (A), of cleaved (B), and of D6 blastocysts (C), however no differences were identified D8 transferable blastocysts per cycle (D), of cleaved (E), and of D6 blastocysts (F) due to GF supplementation.

3.3.2.2 ATP analysis

ATP analyses were restricted to IETS stage 7, 8 and 9 Day 8 whole blastocysts that had not undergone immuno-dissection. Immuno-dissected blastocysts (i.e., separate ICM and TE lineages) were subjected to separate analyses which are not reported in this thesis as they were used for different experiment which is not related with this thesis. This meant that not all treatment combinations were represented at each of the different IETS stages (Fig. 3.11). There was no effect of IVM treatment on ATP production for Stage 7 and Stage 9 Day 8 blastocysts (Fig. 3.10). In contrast, the addition of either GFs or Serum increased (P=0.026) ATP production in Stage 8 blastocysts.

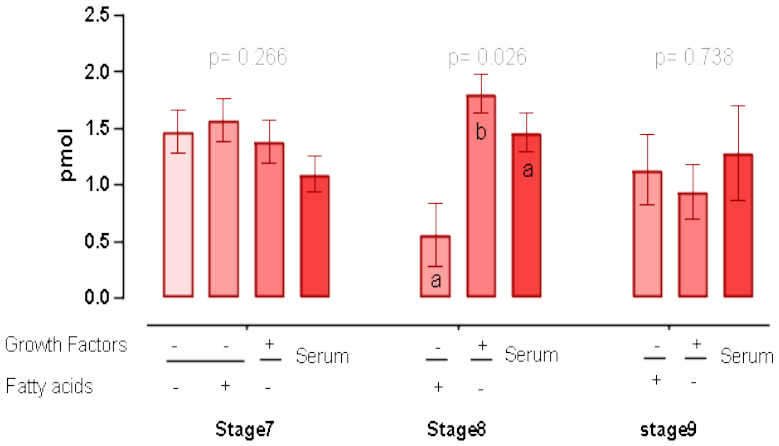


Figure 3. 11 ATP production (pmol/blastocyst) in relation to stage of Day 8 blastocysts.

On D8, stage 7 embryos did not exhibit any difference in ATP production with treatment. Blastocysts treated with GF generated the most ATP (p=0.026) compared to serum and FA groups at the hatching stage (i.e., stage 8). No difference in ATP production were detected in stage 9 blastocysts.

3.3.2.3 RNASeq

RNASeq scatter plot data indicates differentially expressed transcripts in red, and abundance of red plots gives us an idea about treatments effects on gene expressions differences. Differentially gene expression in CC was less between serum and PVP (1A) than between serum and FA (1B), serum and growth factors (1C), and serum and the growth factor/fatty acid combination (1D). However, the greatest number of differentially expressed transcripts was observed between serum and growth factors (1C).

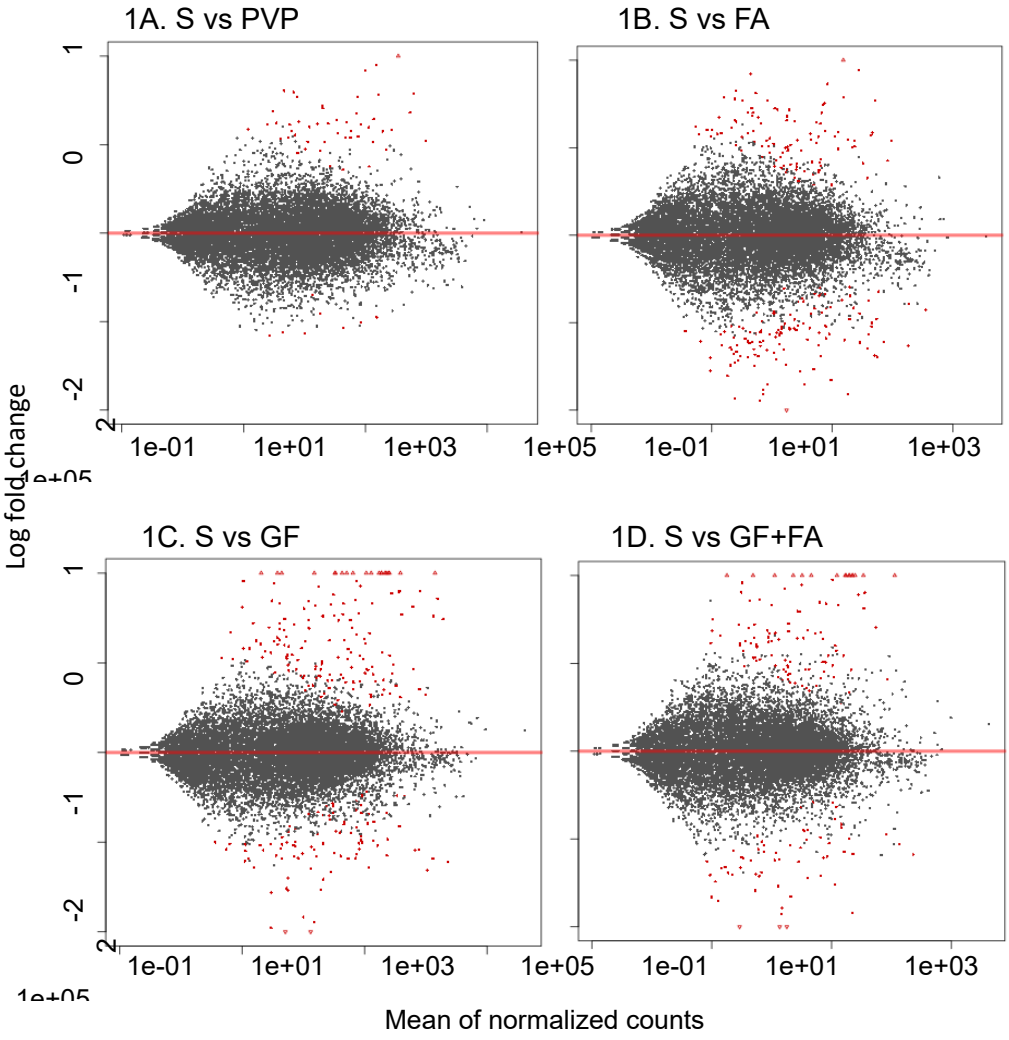


Figure 3. 12 Scatter plots indicate comparisons between serum and the four media conditions which were prepared in house. (A) Differential transcript expression between serum (S) and PVP; (B) S and PVP + fatty acids (FA); (C) S and growth factors (GF); and (D) S and GF+ FA.

No differential transcript expression was observed between the PVP and PVP + FA treatment groups (2A). However, compared to PVP alone, the addition of GFs increased the number of differentially expressed genes (2B). Addition of FA in the media with the presence of GF decreased the number of differentially expressed genes (2C) compared to GF only. On the other hand, the biggest difference was observed between the separate addition of GF and FA (2D).

Intriguingly, this difference was greater than when GFs were added with FA (2E). Finally, the addition of FA with GFs, compared to the addition of GFs alone, did not lead to any differential expression of transcripts (2F).

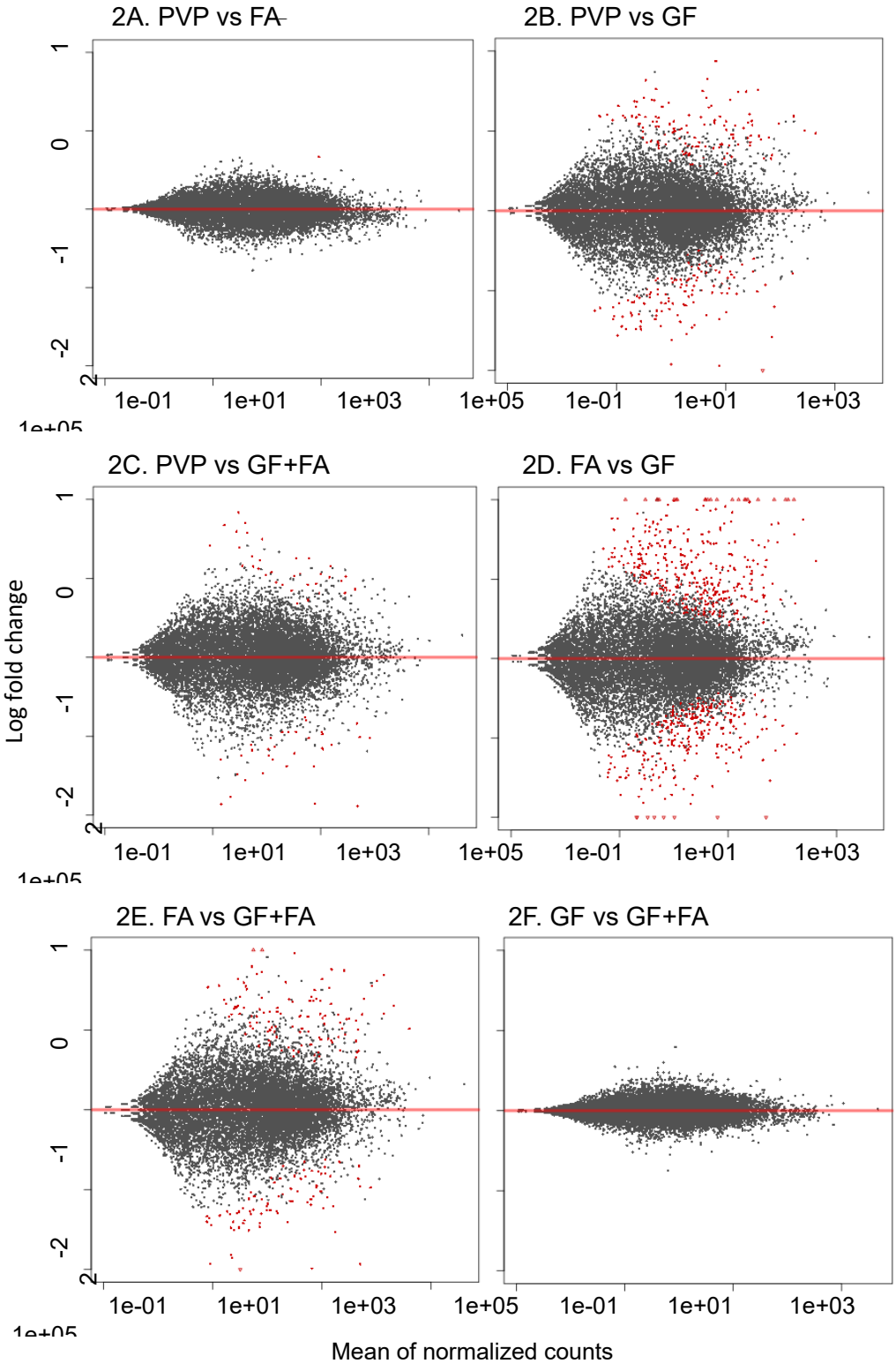


Figure 3. 13 Scatter plots indicate comparisons between serum and the four media conditions which were prepared in house. (A) Differential transcript expression between PVP and fatty acids (FA); (B) PVP and growth factor (GF); (C) PVP and GF+FA; (D) FA and GF; (E) FA and GF+FA; and (F) GF and GF+FA.

Greatest differences of top 8 gene ontology category in transcript expression are listed (Table 3.5). Upregulated genes are coloured with blue and downregulated in red. The comparison between serum and PVP (Table 3.5.A) identified genes related with cholesterol, steroid and lipid metabolism; biosynthesis and regulation were upregulated in the presence of serum. Only *CYP39A1* was downregulated in CC exposed to serum. This gene encodes a member of P450 superfamily enzymes which is an endoplasmic reticulum protein and involved conversion of cholesterol to bile acids.

The comparison between serum and fatty acid treatment groups indicated that whilst the genes which are related with steroid and cholesterol biosynthetic process were upregulated in the presence of serum, genes involved in glycolytic process were downregulated (Table 3.5.B). Apart from *VLDLR*, genes related with cholesterol metabolic process were upregulated compared to fatty acid treated group. *VLDLR* encodes very low-density lipoprotein receptor and plays an important role in VLDL-triglyceride metabolism. That might be seen due to low amount of VLDLs in serum. On the other hand, genes involved in glycolytic process were downregulated in the serum group compared to the FA group. That finding indicates that glycolysis has been highly occurred in CC exposed to fatty acids compared to the serum. Due to glycolysis decreased oxygen level upregulated genes involved in cellular response to hypoxia in FA group compared to the serum.

Genes involved in sterol, steroid, cholesterol and lipid biosynthetic and metabolic process were upregulated when CCs were exposed to serum compared to GFs, apart from *PTGS2* and *ERLIN1* (Table 3.5.C). *PTGS2* encodes prostaglandin-endoperoxide synthase which is the key enzyme in prostaglandin synthesis. Interestingly, *ERLIN1* expression was similar to the serum group when FAs were added (Table 3.5.D). However, *PTGS2* was still over expressed in the GF+FA group compared to serum. Apart from *PTGS2*, genes involved steroid, cholesterol, sterol and lipid biosynthesis and metabolic process were upregulated in the presence of serum.

Table 3. 5 Differentially expressed transcripts in cumulus cells; (A) serum (S) and PVP; (B) S and fatty acids (FA); (C) S and growth factors (GF); and (D) S and GF+FA following *in vitro* maturation. Greatest 7 or 8 changed and most relatively close with oocyte metabolism categories are presented. Upregulated genes are coloured with blue and downregulated in red.

A. S VS PVP

CATEGORY	P value	Changed	Total	Term	GOGenes
GO:0006695	0	7	19	cholesterol biosynthetic process	*DHCR7*, *HMGCR*, *HMGCS1*, *LSS*, *TM7SF2*, *INSIG1*, *FDFT1*
GO:0016126	0	7	14	sterol biosynthetic process	*DHCR7*, *HMGCS1*, *SQLE*, *TM7SF2*, *MSMO1*, *INSIG1*, *FDFT1*
GO:0006694	3,63E-10	5	21	steroid biosynthetic process	*HMGCS1*, *LSS*, *TM7SF2*, *NSDHL*, *FDFT1*
GO:0008203	1,98E-09	6	54	cholesterol metabolic process	*HMGCS1*, *SQLE*, *LDLR*, *TM7SF2*, *SCAP*, *INSIG1*
GO:0006629	2,92E-08	8	236	lipid metabolic process	*HMGCS1*, *PCYT2*, *PTGES2*, *FADS2*, *LDLR*, *ACLY*, *TM7SF2*, *SCAP*
GO:0008610	8,98E-06	3	18	lipid biosynthetic process	*ACLY*, *MSMO1*, *FDFT1*
GO:0042632	1,27E-05	4	64	cholesterol homeostasis	*TMEM97*, *CYP39A1*, *LDLR*, *INSIG1*
GO:0045540	3,55E-05	2	3	regulation of cholesterol biosynthetic process	*LPCAT3*, *SCAP*

B. S VS FA					
GO:0016126	1,58E-13	8	14	sterol biosynthetic process	*DHCR7, HMGCS1, SQLE, TM7SF2, MSMO1, EBP, INSIG1, FDFT1*
GO:0006695	4,07E-12	8	19	Cholesterol biosynthetic process	*DHCR7, HMGCR, HMGCS1, LSS, TM7SF2, EBP, INSIG1, FDFT1*
GO:0006096	4,46E-12	9	29	glycolytic process	*PFKL, PGAM1, PGK1, TPI1, PKM, GAPDH, ENO1, PFKP, GPI*
GO:0006694	2,01E-08	6	21	steroid biosynthetic process	*HMGCS1, LSS, CYP11A1, TM7SF2, NSDHL, FDFT1*
GO:0008203	4,46E-08	8	54	cholesterol metabolic process	*HMGCS1, VLDLR, SQLE, LDLR, CYP11A1, TM7SF2, SCAP, INSIG1*
GO:0071456	8,97E-07	7	55	cellular response to hypoxia	*ZFP36L1, BNIP3, VEGFA, PGK1, EGLN3, AQP3, FAM162A*
GO:0006629	1,56E-05	11	236	lipid metabolic process	*TECR, HMGCS1, PCYT2, VLDLR, FADS2, PLA2G7, LDLR, CYP11A1, ACLY, TM7SF2, SCAP*
GO:0001666	1,59E-05	7	80	response to hypoxia	*SLC2A8, PLOD1, UCP2, VEGFA, EGLN3, SCAP, LONP1*

C. S VS GF

CATEGORY	P value	Changed	Total	Term	GOGenes
GO:0016126	6,35E-20	11	14	sterol biosynthetic process	*DHCR7*, *HMGCS1*, *SQLE*, *MVK*, *TM7SF2*, *IDI1*, *MSMO1*, *SC5D*, *EBP*, *INSIG1*, *FDFT1*
GO:0006695	1,33E-17	11	19	cholesterol biosynthetic process	*DHCR7*, *HMGCR*, *HMGCS1*, *LSS*, *MVK*, *TM7SF2*, *IDI1*, *EBP*, *HSD17B7*, *INSIG1*, *FDFT1*
GO:0006694	2,38E-13	9	21	steroid biosynthetic process	*HMGCS1*, *LSS*, *MVK*, *CYP11A1*, *TM7SF2*, *IDI1*, *NSDHL*, *HSD17B7*, *FDFT1*
GO:0008203	1,17E-07	8	54	cholesterol metabolic process	*HMGCS1*, *SQLE*, *LDLR*, *MVK*, *CYP11A1*, *TM7SF2*, *IDI1*, *INSIG1*
GO:0006629	2,73E-07	14	236	lipid metabolic process	*TECR*, *HMGCS1*, *PCYT2*, *ERG28*, *ELOVL6*, *FADS2*, *FADS3*, *LDLR*, *MVK*, *CYP11A1*, *ACLY*, *TM7SF2*, *PTGS2*, *AACS*
GO:0008610	1,52E-06	5	18	lipid biosynthetic process	*ACSS2*, *ACLY*, *MSMO1*, *SC5D*, *FDFT1*
GO:0008202	2,09E-06	6	41	steroid metabolic process	*HMGCS1*, *MVK*, *CYP11A1*, *TM7SF2*, *ERLIN1*, *IDI1*
GO:0034379	3,42E-05	3	6	VLD lipoprotein particle assembly	*MFSD2A*, *LPCAT3*, *ACSL3*

D. S VS GF+FA					
GO:0006694	0	8	21	steroid biosynthetic process	*HMGCS1*, *LSS*, *MVK*, *TM7SF2*, *IDI1*, *NSDHL*, *HSD17B7*, *FDFT1*
GO:0006695	3,84E-22	12	19	cholesterol biosynthetic process	*DHCR7*, *HMGCR*, *HMGCS1*, *LSS*, *MVK*, *TM7SF2*, *IDI1*, *EBP*, *HSD17B7*, *INSIG1*, *FDFT1*, *DHCR24*
GO:0016126	4,20E-22	11	14	sterol biosynthetic process	*DHCR7*, *HMGCS1*, *SQLE*, *MVK*, *TM7SF2*, *IDI1*, *MSMO1*, *SC5D*, *EBP*, *INSIG1*, *FDFT1*
GO:0008203	3,65E-09	8	54	cholesterol metabolic process	*HMGCS1*, *SQLE*, *LDLR*, *MVK*, *TM7SF2*, *IDI1*, *INSIG1*, *DHCR24*
GO:0006629	8,77E-08	12	236	lipid metabolic process	*HMGCS1*, *PCYT2*, *ERG28*, *ELOVL6*, *FADS2*, *FADS3*, *LDLR*, *MVK*, *ACLY*, *TM7SF2*, *PTGS2*, *AACS*
GO:0008610	1,69E-07	5	18	lipid biosynthetic process	*ACSS2*, *ACLY*, *MSMO1*, *SC5D*, *FDFT1*
GO:0008202	0,000106	4	41	steroid metabolic process	*HMGCS1*, *MVK*, *TM7SF2*, *IDI1*

Sterol, cholesterol and steroid biosynthesis, metabolic homeostatic process genes were upregulated in CC when the COCs were matured in the base media (PVP) compared to GFs apart from *TSKU* and *SCAP* genes (Table 3.6.A).

The PVP vs GF+FA comparison indicated that the presence of FAs reduces differentially gene expression compared to GF alone. However, there remained differential expression for genes involved in sterol, steroid, cholesterol and lipid biosynthesis, which were upregulated in the presence of PVP alone compared to GF+FA (Table 3.6.B).

The comparison between FAs and GF indicated that CC tend to produce ATP via glycolysis in the presence of FAs (Table 3.6.C). As a result of increased glycolysis fructose 1,6-bisphosphate metabolism was increased in FA group. Genes representing cellular responses to hypoxia were upregulated in the FAs group. Additionally, the same three pathway (i.e., sterol, steroid and cholesterol biosynthesis) were upregulated when COCs were matured in FAs compared to GFs alone.

The FA vs GF+FA comparison provided a clue about chemical interactions between GFs and FAs (Table 3.6.D). Genes encoding enzymes and/or proteins required for glycolysis were higher in the FA group compared to GF+FA. Genes for transport proteins which are required for importation of long chain fatty acids to the plasma membrane were upregulated in the FA only group. This may be due to the interaction between GFs and FAs. As a result of this, FA transportation into cells was inhibited in the presence of GFs. The other genes which are involved in cholesterol, sterol, and steroid biosynthesis and metabolism were observed to be highly expressed in the FA group compared to the GF+FA group.

Additionally, there were no differences found between PVP and FA as well as between GF and GF+FA.

Table 3. 6 Differentially expressed transcripts in cumulus cells; (A) PVP and growth factors (GF); (B) PVP and GF+ fatty acids (FA); (C) FA and GF; and (D) FA and GF+FA following *in vitro* maturation. Greatest changed and most relatively close with oocyte metabolism categories are presented.

A. PVP VS GF					
CATEGORY	**P value**	**Changed**	**Total**	**Term**	**GOGenes**
GO:0016126	5,94E-17	9	14	sterol biosynthetic process	*DHCR7*, *HMGCS1*, *SQLE*, *MVK*, *TM7SF2*, *IDI1*, *MSMO1*, *INSIG1*, *FDFT1*
GO:0006695	2,92E-11	7	19	cholesterol biosynthetic process	*DHCR7*, *HMGCS1*, *MVK*, *TM7SF2*, *IDI1*, *INSIG1*, *FDFT1*
GO:0006694	3,73E-09	6	21	steroid biosynthetic process	*HMGCS1*, *MVK*, *TM7SF2*, *IDI1*, *NSDHL*, *FDFT1*
GO:0008203	9,84E-08	7	54	cholesterol metabolic process	*HMGCS1*, *SQLE*, *TSKU*, *MVK*, *TM7SF2*, *IDI1*, *INSIG1*
GO:0008202	5,16E-06	5	41	steroid metabolic process	*HMGCS1*, *MVK*, *TM7SF2*, *SRD5A1*, *IDI1*
GO:0042632	9,11E-05	5	64	cholesterol homeostasis	*TMEM97*, *TSKU*, *LPL*, *INSIG1*, *SCARB1*
GO:0045540	3,55E-05	2	3	regulation of cholesterol biosynthetic process	*LPCAT3*, *SCAP*
B. PVP VS GF+FA					
GO:0016126	1,71E-08	4	14	sterol biosynthetic process	*SQLE*, *IDI1*, *MSMO1*, *FDFT1*
GO:0008610	7,78E-06	3	18	lipid biosynthetic process	*MSMO1*, *FDFT1*, *ACSL6*
GO:0006695	7,38E-04	2	19	cholesterol biosynthetic process	*IDI1*, *FDFT1*
GO:0006694	8,45E-04	2	21	steroid biosynthetic process	*IDI1*, *FDFT1*

C. FA VS GF

CATEGORY	P Value	Changed	Total	Term	GOGenes
GO:0006096	2,30E-15	14	29	glycolytic process	*PFKL*, *HK1*, *ALDOC*, *ALDOA*, *PGAM1*, *PGK1*, *PFKFB2*, *TPI1*, *PKM*, *GAPDH*, *ENO1*, *PFKP*, *GPI*, *HK2*
GO:0016126	1,01E-13	10	14	sterol biosynthetic process	*DHCR7*, *HMGCS1*, *SQLE*, *MVK*, *TM7SF2*, *IDI1*, *MSMO1*, *EBP*, *INSIG1*, *FDFT1*
GO:0006695	3,42E-10	9	19	cholesterol biosynthetic process	*DHCR7*, *HMGCR*, *HMGCS1*, *MVK*, *TM7SF2*, *IDI1*, *EBP*, *INSIG1*, *FDFT1*
GO:0071456	1,11E-06	10	55	cellular response to hypoxia	*ZFP36L1*, *BNIP3*, *NDRG1*, *NPEPPS*, *MTOR*, *VEGFA*, *PGK1*, *EGLN3*, *AQP3*, *FAM162A*
GO:0030388	6,89E-06	4	6	fructose 1,6-bisphosphate metabolic process	*PFKL*, *ALDOC*, *ALDOA*, *PFKP*
GO:0006694	7,64E-06	6	21	steroid biosynthetic process	*HMGCS1*, *MVK*, *TM7SF2*, *IDI1*, *NSDHL*, *FDFT1*
GO:0008203	8,86E-06	9	54	cholesterol metabolic process	*HMGCS1*, *VLDLR*, *SQLE*, *TSKU*, *MVK*, *TM7SF2*, *LCAT*, *IDI1*, *INSIG1*

D. FA VS GF+FA					
GO:0006695	0	8	19	cholesterol biosynthetic process	*DHCR7*, *HMGCS1*, *PMVK*, *MVK*, *TM7SF2*, *IDI1*, *INSIG1*, *FDFT1*
GO:0016126	4,92E-17	9	14	sterol biosynthetic process	*DHCR7*, *HMGCS1*, *SQLE*, *PMVK*, *MVK*, *TM7SF2*, *IDI1*, *INSIG1*, *FDFT1*
GO:0006694	3,92E-11	7	21	steroid biosynthetic process	*HMGCS1*, *PMVK*, *MVK*, *TM7SF2*, *IDI1*, *NSDHL*, *FDFT1*
GO:0006096	3,87E-08	6	29	glycolytic process	*HK1*, *ALDOC*, *PKM*, *ENO1*, *PFKP*, *GPI*
GO:0008203	8,78E-08	7	54	cholesterol metabolic process	*HMGCS1*, *SQLE*, *PMVK*, *MVK*, *TM7SF2*, *IDI1*, *INSIG1*
GO:0008202	1,69E-07	6	41	steroid metabolic process	*HMGCS1*, *PMVK*, *MVK*, *TM7SF2*, *SRD5A1*, *IDI1*
GO:0015911	1,785E-04	2	3	long-chain fatty acid import across plasma membrane	*SLC2A1*, *SLC27A1*

3.4 Discussion

This series of experiments were performed to generate embryos in defined medium to obtain consistent results. The nature of supplements was based on known serum components and the volume of chemicals added were based on previous studies.

In the two preliminary experiments, oocytes were obtained from cycles of DFR. The first of these two experiments assessed specific aspects of albumin removal from IMV media. It was observed that FA supplementation in PVP based media has a beneficial effect on the proportion of advanced zygotes on D2. On the other hand, albumin has an important effect on preventing the degradation of FAs especially oleic acid and PUFAs compared to PVP based media. In the second preliminary experiment, the aim was to observe chemical interactions and its impact on embryo development. Therefore, instead of albumin, FAs was added to PVP, and no treatment effect was observed. The overall data from both experiments indicated that FA supplementation improved slightly embryo development. These findings suggest that albumin does not have any effect on subsequent embryo development in this modified media, however FA supplementation can improve embryo development compared to GF and IAI in PVP-based media.

Based on these preliminary findings, IαI was removed from maturation media in the main experiment due to the effect of IαI on degradation of FAs in maturation media. In the main experiment the aim was to observe the developmental effects of a cocktail of GFs and FAs added to IVM media separately or in combination relative to basal PVP media (negative control) and serum (positive control).

The effects of treatments were observed on D8. Proportion of blastocysts of cleaved was the highest in the presence of serum and followed by FA group. D8 blastocysts of D6 blastocysts was again highest when oocytes were matured in the presence of serum. However, this time followed by the PVP group. Proportion of D8 blastocysts per cycle was significantly increased in the presence of serum as well as transferable blastocysts of cleaved and of D6 embryos. ATP production was significantly higher in Stage 8 embryos which were treated with growth factors. These findings suggest that the GF

cocktail used in this study impairs subsequent embryo development. This could be due to the level and/or interaction of different growth factors added. Additionally, the combination of GFs and FAs has detrimental effect on embryo development.

Sequencing data on transcript expression indicated that, for the most part, cumulus-cell genes involved in steroid, cholesterol, lipid biosynthesis and metabolic processes in general were most affected between treatment groups. In these comparisons, IVM media containing serum served as the positive control. Interestingly, the complete removal of serum (to leave basal PVP media) had less of an effect on differential transcript expression than the other comparisons (i.e., where with FAs or GFs were added). Curiously, the addition of FAs to basal PVP media and FAs to media containing GFs had a minimal effect on the number of differentially expressed transcripts (Figure 3.12).

Collectively these observations indicate that addition of FA in the presence of LC to the PVP-based media can improve embryo development by altering the ATP metabolic genes in CC. However, GF supplementation of maturation media in the presence of FA reverses the beneficial effect of FA on embryo development

3.4.1 Growth Factors and standards in serum replacement

In mammals growth factors play an essential role in ovarian follicle development as well as events within the follicle that prepare the COC for ovulation (Price, 2016). During the natural ovarian folliculogenesis, FGF-2 affects granulosa cells to promote cell proliferation, decrease apoptosis and steroidogenesis (Gospodarowicz and Bialecki, 1979, Baird and Hsueh, 1986, Lavranos et al., 1994). The other mitogenic growth factor is IGF1 (Wetterau et al., 1999) which is known to stimulate mitosis of cultured granulosa (Khamsi and Armstrong, 1997, Spicer and Stewart, 1996) and theca cells, and regulates oestradiol and androgen synthesis (Spicer et al., 1993, Spicer and Stewart, 1996).

In recent studies the effects of growth factors were observed to affect maturation and *in vitro* production of embryos. Up to 100 ng/mL FGF-2 increased maturation rate, moreover inhibition of FGFR (FGF receptor)

inhibited miotic progression in mice (Du et al., 2021). In bovine maturation 1-100 ng/mL FGF-2 increased the proportion of GVBD oocytes (Barros et al., 2019).

Murine oocytes are potent stimulators of cumulus-cell (CC) proliferation. Stimulation was not further increased by IGF-1 (Gilchrist et al., 2001). However, bovine and porcine oocytes require IGF-1 to become potent promoters CC proliferation (Brankin et al., 2003, Li et al., 2000). 10ng/mL IGF-1 supplementation in maturation media increased the proportion matured oocytes and blastocyst formation in pig (Yuan et al., 2017). Moreover, a cocktail of growth factors had a greater beneficial effect on the proportion matured oocytes, blastocysts formation and cell numbers of blastocysts than when they were added separately in pig (Yuan et al., 2017) and bovine IVM media (Stoecklein et al., 2021). However, in our experiments the GF cocktail we used impaired embryo development. Based on current knowledge this might have arisen due to interactions between the specific GFs used, and/or their level, with other components of serum replacement.

Insulin has an effect on lipid and glucose metabolism in mammals (Saltiel and Kahn, 2001). It has been shown that up to 5 ng/mL insulin has a beneficial effect on oocyte maturation (Stefanello et al., 2006). However, supplementation of insulin at supra-levels (0.1 µg/ml and 10 µg/ml) reduced blastocyst formation in cattle (Laskowski et al., 2016). In the other studies when growth factors were supplemented with hormones as a cocktail, their amount was chosen much lower than when they were added separately. In a calf study, embryo proportion was increased when oocytes were matured in enriched medium containing 5 µg/mL insulin, 19 ng/mL IGF-1 and 2.2 ng/mL FGF-2, plus antioxidants and pyruvate (Donnay et al., 2004). Moreover, a combination of insulin and IGFs indicated that IGF1 activity may be reduced when insulin is present at higher concentrations (>10ng/mL) (Entingh-Pearsall and Kahn, 2004). In our experiment even the lower concentrations of GFs (i.e., IGF1 and FGF2) in the presence of 2 ng/ml insulin has detrimental effect on embryo development and oocyte maturation competence as RNASeq data indicates.

In our study the data indicated that combinations of growth factors and serum replacement components did not improve embryo development. It might be due to interactions of chemicals as the result showed that a cocktail of growth

factors and fatty acids were detrimental for embryo developmental competence. RNASeq data supported this notion, indicating that cholesterol, sterol, steroid biosynthesis, glycolysis and long chain fatty acid transportation were all decreased in the presence of GFs compared to FAs.

3.4.2 Fatty acid supplementation in culture system

Lipid metabolism via β-oxidation has been shown to be essential to oocyte developmental competence (Dunning et al., 2010). However, during folliculogenesis lipid proportions can be altered by nutrition and they also change following parturition (i.e., during negative energy balance when FAs are mobilised from body stores) (Leroy et al., 2008). Elevated NEFAs (133 μM palmitic, 67 μM stearic, 200 μM oleic) during NEB reduced fertility by decreasing oocyte maturation competence and blastocyst implantation rate (Leroy et al., 2005). In the basal condition, 50 μM palmitic, 25 μM stearic and 75 μM oleic levels have been suggested to be suitable "physiological levels" (Leroy et al., 2005), and these levels have been used effectively for oocyte maturation (Van Hoeck et al., 2011). In the exploratory experiments our results indicate that FA supplementation at the physiological levels in the presence of GF and IAI have a beneficial effect on embryo development. In the main experiment, serum supplemented media followed by basal media supplemented with FAs gave the best results in terms of embryo development.

Addition of 50 μM α-linolenic acid (C18:3, n-3) improved oocyte maturation compared to FAF-BSA alone, but higher doses were detrimental for oocyte maturation and subsequent embryo development (Marei et al., 2009b, Marei et al., 2012). It has been observed that linoleic acid (C18:2, n-2) inhibited GV breakdown at 50 μM (Homa and Brown, 1992), and has a detrimental effect on oocyte maturation and cumulus expansion (Marei et al., 2010b). The proportion of linoleic acid reduces proportionally to other fatty acids in follicles as they increase in size approaching ovulation (Homa and Brown, 1992). Linoleic acid may be a requirement for oocyte metabolism as it is a major fatty acid component (10% of total FA) of matured oocytes (Fayezi et al., 2018a). There is limited information to indicate safe levels for maturation and subsequent embryo development. It has been used at 9 μM and 43 μM during maturation without detrimental effects on oocyte maturation or cumulus

expansion; however, there was no follow up with regard to fertilisation or embryo development (Carro et al., 2013). Oocyte maturation at 100 μM does not affect oocyte progression or subsequent embryo development if vitamin E or glutathione peroxidase are present, however, cumulus expansion is still inhibited (Khalil et al., 2013). Similarly, for pre-pubertal goat oocytes, high levels of linoleic acid up to 100 μM can be used providing 50 μM of α-Linolenic acid is present (Roura et al., 2018). Alternatively, conjugated linoleic acid could be used in preference to linoleic acid, as up to 200 μM has been used without detrimental effect (Absalón-Medina et al., 2014), and 100 μM at maturation has been reported to improve embryo development (Absalón-Medina et al., 2014). However, the conjugated form of linoleic acid is present at very low levels in follicular fluid (0.1-0.4% of total fatty acids) even in animals fed high levels of conjugated linoleic acid (Castañeda-Gutiérrez et al., 2007b, Sharma et al., 2020).

In the current experiment, addition of fatty acids at physiological levels had a beneficial effect on embryo development in the preliminary experiments. However, the effect was negative in terms of obtaining advance blastocysts in the main experiment. It might be due to the absence of inter-alpha inhibitor in the medium. Additionally, RNASeq data indicated that the genes involved in cellular response to hypoxia and glycolysis were upregulated in the presence of FAs compared to serum due to increased oxidative phosphorylation. As for our media, FAs were free and readily taken up, and the concentration of FAs higher than FAs in serum.

3.5 Conclusion

The current series of studies indicated that the combination of different reagents (i.e., fatty acids and growth factors) used in this study and added during IVM had a detrimental effect on embryo development. It might be due to interactions of chemicals or the volume of some of the chemicals were higher than required when they are combined. However, there is an indication that additional FAs to PVP-based media increases oxidative phosphorylation in cumulus cells compared to serum and this allows to obtain more ATP required for cytoplasmic and nuclear maturation. In the preliminary experiment albumin did not improve embryo development and that might be due to high level of FAs as it is well known that albumin has an important role to carry FAs

in the body. Therefore, albumin needs to be observed in the presence of lower fatty acids to identify its effect on embryo development for further study.

Chapter 4 Effect of L-carnitine in the presence of fatty acids during *in vitro* maturation

4.1 Introduction

In vitro maturation (IVM) is a critical step for the successful fertilization of oocytes and for obtaining viable embryos. Maturational competence relies on media composition, oxygen level and temperature *in vitro* (Nagai, 2001). It is known that IVP embryos contain more lipid droplets than *in vivo* produced embryos in *Bos Taurus* and *Bos Indicus* species (Sudano et al., 2012). Over accumulation of lipids in embryos impairs embryo developmental competence and may cause abnormalities. Therefore, in the last few years attempts have been made to remove serum and/or albumin from culture. Additionally, lipid regulators such as L-carnitine have been investigated in a number of studies to prevent lipid accumulation.

An important goal of IVM is to complete cytoplasmic maturation as well as nuclear maturation in oocytes. Within this context, mitochondria plays an important role to ensure ATP for protein synthesis (Stojkovic et al., 2001). It is known that L-carnitine is a key limiting factor for β-oxidation in mitochondria and ATP production (Adeva-Andany et al., 2019). Therefore, supplementation of L-carnitine during IVM has been found to increase embryo developmental competence in cattle (Knitlova et al., 2017).

One of the key differences between *in vivo* and *in vitro* embryo production is oxygen tension. This difference alters ROS level and subsequently embryo development. To reverse it, antioxidants are commonly used during culture (Agarwal et al., 2005b). L- carnitine has an effect on preventing ROS formation and improving embryo development (Shafiei et al., 2020).

In the recent decade, the importance of fatty acids in IVM and subsequent embryo development has received attention. Adding fatty acids individually has been studied. Mostly their presence increases embryo development, however beneficial effects are observed in a dose-dependent manner. Mainly palmitic, oleic, stearic, α-linolenic and linoleic acids were added during maturation as they are the main long-chain fatty acids in follicular fluid. Whilst adding oleic acid supported embryo development (Tsujii et al., 2001),

overdosing with palmitic acid has caused endoplasmic reticulum stress (Sutton-McDowall et al., 2016), mitochondrial dysfunction (Itami et al., 2018) and subsequently impaired embryo development (Wu et al., 2012). High levels of stearic acid also had a detrimental effect on embryo development (Mirabi et al., 2017). Fatty acid supplementation is also important for cell signalling, ATP production and membrane formation especially in the absence of serum or albumin.

The study reported in this chapter sought to increase embryo developmental competence when oocytes are matured in chemically defined medium. Within this content, serum is removed. However, as demonstrated Chapter 3, albumin is important for PUFA uptake into COCs. Therefore, in the current experiment FA levels, equivalent to 10% fetal calf serum (FSA) were conjugated with BSA in the presence and/or absence of LC. To understand physiological changes in maturing COCs when matured in LC+FA, gene expression, ATP production and mtDNA copy number were assessed.

4.2 Material and Method

4.2.1 Experimental Design

Four 10–13-month-old heifers underwent repeated cycles of OPU. Animal preparation, oocyte collection and IVP procedures were described previously (Chapter 3, Section 3.3.1.2). OPU-derived oocytes were matured in the presence of FAs (25 µM palmitic,15 µM stearic and oleic, 5 µM linoleic and alpha-linolenic acids) in the presence or absence of 2.5 mM L-carnitine. Oocytes were matured at 20 µl/per oocyte and the experiment replicated six times. In the first four cycles, oocytes were cultured through to the D8 blastocyst stage and development was assessed. Following maturation, CCs were collected for RNASeq as described previously (Chapter 3, Section 3.3.1.3) and spent maturation media was retained for fatty acid analyses as explained previously (Chapter 3, Section 3.2.1.4). Glucose uptake and lactate production were also determined for the last two cycles. When the samples were prepared for fatty acid analyses, media were separately diluted with water for glucose and lactate analysis. According to test run, 20 µl per oocyte is the optimum dilution for analysis. Glucose concentration in the media was

measured by using the Glucose-hexokinase kit (GL3816, Randox Laboratories Ltd) and analysed by Imola (RX Imola, Randox Laboratories Ltd). Glucose uptake was calculated by difference between stock and spent media. Lactate production in spent media was determined by L-lactate kit (LC3980, Randox Laboratories Ltd) and measured by Imola. Following maturation, oocytes were denuded for ATP and mtDNA copy number determination.

4.2.2 ATP assay and mtDNA copy number

After maturation, CCs were removed by vortexing for 10 min. Denuded oocytes were washed in 0.1%PVP-PBS solution and placed individually in tubes with 25ul 0.1%PVP-PBS. Tubes were heated to 100°C in a heating block for 3 min, then stored at -80°C until analysis. Based on previous tests for optimization, eight different levels (5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 and 0 pmol/oocyte) were determined as standards. On the day of assay, according to manufacturer instructions, 50 µl somatic cell releasing agent and 1.875 µl of 200 ng/µl tRNA were added to each tube. After vortex, samples were incubated on ice for 5 min. 4ul was taken from each lysed oocyte for mtDNA assay. The remaining elements of the protocol were described previously for ATP (Chapter 3, Section 3.3.1.4) and for mtDNA copy number (Chapter 2, Section 2.2.6).

4.2.3 Statistical analyses

Embryo production data were analysed using Generalised Linear Mixed Models within GenStat (GenStat 19th Edition, 2018, VSN International Ltd, Hemel Hempstead, UK) assuming binomial errors and with logit link functions. Terms fitted to the fixed model were OPU-cycle, fatty acids, fatty acids with LC and interactions between these terms. Donor was included as a random effect in these models. Data are presented as means ± Standard Error of Means (SEM). ATP generation and mtDNA copy number in oocytes, fatty acid and glucose uptake and lactate production in spent media were analysed using similar Mixed Linear Models but assumed normal errors. Data are also presented as means ± Standard Error of Means (SEM).

4.3 Results

4.3.1 Embryo Development

Day 2 assessment indicated that the proportion cleaved was reduced (P = 0.009) in the presence of L-carnitine (Figure 4.1A). However, the inclusion of L-carnitine improved the proportion of developmentally advanced embryos amongst this pool (Figure 4.1 B-E).

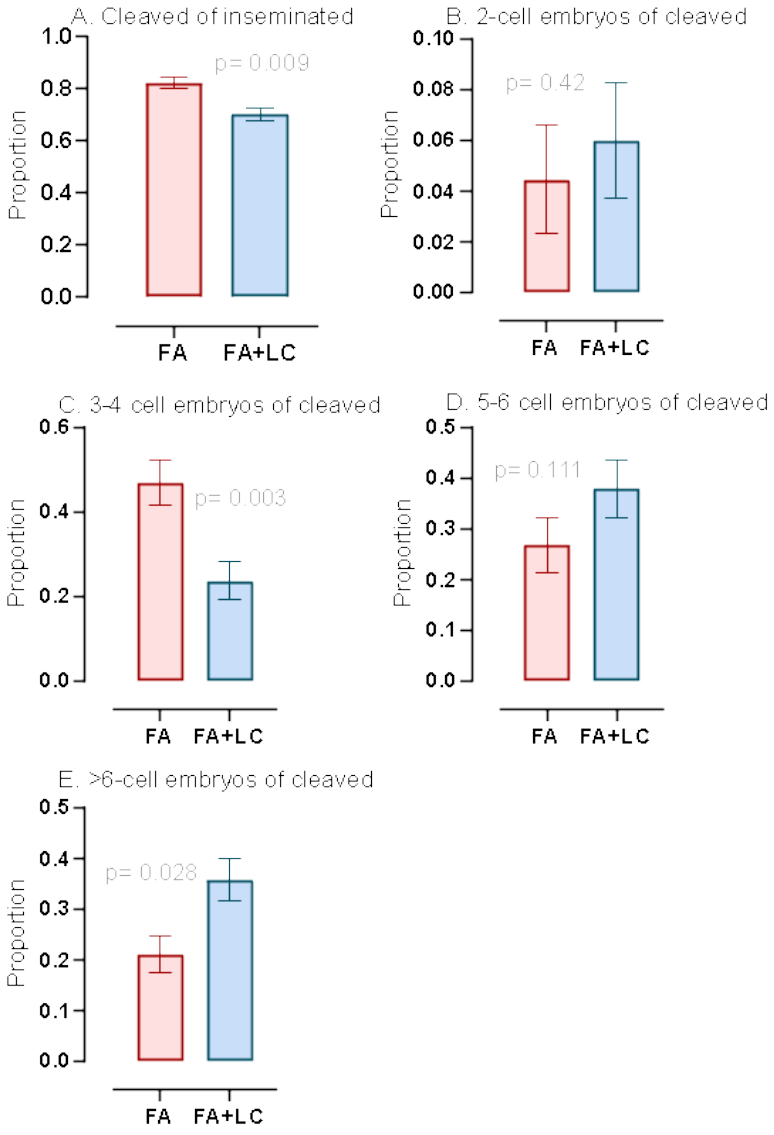


Figure 4. 1 Day 2 embryo development following insemination; total cleavage (A), and cell number distribution (B-E). FA = Fatty acids, FA+LC = FA and L-carnitine. See text for details.

By Day 8, the proportion blastocysts of matured (P = 0.012) (Figure 4.2A) and of cleaved (P = 0.008) (Figure 4.2B) were increased with LC supplementation as well as transferable blastocysts of matured (P = 0.045) (Figure 4.2D) and of cleaved (P = 0.035) (Figure 4.2E). No treatment effect was observed on the proportion Day 8 blastocysts of D6 embryos (Figure 4.2C).

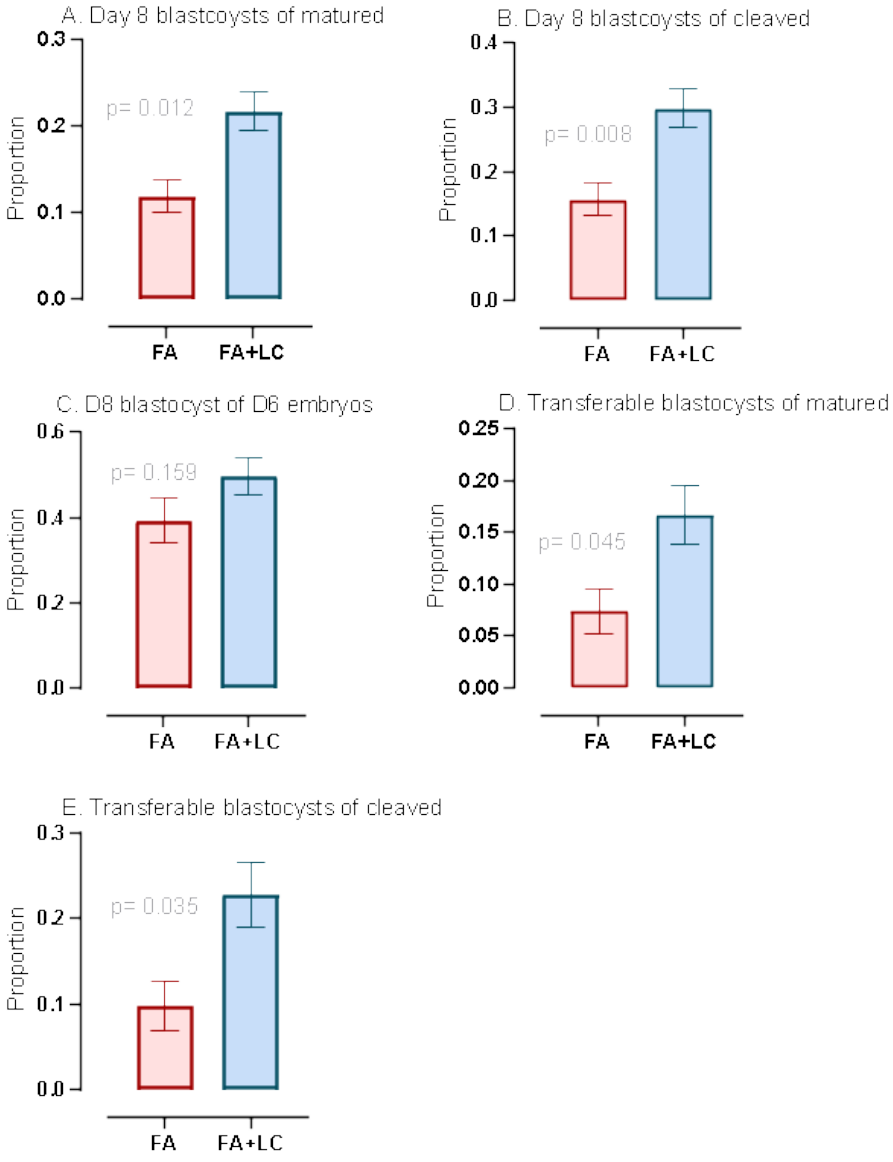


Figure 4. 2 Proportion D8 blastocysts of matured (A), cleaved (B), D6 embryos (C), and transferable (IETS Stage 7 to 9, Grades 1 and 2) blastocysts of matured (D) and of cleaved (E). FA = Fatty acids, FA+LC = FA and L-carnitine. See text for details.

4.3.2 ATP Assay

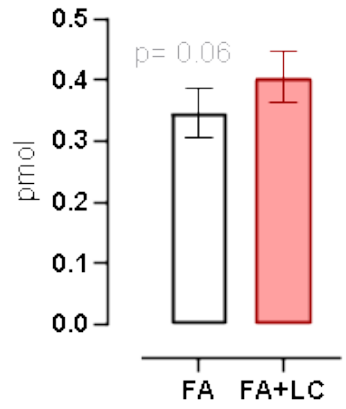


Figure 4. 3 ATP production per oocyte following maturation.

Following *in vitro* maturation, there was an indication that ATP production per denuded oocyte was increased (P = 0.06) in the presence of LC.

4.3.3 mtDNA copy number

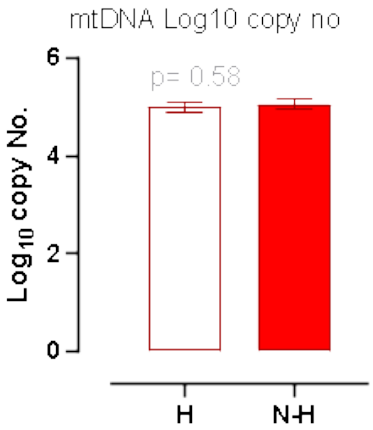


Figure 4. 4 Log mtDNA copy number between heated and non-heated oocytes following maturation.

As oocytes were heated to 100ºC for ATP analysis as per the manufacturer's instructions, the effects of heating on mtDNA copy number was assessed. This assessment revealed that there was no difference between heated and non-heated oocytes in terms of copy number (Figure 4.4).

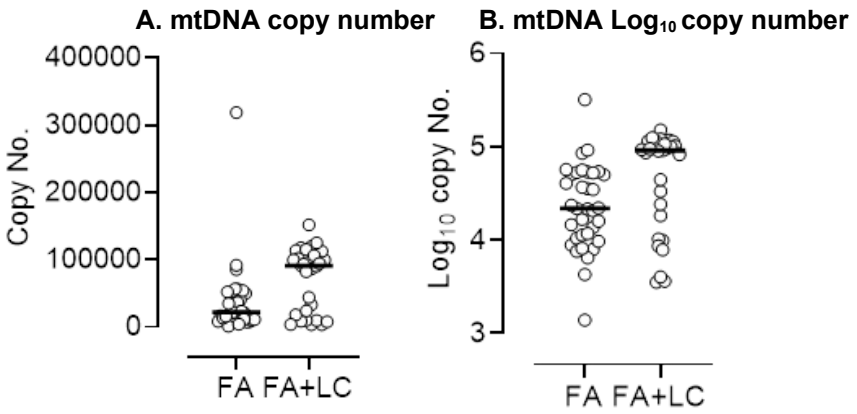


Figure 4. 5 mtDNA copy number (A) and Log_{10} copy number (B) in oocytes following maturation with fatty acids (FA) in the presence or absence of L-carnitine (LC).

mtDNA copy number was less than 50,000 in oocytes matured in the presence of FAs alone but greater (P = 0.022) at around 100,000 in oocytes matured in the presence of FA and LC.

4.3.4 Spent Media Analysis

Analyses of FA uptake during IVM indicated that, in the presence of LC, oocytes released palmitic (P = 0.07), stearic (P = 0.035) and oleic (P = 0.002) acids to the media rather taking them up. However, no treatment differences were observed for LA and ALA uptake (Figure 4.6 A). Glucose uptake and lactate formation was decreased in the presence of LC, however these changes were not statistically significant (Figure 4.6 B, C).

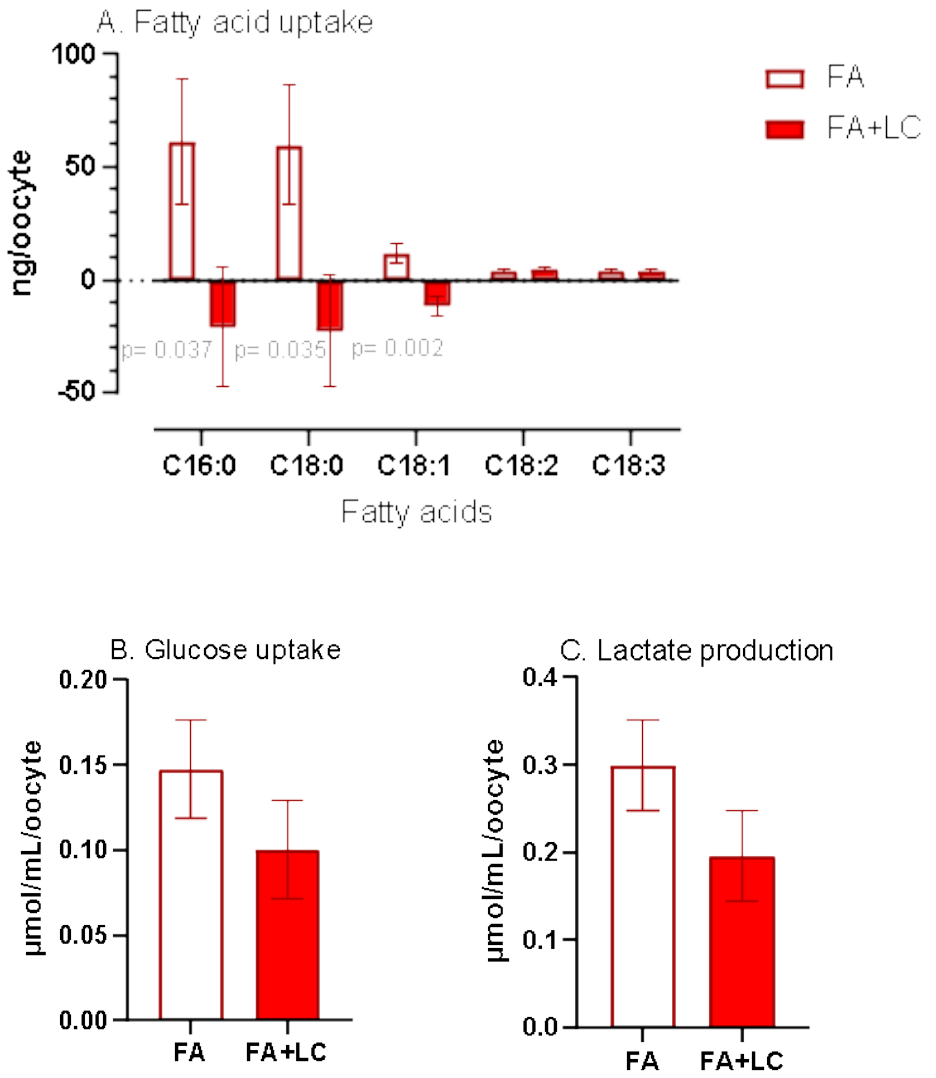


Figure 4. 6 Analyses of fatty acids (FAs) (A), glucose (B) and lactate (C) in media following *in vitro* maturation. Oocytes were matured in media supplemented with FAs alone or with L-carnitine (LC).

4.3.5 RNASeq

RNAseq analyses indicated that, apart from *BOLA,* extracellular space genes were upregulated in the presence of LC. Secreted genes such as *PLAT*, which plays a role in the cortical granule reaction in zona block to polyspermy, in CCs were upregulated with LC supplementation as well as cell surface genes. For the cell surface gene, mainly downregulation was observed when the COCs were treated with FA only in the absence of LC. Only *BOLA* and *ITGA4* genes were upregulated in this group. Glycosaminoglycan metabolism is a broad pathway which includes hyaluronan uptake and degradation,

mucopolysaccharidoses, carbohydrate metabolism and so on. LC supplementation upregulated the genes which are related with metabolism of glycosaminoglycan. This is possible due to effect of LC on mitochondrial activity.

Table 4. 1 RNASeq data comparison between FA and FA+LC. Downregulated genes are indicated red and upregulated are blue.

FA VS FA+LC				
TERM	**P value**	**Changed**	**Total**	**GOGenes**
EXTRACELLULAR SPACE	0.015	9	293	*PLAT, BOLA, NGF, ANGPTL2, ANPEP, IL33, JSP.1, PRSS2, PROS1*
CELL SURFACE	0.01	8	200	*CXCR4, PLAT, BOLA, ITGA4, SDC1, ANPEP, JSP.1*
GLYCOSAMINOGLYCAN METABOLISM	0.01	5	52	*LUM, DCN, EXT1, SDC1, CHST2*

4.4 Discussion

In this chapter the effect of LC supplementation in maturation media in the presence of selected long-chain fatty acids was assessed with respect to molecular changes in cumulus cells, embryo development, and metabolic markers. The benefit of added LC during IVM was observed on D2, with more advanced zygotes, and D8, with improved blastocyst formation. mtDNA copy number was greater and ATP production increased in the presence of LC. Furthermore, increased release in palmitic, oleic and stearic acid to the media was observed when oocytes were treated with LC. Genes which are involved in extracellular space, cell surface and glycosaminoglycan metabolism were upregulated following LC treatment. Collectively these results indicate that LC improves aspects of cytoplasmic maturation by increasing mtDNA copy number and ATP production in matured oocytes and subsequent embryo development.

4.4.1 L-carnitine impacts on maturation and embryo development

In vitro maturation is a crucial step in bovine IVP, required to prepare oocytes for fertilization and subsequent embryo development (Albuz et al., 2010). Oocyte maturational competence is altered by metabolite content of maturation media, oxygen concentration, timing, and temperature (Nagai, 2001). Several studies have demonstrated that supplementation of L-carnitine to either IVM or IVC media has beneficial effects on oocyte maturation and embryo development in cattle (Carrillo-González and Maldonado-Estrada, 2020), mice (Abdelrazik et al., 2009), pigs (You et al., 2012), canines (Moawad et al., 2021) and camels (Fathi and El-Shahat, 2017).

Supplementation of L-carnitine to maturation media increases proportion of oocytes which reach MII (Knitlova et al., 2017). Moreover, addition of 3.8 mM L-carnitine during IVM and 1.5 mM during IVC significantly improved implantation and pregnancy following embryo transfer in cattle (Carrillo-González and Maldonado-Estrada, 2020).

In our experiment 2.5 mM L-carnitine added to maturation media increased the proportion of advanced zygotes on D2 and blastocyst formation on D8.

4.4.2 L-carnitine as a lipid metabolism regulator

It is known that carnitines play a crucial role in β-oxidation by carrying fatty acids to mitochondria. The electron transport chain follows β-oxidation to produce ATP in the mitochondrial matrix (Hashimoto, 2009) . Moreover, LC protects cells and their DNA against ROS which are formed naturally as a result of oxidative phosphorylation (Vanella et al., 2000).

Fatty acids are major energy sources for oocytes and at certain stages of embryo development (Bertevello et al., 2018). *In vivo* FFAs are taken up by somatic cells then esterified to TAG and stored as neutral lipids in LDs (Carro et al., 2013). Their oxidation is crucial for oocyte nuclear maturation and competence acquisition (Paczkowski et al., 2013, Dunning et al., 2014b). It has been observed that L-carnitine supplementation decreases lipid accumulation by increasing beta oxidation (Dunning and Robker, 2012a). L-carnitine supplementation to IVC media significantly decreased lipid content of bovine embryos (Somfai et al., 2011) and increased blastocyst development (Wu et al., 2011).

On the other hand, oxygen species are formed by the ETC, and increased beta-oxidation breaks the balance between ROS and antioxidants. Whilst β-oxidation is important for oocyte developmental competence acquisition, without oxidative stress defenders, increased oxidative stress triggers an increase in lipid peroxidation and ROS are released (Seidel, 2006). Supplementation of LC to maturation media increases GSH levels in porcine oocytes (Somfai et al., 2011). When bovine oocytes from aged donors were matured in the presence of LC, GSH levels and *GPX4* and *SOD1* expression were increased in D8 blastocysts, and developmental capacity was improved most likely by reducing oxidative stress and autophagy (Jiang et al., 2020).

Additionally, maturation of porcine oocytes in the presence of LC increased the proportion of active mitochondria and reduced lipid droplets (Somfai et al., 2011). Dietary LC supplementation increased porcine oocyte active mitochondria number following ovulation, and supplementation of LC to IVM media significantly increased the number of MII oocytes, most likely by increasing mitochondrial activity and decreasing apoptosis (Hashimoto, 2009). During IVP (i.e., IVM, IVF and IVC) the presence of LC significantly reduced apoptosis in porcine blastocysts (Wu et al., 2011).

In our experiment L-carnitine significantly increased mtDNA copy number and ATP production. Moreover, in the presence of LC palmitic, stearic and oleic acid uptake were significantly lower compared to the FA group. The data indicated that those 3 fatty acids were released to media from COCs which were treated with LC. These findings indicate that LC reduced lipid accumulation during maturation and increased ATP production by increasing mtDNA copy number.

4.4.3 mtDNA in fertility

Oocytes contain a large stock of organelles and molecules to be used until the early embryo stage. Until embryonic mRNAs are produced, oocytes provide stock mRNA for zygotes. Therefore, oocyte mtDNA accumulation becomes important to encode mRNA for mitochondrial respiratory to generate ATP by oxidative phosphorylation (Dumollard et al., 2007, St John et al., 2010) until implantation, as embryonic cells do not replicate mtDNA (Thundathil et al., 2005, El Shourbagy et al., 2006). Accordingly, until fertilization the oocyte must produce large quantities of mtDNA to provide enough molecules to each cell of the blastocyst. Decreased mitochondrial function, as a consequence of insufficient mtDNA replication, decreases oocyte developmental competence which may occur during oocyte aging in some cases (Van Blerkom, 2011) or by stress (Van Blerkom, 2009).

mtDNA copy number varies in mature oocytes between species (mouse (Cao et al., 2007), human (Zeng et al., 2007), pig (El Shourbagy et al., 2006), cow (May-Panloup et al., 2007) and rat (Kameyama et al., 2007)) and, in the same species, lower copy number induces less developmental competence. Age, season and genetic factors can affect aspects of oocyte developmental competence such as mRNA abundance, metabolites and mtDNA copy number (Podolak et al., 2022). Therefore, some studies have tried to improve mtDNA accumulation during maturation or during antral follicle development (Kirillova et al., 2021).

In our experiment LC supplementation increased mtDNA copy number and ATP production correspondingly. Improved mtDNA copy number and ATP production increased the proportion of advanced zygotes on D2 and transferable blastocysts on D8.

4.5 Conclusion

This chapter assessed the beneficial effects of 2.5 mM L-carnitine in the presence of certain fatty acids (25 µM palmitic,15 µM stearic and oleic, 5 µM linoleic and alpha-linolenic acids) on embryo development. L-carnitine has a beneficial effect on bovine embryo development. It is possibly due to increased mitochondria and therefore increased ATP production which is required for blastocysts formation and development. Moreover, it was observed that in the presence of LC, oocytes released palmitic, stearic and oleic acids to the media rather taking them up to avoid fatty acid accumulation. However, there was not a chance to observe implantation and healthy calving proportions following embryo transfer in this study. Instead, *in vitro* techniques were developed to assess treatment effects on embryo quality up to D8, a time point when most developmentally competent bovine embryos hatch. An extension of these observations would be to generate stem cells and then to derive gastruloids. This would allow the *in vitro* assessment of later stages of development without the need for embryo transfer and further animal experimentation. However, the derivation of gastruloids has not yet been successfully achieved in cattle. This is the topic of consideration of Chapter 5.

Chapter 5 Bovine Embryonic Stem Cell Derivation and Gastruloid Formation

5.1 Introduction

Stem cells represent a unique population of cells that can self-renew and also differentiate into many specialised cell types (Kuijk et al., 2011). They are classified by their ability to differentiate or by their origin (Figure 1.8). Embryonic stem cells (ESC) are derived from the inner cell mass of preimplantation embryos and are classified as pluripotent (PSCs) as they can differentiate into all three germ layers (i.e., ectoderm, mesoderm and endoderm (Biswas and Hutchins, 2007)). Livestock ESCs lend themselves to being genetically modified with agricultural and biomedical applications, with the promise of generating gametes *in vitro*, and additionally for understanding cell-fate decision during early embryo development (Soto and Ross, 2016). ESCs are classified according to the cells from which they are derived; those derived from the ICM of early embryos are referred to as 'naïve' and those from the epiblast of advanced embryos (EpiSC) are referred to as being 'primed' (Nichols and Smith, 2009). Due to stability or long-term maintenance of pluripotency, the establishment of naïve ESCs remains challenging in cattle (Blomberg and Telugu, 2012). Derivation of stable bovine EpiSCs has been attempted for many years, but successful derivation has only recently been reported (Bogliotti et al., 2018). Inhibition of the WNT signalling pathway and stimulation of FGF signalling were combined with mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers to establish primed bESC lines from *in vitro* produced embryos (Bogliotti et al., 2018). However, due to MEF cells representing an undefined culture environment, more defined media and synthetic biomolecules have since been employed for coating plates and culturing cells. And recently, under the defined media condition EpiSC were successfully derived from advanced bovine, porcine and ovine blastocyst (Kinoshita et al., 2021a).

Mouse embryonic stem cell research led this field (Evans and Kaufman, 1981, Evans, 2011) and stimulated interest in deriving such cells from other species. However, due to differences in both embryo development and cellular signals, it has not been a good model for bovine stable stem cell derivation (Blomberg and Telugu, 2012). Whilst cell polarization starts at the 8-cell stage in the

mouse, it occurs at the 16-cell stage in human, pig and cattle embryos (Alberio, 2020). Moreover, the epiblasts exhibit an egg-cylinder shape in the mouse. However, in other animals including pig and human embryos the epiblast forms a flat disc (Alberio et al., 2021) as well as cattle (Maruotti et al., 2012). These similarities between pigs, cattle and humans may allow us to understand human gastrulation as accessibility to human embryos are restricted due to ethical issues.

During early embryo development, following cell polarization at the morula stage, the first lineage specification occurs, and the outer cells split from the remaining cells to form the trophectoderm (TE); the remaining cells form the inner cell mass. The second lineage specification occurs within the ICM at the blastocyst stage, and specific cell types are formed which give rise to the pluripotent epiblast (EPI) and hypoblast (Springer et al., 2021). During bovine blastocyst development, inhibition of ERK and WNT signalling prevents hypoblast formation without affecting the epiblast (Canizo et al., 2019). However, during stem cell culture for maintenance of the undifferentiated state, FGF/ERK signalling and activin A are required in human and mouse EpiSC (Greber et al., 2010). Lack of these signals leads to EpiSCs reverting back to pre-implantation ESC-like stages and/or to differentiate to neuronal cells when cultured in MEF-conditions or in N2B27 medium (Lanner and Rossant, 2010).

Stem cell culture can lead to apoptosis during splitting to dissociate colonies. In hESCs, addition of 10 μM ROCKi reduces apoptosis and increases colony formation following splitting by increasing the survival rate of dissociated cells (Watanabe et al., 2007). Therefore, while plating ICMs, and following splitting of each passage, ROCKi is widely used in bEpiSC culture (Xiao et al., 2021).

Characterization of stem cells is done by various methods such as immunostaining, gene expression analysis and embryoid body (EB) formation. Immunostaining determines the presence of markers as well as gene expression. It is known that *OCT4* and *SOX2* are expressed in the epiblast and *NANOG* is expressed in the ICM in cattle (Aguila et al., 2022). Based on the characteristic features of these cells, these proteins/genes are known as pluripotency markers. One of the surface markers is SSEA-4; expression of which commences with differentiation in mouse ESC (mESC) (Martin, 1981). Human ESC (hESC) typically express SSEA-4 during the pluripotent stage

and its expression disappears during differentiation (Fenderson et al., 1987). In cattle, SSEA-4 expression was observed during the pluripotent stage (Roach et al., 2006). Other surface markers include TRA-1-60 and TRA-1-81, which are also expressed characteristically in hESC (Henderson et al., 2002) and their expression was determined also in buffalo EpiSC (Shah et al., 2015). ESCs should give rise to the three germ layers. Therefore, *in vivo* teratoma test has been applied for many years as it includes all germ layers. However, to decrease animal testing, *in vitro* techniques were developed and embryoid bodies (EB) generated (Stachelscheid et al., 2013). EBs are formed under specific conditions by aggregation and differentiation of stem cells, and they include the three germ layers. It was used a model of early embryo development; however, they were typically disorganized three-dimensional aggregates (Simunovic and Brivanlou, 2017). More recently, gastruloid formation has emerged as a model of differentiation into mesoderm and endoderm layers. Therefore, *in vitro* gastruloid generation allows us to understand the gastrulation process in terms of signalling, metabolic features and progression prior to organogenesis. Human and mouse gastruloids have been generated based on a protocol where SCs are treated with the WNT signalling agonist, Chiron. They are compacted and aggregate within a few hours (Moris et al., 2020); and after 24 hr are termed 'pre-treatment' (PT). Following aggregation, once the cells are seeded, they break symmetry and start to elongate. Specific genes of the three germ layers have been monitored and the migration of cells to specific ends recorded (Martyn et al., 2019). This gives us an idea about body formation and cell-fate decisions determining the subsequent steps of embryogenesis. However, due to limitations of human sources and ethical issues, bovine gastruloids should be considered as there are differences between humans and mice.

To the best of our knowledge, no previous studies have successfully generated bovine gastruloids. The current study, therefore, sought to derive stable bovine stem cells from advanced blastocysts in defined media. This was undertaken to establish gastruloids to better understand developmental processes beyond the implantation stage. In turn, this would facilitate future studies to assess long-term developmental implications of alterations in, for example, lipid metabolism during pre-implantation development, the subject of investigation in earlier chapters in this thesis.

5.2 Material and Methods

In this chapter Day 8 blastocysts (IETS Stage 7, 8 and 9) were generated from OPU-derived oocytes matured in the PVP-based serum free defined medium as described previously (Chapter 3; Section 3.3.1). Two donors were selected from the OPU study, and their blastocysts used to derive stem cells. Animal numbers were identified based on the last two digits of their ear-tag; i.e., 99 and 30. Donor 99 was 15 months old, donor 30 was 16 months old. Both were Holstein-Friesians. Both of their blastocysts were identified as male. Following stable stem-cell derivation, gastruloid formation was performed and the process repeated three times. In each replicate stem cells, pre-treated cells, 48h and 72h gastruloids were collected for gene expression and for immuno-staining. Also, the antero-posterior (A-P) axis of 48h and 72h gastruloids were observed. The ploidy status of stem cells was determined by Karyo-mapping (Tutt et al., 2021).

5.2.1 Stem Cell Derivation

5.2.1.1 Inner Cell Mass (ICM) Plating

The method for stem cell establishment was based on the recent report by Kinoshita et al. (2021b), and is described in the following paragraph. On the day of immunosurgery, 4-well plates were coated with a mixture of 500 µl L511 (Cat No AMS 892.021, Amsbio, UK) and PBS per well and incubated for 1 h at 39ºC. Meanwhile, culture media was prepared (Table 5.1) containing with 10 µM Y-27632 dihydrochloride, which is known as ROCK inhibitor (ROCKi) (Cat no 1254, Tocris, UK). Following incubation, coating media was removed by vacuum pump, and culture media placed into the wells. For immunosurgery (IS), Day 8 blastocysts (IETS Stage 7, 8 and 9) were selected. While Stage 9 embryos were directly placed into 50% anti-bovine sera (Sigma - Anti-Bovine Serum antibody produced in rabbit, whole antiserum, Cat No B8270-2ML) containing drops for 30 min, Stages 7 and 8 embryos were placed after zona pellucida removal (Chapter 2; Section 2.2.4). Blastocysts were washed with wash media (DMEM-0.3 mg/ml BSA) three times and incubated in complement (Sigma - Complement sera from guinea pig, lyophilized powder, Cat No S1639-5ML) for 15 min, then washed again three

times with fresh wash media. In the last drop, lysed trophectoderm were removed by mouth pipetting and pure ICM was obtained. Before plating, the ICMs were washed with culture media to remove the wash media around the ICMs, then incubated at 39˚C (7% CO_2, 5% O_2) for 48h continually. At the end of incubation, hypoblasts were scratched to remove with a glass pipette and, after refreshing the culture medium, they were returned to the incubator. Until the colony became confluent, media was changed, and cells checked every two days.

When the colonies became confluent, 12-well plates were coated on the day of splitting. To split the colony, spent media was removed by vacuum pump and the colony washed once with PBS. After removing PBS by vacuum pump, 500 µl EDTA was added to each colony and incubated for 3 min. To block the activation of EDTA, 4.5 ml standard media was added. The solution, with a final volume of 5 ml, was placed into falcon tubes for centrifuging at 300 x g for 3.5 min. Following centrifugation, supernatants were removed by pouring. Then, cell pellets were resuspended with culture medium and plated in coated dishes and media changed daily. Only on each day of splitting, ROCKi was added to the culture media. In subsequent passages, colonies were split into 6-well plates at a 1:10/1:20 ratio depending on growth rate.

Table 5. 1 Stem cell culture medium ingredients with catalogue numbers. Base media was mixed with AFX to obtain culture media

N2B27- Base Media	**For 50 ml**
DMEM/F12 medium (Gibco 11320074)	24 ml
Neurobasal medium (Gibco 21103049)	24 ml
B-27 supplement (Gibco 17504001)	1.0 ml
N-2 supplement (Gibco 17502001)	0.5 ml
GlutaMAX (Gibco 35050038)	0.5 ml
2-Mercaptoethanol (50 mM) (Gibco 31350010)	0.1 ml
AFX	
Activin A (Qkine Qk001) (500 µg/ml)	20 ng/ml
zFGF2 (Qkine Qk002) (80 µg/ml)	12.5 ng/ml
XAV939 (Sigma X3004) (20 mM)	2 µM

5.2.1.2 Cryopreservation and thawing

The colonies were dissociated and centrifuged as described previously for stem cell derivation for splitting. Supernatants were decanted and cell pellets resuspended with 500 µl NutriFreez™ D10 Cryopreservation Medium (Cat No 05-713-1, Sartorius, UK) per colony, then transferred to cryovials (Cat No 72.380.002, Sarstedt UK) and frozen in CoolCell containers (Cat Number; CLS432002, Sigma Aldrich (Dorset, England)). Containers were stored in -80ºC for up to 48h then transferred to liquid nitrogen for long term storage.

Vials were thawed in a 37˚C water bath until only a small piece of ice was left in the tube, then cell-cryopreservation media mixture were transferred to 4.5 ml wash media and spun 3.5 min at 300 x g. Supernatants were decanted, and cells resuspended with culture medium which contained 10 µM ROCKi. Colonies were placed carefully into pre-coated dishes to avoid disrupting the clumps.

5.2.1.3 Immunocytochemistry

5.2.1.3.1 Loading Chamber

Colonies were split into coated L511 chamber slides (Nunc Lab Tek 8 Well Glass Slide) which were purchased from SLS, UK (Cat No 177402). Splitting amounts were calculated for chamber surface (i.e., 0.81cm^2 per well surface of chamber). For a single chamber it was 8 x 0.81= 6.5 cm^2. For a 1.6 splitting ratio; 0.8 µl is required for 9.6 cm^2 (6 well plate). For 6.5 cm^2 0.5µl was required. Following incubation with EDTA and blocking EDTA with washing media, the required amounts of cells were transferred to 15ml tubes and spun down. After discarding the liquid, cells were resuspended with culture media (300µl per each well) and plated in the chamber. Until establishing sufficient colonies, media was changed daily, and incubation continued.

5.2.1.3.2 Fixation

Four consistent wells were chosen to fix, then the spent media were aspirated. Therefore, colonies were washed once with 300µl PBS. 200µl 4%PFA was added per well after removing PBS. Chamber slides were placed on the rocker for 10-15 min while covered by aluminium. PFA was removed and

colonies were washed twice with PBS. On the 3rd occasion, 400 µl PBS was added to colonies and chambers wrapped with film then stored in the fridge for up to one week.

5.2.1.3.3 Staining

After pouring the PBS, 0.5% Triton-PBS was added to each well for permeabilization for 10-15 min. Permeabilisation solution was discarded and the lines were blocked with 5% BSA-PBS+10% Donkey Serum for 45 min at room temperature. Fixed wells were incubated with a combination of primary antibodies (Appendix IV) overnight at 4 °C.

After removing the chambers slides were washed three times for 5 min with PBS- 0.1% Tween-20. Cell lines were incubated with secondary antibodies in the dark for 45 min at room temperature. Another wash was applied (three times for 5 min with PBS- 0.1% Tween-20).

3 µl mounting media (Fluoroshield with DAPI) was placed on each defined wells of the slide, coverslipped and then sealed with nail varnished. Before imaging, slides were incubated in the fridge for 15-30 min, for imaging ZEISS microscope was used.

Primary antibodies were tested on control tissues (fibroblasts) and the secondary antibodies showed no unspecific binding.

5.2.2 Karyo-mapping

Karyo-mapping (Tutt et al., 2021) was undertaken to identify the ploidy status of cell lines. Samples were collected at passage 6, 12 and 24. For whole genome amplification (WGA) from single cells, REPLI-g single cell kit (Qiagen, Cat No 150345) was used. Sample was collected in 4 µl PBS which was supplied by company. On the day of assay 3 µl buffer D2 (denaturation buffer) was added on the samples, they were mixed and quickly centrifuged. Samples were incubated at 65°C for 10 min to desaturate the DNA. Following the incubation 3 µl of stop solution was added and they were mixed and quickly centrifuged. 40 µl master mix was added to each sample, they were mixed and centrifuged briefly again. Samples were incubated at 30°C for 4 h and to inactivate the enzymes at 65°C for 3 min again. From 50 µl of samples, 22 µl were placed into 96 well plate and store at -80°C until to be sent to Neogen to

do SNP analysis (GGP 100K), which was then Karyo-mapped by the group at the University of Kent.

5.2.3 Gastruloid Formation

5.2.3.1 Pre-treatment

Stem cell colonies were thawed and passaged twice before pre-treatment (PT) to adapt. PT was performed when they became 60% confluence. 5 µM Chiron (30 mM stock), 12.5 ng/ml (80 µg/ml stock) FGF in N2B27 was prepared as PT media and allowed to reach room temperature. Spent media was aspirated and colonies washed once with PBS. PT media was added to wells and the time recorded.

5.2.3.2 Seeding

24 hr after PT, the required volume of E6 media (40 µl/well) (Cat No A1516401, Thermo Fisher, UK) including 3 µM Chiron and 10 µM ROCKi (ROCK inhibitor) was prepared. Spent media was aspirated and colonies washed with PBS. 0.5 ml TrypLE Express Enzyme (ThermoFisher, Cat No 12604013) was added and incubated for 8 min to dissociate the cells. During incubation, sterile Strainer (Cat No 22363547, Thermo Fisher, UK) was placed on 50 ml tube and 3.5 ml wash media was added to top up final volume to 5 ml (1.5 ml solution will be added) and to make the Strainer wet to prevent cell adhesions to surface of Strainer. 1 ml pre-warmed wash media was added to each dish to block triple activation and pipetted many times to make the cells single. 1.5 ml solution (cells, triple, and wash) was filtered through Strainer and centrifuged at 300 x g for 3.5 min. Supernatants were discarded, and cells was mixed with the leftover wash media. To determine cell concentration, 1 µl cell suspension was diluted with standard media at 1:20. According to cell concentration, required volume of cells (final concentration of 20 cells/µl) was added to media and mixed. To load 96 well plates (Cat No 650970, Greiner Bio-One Ltd, UK), cells were poured into reservoirs (Cat No DD97801, SLS, UK), 40 µl was added to each well using a multi-channel pipette. Plates were wrapped with stretch film to avoid contamination for centrifugation. Plates

were centrifuged at 16 x g for 2 min. After capturing the pictures of the seeded cells, plate was returned to incubator.

5.2.3.3 Media changing and gastruloid collection

Following 24 h after seeding, 150 µl pre-warmed E6 was added to each well. Media was changed daily using multichannel pipette. While aspirating, the pipette was inclined at 45 degrees to the plate to avoid collecting gastruloids.

To investigate the developmental process of gastruloids, they were collected at two different time points; 48 h and 72 h for staining and gene expression. Gastruloids were transferred to 100mm dishes containing 4 ml wash media using a multi-channel pipette under the microscope. At each time point, four gastruloids were washed with BSA twice and fixed in 4%PFA-PBS solution for 2 h at 4°C for staining. Remaining gastruloids were snap frozen for qPCR.

5.2.3.4 Staining

Gastruloids were washed in 400 µl washing solution (0.2% triton with PBS) three times each for 20 min on the rocker, then blocked in blocking solution for 1 h at room temperature (10% Donkey serum in 5%BSA-PBS and add 0.2% triton). They were incubated with pre-selected primary antibodies overnight in the fridge. The following day, they were washed with washing solution three times each for 20 min, and incubated in secondary antibodies (Appendix IV), DAPI mixture for 2 h at room temperature. Following incubation, they were washed again as previously described and mounted with VECTASHIELD ® Antifade mounting medium (Cat No H-1000-10, 2B Scientific, UK). Tape was folded and stuck on the slides to avoid squeezing gastruloids.

Primary antibodies were tested on control tissues (liver for T and EpiSC for SOX17) for and the secondary antibodies showed no unspecific binding.

5.2.4 Gene Expression

5.2.4.1 Sample preparation and RNA extraction

For gene expression analysis, EpiSc were collected before and after pre-treatment and gastruloids at 48 h and 72 h after pre-treatment. Following splitting of EpiSc, the leftover cells were collected into tubes and centrifuged for 2 min with 13400 x g. Media were removed by pipetting without disturbing cell pellets, then pellets were washed with PBS-PVP solution and centrifuged again. Following the final spin, PBS-PVS was removed, and pellets snap frozen. At 48 h, 50 gastruloids were split into two tubes as a duplicate and at 72 h 34 gastruloids were collected into two tubes and prepared in the same manner as stem cells. Before snap-freezing, lysis buffer was added and the tubes vortexed in the cold room. On the day of assay, dynabeads was used to extract RNA and QuantiTect reverse transcription kit (Qiagen Ltd., West Sussex, UK) to synthesis cDNA as described previously (Chapter 2; Section 2.2.5.3). These analyses were replicated three times.

The expression profile of selected genes (Brachyury (*T/BRA*), *EOMES*, *TBX6*, *SOX2* and *FOXA2*; Table 5.3) was established to characterise gastruloid development. *BRA*, *TBX6* and *EOMES* were selected as mesoderm markers, *SOX2* represents the ectoderm and *FOXA2* was chosen as an endoderm marker.

5.2.4.2 Housekeeping gene selection (HKG)

Following RNA extraction, all the samples were diluted to 7 ng/µl. Based on this calculation each reaction consisted of 14 ng/µl RNA. To identify the most stable HKG(s) for normalisation, two ribosomal genes (*RSP18* and *RPL19*) and β-Actin (*ACTB*) were run by qPCR. Zhou et al. (2010) indicated that ribosomal genes are more stable during differentiation, and ACTB is commonly used in many studies as a reference gene (Lin and Redies, 2012).

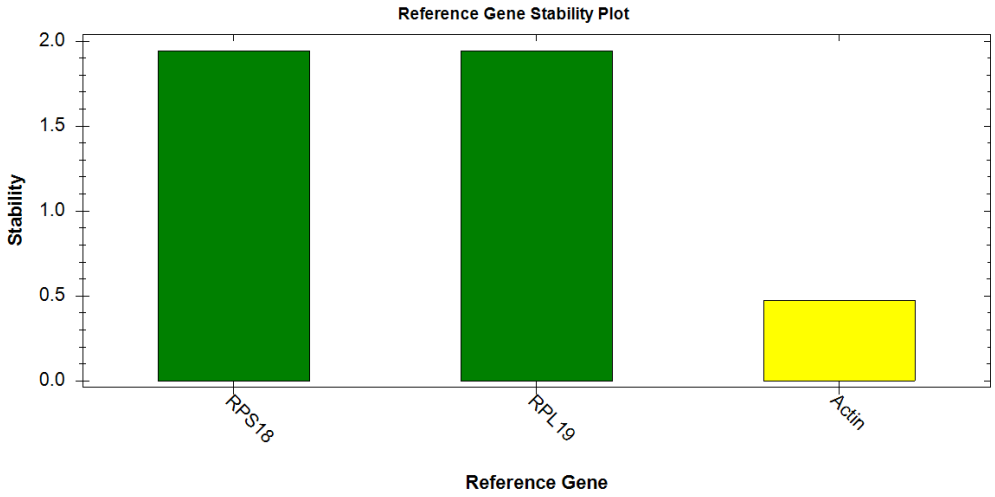


Figure 5. 1 Stability of reference genes (HKG) was measured in Bio-Rad system based on the changes of candidate HKGs in stem cells, in pre-treated cells, and in 48hr and 72hr gastruloids. Based on the qPCR gene study, *RPS18* was selected as a reference gene.

Table 5. 2 Selected HKGs, M values and stability outputs.

Gene Name	Evaluation	Average M Value (Acceptable range <1.5)	Stability (Ln(1/AvgM))
RPS18	İdeal (Santiquet et al.)	0.143	1.943
RPL19	İdeal (Santiquet et al.)	0.143	1.943
ACTB	Acceptable (Yellow)	0.622	0.474

Table 5. 3 Marker genes selected for expression analysis by qPCR.

Gene	Accession Number	Primer Sequence	Product Length (bp)	PCR Efficiency (%)
BRA	NM_001192985	F: TTGAGCTTGTTGGTGAGCTTG	203	104.4
		R: GCCATGTACTCCTTCCTGCT		
EOMES	NM_001191188	F: TCCCATGCCCTGGGGTACTA	107	89.0
		R: GAGGTCCATGGGAGTCCAGC		
TBX6	NM_001192630	F: CATACACTTGGTGCGGGCAG	121	99.8
		R: TTGCGTGATCCGTGGGTTCT		
SOX2	NM_001105463	F: ACATGAATGGCTGGAGCAAC	166	96.5
		R: ATGTAGGTCTGCGAGCTGGT		
FOXA2	XR_003037795	F: GCAGCACCATCACAGCCATC	150	91.2
		R: GAGGTATCCAGGCCCGCTTT		
RPS18	NM_001033614	F: GGATCAGGTGGAACGTGTGAT	101	100.1
		R: ATTTCCCGTCCTTCACGTCCT		

5.2.5 Statistical analyses

Transcript expression was analysed using general linear models within GenStat (GenStat 19th Edition, 2018, VSN International Ltd, Hemel Hempstead, UK). Terms fitted to these models were ‘Donor’ (of the cell lines) and ‘Stage’, representing the extent of cell differentiation. The experiment was replicated three times and therefore blocked by replicate. Residual plots confirmed that the data were normally distributed, the variances homogeneous and mean differences proportional. Data are presented as means ± Standard Error of Means (SEM).

5.3 Results

5.3.1 Stem Cell Derivation

Following immunosurgery, ICMs were incubated with ROCKi, XAV939, actin and FGF for 48hr. At the end of incubation, it was observed that round cells, which are hypoblasts, were formed (Figure 5.2), and they were scratched using a sterile pipette to remove them. After incubation for another 24hr no more hypoblasts formed, as XAV939 would have inhibited WNT signalling required for hypoblast formation. Cell lines underwent media change at 48hr intervals, and pure epiblasts obtained within two weeks. These flat and attached cells formed colonies (Figure 5.3A). Cell shape did not change following splitting and these cells continued to grow (Figure 5.3B) until passage 24 (P24). Karyo-mapping data indicated that all cell lines were euploid.

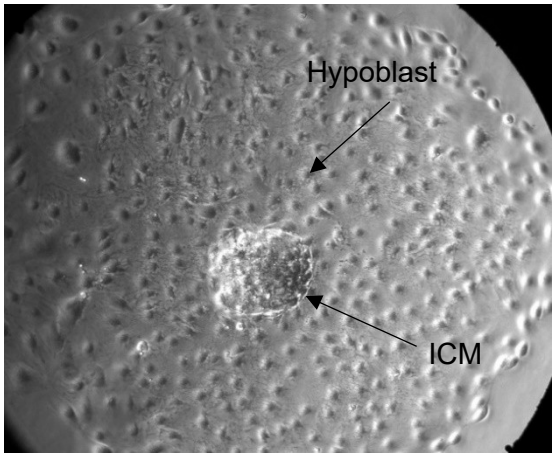


Figure 5. 2 48 hr after ICM was plated following IS. (X20 magnification). Hypoblasts formed during the 48hr incubation period. Prior to media change they were removed by scratching with a sterile glass pipette.

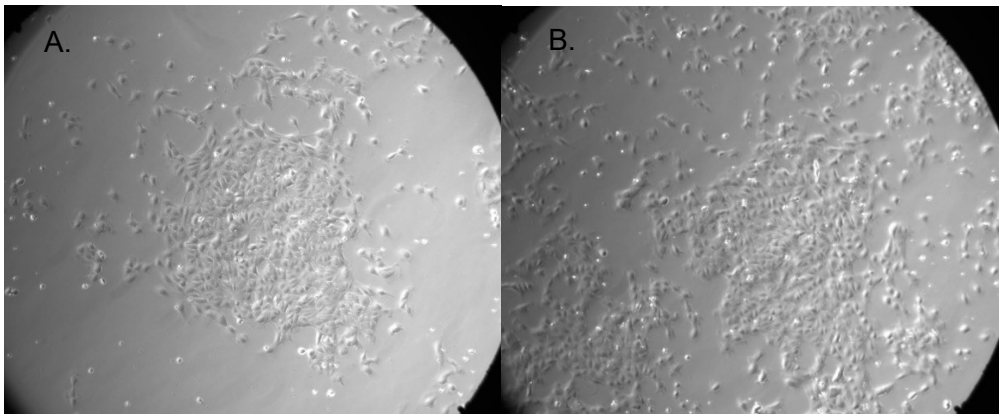


Figure 5. 3 bEpiSC colonies; A, Passage 1 (X10 magnification) on day 10 (X10 magnification) and B, P2 (x10 magnification)

5.3.2 Stem Cell immunostaining

Immunostaining data indicated that derived stem cell lines express pluripotency (Figure 5.4) and surface (Figure 5.5-7) markers which are the characteristic of stable stem cell lines.

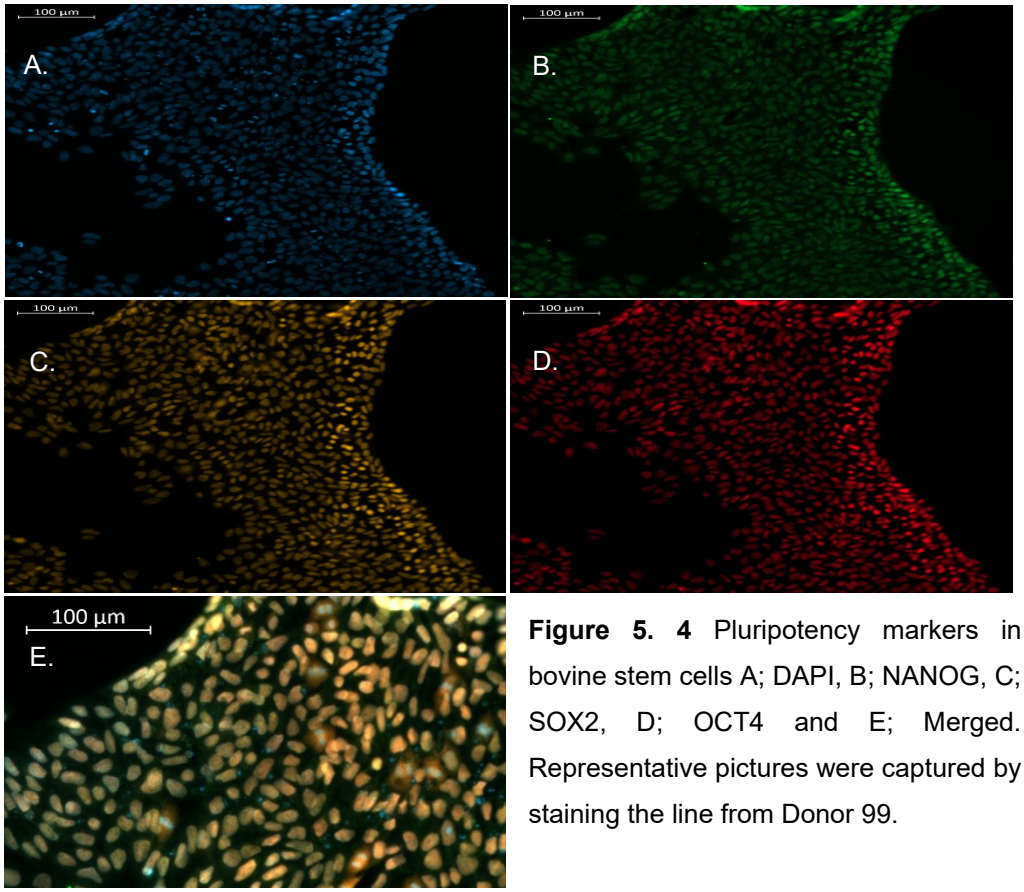


Figure 5. 4 Pluripotency markers in bovine stem cells A; DAPI, B; NANOG, C; SOX2, D; OCT4 and E; Merged. Representative pictures were captured by staining the line from Donor 99.

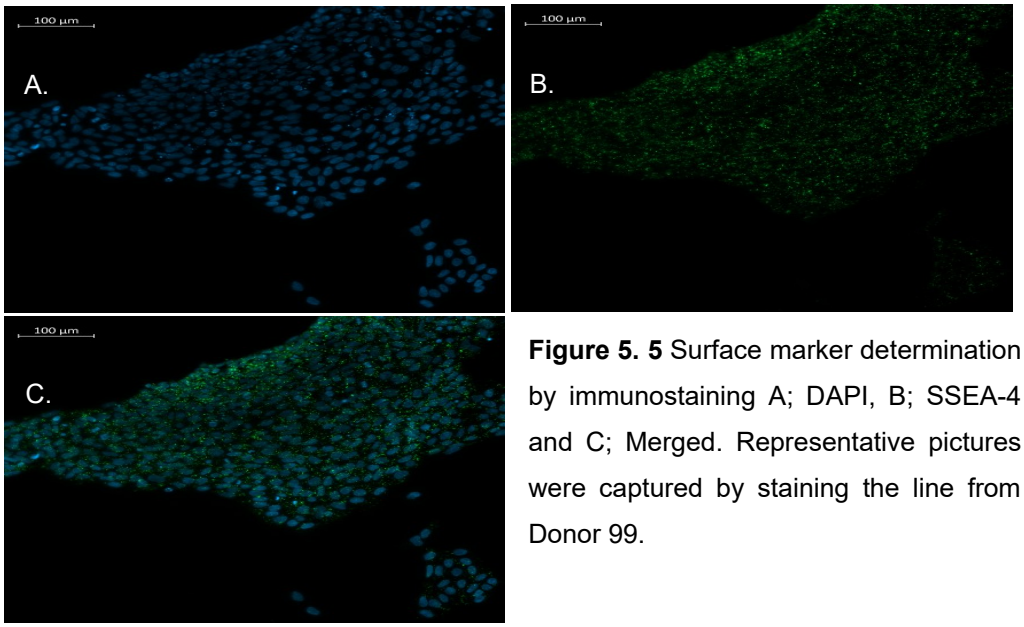


Figure 5. 5 Surface marker determination by immunostaining A; DAPI, B; SSEA-4 and C; Merged. Representative pictures were captured by staining the line from Donor 99.

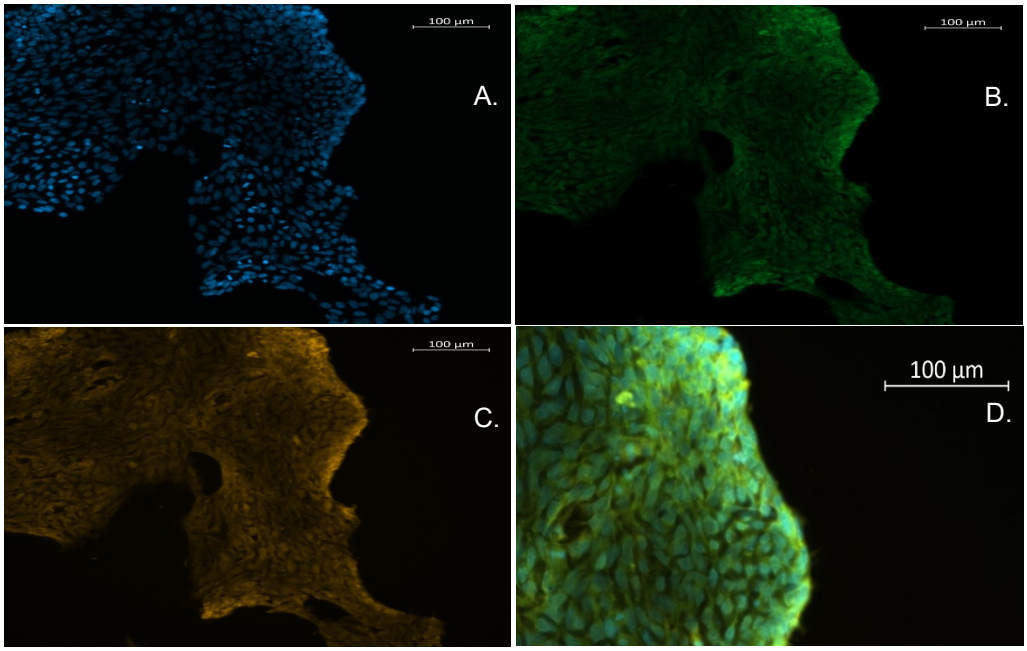


Figure 5. 6 Immunostaining images. A; DAPI, B; FOXA2, C; TRA-1-60 and D; Merged. Representative pictures were captured by staining the line from Donor 99.

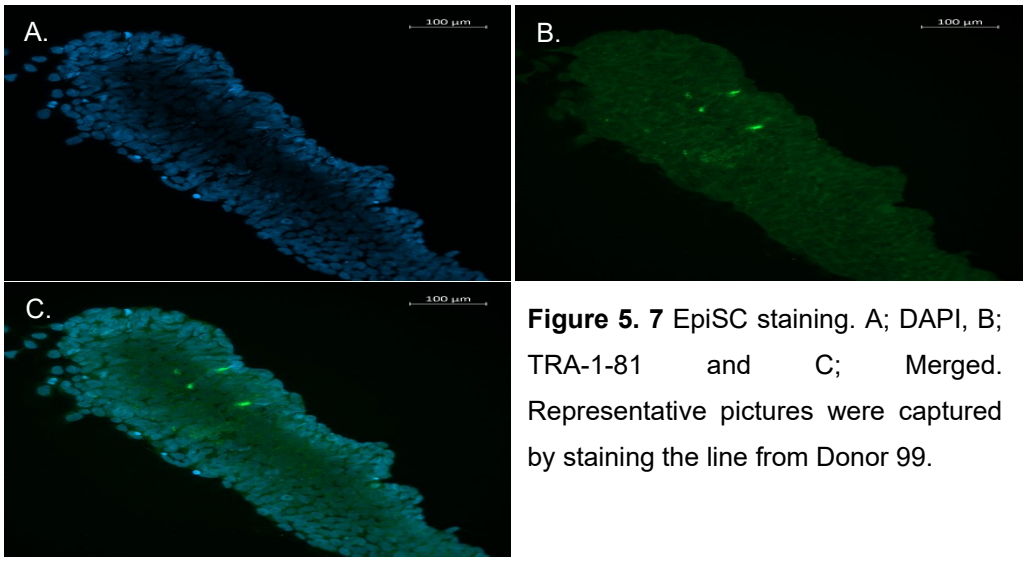


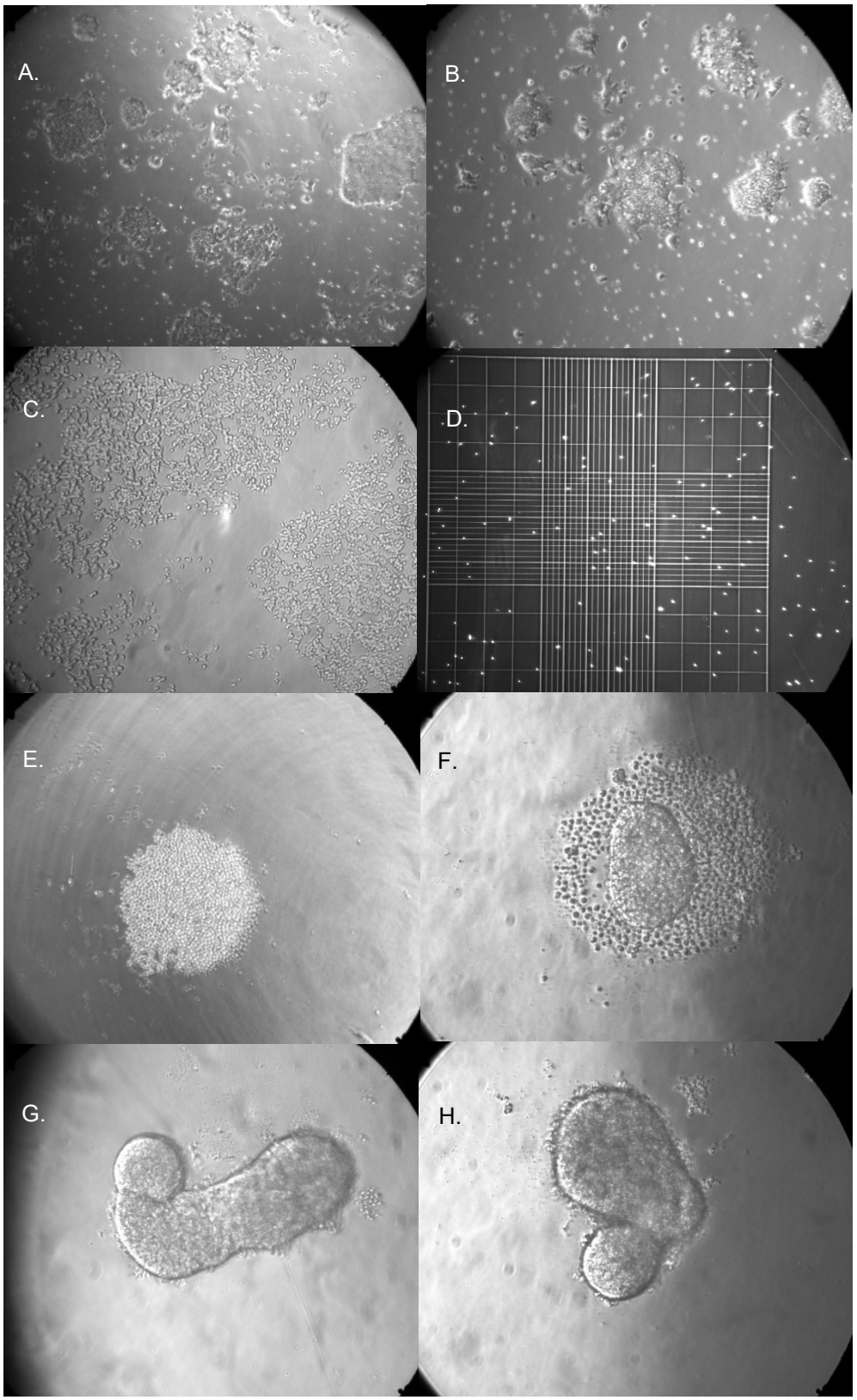
Figure 5. 7 EpiSC staining. A; DAPI, B; TRA-1-81 and C; Merged. Representative pictures were captured by staining the line from Donor 99.

5.3.3 Gastruloid Formation

In the current study the very first bovine gastruloid formation was observed and the findings indicate that, during pre-treatment, 5µM Chrion and FGF concentration in N2B27 medium are sufficient to switch on WNT signal in bovine stem cells. Following pre-treatment cell shape changed (Figure 5.8 B). Whilst stem cells are flat (Figure 5.8 A), pre-treated cells are chambered, and the edges of colonies are not smooth as seen in stem cells. To seed these cells, they were incubated with TrypLE Express (Cat No: 12604013 Thermo Fisher, UK) for 8 min and cells looked shiny (Figure 5.8 C). This view is an indicator that cells are dissociated and ready to be counted (Figure 5.8 D). Following centrifugation, seeded cells were gathered in the middle of well (Figure 5.8 E) as that configuration is also important for aggregation. 24hr gastruloids were observed as egg-shaped (Figure 5.8 F). Gastruloid elongation was observed visibly at 48hr stage (Figure 5.8 G) and some of the structures remained elongated until the 72hr stage (Figure 5.8 H).

As with stem cell culture medium, no undefined chemicals such as serum were added, therefore gastruloid shapes were smooth and no abnormalities were observed.

Figure



5. 8 Gastruloid formation. A; before PT x5, B; after PT x10, C; following incubation with TrypLE Express x10, D; cell counting, E; seeding (0hr), F; 24hr gastruloid x20, G; 48hr gastruloid x20, H; 72hr gastruloid x20

5.3.4 Gastruloid Gene Expression

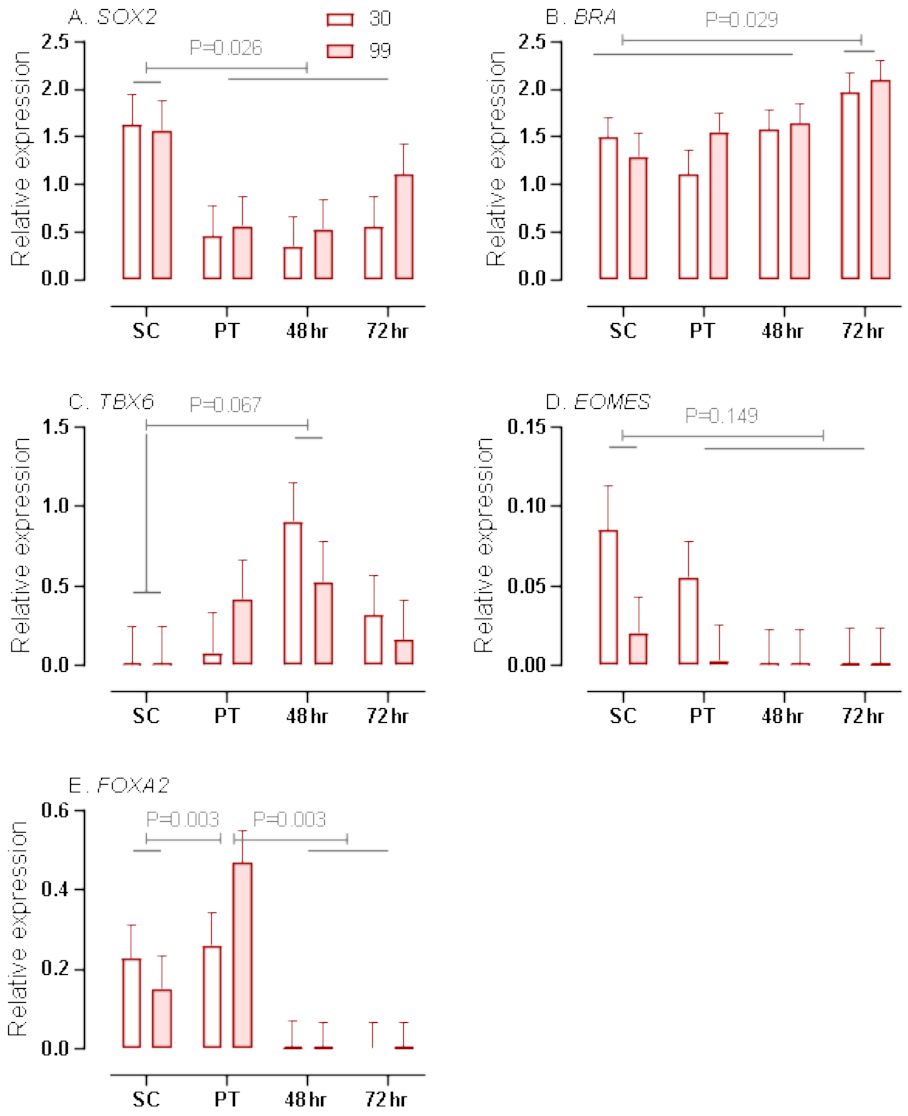


Figure 5. 9 Relative gene expression in stem cells (SC), following pre-treatment (PT), and 48 and 72 hr gastruloids. Lines generated from Donors 30 and 99.

Transcript expression (qPCR) data indicated that, as an ectodermal marker, *SOX2* expression was highest in undifferentiated stem cells (Figure 5.9A). However, its expression decreased (P=0.026) following pre-treatment and no difference was observed between 48hr and 72hr gastruloids. These changes were consistent in both donor lines.

One of the mesodermal markers, *BRA* expression (Figure 5.9B) was observed from stem cell stage to 48hr gastruloid, and its expression was not altered by differentiation. In 72hr gastruloids *BRA* expression level was statistically

upregulated compared to other stages (p=0.029). Between donors no difference was observed.

Another mesodermal marker, *TBX6* (Figure 5.9C) was not observed at the SC stage and its expression appeared to increase (P=0.067) following PT until the 48hr gastruloid stage. Following the 48hr stage its expression appeared to decrease up to the 72 hr gastruloid stage. The expression of a further mesodermal marker, *EOMES,* appeared to decrease (P=0.149) from the SC stage through to the 48 and 72 hr gastruloid stages (Figure 5.9D).

Expression of the endodermal marker *FOXA2* increased (P=0.003) between the SC and PT stages of development before decreasing again up to the 72 hr gastruloid stage (Figure 5.9E).

5.3.5 Gastruloid Immuno Staining Result

Bovine gastruloid staining indicated that, in 48 hr gastruloids, BRA and SOX2 expression was located at the same pole which is expected to be posterior pole (Figure 5.10). Limited migration of cells expressing BRA and SOX2 was observed at 72hr. SOX17 expression was not observed in 48h gastruloids, however, it was visible throughout the gastruloids by 72h.

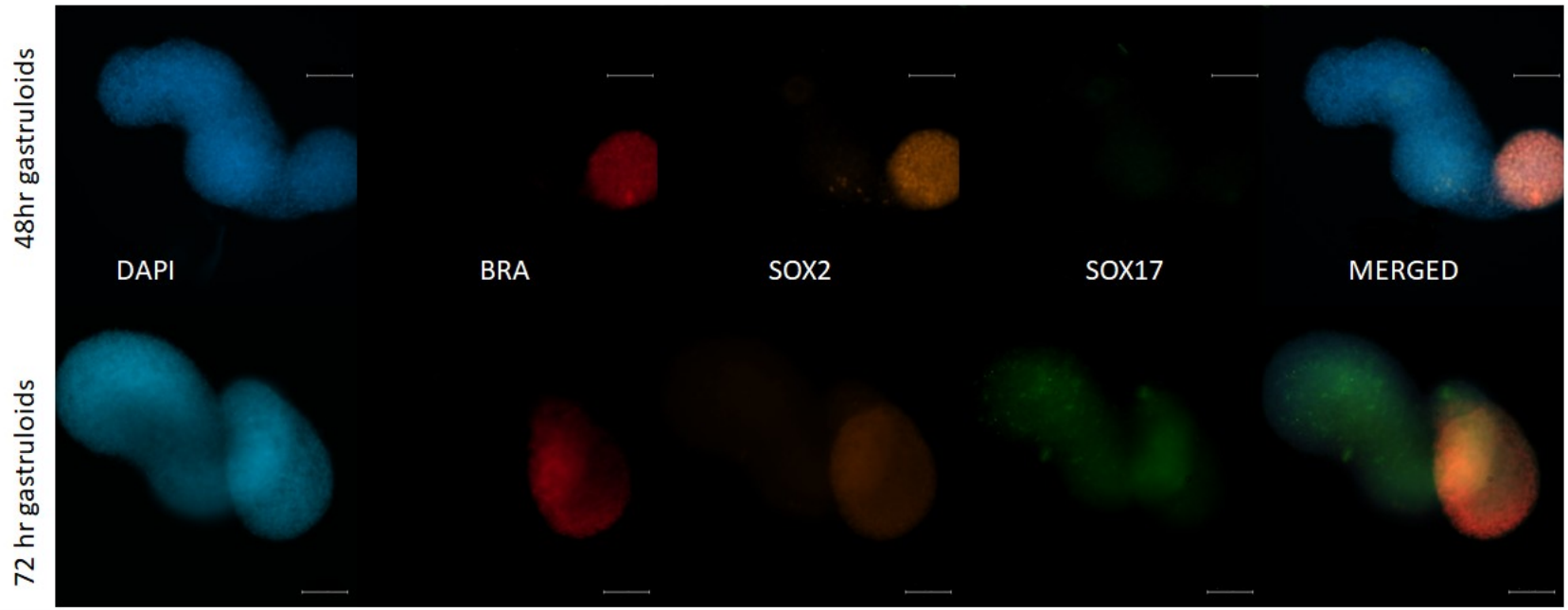


Figure 5. 10 Representative immuno-staining gastruloid which were generated from donor 99 at 48hr and 72hr stages, Scale bars are 100μm.

5.4 Discussion

In this chapter, bEpiSC were derived from advanced D8 blastocysts which were generated in defined media (PVP-based only). Cells were cultured until passage 24 and they remained undifferentiated state and were euploid. Characterisation of bEpiSC indicated that they have pluripotency and surface markers. By differentiating bEpiSCs with Chiron and FGF, very first bovine gastruloids were formed and their developments were observed morphologically up to 72 hr stage and their gene expressions change and line formations were monitored.

5.4.1 Embryonic stem cell derivation

EpiSC derivation differs between species as the required cell signalling pathways appear to differ. Since the discovery that 2i derivation methods (which inhibit mitogen activated protein kinase (MAPK) and glycogen synthase kinase-3 (GSK-3), and activates WNT signalling), can be used for naïve ESC in mice (Mulas et al., 2019), these approaches were tried in other species including cattle (Ozawa et al., 2012) and pigs (Haraguchi et al., 2012). However, these studies failed to maintain viable ESCs for long periods. In humans it was understood that MAPK activation is required for derivation of hEpiSC (Gafni et al., 2013). Derivation of EpiSC occurs successfully under serum-free conditions in cattle (Bogliotti et al., 2018) and pig embryos (Choi et al., 2019). In cattle and pigs, derived stem cells were cultured longer than 40 passages and maintained their undifferentiated stage successfully during this period. However, MEF or serum replacement were still required to maintain pluripotency. Here we followed the protocol published by Kinoshita et al. (2021b), where fully defined conditions were reported and obtained undifferentiated state bEpiSC. Differentiation leads to the formation of cells of the three germ layers. It is known that culture environments cause epigenetic changes and affect cell fate (Badr et al., 2007). Because of this in this experiment, serum was removed from culture media in the current study and replaced with mostly defined chemicals and the result indicates that cells were euploid until the last passage.

WNT signalling commences when the WNT protein attaches to cell surface receptors. When ligand-receptor attachment occurs the beta-catenin destruction complex (which consists of cytosolic proteins glycogen synthase kinase 3 (GSK3), AXIN, adenomatous polyposis coli (APC) protein and casein kinase 1 (CK1)) is blocked by TNKS (Huang et al., 2009). Increased beta-catenin can reach the nucleus and bind to T-cell factor (TCF), which is known as a transcriptional receptor of DNA-binding transcriptional factor (Moon, 2005).

WNT signalling plays an important role in the derivation of naïve ESCs in humans and mice (Ying et al., 2008, Gafni et al., 2013). In serum-free media, inhibition of the WNT signalling by the addition of inhibitor of WNT production 2 (IWP-2) (which blocks the secretion of WNT protein) changes naïve to primed ESCs in both humans and mice (Xu et al., 2016, Gafni et al., 2013). Addition of XAV939 (which is known as WNT signal inhibitor (i.e., it inhibits tankyrase (TNKS)) to naïve hESC culture decreases naïve pluripotency factors (Rostovskaya et al., 2019). However, addition of XAV939 with Activin and FGF2 maintains stem cells in a primed pluripotent state. Moreover, mESC and mEpiSC comparison studies indicate that, while mESC requires WNT signalling to be triggered, mEpiSC requires activin/nodal signalling to maintain their naïve and primed states (Brons et al., 2007). Activin/nodal signalling is required to maintain pluripotent primed state of epiblasts during development in humans and pigs (Vallier et al., 2004, Alberio et al., 2010). Moreover, absence of activin/nodal signalling causes failure of mesoderm and definitive endoderm formation following implantation *in vivo* (Camus et al., 2006). Additionally, the culture of bEpiSC in the presence of activin and FGF indicated that maintenance of pluripotency was higher than activin alone (Maruotti et al., 2012), and this suggests that these two factors are synergetic.

OCT4, SOX2 and NANOG are transcriptional factors that are required for maintenance of pluripotency (Silva and Smith, 2008), and these markers were detected in bEpiSC. As a classification method, immunostaining is widely used to determine pluripotency of stem cells (Roach et al., 2006). In bEpiSC, OCT4, NANOG and SOX2 were identified as strong pluripotency markers. Moreover, surface markers became hallmarks for identification of stem cells (Roach et al., 2006). In this sense, SSEA-4, TRA-1-60, TRA-1-81 markers are important, and their presence was detected in our bEpiSC.

5.4.2 Gastruloid Formation

Stem cell classification was confirmed by the *in vivo* teratoma test for many years. However, due to the desire to reduce animal testing, *in vitro* approaches are now favoured. Over the years embryoid body (EB) formation has been used to determine the differentiation ability of stem cells into the three germ layers (Itskovitz-Eldor et al., 2000). Under non-adhesive, undefined culture conditions, stem cells form three-dimensional aggregates which are called EBs, and their development stage represents early embryogenesis by expressing key genes that are observed during embryo development (Stachelscheid et al., 2013).

Under defined culture conditions mESC form round aggregates which break the symmetry, and antero-posterior polarity and primitive streaks are formed (Martyn et al., 2019). These aggregates are called gastruloids and their development represents embryo gastrulation. Symmetry-breaking processes in the mouse are controlled by extra-embryonic tissues (especially the visceral endoderm) by releasing transforming growth factor β (TGF-β), BMP and activating nodal and WNT signalling pathways (Tam et al., 2006). Additionally, it was observed that for symmetry-breaking FGF and WNT signalling actions are required in epiblasts (Barrow et al., 2007).

Based on these *in vivo* findings, gastruloids were generated *in vitro*. After a number of attempts, it was observed that gastruloid formation depends on confluence of the cells or diameter of the colonies before pre-treatment, level of WNT signalling during the pre-treatment period and cell concentration before seeding (Cermola et al., 2021). Moreover, it was observed that culture conditions may affect gastruloid formation such as multiple aggregates formation in one well, and multiple poles and ragged aggregates (Baillie-Johnson et al., 2015, Turner et al., 2017). The reason for failed attempts, however, is still largely unknown. Nevertheless, it is known that stem cell culture conditions affect cell fate and their differentiation ability (Mulas et al., 2019). Failure may be due to undefined stem-cell culture media which contains serum and LIF. Therefore, under defined culture condition, derived bEpiSCs were differentiated by Chiron and very first bovine gastruloids were obtained in this chapter.

In vitro generated gastruloids have been developed as gastrulating embryos and indicate body plan formation. This gives an opportunity to observe gastrulating embryo fate following treatment during IVP. 24hr gastruloids were observed as egg-shaped, elongation was observed visibly at 48hr stage and some of the structures remained elongated until the 72hr stage. 24hr gastruloids were larger and more developed compared to human 24hr gastruloids (Moris et al., 2020).

SOX2 expression decreased following pre-treatment and no difference was observed between 48hr and 72hr bovine gastruloids. Decreased *SOX2* expression following PT indicates that these cells are losing their pluripotency features (Zhang and Cui, 2014).

In a human study it was observed that PT increases expression of mesodermal markers such as *BRA* and *EOMES* (Moris et al., 2020). However, in bovine *BRA* expression was found stable up to 72 hr gastruloid, in which upregulation was observed. Additionally, *EOMES* expression was downregulated with PT in both donors. On the other hand, increased TBX6 with PT may explain the decrease in *EOMES* expression, as *TBX6* encodes transcriptional factors which inhibit neuronal development. In this sense, *TBX6* inhibits *EOMES* expression to prevent early neuronal development (Hadjantonakis et al., 2008).

Expression of the endodermal marker *FOXA2* increased between the SC and PT stages of development before decreasing again up to the 72 hr gastruloid stage. In contrast, a human study indicated that 44hr gastruloids express *FOXA2* (Minn et al., 2021).

In human gastruloids following pre-treatment the presence of mesodermal markers such as BRA and EOMES are observed by immuno-staining (Moris et al., 2020). In the same study, the time-course of gastruloid development was monitored and BRA and SOX2 proteins were identified in posterior cells at 48 and 72h. Similar localisation of the cells which express BRA and SOX2 were identified in the mouse (Beccari et al., 2018). In both experiments these findings were similar to gastrulating embryo changes.

5.5 Conclusion and perspective

This chapter reports the successful derivation bovine EpiSCs from advanced (Day 8) blastocysts in defined medium. Moreover, an advanced method for cell characterization in the gastrulating embryo was achieved. The generation of gastruloids from bovine EpiSC is, as far as we know, the first report in this species. Thus, in future, signalling pathways during gastrulation, antero-posterior axis and body plan can be monitored closely in cattle. Future studies investigating fatty acid metabolism can also be undertaken by addition of lipid metabolic precursors and/or regulators, as gastruloid metabolism up to placenta formation is not well understood.

Chapter 6 General Discussion and Conclusions

6.1 General Discussion

This thesis aimed to improve embryo development by altering the lipid metabolism. Therefore, mainly carnitine groups was under the focus to improve β-oxidation and subsequently increase in ATP production which is required for maturation and embryo development. Following the metabolic experiments, new *in vitro* technique was developed in cattle to assess the further embryo development.

6.1.1 Acetyl L-carnitine

There is evidence that addition of L-carnitine (LC) during IVM improves the proportion of matured oocytes and cleaved embryos in camels (Fathi and El-Shahat, 2017), pigs (Wu et al., 2011), cattle (Phongnimitr et al., 2013) and sheep (Mishra et al., 2016a). Moreover, LC supplementation during IVC improves embryo development and ATP production in cattle (Sutton-Mcdowall et al., 2012).

For the most part, LC is added at between 2-3 mM during IVM or IVC. However, in mice acetyl L-carnitine (ALC) has been successfully used at 10 μM during IVF and IVC leading to enhanced embryo development and reduced concentration of ROS (Truong and Gardner, 2017). Also, data collected from women indicate that physiological level of L-carnitine in follicular fluid between 0.6 μmol/L to 46 μmol/L (Montjean et al., 2012).

Therefore, Chapter 2 sought to establish the optimal concentration of during IVM and IVC on embryo development. Incremental increases of ALC within physiological concentrations were assessed, however, no difference was observed on embryo development. On the other hand, molecular markers on CC indicated that antioxidant genes, such as *CAT* and *SOD2*, were altered by ALC treatment. Whilst *CAT* expression was decreased, *SOD2* expression was increased with ALC treatment compared to the control group. The reason is possibly due to the effect of ALC on antioxidation; i.e., ALC is known the

prevent formation of hydroxyl free radicals (Loots et al., 2004). As they are normally formed from hydrogen peroxide in the presence of ferrous ions the reaction of CAT inactivates hydrogen peroxide. However, treatment with ALC reduces the expression of *CAT* as ALC acts as an ROS scavenger and prevents the formation of hydroxyl free radicals instead of CAT. Expression of *PRKAA1*, which encodes the AMPK, was increased in the presence of ALC. Its activation depends on increased levels of AMP, resulting in ATP production by fatty acid oxidation (Watt et al., 2006). Its activation also inhibits fatty acid synthesis by inhibiting acetyl-CoA activation thereby blocking Malonyl-CoA formation. This statement explains the highest abundance of *ACACA* gene in 0.01mM compared to others. Cumulative effects of ALC treatment were observed in the form of blastocyst gene expression as the highest *CAT* and *PLIN3* abundance was obtained in D8 blastocysts which were treated with 0.1mM ALC. This may suggest that addition of 0.1mM ALC during IVM and IVC increase the protection of cells against to ROS and decreases lipid accumulation. However, there needs to be specific analysis to support this statement such as analysis of ROS and lipid droplets. Moreover, mtDNA copy number increased in oocytes following maturation as ALC concentration increased. These data suggest that ALC has a beneficial effect on cytoplasmic maturation even at lower concentrations, which contrast to other studies which used higher concentrations of ALC (2.5mM) (Xu et al., 2018a). However, apart from cytoplasmic maturation no beneficial effect was observed on embryo development. Therefore, in the following chapters LC, rather than ALC, was investigated in terms of its impact on embryo development, cytoplasmic maturation and development competence of bovine blastocysts.

6.1.2 Replacing serum with fatty acids and growth factors during IVM

Following the first study (Chapter 2), this thesis next sought to obtain defined maturation medium including LC (Chapter 3). Serum is commonly added to maturation media to improve the proportion of matured oocytes and subsequent embryo development (Thompson, 2000). On one hand, as a result of serum supplementation during bovine oocyte *in vitro* maturation, the proportion of blastocysts is enhanced (Mingoti et al., 2011). However, *in vivo* produced embryos never normally come into direct contact with serum in the uterus (Moore et al., 2007). On the other hand, serum supplementation

causes variable results due to the undefined components it contains (Moore et al., 2007). It is known that serum supplementation induces lipid accumulation and impairs cryotolerance of embryos (Abe et al., 2002). When bovine blastocysts are cultured in the presence of serum they exhibit more chromosomal abnormalities compared to other *in vitro* culture systems (Lonergan et al., 2004). Moreover, serum supplementation to culture media can lead to the Large Offspring Syndrome (LOS) (Young et al., 1998, Hill, 2014).

Therefore, in the series of experiments (Chapter 3) a cocktail of growth factors (GF), which also included serum replacement components and/or fatty acids (FA), were added to PVP-based maturation medium in the presence of LC. However, its known that albumin is a carrier protein for fatty acids, steroids and hormones. To ensure fatty acid uptake into the cells, albumin is required to be added to maturation media (Hughes et al., 2010). Therefore, in the first exploratory experiment the efficiency of albumin for transport of fatty acids in our enriched media was evaluated. In the first experiment, (FA in the presence of PVP and/or GF and inter alpha inhibitor (IAI) were compared with GF, FA and IAI in the presence of BSA. FA increased the proportion of advanced embryos (>4-cell embryos) compared to GF-IAI group by D2. However, the difference disappeared by D8. Spent maturation media analysis indicated that, proportionally, uptake of saturated fatty acids (i.e., palmitic and stearic) was greater for PVP, whilst uptake of linoleic acid (C18:2) was greater for BSA. Based on the outcome of the first experiment, the second exploratory experiment focused on effect of FA on embryo development compared to other groups which contains GF-IAI and/or FA. Embryo development was not altered by treatment. However, there was evidence that the uptake of PUFAs (i.e., linoleic and α-linolenic acids) was greater for the FA than the GF-IAI-FA treatment group. Data which was collected from these two exploratory experiments were evaluated and it was observed that fatty acid supplementation has a beneficial effect on the proportion of D8 blastocyst of cleaved. This might be due to uptake of PUFAs as they enhance embryo development (Amini et al., 2016, Lee et al., 2017).

Based on these two exploratory experiments, the main experiment in this chapter was designed; GF and/or FA cocktails were prepared in PVP based media and treatment groups were compared with serum and PVP based media in terms of molecular markers in CC following maturation, and embryo

development to D8. Supplementation of GF had a detrimental effect on embryo development. Moreover, blastocysts that were generated from oocytes matured in the presence of serum exhibited advanced embryo development compared to other groups.

It is known that under the control of gonadotropins CC release IGFs and they promote PI3K and Akt activation in oocytes which are required for maturation (Javvaji et al., 2021). Insulin has the same effect on PI3K activation, however, it acts downstream of MAPK (Saltiel and Kahn, 2001) which is also required for meiosis. Therefore, FGF was added to maturation media in our experiment so that MAPK was activated under the control of FGF (Du et al., 2021). Moreover, insulin regulates glucose and lipid metabolism by inhibiting glucogenesis and increasing glycolytic enzymes and fatty acid synthesis (Saltiel and Kahn, 2001). However, according to our RNASeq analyses, glycolysis was increased in the presence of FA compared to GF in our experiment. Moreover, comparison FA vs GF+FA indicated that long-chain fatty acids are transported less in GF+FA compared to FA; this might be due to accumulation of fatty acids by activated lipogenic enzymes under the control of insulin, and lipid accumulation explains impaired embryo development when the COCs were treated with GF+FA. Additionally spent media analysis supported this statement as uptake of PUFAs was observed to be lower in the presence of GF and IAI. Previous studies indicated that lack of PUFA impairs fertility by altering oocyte quality and subsequent embryo development (Bayar et al., 2021). Limited embryo production due to the treatment effect of GF+FA group did not allow us to compare ATP production on D8 blastocysts properly. However, data supports lipid accumulation in blastocysts when the COCs were treated with GF, as stage 8 embryos exhibited higher ATP production in the GF group. Fatty acid analysis needs to be done to confirm lipid accumulation in blastocysts per stage.

6.1.3 Molecular and metabolic effects of L-carnitine during IVM

Intracellular lipids serve as an energy source for ATP production via β-oxidation as well as being involved in cell signalling processes, sterols and steroid hormones biosynthesis (de Carvalho and Caramujo, 2018). Enhancing lipid metabolism improves oocyte competence and embryo development (Somfai et al., 2011). L-carnitine is a rate limiting factor for β-oxidation in

mammals. It was observed that LC supplementation improves embryo development in the presence of fetal calf serum compared to the absence of fatty acids (Dubeibe Marin et al., 2020). However, the effect of LC in the presence of FAs only has not been investigated. To understand molecular changes in COCs when they are matured in presence of LC, the next experiment (Chapter 4) focused on LC supplementation in the presence of specific fatty acids which are prevalent in follicular fluid. In Chapter 4, they were added at lower concentrations (equivalent to 10% of FBS), in contrast to the previous chapter (as in the preliminary experiment in Chapter 3 albumin did not improve embryo development and that might be due to high level of FAs), and conjugated with BSA. It was observed that oocytes matured in the presence of FAs exhibited a higher proportion of cleaved embryos on D2; an observation that corresponds to other work (Knitlova et al., 2017, Ghanem et al., 2014). However, distribution of embryos by cell number indicated that FAs group accumulated mostly in 3-4 cell and higher than FA+LC group and LC supplementation enhanced the proportion of greater than 6-cell zygotes. Moreover, LC increased embryo development on D8. During IVM, cytoplasmic maturation increases oocytes competence together with nuclear maturation (Ferreira et al., 2009). Therefore, protein, nutrition, mRNA and organelle accumulations are important for oocyte developmental competence. In the current experiment, mtDNA copy number was increased in oocytes following maturation. In mammalian somatic cells, each mitochondria includes 1-15 mtDNA copy (Satoh and Kuroiwa, 1991), however germ cells such as oocytes and sperm, contains only one mtDNA copy per organelle (Pikó and Matsumoto, 1976, Wai et al., 2008). Additionally, during fertilisation, paternal mtDNA are eliminated and only maternal mtDNA are transferred to offspring (May-Panloup et al., 2021). However, mtDNA copy number does not give a clue about active mitochondria distribution. Therefore, ATP analysis was undertaken in the current experiment which indicated that ATP production is increased in the presence of LC. Moreover, spent media analysis indicated that in the presence of LC oocyte released palmitic, stearic and oleic acids to the media, however no difference was found in LA and ALA uptake.

6.1.4 Post-implantation model of embryo development: generation of gastruloids

In previous experiments there were no opportunities to observe embryo development following the 8th day of culture. A recently published paper indicated that it is possible to observe later stages of embryo development by deriving and differentiating ESC to form organoids (Khurana et al., 2022). Furthermore, recently a developed *in vitro* technique allows us to generate gastruloids which helps us to understand further embryo development as their behaviors represent gastrulating embryos (Moris et al., 2020). Additionally, this technique allows us to observe germ layer differentiation and cell migration (Moris et al., 2020). However, there is no published study about bovine gastruloids and so, therefore, the last experiment considerd bovine gastruloid generation.

Derivation of EpiSC occurs successfully under serum-free conditions in cattle (Bogliotti et al., 2018) and pig embryos (Choi et al., 2019). However, MEF or serum replacement were still required to maintain pluripotency. In this experiment following immunosurgery, ICMs were incubated with defined medium including ROCKi, XAV939, actin and FGF in N2B27-base. Pure epiblasts were obtained within two weeks, cell shape did not change following splitting and these euploid (confirmed by karyomapping) cells continued to grow until passage 24 (P24).

Immunostaining is widely used to determine pluripotency of stem cells (Roach et al., 2006). Transcriptional factors (i.e., OCT4, SOX2 and NANOG) are required for maintenance of pluripotency (Silva and Smith, 2008). Moreover, surface markers (i.e., SSEA-4, TRA-1-60 and TRA-1-81) became hallmarks for identification of stem cells (Roach et al., 2006). In the current experiment immunostaining data indicated that derived stem cell lines expressed pluripotency and surface markers.

In vitro generated gastruloids have been developed, resembling gastrulating embryos and indicate body plan formation (Cermola et al., 2021). In this study the very first bovine gastruloid formation was observed and the findings indicate that, during pre-treatment, 5μM Chrion and FGF concentration in N2B27 medium are sufficient to switch on WNT signal in bovine stem cells. Following pre-treatment cell shape changed. 24h after seeding the cells,

gastruloids appeared as egg-shaped structures, and larger and more developed compared to human 24h gastruloids (Moris et al., 2020). Gasrtuloid elongation was observed visibly at the 48h stage and some of the structures remained elongated until the 72h stage.

Transcript expression (qPCR) data indicated that *SOX2* expression was highest in undifferentiated stem cells. However, its expression decreased following pre-treatment and no difference was observed between 48h and 72h gastruloids. Decreased *SOX2* expression following PT indicates that these cells are losing their pluripotency features (Zhang and Cui, 2014) which is an indicator for differentiation. *BRA* expression was observed from the stem cell stage to the 48h gastruloid stage. Its expression was not altered by differentiation. In 72h gastruloids, *BRA* expression was upregulated compared to other stages. *TBX6* was not observed at the SC stage and its expression appeared to increase following PT until the 48h gastruloid stage. Following the 48h stage its expression appeared to decrease up to the 72 h gastruloid stage. *EOMES,* appeared to decrease from the SC stage through to the 48 and 72 h gastruloid stages. In a human study it was observed that PT increases expression of mesodermal markers such as *BRA* and *EOMES* (Moris et al., 2020). However, in bovine *BRA* expression was found stable up to 72h gastruloid, in which upregulation was observed. Additionally, *EOMES* expression was downregulated with PT in both donors. On the other hand, increased *TBX6* with PT may explain the decrease in *EOMES* expression, as *TBX6* encodes transcriptional factors which inhibit neuronal development. In this sense, *TBX6* inhibits *EOMES* expression to prevent early neuronal development (Hadjantonakis et al., 2008). Expression of *FOXA2* increased between the SC and PT stages of development before decreasing again up to the 72 hr gastruloid stage. In contrast, a human study indicated that 44h gastruloids express *FOXA2* (Minn et al., 2021).

Bovine gastruloid staining indicated that, in 48 h gastruloids, BRA and SOX2 expression was located at the same pole which is expected to be posterior pole. Limited migration of cells expressing *BRA* and *SOX2* was observed at 72h. SOX17 expression was not observed in 48h gastruloids, however, it was visible throughout the gastruloids by 72h.

6.2 General Conclusions

This thesis initially focused on the effects of acetylated and free LC on fatty acid metabolism and subsequent effects on gene expression in bovine cumulus cells and oocytes and the pre-implantation embryo. Additional to this, defined media was formulated, and the effects of media components assessed in oocytes, cumulus cells and *in vitro* produced embryos by spent media analysis, gene expression of CC and pre-implantation embryos, mtDNA copy number and total cell number of D8 blastocysts. Additionally, a new technique was developed by differentiating bEpiSC and the process was assessed by gene expression analyses and immunostaining.

This thesis indicated that:

i. ALC has a beneficial effect on antioxidation and cytoplasmic maturation of oocytes, however embryo development is not altered by lower concentrations of ALC.
ii. BSA improves uptake of linoleic acid into cells compared to PVP based media, and combination of GF and IAI restricts uptake of omega fatty acids.
iii. In the presence of IAI, FA supplementation in IVM has positive effect on preimplantation embryos in cattle.
iv. LC supplementation in the presence of long chain fatty acids increases bovine embryo development.
v. Inclusion of LC during IVM improves cytoplasmic maturation by increasing ATP production and mtDNA copy number in bovine oocytes.
vi. Bovine EpiSC can be derived from advanced peri-implantation embryos in defined medium in the presence of XAV, ROCKi, Actin, FGF and N2B27 based medium.
vii. Very first attempt indicated that bovine gastruloids can be generated from bEpiSC and it allows us to understand developmental processes of gastrulating embryos.

In conclusion, it was observed that ALC supplementation during IVP (i.e., IVM and IVC) at low concentrations (<1mM) increases antioxidation, however it does not alter embryo development. In contrast LC supplementation to maturation media improves cytoplasmic maturation and subsequent embryo

development. On the other hand, gene expression in CC is altered by defined medium compared to serum or PVP-based media, and it was observed that FA addition at the physiological levels in the presence of growth factors cocktails impairs embryo development.

This thesis has highlighted the importance of gastruloid production and its impact on observation of further embryo development in defined media. To understand the metabolism of gastruloids, modulators need to be supplemented and gastruloid behaviors to be observed.

REFERENCES

AARDEMA, H., LOLICATO, F., VAN DE LEST, C. H. A., BROUWERS, J. F., VAANDRAGER, A. B., VAN TOL, H. T. A., ROELEN, B. A. J., VOS, P. L. A. M., HELMS, J. B. & GADELLA, B. M. 2013. Bovine Cumulus Cells Protect Maturing Oocytes from Increased Fatty Acid Levels by Massive Intracellular Lipid Storage1. *Biology of Reproduction,* 88.

AARDEMA, H., VOS, P. L. A. M., LOLICATO, F., ROELEN, B. A. J., KNIJN, H. M., VAANDRAGER, A. B., HELMS, J. B. & GADELLA, B. M. 2011. Oleic Acid Prevents Detrimental Effects of Saturated Fatty Acids on Bovine Oocyte Developmental Competence1. *Biology of Reproduction,* 85**,** 62-69.

ABDELRAZIK, H., SHARMA, R., MAHFOUZ, R. & AGARWAL, A. 2009. L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. *Fertil Steril,* 91**,** 589-96.

ABE, H., YAMASHITA, S., SATOH, T. & HOSHI, H. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Molecular Reproduction and Development,* 61**,** 57-66.

ABSALON-MEDINA, V. A., BEDFORD-GUAUS, S. J., GILBERT, R. O., SIQUEIRA, L. C., ESPOSITO, G., SCHNEIDER, A., CHEONG, S. H. & BUTLER, W. R. 2014. The effects of conjugated linoleic acid isomers cis-9,trans-11 and trans-10,cis-12 on in vitro bovine embryo production and cryopreservation. *J Dairy Sci,* 97**,** 6164-76.

ABSALÓN-MEDINA, V. A., BEDFORD-GUAUS, S. J., GILBERT, R. O., SIQUEIRA, L. C., ESPOSITO, G., SCHNEIDER, A., CHEONG, S. H. & BUTLER, W. R. 2014. The effects of conjugated linoleic acid isomers cis-9,trans-11 and trans-10,cis-12 on in vitro bovine embryo production and cryopreservation. *J Dairy Sci,* 97**,** 6164-76.

ACUÑA-CASTROVIEJO, D., ESCAMES, G., VENEGAS, C., DÍAZ-CASADO, M. E., LIMA-CABELLO, E., LÓPEZ, L. C., ROSALES-CORRAL, S., TAN, D. X. & REITER, R. J. 2014. Extrapineal melatonin: sources, regulation, and potential functions. *Cell Mol Life Sci,* 71**,** 2997-3025.

ADAMIAK, S. J., POWELL, K., ROOKE, J. A., WEBB, R. & SINCLAIR, K. D. 2006. Body composition, dietary carbohydrates and fatty acids determine post-fertilisation development of bovine oocytes in vitro. *Reproduction,* 131**,** 247-58.

ADEVA-ANDANY, M. M., CALVO-CASTRO, I., FERNÁNDEZ-FERNÁNDEZ, C., DONAPETRY-GARCÍA, C. & PEDRE-PIÑEIRO, A. M. 2017. Significance of l-carnitine for human health. *IUBMB Life,* 69**,** 578-594.

ADEVA-ANDANY, M. M., CARNEIRO-FREIRE, N., SECO-FILGUEIRA, M., FERNÁNDEZ-FERNÁNDEZ, C. & MOURIÑO-BAYOLO, D. 2019. Mitochondrial β-oxidation of saturated fatty acids in humans. *Mitochondrion,* 46**,** 73-90.

ADLER, S., PELLIZZER, C., PAPARELLA, M., HARTUNG, T. & BREMER, S. 2006. The effects of solvents on embryonic stem cell differentiation. *Toxicol In Vitro,* 20**,** 265-71.

AGARWAL, A., DURAIRAJANAYAGAM, D. & DU PLESSIS, S. S. 2014. Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reprod Biol Endocrinol,* 12**,** 112.

AGARWAL, A., GUPTA, S. & SHARMA, R. 2005a. Oxidative stress and its implications in female infertility - a clinician's perspective. *Reprod Biomed Online,* 11**,** 641-50.
AGARWAL, A., GUPTA, S. & SHARMA, R. K. 2005b. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol,* 3**,** 28.
AGARWAL, A., SENGUPTA, P. & DURAIRAJANAYAGAM, D. 2018. Role of L-carnitine in female infertility. *Reproductive biology and endocrinology : RB&E,* 16**,** 5-5.
AGUILA, L., OSYCKA-SALUT, C., TREULEN, F. & FELMER, R. 2022. Pluripotent Core in Bovine Embryos: A Review. *Animals (Basel),* 12.
AKRAM, M. 2014. Citric Acid Cycle and Role of its Intermediates in Metabolism. *Cell Biochemistry and Biophysics,* 68**,** 475-478.
AL DARWICH, A., PERREAU, C., PETIT, M. H., PAPILLIER, P., DUPONT, J., GUILLAUME, D., MERMILLOD, P. & GUIGNOT, F. 2010. Effect of PUFA on embryo cryoresistance, gene expression and AMPKalpha phosphorylation in IVF-derived bovine embryos. *Prostaglandins Other Lipid Mediat,* 93**,** 30-6.
ALASBAHI, R. & MELZIG, M. 2012. Forskolin and derivatives as tools for studying the role of cAMP. *Die Pharmazie,* 67**,** 5-13.
ALBERIO, R. 2020. Regulation of Cell Fate Decisions in Early Mammalian Embryos. *Annual Review of Animal Biosciences,* 8.
ALBERIO, R., CROXALL, N. & ALLEGRUCCI, C. 2010. Pig epiblast stem cells depend on activin/nodal signaling for pluripotency and self-renewal. *Stem Cells Dev,* 19**,** 1627-36.
ALBERIO, R., KOBAYASHI, T. & SURANI, M. A. 2021. Conserved features of non-primate bilaminar disc embryos and the germline. *Stem cell reports,* 16**,** 1078-1092.
ALBUZ, F. K., SASSEVILLE, M., LANE, M., ARMSTRONG, D. T., THOMPSON, J. G. & GILCHRIST, R. B. 2010. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod,* 25**,** 2999-3011.
ALESSANDRA CORALLO NICACIO2, RENATA SIMÕES2 , FABIOLA FREITAS DE PAULA-LOPES2 ,, FLAVIA REGINA OLIVEIRA DE BARROS2 , M. A. P., MAYRA ELENA ORTIZ D'AVILA ASSUMPÇÃO2 & VISINTIN1, A. J. A. 16 March 2011. Effects of different cryopreservation methods on post-thaw culture

conditions of in vitro produced bovine embryos.
ALIABADI, E., SOLEIMANI MEHRANJANI, M., BORZOEI, Z., TALAEI-KHOZANI, T., MIRKHANI, H. & TABESH, H. 2012. Effects of L-carnitine and L-acetyl-carnitine on testicular sperm motility and chromatin quality. *Iran J Reprod Med,* 10**,** 77-82.
AMINI, E., ASADPOUR, R., ROSHANGAR, L. & JAFARI-JOOZANI, R. 2016. Effect of linoleic acid supplementation on in vitro maturation, embryo development and apoptotic related gene expression in ovine. *Int J Reprod Biomed,* 14**,** 255-62.
ANCKAERT, E., DE RYCKE, M. & SMITZ, J. 2012. Culture of oocytes and risk of imprinting defects. *Human Reproduction Update,* 19**,** 52-66.
ANNES, K., MÜLLER, D. B., VILELA, J. A. P., VALENTE, R. S., CAETANO, D. P., CIBIN, F. W. S., MILAZZOTTO, M. P., MESQUITA, F. S., BELAZ, K. R. A., EBERLIN, M. N. & SUDANO, M. J. 2019. Influence of follicle size on bovine oocyte lipid composition, follicular metabolic and stress markers, embryo development and blastocyst lipid content. *Reprod Fertil Dev,* 31**,** 462-472.

ANNESLEY, S. J. & FISHER, P. R. 2019. Mitochondria in Health and Disease. *Cells,* 8.

ARRIGONI, O. & DE TULLIO, M. C. 2002. Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1569**,** 1-9.

AUCLAIR, S., UZBEKOV, R., ELIS, S., SANCHEZ, L., KIREEV, I., LARDIC, L., DALBIES-TRAN, R. & UZBEKOVA, S. 2013. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. *Am J Physiol Endocrinol Metab,* 304**,** E599-613.

BADR, H., BONGIONI, G., ABDOON, A. S., KANDIL, O. & PUGLISI, R. 2007. Gene expression in the in vitro-produced preimplantation bovine embryos. *Zygote,* 15**,** 355-67.

BAILLARGEON, P., FECTEAU, G., PARÉ, J., LAMOTHE, P. & SAUVÉ, R. 2001. Evaluation of the embryo transfer procedure proposed by the International Embryo Transfer Society as a method of controlling vertical transmission of Neospora caninum in cattle. *Journal of the American Veterinary Medical Association,* 218**,** 1803-1806.

BAILLIE-JOHNSON, P., VAN DEN BRINK, S. C., BALAYO, T., TURNER, D. A. & MARTINEZ ARIAS, A. 2015. Generation of Aggregates of Mouse Embryonic Stem Cells that Show Symmetry Breaking, Polarization and Emergent Collective Behaviour In Vitro. *Journal of visualized experiments : JoVE***,** 53252.

BAIRD, A. & HSUEH, A. 1986. Fibroblast growth factor as an intraovarian hormone: differential regulation of steroidogenesis by an angiogenic factor. *Regulatory Peptides,* 16**,** 243-250.

BARROS, R. G., LIMA, P. F., SOARES, A. C. S., SANCHES, L., PRICE, C. A. & BURATINI, J. 2019. Fibroblast growth factor 2 regulates cumulus differentiation under the control of the oocyte. *Journal of assisted reproduction and genetics,* 36**,** 905-913.

BARROW, J. R., HOWELL, W. D., RULE, M., HAYASHI, S., THOMAS, K. R., CAPECCHI, M. R. & MCMAHON, A. P. 2007. Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. *Developmental Biology,* 312**,** 312-320.

BAUMAN, D. & LOCK, A. 2007. Conjugated Linoleic Acid: Biosynthesis and Nutritional Significance.

BAYAR, E., SASO, S., GALAZIS, N., JONES, B., BRACEWELL-MILNES, T., CHAWLA, M., AHMED-SALIM, Y. & NAGI, J. B. 2021. Impact of polyunsaturated fatty acid supplementation on assisted reproductive technology outcomes: a systematic review. *Hum Fertil (Camb)***,** 1-9.

BECCARI, L., MORIS, N., GIRGIN, M., TURNER, D. A., BAILLIE-JOHNSON, P., COSSY, A.-C., LUTOLF, M. P., DUBOULE, D. & ARIAS, A. M. 2018. Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. *Nature,* 562**,** 272-276.

BEG, M. A., BERGFELT, D. R., KOT, K., WILTBANK, M. C. & GINTHER, O. J. 2001. Follicular-Fluid Factors and Granulosa-Cell Gene Expression Associated with Follicle Deviation in Cattle1. *Biology of Reproduction,* 64**,** 432-441.

BELURY, M. A., MOYA-CAMARENA, S. Y., LU, M., SHI, L., LEESNITZER, L. M. & BLANCHARD, S. G. 2002. Conjugated linoleic acid is an activator and ligand for peroxisome proliferator-activated receptor-gamma (PPARγ). *Nutrition Research,* 22**,** 817-824.

BENDER, K., WALSH, S., EVANS, A. C., FAIR, T. & BRENNAN, L. 2010. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. *Reproduction,* 139**,** 1047-55.

BERTEVELLO, P. S., TEIXEIRA-GOMES, A.-P., SEYER, A., VITORINO CARVALHO, A., LABAS, V., BLACHE, M.-C., BANLIAT, C., CORDEIRO, L. A. V., DURANTHON, V., PAPILLIER, P., MAILLARD, V., ELIS, S. & UZBEKOVA, S. 2018. Lipid Identification and Transcriptional Analysis of Controlling Enzymes in Bovine Ovarian Follicle. *International journal of molecular sciences,* 19**,** 3261.

BILODEAU-GOESEELS, S. 2012. Bovine oocyte meiotic inhibition before in vitro maturation and its value to in vitro embryo production: does it improve developmental competence? *Reprod Domest Anim,* 47**,** 687-93.

BISWAS, A. & HUTCHINS, R. 2007. Embryonic stem cells. *Stem Cells Dev,* 16**,** 213-22.

BLOMBERG, L. A. & TELUGU, B. P. 2012. Twenty years of embryonic stem cell research in farm animals. *Reprod Domest Anim,* 47 Suppl 4**,** 80-5.

BOGLIOTTI, Y. S., WU, J., VILARINO, M., OKAMURA, D., SOTO, D. A., ZHONG, C., SAKURAI, M., SAMPAIO, R. V., SUZUKI, K., IZPISUA BELMONTE, J. C. & ROSS, P. J. 2018. Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proceedings of the National Academy of Sciences of the United States of America,* 115**,** 2090-2095.

BOIVIN, J., BUNTING, L., COLLINS, J. A. & NYGREN, K. G. 2007. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Human Reproduction,* 22**,** 1506-1512.

BOND, L. M., MIYAZAKI, M., O'NEILL, L. M., DING, F. & NTAMBI, J. M. 2016. Chapter 6 - Fatty Acid Desaturation and Elongation in Mammals. *In:* RIDGWAY, N. D. & MCLEOD, R. S. (eds.) *Biochemistry of Lipids, Lipoproteins and Membranes (Sixth Edition).* Boston: Elsevier.

BONI, R., CUOMO, A. & TOSTI, E. 2002. Developmental Potential in Bovine Oocytes Is Related to Cumulus-Oocyte Complex Grade, Calcium Current Activity, and Calcium Stores. *Biology of Reproduction,* 66**,** 836-842.

BONNEFONT, J.-P., DJOUADI, F., PRIP-BUUS, C., GOBIN, S., MUNNICH, A. & BASTIN, J. 2004. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Molecular aspects of medicine,* 25**,** 495-520.

BRADLEY, J., POPE, I., MASIA, F., SANUSI, R., LANGBEIN, W., SWANN, K. & BORRI, P. 2016. Quantitative imaging of lipids in live mouse oocytes and early embryos using CARS microscopy. *Development,* 143**,** 2238-47.

BRADLEY, J. & SWANN, K. 2019. Mitochondria and lipid metabolism in mammalian oocytes and early embryos. *Int J Dev Biol,* 63**,** 93-103.

BRANKIN, V., MITCHELL, M. R. P., WEBB, B. & HUNTER, M. G. 2003. Paracrine effects of oocyte secreted factors and stem cell factor on porcine granulosa and theca cells in vitro. *Reproductive Biology and Endocrinology,* 1**,** 55.

BRAUN, K., OECKL, J., WESTERMEIER, J., LI, Y. & KLINGENSPOR, M. 2018. Non-adrenergic control of lipolysis and thermogenesis in adipose tissues. *The Journal of Experimental Biology,* 221**,** jeb165381.

BRIGGS, D. A., SHARP, D. J., MILLER, D. & GOSDEN, R. G. 1999. Transferrin in the developing ovarian follicle: evidence for de-novo expression by granulosa cells. *Mol Hum Reprod,* 5**,** 1107-14.

BRONS, I. G. M., SMITHERS, L. E., TROTTER, M. W. B., RUGG-GUNN, P., SUN, B., CHUVA DE SOUSA LOPES, S. M., HOWLETT, S. K., CLARKSON, A., AHRLUND-RICHTER, L., PEDERSEN, R. A. &

VALLIER, L. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature,* 448**,** 191-195.
BROWN, H. M., DUNNING, K. R., SUTTON-MCDOWALL, M., GILCHRIST, R. B., THOMPSON, J. G. & RUSSELL, D. L. 2017. Failure to launch: aberrant cumulus gene expression during oocyte in vitro maturation. *Reproduction,* 153**,** R109-R120.
BROWN, M. S. & GOLDSTEIN, J. L. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell,* 89**,** 331-40.
CAGNONE, G. & SIRARD, M. A. 2014. The impact of exposure to serum lipids during in vitro culture on the transcriptome of bovine blastocysts. *Theriogenology,* 81**,** 712-22.e1-3.
CAMUS, A., PEREA-GOMEZ, A., MOREAU, A. & COLLIGNON, J. 2006. Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo. *Dev Biol,* 295**,** 743-55.
CANEL, N. G., SUVÁ, M., BEVACQUA, R. J., ARIAS, M. E., FELMER, R. & SALAMONE, D. F. 2018. Improved embryo development using high cysteamine concentration during IVM and sperm co-culture with COCs previous to ICSI in bovine. *Theriogenology,* 117**,** 26-33.
CANIZO, J. R., YNSAURRALDE RIVOLTA, A. E., VAZQUEZ ECHEGARAY, C., SUVÁ, M., ALBERIO, V., ALLER, J. F., GUBERMAN, A. S., SALAMONE, D. F., ALBERIO, R. H. & ALBERIO, R. 2019. A dose-dependent response to MEK inhibition determines hypoblast fate in bovine embryos. *BMC developmental biology,* 19**,** 13-13.
CAO, L., SHITARA, H., HORII, T., NAGAO, Y., IMAI, H., ABE, K., HARA, T., HAYASHI, J. & YONEKAWA, H. 2007. The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat Genet,* 39**,** 386-90.
CARRILLO-GONZÁLEZ, D. F. & MALDONADO-ESTRADA, J. G. 2020. L-carnitine supplementation in culture media improves the pregnancy rate of in vitro produced embryos with sexed semen from Bos taurus indicus cows. *Trop Anim Health Prod,* 52**,** 2559-2565.
CARRO, M., BUSCHIAZZO, J., RÍOS, G. L., ORESTI, G. M. & ALBERIO, R. H. 2013. Linoleic acid stimulates neutral lipid accumulation in lipid droplets of maturing bovine oocytes. *Theriogenology,* 79**,** 687-94.
CASTAÑEDA-GUTIÉRREZ, E., BENEFIELD, B. C., DE VETH, M. J., SANTOS, N. R., GILBERT, R. O., BUTLER, W. R. & BAUMAN, D. E. 2007a. Evaluation of the mechanism of action of conjugated linoleic acid isomers on reproduction in dairy cows. *J Dairy Sci,* 90**,** 4253-64.
CASTAÑEDA-GUTIÉRREZ, E., BENEFIELD, B. C., DE VETH, M. J., SANTOS, N. R., GILBERT, R. O., BUTLER, W. R. & BAUMAN, D. E. 2007b. Evaluation of the Mechanism of Action of Conjugated Linoleic Acid Isomers on Reproduction in Dairy Cows1. *Journal of Dairy Science,* 90**,** 4253-4264.
CASTAÑEDA-GUTIÉRREZ, E., OVERTON, T. R., BUTLER, W. R. & BAUMAN, D. E. 2005. Dietary Supplements of Two Doses of Calcium Salts of Conjugated Linoleic Acid During the Transition Period and Early Lactation*. *Journal of Dairy Science,* 88**,** 1078-1089.
CEREZO, A. B., LEAL, Á., ÁLVAREZ-FERNÁNDEZ, M. A., HORNEDO-ORTEGA, R., TRONCOSO, A. M. & GARCÍA-PARRILLA, M. C. 2016. Quality control and determination of melatonin in food supplements. *Journal of Food Composition and Analysis,* 45**,** 80-86.
CERMOLA, F., D'ANIELLO, C., TATÈ, R., DE CESARE, D., MARTINEZ-ARIAS, A., MINCHIOTTI, G. & PATRIARCA, E. J. 2021. Gastruloid

Development Competence Discriminates Different States of Pluripotency. *Stem cell reports,* 16**,** 354-369.
CETICA, P., PINTOS, L., DALVIT, G. & BECONI, M. 2002. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. *Reproduction,* 124**,** 675-81.
CHASON, R. J., CSOKMAY, J., SEGARS, J. H., DECHERNEY, A. H. & ARMANT, D. R. 2011. Environmental and epigenetic effects upon preimplantation embryo metabolism and development. *Trends in Endocrinology & Metabolism,* 22**,** 412-420.
CHEN, L., MAO, S. J. & LARSEN, W. J. 1992. Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family. *J Biol Chem,* 267**,** 12380-6.
CHOI, K.-H., LEE, D.-K., KIM, S. W., WOO, S.-H., KIM, D.-Y. & LEE, C.-K. 2019. Chemically Defined Media Can Maintain Pig Pluripotency Network In Vitro. *Stem Cell Reports,* 13**,** 221-234.
CHOI, Y., PARK, Y., PARIZA, M. W. & NTAMBI, J. M. 2001. Regulation of Stearoyl-CoA Desaturase Activity by the trans-10,cis-12 Isomer of Conjugated Linoleic Acid in HepG2 Cells. *Biochemical and Biophysical Research Communications,* 284**,** 689-693.
CHUNG, J.-T., TOSCA, L., HUANG, T.-H., XU, L., NIWA, K. & CHIAN, R.-C. 2007. Effect of polyvinylpyrrolidone on bovine oocyte maturation in vitro and subsequent fertilization and embryonic development. *Reproductive BioMedicine Online,* 15**,** 198-207.
COELHO, M., RODRIGUES-CUNHA, M., FERREIRA, C., CABRAL, E., NOGUEIRA, G., EBERLIN, M., LEAL, C. & SIMAS, R. 2013. Assessing melatonin and its oxidative metabolites amounts in biological fluid and culture medium by liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). *Analytical Methods,* 5**,** 6911.
COLLADO-FERNANDEZ, E., PICTON, H. M. & DUMOLLARD, R. 2012. Metabolism throughout follicle and oocyte development in mammals. *Int J Dev Biol,* 56**,** 799-808.
COMBELLES, C. M. H., GUPTA, S. & AGARWAL, A. 2009. Could oxidative stress influence the in-vitro maturation of oocytes? *Reproductive biomedicine online,* 18**,** 864-880.
CONTI, M., ANDERSEN, C. B., RICHARD, F., MEHATS, C., CHUN, S. Y., HORNER, K., JIN, C. & TSAFRIRI, A. 2002. Role of cyclic nucleotide signaling in oocyte maturation. *Mol Cell Endocrinol,* 187**,** 153-9.
CORDEIRO, F. B., MONTANI, D. A., PILAU, E. J., GOZZO, F. C., FRAIETTA, R. & TURCO, E. G. L. 2018. Ovarian environment aging: follicular fluid lipidomic and related metabolic pathways. *Journal of assisted reproduction and genetics,* 35**,** 1385-1393.
CÓRDOVA, B., MORATÓ, R., IZQUIERDO, D., PARAMIO, T. & MOGAS, T. 2010. Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the in vitro maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development. *Theriogenology,* 74**,** 1341-8.
COULL, G. D., SPEAKE, B. K., STAINES, M. E., BROADBENT, P. J. & MCEVOY, T. G. 1998. Lipid and fatty acid composition of zona-intact sheep oocytes. *Theriogenology,* 49**,** 179.
CURNOW, E. C., RYAN, J., SAUNDERS, D. & HAYES, E. S. 2008. Bovine in vitro oocyte maturation as a model for manipulation of the gamma-glutamyl cycle and intraoocyte glutathione. *Reprod Fertil Dev,* 20**,** 579-88.

DE CARVALHO, C. & CARAMUJO, M. J. 2018. The Various Roles of Fatty Acids. *Molecules,* 23.

DE MATOS, D. G. & FURNUS, C. C. 2000. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenology,* 53**,** 761-71.

DEL COLLADO, M., DA SILVEIRA, J. C., OLIVEIRA, M. L. F., ALVES, B., SIMAS, R. C., GODOY, A. T., COELHO, M. B., MARQUES, L. A., CARRIERO, M. M., NOGUEIRA, M. F. G., EBERLIN, M. N., SILVA, L. A., MEIRELLES, F. V. & PERECIN, F. 2017. In vitro maturation impacts cumulus-oocyte complex metabolism and stress in cattle. *Reproduction,* 154**,** 881-893.

DEL COLLADO, M., SARAIVA, N. Z., LOPES, F. L., GASPAR, R. C., PADILHA, L. C., COSTA, R. R., ROSSI, G. F., VANTINI, R. & GARCIA, J. M. 2015. Influence of bovine serum albumin and fetal bovine serum supplementation during in vitro maturation on lipid and mitochondrial behaviour in oocytes and lipid accumulation in bovine embryos. *Reprod Fertil Dev*.

DELEUZE, S. & GOUDET, G. 2010. Cysteamine Supplementation of In vitro Maturation Media: A Review. *Reproduction in Domestic Animals,* 45**,** e476-e482.

DESVERGNE, B. & WAHLI, W. 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev,* 20**,** 649-88.

DISKIN, M. G., MURPHY, J. J. & SREENAN, J. M. 2006. Embryo survival in dairy cows managed under pastoral conditions. *Animal Reproduction Science,* 96**,** 297-311.

DOKMECI, D. 2005. Oxidative stress, male infertility and the role of carnitines. *Folia Med (Plovdiv),* 47**,** 26-30.

DONNAY, I., FAERGE, I., GRØNDAHL, C., VERHAEGHE, B., SAYOUD, H., PONDERATO, N., GALLI, C. & LAZZARI, G. 2004. Effect of prematuration, meiosis activating sterol and enriched maturation medium on the nuclear maturation and competence to development of calf oocytes. *Theriogenology,* 62**,** 1093-1107.

DOWNS, S. M., MOSEY, J. L. & KLINGER, J. 2009. Fatty acid oxidation and meiotic resumption in mouse oocytes. *Mol Reprod Dev,* 76**,** 844-53.

DU, C., DAVIS, J. S., CHEN, C., LI, Z., CAO, Y., SUN, H., SHAO, B. S., LIN, Y. X., WANG, Y. S., YANG, L. G. & HUA, G. H. 2021. FGF2/FGFR signaling promotes cumulus-oocyte complex maturation in vitro. *Reproduction,* 161**,** 205-214.

DUBEIBE MARIN, D. F., NOGUEIRA DA COSTA, N., DI PAULA BESSA SANTANA, P., BAIA DE SOUZA, E., ROLIM FILHO, S. T., DA SILVA CORDEIRO, M. & OHASHI, O. M. 2020. Influence of l-carnitine on lipid metabolism of buffalo cumulus-oocyte complexes matured in either fetal bovine serum or fatty acid-free bovine serum albumin. *Theriogenology,* 158**,** 382-390.

DUMOLLARD, R., DUCHEN, M. & CARROLL, J. 2007. The role of mitochondrial function in the oocyte and embryo. *Curr Top Dev Biol,* 77**,** 21-49.

DUNNING, K. R., AKISON, L. K., RUSSELL, D. L., NORMAN, R. J. & ROBKER, R. L. 2011. Increased Beta-Oxidation and Improved Oocyte Developmental Competence in Response to l-Carnitine During Ovarian In Vitro Follicle Development in Mice1. *Biology of Reproduction,* 85**,** 548-555.

DUNNING, K. R., ANASTASI, M. R., ZHANG, V. J., RUSSELL, D. L. & ROBKER, R. L. 2014a. Regulation of fatty acid oxidation in mouse

cumulus-oocyte complexes during maturation and modulation by PPAR agonists. *PloS one,* 9**,** e87327-e87327.
DUNNING, K. R., CASHMAN, K., RUSSELL, D. L., THOMPSON, J. G., NORMAN, R. J. & ROBKER, R. L. 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol Reprod,* 83**,** 909-18.
DUNNING, K. R. & ROBKER, R. L. 2012a. Promoting lipid utilization with l-carnitine to improve oocyte quality. *Anim Reprod Sci,* 134**,** 69-75.
DUNNING, K. R. & ROBKER, R. L. 2012b. Promoting lipid utilization with l-carnitine to improve oocyte quality. *Animal Reproduction Science,* 134**,** 69-75.
DUNNING, K. R., RUSSELL, D. L. & ROBKER, R. L. 2014b. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. *Reproduction,* 148**,** R15-27.
DUQUE, P., GÓMEZ, E., DíAZ, E., FACAL, N., HIDALGO, C. & DíEZ, C. 2003. Use of two replacements of serum during bovine embryo culture in vitro. *Theriogenology,* 59**,** 889-899.
EBERLÉ, D., HEGARTY, B., BOSSARD, P., FERRÉ, P. & FOUFELLE, F. 2004. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie,* 86**,** 839-48.
EL SHOURBAGY, S. H., SPIKINGS, E. C., FREITAS, M. & ST JOHN, J. C. 2006. Mitochondria directly influence fertilisation outcome in the pig. *Reproduction,* 131**,** 233-45.
ENTINGH-PEARSALL, A. & KAHN, C. R. 2004. Differential roles of the insulin and insulin-like growth factor-I (IGF-I) receptors in response to insulin and IGF-I. *J Biol Chem,* 279**,** 38016-24.
EPPIG, J. J. & O'BRIEN, M. J. 1998. Comparison of preimplantation developmental competence after mouse oocyte growth and development in vitro and in vivo. *Theriogenology,* 49**,** 415-22.
ESPOSITO, G., IRONS, P. C., WEBB, E. C. & CHAPWANYA, A. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. *Anim Reprod Sci,* 144**,** 60-71.
EVANS, M. 2011. Discovering pluripotency: 30 years of mouse embryonic stem cells. *Nat Rev Mol Cell Biol,* 12**,** 680-6.
EVANS, M. J. & KAUFMAN, M. H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292**,** 154-6.
FAIR, T., HULSHOF, S. C., HYTTEL, P., GREVE, T. & BOLAND, M. 1997. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anat Embryol (Berl),* 195**,** 327-36.
FATHI, M. & EL-SHAHAT, K. H. 2017. L-carnitine enhances oocyte maturation and improves in vitro development of embryos in dromedary camels (Camelus dromedaries). *Theriogenology,* 104**,** 18-22.
FAYEZI, S., LEROY, J., GHAFFARI NOVIN, M. & DARABI, M. 2018a. Oleic acid in the modulation of oocyte and preimplantation embryo development. *Zygote,* 26**,** 1-13.
FAYEZI, S., LEROY, J. L. M. R., GHAFFARI NOVIN, M. & DARABI, M. 2018b. Oleic acid in the modulation of oocyte and preimplantation embryo development. *Zygote,* 26**,** 1-13.
FEIGE, J. N., GELMAN, L., TUDOR, C., ENGELBORGHS, Y., WAHLI, W. & DESVERGNE, B. 2005. Fluorescence imaging reveals the nuclear behavior of peroxisome proliferator-activated receptor/retinoid X receptor heterodimers in the absence and presence of ligand. *J Biol Chem,* 280**,** 17880-90.

FENDERSON, B. A., ANDREWS, P. W., NUDELMAN, E., CLAUSEN, H. & HAKOMORI, S. 1987. Glycolipid core structure switching from globo- to lacto- and ganglio-series during retinoic acid-induced differentiation of TERA-2-derived human embryonal carcinoma cells. *Dev Biol,* 122**,** 21-34.

FERGUSON, E. M. & LEESE, H. J. 1999. Triglyceride content of bovine oocytes and early embryos. 116**,** 373.

FERGUSON, E. M. & LEESE, H. J. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Mol Reprod Dev,* 73**,** 1195-201.

FERREIRA, E. M., VIREQUE, A. A., ADONA, P. R., MEIRELLES, F. V., FERRIANI, R. A. & NAVARRO, P. A. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology,* 71**,** 836-48.

FROMENT, P., GIZARD, F., DEFEVER, D., STAELS, B., DUPONT, J. & MONGET, P. 2006. Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. *J Endocrinol,* 189**,** 199-209.

FU, X. W., WU, G. Q., LI, J. J., HOU, Y. P., ZHOU, G. B., LUN, S., WANG, Y. P. & ZHU, S. E. 2011. Positive effects of Forskolin (stimulator of lipolysis) treatment on cryosurvival of in vitro matured porcine oocytes. *Theriogenology,* 75**,** 268-75.

FURNUS, C. C., DE MATOS, D. G., PICCO, S., GARCÍA, P. P., INDA, A. M., MATTIOLI, G. & ERRECALDE, A. L. 2008. Metabolic requirements associated with GSH synthesis during in vitro maturation of cattle oocytes. *Anim Reprod Sci,* 109**,** 88-99.

GAFNI, O., WEINBERGER, L., MANSOUR, A. A., MANOR, Y. S., CHOMSKY, E., BEN-YOSEF, D., KALMA, Y., VIUKOV, S., MAZA, I., ZVIRAN, A., RAIS, Y., SHIPONY, Z., MUKAMEL, Z., KRUPALNIK, V., ZERBIB, M., GEULA, S., CASPI, I., SCHNEIR, D., SHWARTZ, T., GILAD, S., AMANN-ZALCENSTEIN, D., BENJAMIN, S., AMIT, I., TANAY, A., MASSARWA, R., NOVERSHTERN, N. & HANNA, J. H. 2013. Derivation of novel human ground state naive pluripotent stem cells. *Nature,* 504**,** 282-6.

GAMBINI, J., INGLÉS, M., OLASO, G., LOPEZ-GRUESO, R., BONET-COSTA, V., GIMENO-MALLENCH, L., MAS-BARGUES, C., ABDELAZIZ, K. M., GOMEZ-CABRERA, M. C., VINA, J. & BORRAS, C. 2015. Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and Humans. *Oxid Med Cell Longev,* 2015**,** 837042.

GARDINER, C. S. & REED, D. J. 1994. Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. *Biol Reprod,* 51**,** 1307-14.

GARDNER, D. K. 2011. Embryo metabolism and in vitro culture. *Physiologie, pathologie et thérapie de la reproduction chez l'humain.* Paris: Springer Paris.

GARNSWORTHY, P. C., FOULADI-NASHTA, A. A., MANN, G. E., SINCLAIR, K. D. & WEBB, R. 2009. Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. *Reproduction,* 137**,** 759-68.

GHANEM, N., HA, A. N., FAKRUZZAMAN, M., BANG, J. I., LEE, S. C. & KONG, I. K. 2014. Differential expression of selected candidate genes in bovine embryos produced in vitro and cultured with chemicals modulating lipid metabolism. *Theriogenology,* 82**,** 238-50.

GILCHRIST, R. B., RITTER, L. J. & ARMSTRONG, D. T. 2001. Mouse Oocyte Mitogenic Activity Is Developmentally Coordinated throughout Folliculogenesis and Meiotic Maturation. *Developmental Biology,* 240**,** 289-298.

GLUJOVSKY, D., FARQUHAR, C., QUINTEIRO RETAMAR, A. M., ALVAREZ SEDO, C. R. & BLAKE, D. 2016. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database of Systematic Reviews*.

GÓMEZ, E., DUQUE, P., DÍAZ, E. & DÍEZ, C. 2001. Effects of Acetoacetate on in vitro Development of Bovine Embryos in Medium Containing Citrate and Myo-inositol. *Reproduction in Domestic Animals,* 36**,** 189-194.

GOMEZ, E., DUQUE, P., DIAZ, E., FACAL, N., ANTOLIN, I., HIDALGO, C. & DIEZ, C. 2002. Effects of acetoacetate and D-beta-hydroxybutyrate on bovine in vitro embryo development in serum-free medium. *Theriogenology,* 57**,** 1551-62.

GONZALEZ-AÑOVER, P., ENCINAS, T., TORRES-ROVIRA, L., PALLARES, P., MUÑOZ-FRUTOS, J., GOMEZ-IZQUIERDO, E., SANCHEZ-SANCHEZ, R. & GONZALEZ-BULNES, A. 2011. Ovulation rate, embryo mortality and intrauterine growth retardation in obese swine with gene polymorphisms for leptin and melanocortin receptors. *Theriogenology,* 75**,** 34-41.

GONZÁLEZ-SERRANO, A. F., FERREIRA, C. R., PIRRO, V., LUCAS-HAHN, A., HEINZMANN, J., HADELER, K. G., BAULAIN, U., ALDAG, P., MEYER, U., PIECHOTTA, M., JAHREIS, G., DÄNICKE, S., COOKS, R. G. & NIEMANN, H. 2015. Effects of long-term dietary supplementation with conjugated linoleic acid on bovine oocyte lipid profile. *Reprod Fertil Dev*.

GOSPODAROWICZ, D. & BIALECKI, H. 1979. Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine, and human origin. *Endocrinology,* 104**,** 757-764.

GRAY, L. R., TOMPKINS, S. C. & TAYLOR, E. B. 2014. Regulation of pyruvate metabolism and human disease. *Cellular and Molecular Life Sciences,* 71**,** 2577-2604.

GREBER, B., WU, G., BERNEMANN, C., JOO, J. Y., HAN, D. W., KO, K., TAPIA, N., SABOUR, D., STERNECKERT, J., TESAR, P. & SCHÖLER, H. R. 2010. Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell,* 6**,** 215-26.

GROSS, C. J. & HENDERSON, L. M. 1984. Absorption of d- and l-carnitine by the intestine and kidney tubule in the rat. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 772**,** 209-219.

GU, L., LIU, H., GU, X., BOOTS, C., MOLEY, K. H. & WANG, Q. 2015. Metabolic control of oocyte development: linking maternal nutrition and reproductive outcomes. *Cellular and Molecular Life Sciences,* 72**,** 251-271.

GUO, J., LU, W. F., LIANG, S., CHOI, J. W., KIM, N. H. & CUI, X. S. 2017. Peroxisome proliferator-activated receptor δ improves porcine blastocyst hatching via the regulation of fatty acid oxidation. *Theriogenology,* 90**,** 266-275.

HADJANTONAKIS, A.-K., PISANO, E. & PAPAIOANNOU, V. E. 2008. Tbx6 regulates left/right patterning in mouse embryos through effects on nodal cilia and perinodal signaling. *PloS one,* 3**,** e2511-e2511.

HARAGUCHI, S., KIKUCHI, K., NAKAI, M. & TOKUNAGA, T. 2012. Establishment of self-renewing porcine embryonic stem cell-like cells by signal inhibition. *J Reprod Dev,* 58**,** 707-16.

HASHIMOTO, S. 2009. Application of In Vitro Maturation to Assisted Reproductive Technology. *Journal of Reproduction and Development,* 55**,** 1-10.

HAYASHI, K., OHTA, H., KURIMOTO, K., ARAMAKI, S. & SAITOU, M. 2011. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell,* 146**,** 519-32.

HE, B., YIN, C., GONG, Y., LIU, J., GUO, H. & ZHAO, R. 2018. Melatonin-induced increase of lipid droplets accumulation and in vitro maturation in porcine oocytes is mediated by mitochondrial quiescence. *J Cell Physiol,* 233**,** 302-312.

HELD-HOELKER, E., KLEIN, S. L., RINGS, F., SALILEW-WONDIM, D., SAEED-ZIDANE, M., NEUHOFF, C., TESFAYE, D., SCHELLANDER, K. & HOELKER, M. 2017. Cryosurvival of in vitro produced bovine embryos supplemented with l-Carnitine and concurrent reduction of fatty acids. *Theriogenology,* 96**,** 145-152.

HEMMINGS, K. E., LEESE, H. J. & PICTON, H. M. 2012. Amino Acid Turnover by Bovine Oocytes Provides an Index of Oocyte Developmental Competence In Vitro1. *Biology of Reproduction,* 86.

HENDERSON, J. K., DRAPER, J. S., BAILLIE, H. S., FISHEL, S., THOMSON, J. A., MOORE, H. & ANDREWS, P. W. 2002. Preimplantation Human Embryos and Embryonic Stem Cells Show Comparable Expression of Stage-Specific Embryonic Antigens. *Stem Cells,* 20**,** 329-337.

HICKMAN, A. B., KLEIN, D. C. & DYDA, F. 1999. Melatonin Biosynthesis: The Structure of Serotonin N-Acetyltransferase at 2.5 Å Resolution Suggests a Catalytic Mechanism. *Molecular Cell,* 3**,** 23-32.

HILL, J. R. 2014. Incidence of abnormal offspring from cloning and other assisted reproductive technologies. *Annu Rev Anim Biosci,* 2**,** 307-21.

HIRAISHI, H., TERANO, A., OTA, S., MUTOH, H., RAZANDI, M., SUGIMOTO, T. & IVEY, K. J. 1991. Role for iron in reactive oxygen species-mediated cytotoxicity to cultured rat gastric mucosal cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 260**,** G556-G563.

HOMA, S. T. & BROWN, C. A. 1992. Changes in linoleic acid during follicular development and inhibition of spontaneous breakdown of germinal vesicles in cumulus-free bovine oocytes. 94**,** 153.

HORTON, J. D., GOLDSTEIN, J. L. & BROWN, M. S. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of clinical investigation,* 109**,** 1125-1131.

HOTA, K. B., HOTA, S. K., CHAURASIA, O. P. & SINGH, S. B. 2012. Acetyl-L-carnitine-mediated neuroprotection during hypoxia is attributed to ERK1/2-Nrf2-regulated mitochondrial biosynthesis. *Hippocampus,* 22**,** 723-36.

HOUTEN, S. M. & WANDERS, R. J. 2010. A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation. *J Inherit Metab Dis,* 33**,** 469-77.

HUANG, S. M., MISHINA, Y. M., LIU, S., CHEUNG, A., STEGMEIER, F., MICHAUD, G. A., CHARLAT, O., WIELLETTE, E., ZHANG, Y., WIESSNER, S., HILD, M., SHI, X., WILSON, C. J., MICKANIN, C., MYER, V., FAZAL, A., TOMLINSON, R., SERLUCA, F., SHAO, W., CHENG, H., SHULTZ, M., RAU, C., SCHIRLE, M., SCHLEGL, J.,

GHIDELLI, S., FAWELL, S., LU, C., CURTIS, D., KIRSCHNER, M. W., LENGAUER, C., FINAN, P. M., TALLARICO, J. A., BOUWMEESTER, T., PORTER, J. A., BAUER, A. & CONG, F. 2009. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature,* 461**,** 614-20.

HUGHES, J., KWONG, W., LI, D., SALTER, A., LEA, R. & SINCLAIR, K. 2010. Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. *Reproduction (Cambridge, England),* 141**,** 105-18.

HWANG, I.-S. & HOCHI, S. 2014. Recent Progress in Cryopreservation of Bovine Oocytes. *BioMed Research International,* 2014**,** 570647.

IDREES, M., XU, L., EL SHEIKH, M., SIDRAT, T., SONG, S. H., JOO, M. D., LEE, K. L. & KONG, I. K. 2019. The PPARδ Agonist GW501516 Improves Lipolytic/Lipogenic Balance through CPT1 and PEPCK during the Development of Pre-Implantation Bovine Embryos. *Int J Mol Sci,* 20.

INANO, A., SAI, Y., NIKAIDO, H., HASIMOTO, N., ASANO, M., TSUJI, A. & TAMAI, I. 2003. Acetyl-L-carnitine permeability across the blood-brain barrier and involvement of carnitine transporter OCTN2. *Biopharm Drug Dispos,* 24**,** 357-65.

ISHII, T. & BANNAI, S. 1985. The synergistic action of the copper chelator bathocuproine sulphonate and cysteine in enhancing growth of L1210 cells in vitro. *J Cell Physiol,* 125**,** 151-5.

ISMAIL, A. M., HAMED, A. H., SASO, S. & THABET, H. H. 2014. Adding L-carnitine to clomiphene resistant PCOS women improves the quality of ovulation and the pregnancy rate. A randomized clinical trial. *Eur J Obstet Gynecol Reprod Biol,* 180**,** 148-52.

ITAMI, N., SHIRASUNA, K., KUWAYAMA, T. & IWATA, H. 2018. Palmitic acid induces ceramide accumulation, mitochondrial protein hyperacetylation, and mitochondrial dysfunction in porcine oocytes†. *Biology of Reproduction,* 98**,** 644-653.

ITSKOVITZ-ELDOR, J., SCHULDINER, M., KARSENTI, D., EDEN, A., YANUKA, O., AMIT, M., SOREQ, H. & BENVENISTY, N. 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med,* 6**,** 88-95.

JAVVAJI, P. K., DHALI, A., FRANCIS, J. R., KOLTE, A. P., ROY, S. C., SELVARAJU, S., MECH, A. & SEJIAN, V. 2021. IGF-1 treatment during in vitro maturation improves developmental potential of ovine oocytes through the regulation of PI3K/Akt and apoptosis signaling. *Anim Biotechnol,* 32**,** 798-805.

JEON, S.-M. 2016. Regulation and function of AMPK in physiology and diseases. *Experimental & molecular medicine,* 48**,** e245-e245.

JEONG, Y. W., HOSSEIN, M. S., BHANDARI, D. P., KIM, Y. W., KIM, J. H., PARK, S. W., LEE, E., PARK, S. M., JEONG, Y. I., LEE, J. Y., KIM, S. & HWANG, W. S. 2008. Effects of insulin-transferrin-selenium in defined and porcine follicular fluid supplemented IVM media on porcine IVF and SCNT embryo production. *Anim Reprod Sci,* 106**,** 13-24.

JI-EUN, L., HWANGBO, Y., HWA-YOUNG, K., WON-HEE, L., HEE-TAE, C., BOO-KEUN, Y. & CHOON-KEUN, P. 2017. Effect of Alpha-Linolenic Acid on Oocyte Maturation and Embryo Development in Pigs. *Development & Reproduction,* 21**,** 205-213.

JIA, B., WU, G., FU, X., MO, X., DU, M., HOU, Y. & ZHU, S. 2014. trans-10, cis-12 conjugated linoleic acid enhances in vitro maturation of porcine oocytes. *Mol Reprod Dev,* 81**,** 20-30.

JIANG, W.-J., YAO, X.-R., ZHAO, Y.-H., GAO, Q.-S., JIN, Q.-G., LI, Y.-H., YAN, A.-G. & XU, Y.-N. 2019. L-carnitine prevents bovine oocyte aging and promotes subsequent embryonic development. *Journal of Reproduction and Development,* 65**,** 499-506.

JIANG, W., LI, Y., ZHAO, Y., GAO, Q., JIN, Q., YAN, C. & XU, Y. 2020. l-carnitine supplementation during in vitro culture regulates oxidative stress in embryos from bovine aged oocytes. *Theriogenology,* 143**,** 64-73.

JIN, J. X., LEE, S., TAWEECHAIPAISANKUL, A., KIM, G. A. & LEE, B. C. 2017. Melatonin regulates lipid metabolism in porcine oocytes. *J Pineal Res,* 62.

JOUANNET, P. 2009. [Evolution of assisted reproductive technologies]. *Bulletin de l'Academie nationale de medecine,* 193**,** 573-582.

KAMEYAMA, Y., FILION, F., YOO, J. G. & SMITH, L. C. 2007. Characterization of mitochondrial replication and transcription control during rat early development in vivo and in vitro. *Reproduction,* 133**,** 423-32.

KANG, J.-T., KOO, O.-J., KWON, D.-K., PARK, H.-J., JANG, G., KANG, S.-K. & LEE, B.-C. 2009. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. *Journal of Pineal Research,* 46**,** 22-28.

KASTANIOTIS, A. J., AUTIO, K. J., KERÄTÄR, J. M., MONTEUUIS, G., MÄKELÄ, A. M., NAIR, R. R., PIETIKÄINEN, L. P., SHVETSOVA, A., CHEN, Z. & HILTUNEN, J. K. 2017. Mitochondrial fatty acid synthesis, fatty acids and mitochondrial physiology. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1862**,** 39-48.

KAUSHIK, S. & CUERVO, A. M. 2015. Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis. *Nat Cell Biol,* 17**,** 759-70.

KHAJEH, M., RAHBARGHAZI, R., NOURI, M. & DARABI, M. 2017. Potential role of polyunsaturated fatty acids, with particular regard to the signaling pathways of arachidonic acid and its derivatives in the process of maturation of the oocytes: Contemporary review. *Biomed Pharmacother,* 94**,** 458-467.

KHALIL, W., MAREI, W., FAROOQ, H. & KHALID, M. 2014. A COMPARISON BETWEEN THE EFFECT OF LINOLEIC ACID AND CONJUGATED LINOLEIC ACID ON BOVINE OOCYTE DEVELOPMENTAL COMPETENCE. *Journal of Animal and Poultry Production,* 5**,** 115-125.

KHALIL, W. A., MAREI, W. F. & KHALID, M. 2013. Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. *Theriogenology,* 80**,** 161-8.

KHAMSI, F. & ARMSTRONG, D. T. 1997. Interactions between Follicle-Stimulating Hormone and Growth Factors in Regulation of Deoxyribonucleic Acid Synthesis in Bovine Granulosa Cells1. *Biology of Reproduction,* 57**,** 684-688.

KHAN, D. R., LANDRY, D. A., FOURNIER, É., VIGNEAULT, C., BLONDIN, P. & SIRARD, M.-A. 2016. Transcriptome meta-analysis of three follicular compartments and its correlation with ovarian follicle maturity and oocyte developmental competence in cows. *Physiological genomics,* 48**,** 633-643.

KHANMOHAMMADI, N., MOVAHEDIN, M., SAFARI, M., SAMENI, H., YOUSEFI, B., JAFARI, B. & ZARBAKHSH, S. 2016. Effect of L-carnitine on in vitro developmental rate, the zona pellucida and hatching of blastocysts and their cell numbers in mouse embryos. *International Journal of Reproductive Biomedicine,* 14**,** 649-656.

KHURANA, N. K. & NIEMANN, H. 2000a. Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology,* 54**,** 741-56.

KHURANA, N. K. & NIEMANN, H. 2000b. Energy Metabolism in Preimplantation Bovine Embryos Derived In Vitro or In Vivo1. *Biology of Reproduction,* 62**,** 847-856.

KHURANA, P., SMYTH, N. R., SHETH, B., VELAZQUEZ, M. A., ECKERT, J. J. & FLEMING, T. P. 2022. Advanced maternal age perturbs mouse embryo development and alters the phenotype of derived embryonic stem cells. *Journal of Developmental Origins of Health and Disease,* 13**,** 395-405.

KIM, J. Y., KINOSHITA, M., OHNISHI, M. & FUKUI, Y. 2001. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. *Reproduction,* 122**,** 131-8.

KINOSHITA, M., BARBER, M., MANSFIELD, W., CUI, Y., SPINDLOW, D., STIRPARO, G. G., DIETMANN, S., NICHOLS, J. & SMITH, A. 2021a. Capture of Mouse and Human Stem Cells with Features of Formative Pluripotency. *Cell Stem Cell,* 28**,** 453-471.e8.

KINOSHITA, M., KOBAYASHI, T., PLANELLS, B., KLISCH, D., SPINDLOW, D., MASAKI, H., BORNELÖV, S., STIRPARO, G. G., MATSUNARI, H., UCHIKURA, A., LAMAS-TORANZO, I., NICHOLS, J., NAKAUCHI, H., NAGASHIMA, H., ALBERIO, R. & SMITH, A. 2021b. Pluripotent stem cells related to embryonic disc exhibit common self-renewal requirements in diverse livestock species. *Development,* 148.

KIRILLOVA, A., SMITZ, J. E. J., SUKHIKH, G. T. & MAZUNIN, I. 2021. The Role of Mitochondria in Oocyte Maturation. *Cells,* 10.

KLEIN, D. C. & WELLER, J. L. 1970. Indole metabolism in the pineal gland: a circadian rhythm in N-acetyltransferase. *Science,* 169**,** 1093-5.

KLOSE, C., SURMA, M. A. & SIMONS, K. 2013. Organellar lipidomics--background and perspectives. *Curr Opin Cell Biol,* 25**,** 406-13.

KNIGHT, P. G. & GLISTER, C. 2003. Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Animal Reproduction Science,* 78**,** 165-183.

KNITLOVA, D., HULINSKA, P., JESETA, M., HANZALOVA, K., KEMPISTY, B. & MACHATKOVA, M. 2017. Supplementation of l-carnitine during in vitro maturation improves embryo development from less competent bovine oocytes. *Theriogenology,* 102**,** 16-22.

KOLIOS, G. & MOODLEY, Y. 2013. Introduction to Stem Cells and Regenerative Medicine. *Respiration,* 85**,** 3-10.

KOMAR, C. M. 2005. Peroxisome proliferator-activated receptors (PPARs) and ovarian function--implications for regulating steroidogenesis, differentiation, and tissue remodeling. *Reproductive biology and endocrinology : RB&E,* 3**,** 41-41.

KORHONEN, K., KANANEN, K., KETOJA, E., MATOMÄKI, J., HALMEKYTÖ, M. & PEIPPO, J. 2010. Effects of serum-free in vitro maturation of bovine oocytes on subsequent embryo development and cell allocation in two developmental stages of day 7 blastocysts. *Reprod Domest Anim,* 45**,** 42-9.

KRISHER, R. L. & BAVISTER, B. D. 1998. Responses of oocytes and embryos to the culture environment. *Theriogenology,* 49**,** 103-114.

KUIJK, E. W., CHUVA DE SOUSA LOPES, S. M., GEIJSEN, N., MACKLON, N. & ROELEN, B. A. J. 2011. The different shades of mammalian pluripotent stem cells. *Human reproduction update,* 17**,** 254-271.

KUSUMI, A., FUJIWARA, T. K., CHADDA, R., XIE, M., TSUNOYAMA, T. A., KALAY, Z., KASAI, R. S. & SUZUKI, K. G. N. 2012. Dynamic Organizing Principles of the Plasma Membrane that Regulate Signal Transduction: Commemorating the Fortieth Anniversary of Singer and Nicolson's Fluid-Mosaic Model. *Annual Review of Cell and Developmental Biology,* 28**,** 215-250.

KWAK, S. S., CHEONG, S. A., JEON, Y., LEE, E., CHOI, K. C., JEUNG, E. B. & HYUN, S. H. 2012. The effects of resveratrol on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic activation and in vitro fertilization. *Theriogenology,* 78**,** 86-101.

LANDAU, S., BRAW-TAL, R., KAIM, M., BOR, A. & BRUCKENTAL, I. 2000. Preovulatory follicular status and diet affect the insulin and glucose content of follicles in high-yielding dairy cows. *Anim Reprod Sci,* 64**,** 181-97.

LANNER, F. & ROSSANT, J. 2010. The role of FGF/Erk signaling in pluripotent cells. *Development,* 137**,** 3351-3360.

LAPA, M., MARQUES, C. C., ALVES, S. P., VASQUES, M. I., BAPTISTA, M. C., CARVALHAIS, I., SILVA PEREIRA, M., HORTA, A. E., BESSA, R. J. & PEREIRA, R. M. 2011. Effect of trans-10 cis-12 conjugated linoleic acid on bovine oocyte competence and fatty acid composition. *Reprod Domest Anim,* 46**,** 904-10.

LASKOWSKI, D., ANDERSSON, G., HUMBLOT, P., SIRARD, M.-A., SJUNNESSON, Y., FERREIRA, C. R., PIRRO, V. & BÅGE, R. 2018. Lipid profile of bovine blastocysts exposed to insulin during in vitro oocyte maturation. *Reproduction, Fertility and Development,* 30**,** 1253-1266.

LASKOWSKI, D., SJUNNESSON, Y., HUMBLOT, P., SIRARD, M. A., ANDERSSON, G., GUSTAFSSON, H. & BÅGE, R. 2016. Insulin exposure during in vitro bovine oocyte maturation changes blastocyst gene expression and developmental potential. *Reprod Fertil Dev*.

LAVRANOS, T., RODGERS, H., BERTONCELLO, I. & RODGERS, R. 1994. Anchorage-independent culture of bovine granulosa cells: the effects of basic fibroblast growth factor and dibutyryl cAMP on cell division and differentiation. *Experimental Cell Research,* 211**,** 245-251.

LAZZARI, G., WRENZYCKI, C., HERRMANN, D., DUCHI, R., KRUIP, T., NIEMANN, H. & GALLI, C. 2002. Cellular and Molecular Deviations in Bovine In Vitro-Produced Embryos Are Related to the Large Offspring Syndrome1. *Biology of Reproduction,* 67**,** 767-775.

LEE CARTER, A., ABNEY, T. O. & LAPP, D. F. 1995. Biosynthesis and Metabolism of Carnitine. *Journal of Child Neurology,* 10**,** 2S3-2S7.

LEE, C. J., DO, B. R., LEE, Y. H., PARK, J. H., KIM, S. J., KIM, J. K., ROH, S. I., YOON, Y.-D. & YOON, H. S. 2001. Ovarian expression of melatonin Mel1a receptor mRNA during mouse development. *Molecular Reproduction and Development,* 59**,** 126-132.

LEE, J. E., YONG, H., KIM, H. Y., LEE, W. H., CHEONG, H. T., YANG, B. K. & PARK, C. K. 2017. Effect of Alpha-Linolenic Acid on Oocyte Maturation and Embryo Development in Pigs. *Dev Reprod,* 21**,** 205-213.

LEE, K., WANG, C., CHAILLE, J. M. & MACHATY, Z. 2010. Effect of resveratrol on the development of porcine embryos produced in vitro. *J Reprod Dev,* 56**,** 330-5.

LEESE, H. J. 2015. History of oocyte and embryo metabolism. *Reproduction, Fertility and Development,* 27**,** 567-571.

LEESE, H. J., HUGENTOBLER, S. A., GRAY, S. M., MORRIS, D. G., STURMEY, R. G., WHITEAR, S.-L. & SREENAN, J. M. 2007. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction, Fertility and Development,* 20**,** 1-8.

LEROY, J. L., OPSOMER, G., VAN SOOM, A., GOOVAERTS, I. G. & BOLS, P. E. 2008. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part I. The importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cows. *Reprod Domest Anim,* 43**,** 612-22.

LEROY, J. L., STURMEY, R. G., VAN HOECK, V., DE BIE, J., MCKEEGAN, P. J. & BOLS, P. E. 2014. Dietary fat supplementation and the consequences for oocyte and embryo quality: hype or significant benefit for dairy cow reproduction? *Reprod Domest Anim,* 49**,** 353-61.

LEROY, J. L., VANHOLDER, T., MATEUSEN, B., CHRISTOPHE, A., OPSOMER, G., DE KRUIF, A., GENICOT, G. & VAN SOOM, A. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction,* 130**,** 485-95.

LEVENTHAL, A., CHEN, G., NEGRO, A. & BOEHM, M. 2012. The benefits and risks of stem cell technology. *Oral diseases,* 18**,** 217-222.

LI, H. J., SUTTON-MCDOWALL, M. L., WANG, X., SUGIMURA, S., THOMPSON, J. G. & GILCHRIST, R. B. 2016. Extending prematuration with cAMP modulators enhances the cumulus contribution to oocyte antioxidant defence and oocyte quality via gap junctions. *Hum Reprod,* 31**,** 810-21.

LI, R., NORMAN, R. J., ARMSTRONG, D. T. & GILCHRIST, R. B. 2000. Oocyte-Secreted Factor(s) Determine Functional Differences Between Bovine Mural Granulosa Cells and Cumulus Cells1. *Biology of Reproduction,* 63**,** 839-845.

LI, S., GUO, Q., WANG, Y. M., LI, Z. Y., KANG, J. D., YIN, X. J. & ZHENG, X. 2018. Glycine treatment enhances developmental potential of porcine oocytes and early embryos by inhibiting apoptosis. *J Anim Sci,* 96**,** 2427-2437.

LI, Y.-X., GUO, X., GULAPPA, T., MENON, B. & MENON, K. M. J. 2019. SREBP Plays a Regulatory Role in LH/hCG Receptor mRNA Expression in Human Granulosa-Lutein Cells. *The Journal of clinical endocrinology and metabolism,* 104**,** 4783-4792.

LIN, J. & REDIES, C. 2012. Histological evidence: housekeeping genes beta-actin and GAPDH are of limited value for normalization of gene expression. *Dev Genes Evol,* 222**,** 369-76.

LIU, G., DAVID, B. T., TRAWCZYNSKI, M. & FESSLER, R. G. 2020. Advances in Pluripotent Stem Cells: History, Mechanisms, Technologies, and Applications. *Stem Cell Rev Rep,* 16**,** 3-32.

LIU, J., HEAD, E., KURATSUNE, H., COTMAN, C. W. & AMES, B. N. 2004a. Comparison of the effects of L-carnitine and acetyl-L-carnitine on carnitine levels, ambulatory activity, and oxidative stress biomarkers in the brain of old rats. *Ann N Y Acad Sci,* 1033**,** 117-31.

LIU, J., HEAD, E., KURATSUNE, H., COTMAN, C. W. & AMES, B. N. 2004b. Comparison of the Effects of l-Carnitine and Acetyl-l-Carnitine on Carnitine Levels, Ambulatory Activity, and Oxidative Stress Biomarkers in the Brain of Old Rats. *Annals of the New York Academy of Sciences,* 1033**,** 117-131.

LIZARRAGA, R. M., ANCHORDOQUY, J. M., GALARZA, E. M., FARNETANO, N. A., CARRANZA-MARTIN, A., FURNUS, C. C., MATTIOLI, G. A. & ANCHORDOQUY, J. P. 2020. Sodium Selenite Improves In Vitro Maturation of Bos primigenius taurus Oocytes. *Biol Trace Elem Res,* 197**,** 149-158.

LOEWENSTEIN, J. E. & COHEN, A. I. 1964. DRY MASS, LIPID CONTENT AND PROTEIN CONTENT OF THE INTACT AND ZONA-FREE MOUSE OVUM. *J Embryol Exp Morphol,* 12**,** 113-21.

LONERGAN, P., PEDERSEN, H. G., RIZOS, D., GREVE, T., THOMSEN, P. D., FAIR, T., EVANS, A. & BOLAND, M. P. 2004. Effect of the Post-Fertilization Culture Environment on the Incidence of Chromosome Aberrations in Bovine Blastocysts1. *Biology of Reproduction,* 71**,** 1096-1100.

LONG, Y. C. & ZIERATH, J. R. 2006. AMP-activated protein kinase signaling in metabolic regulation. *J Clin Invest,* 116**,** 1776-83.

LONGO, N., FRIGENI, M. & PASQUALI, M. 2016. Carnitine transport and fatty acid oxidation. *Biochim Biophys Acta,* 1863**,** 2422-35.

LOOTS, D. T., MIENIE, L. J., BERGH, J. J. & VAN DER SCHYF, C. J. 2004. Acetyl-L-carnitine prevents total body hydroxyl free radical and uric acid production induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the rat. *Life sciences,* 75**,** 1243-1253.

LOPES, C. M. A. & FELISBERTI, M. I. 2003. Mechanical behaviour and biocompatibility of poly(1-vinyl-2-pyrrolidinone)-gelatin IPN hydrogels. *Biomaterials,* 24**,** 1279-1284.

LOWE, J. L., BARTOLAC, L. K., BATHGATE, R. & GRUPEN, C. G. 2017. Supplementation of culture medium with L-carnitine improves the development and cryotolerance of in vitro-produced porcine embryos. *Reproduction, Fertility and Development,* 29**,** 2357-2366.

LOWE, J. L., BATHGATE, R. & GRUPEN, C. G. 2018. Effect of carbohydrates on lipid metabolism during porcine oocyte IVM. *Reproduction, Fertility and Development,* 31**,** 557-569.

MACHADO, G., FERREIRA, A., PIVATO, I., FIDELIS, A., SPRICIGO, J., PAULINI, F., LUCCI, C., FRANCO, M. & DODE, M. 2013. Post-hatching development of in vitro bovine embryos from day 7 to 14 in vivo versus in vitro. *Molecular Reproduction and Development,* 80**,** 936-947.

MACLAREN, L. A., GUZELOGLU, A., MICHEL, F. & THATCHER, W. W. 2006. Peroxisome proliferator-activated receptor (PPAR) expression in cultured bovine endometrial cells and response to omega-3 fatty acid, growth hormone and agonist stimulation in relation to series 2 prostaglandin production. *Domest Anim Endocrinol,* 30**,** 155-69.

MAGDANZ, V., BORYSHPOLETS, S., RIDZEWSKI, C., ECKEL, B. & REINHARDT, K. 2019. The motility-based swim-up technique separates bull sperm based on differences in metabolic rates and tail length. *PLOS ONE,* 14**,** e0223576.

MAHDAVINEZHAD, F., KAZEMI, P., FATHALIZADEH, P., SARMADI, F., SOTOODEH, L., HASHEMI, E., HAJARIAN, H. & DASHTIZAD, M. 2019. In vitro versus In vivo: Development-, Apoptosis-, and Implantation- Related Gene Expression in Mouse Blastocyst. *Iranian journal of biotechnology,* 17**,** e2157-e2157.

MAO, L., LOU, H., LOU, Y., WANG, N. & JIN, F. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reprod Biomed Online,* 28**,** 284-99.

MAPLETOFT, R. J. & HASLER, J. F. 2005. Assisted reproductive technologies in cattle: a review. *Rev Sci Tech,* 24**,** 393-403.

MAREI, W. F., WATHES, D. C. & FOULADI-NASHTA, A. A. 2009a. The effect of linolenic Acid on bovine oocyte maturation and development. *Biol Reprod,* 81**,** 1064-72.
MAREI, W. F., WATHES, D. C. & FOULADI-NASHTA, A. A. 2009b. The Effect of Linolenic Acid on Bovine Oocyte Maturation and Development. *Biology of Reproduction,* 81**,** 1064-1072.
MAREI, W. F., WATHES, D. C. & FOULADI-NASHTA, A. A. 2010a. Impact of linoleic acid on bovine oocyte maturation and embryo development. 139**,** 979.
MAREI, W. F., WATHES, D. C. & FOULADI-NASHTA, A. A. 2010b. Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction,* 139**,** 979-88.
MAREI, W. F., WATHES, D. C. & FOULADI-NASHTA, A. A. 2012. Differential effects of linoleic and alpha-linolenic fatty acids on spatial and temporal mitochondrial distribution and activity in bovine oocytes. *Reprod Fertil Dev,* 24**,** 679-90.
MARTIN, G. R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences,* 78**,** 7634-7638.
MARTÍNEZ-IBARRA, J. L., ESPINOZA-MENDOZA, E. A., RANGEL-SANTOS, R., AMBRIZ-GARCIA, D. A. & NAVARRO-MALDONADO, M. D. C. 2018. Effect of resveratrol on the in vitro maturation of ovine (Ovis aries) oocytes and the subsequent development of handmade cloned embryos. *Veterinaria México OA,* 5**,** 0-0.
MARTYN, I., SIGGIA, E. D. & BRIVANLOU, A. H. 2019. Mapping cell migrations and fates in a gastruloid model to the human primitive streak. *Development (Cambridge, England),* 146**,** dev179564.
MARUOTTI, J., MUÑOZ, M., DEGRELLE, S. A., GÓMEZ, E., LOUET, C., DÍEZ, C., DE LONGCHAMP, P. H., BROCHARD, V., HUE, I., CAAMAÑO, J. N. & JOUNEAU, A. 2012. Efficient derivation of bovine embryonic stem cells needs more than active core pluripotency factors. *Mol Reprod Dev,* 79**,** 461-77.
MASTROIANNI, L., JR. & JONES, R. 1965. OXYGEN TENSION WITHIN THE RABBIT FALLOPIAN TUBE. *J Reprod Fertil,* 9**,** 99-102.
MATTOS, R., STAPLES, C. & THATCHER, W. 2000. Effects of dietary fatty acids on reproduction in ruminants. *Reviews of reproduction,* 5**,** 38-45.
MATTOS, R., STAPLES, C. R., WILLIAMS, J., AMOROCHO, A., MCGUIRE, M. A. & THATCHER, W. W. 2002. Uterine, ovarian, and production responses of lactating dairy cows to increasing dietary concentrations of menhaden fish meal. *J Dairy Sci,* 85**,** 755-64.
MAY-PANLOUP, P., BOGUENET, M., HACHEM, H. E., BOUET, P. E. & REYNIER, P. 2021. Embryo and Its Mitochondria. *Antioxidants (Basel),* 10.
MAY-PANLOUP, P., CHRETIEN, M. F., MALTHIERY, Y. & REYNIER, P. 2007. Mitochondrial DNA in the oocyte and the developing embryo. *Curr Top Dev Biol,* 77**,** 51-83.
MCEVOY, T. G., COULL, G. D., BROADBENT, P. J., HUTCHINSON, J. S. & SPEAKE, B. K. 2000. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *J Reprod Fertil,* 118**,** 163-70.
MCKEEGAN, P. & STURMEY, R. 2011. The role of fatty acids in oocyte and embryo development. *Reproduction, fertility, and development,* 24**,** 59-67.

MCNAMARA, S., BUTLER, T., RYAN, D. P., MEE, J. F., DILLON, P., O'MARA, F. P., BUTLER, S. T., ANGLESEY, D., RATH, M. & MURPHY, J. J. 2003. Effect of offering rumen-protected fat supplements on fertility and performance in spring-calving Holstein-Friesian cows. *Anim Reprod Sci,* 79**,** 45-56.

MCNATTY, K. P., JUENGEL, J. L., READER, K. L., LUN, S., MYLLYMAA, S., LAWRENCE, S. B., WESTERN, A., MEERASAHIB, M. F., MOTTERSHEAD, D. G., GROOME, N. P., RITVOS, O. & LAITINEN, M. P. E. 2005. Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function in ruminants. 129**,** 481.

MCNATTY, K. P., JUENGEL, J. L., WILSON, T., GALLOWAY, S. M., DAVIS, G. H., HUDSON, N. L., MOELLER, C. L., CRANFIELD, M., READER, K. L., LAITINEN, M. P., GROOME, N. P., SAWYER, H. R. & RITVOS, O. 2003. Oocyte-derived growth factors and ovulation rate in sheep. *Reprod Suppl,* 61**,** 339-51.

MENEZO, Y. J. & HERUBEL, F. 2002. Mouse and bovine models for human IVF. *Reprod Biomed Online,* 4**,** 170-5.

MI, Y., TAN, D., HE, Y., ZHOU, X., ZHOU, Q. & JI, S. 2018. Melatonin Modulates lipid Metabolism in HepG2 Cells Cultured in High Concentrations of Oleic Acid: AMPK Pathway Activation may Play an Important Role. *Cell Biochem Biophys,* 76**,** 463-470.

MINGOTI, G. Z., CASTRO, V. S., MÉO, S. C., LS, S. B. & GARCIA, J. M. 2011. The effects of macromolecular and serum supplements and oxygen tension during bovine in vitro procedures on kinetics of oocyte maturation and embryo development. *In Vitro Cell Dev Biol Anim,* 47**,** 361-7.

MINN, K. T., DIETMANN, S., WAYE, S. E., MORRIS, S. A. & SOLNICA-KREZEL, L. 2021. Gene expression dynamics underlying cell fate emergence in 2D micropatterned human embryonic stem cell gastruloids. *Stem cell reports,* 16**,** 1210-1227.

MIRABI, P., CHAICHI, M. J., ESMAEILZADEH, S., ALI JORSARAEI, S. G., BIJANI, A., EHSANI, M. & HASHEMI KAROOEE, S. F. 2017. The role of fatty acids on ICSI outcomes: a prospective cohort study. *Lipids in Health and Disease,* 16**,** 18.

MISHRA, A., REDDY, I. J., GUPTA, P. & MONDAL, S. 2016a. Developmental regulation and modulation of apoptotic genes expression in sheep oocytes and embryos cultured in vitro with L-carnitine. *Reprod Domest Anim,* 51**,** 1020-1029.

MISHRA, A., REDDY, I. J., GUPTA, P. S. & MONDAL, S. 2016b. L-carnitine Mediated Reduction in Oxidative Stress and Alteration in Transcript Level of Antioxidant Enzymes in Sheep Embryos Produced In Vitro. *Reprod Domest Anim,* 51**,** 311-21.

MOAWAD, A. R., SALAMA, A., BADR, M. R. & FATHI, M. 2021. Beneficial Effects of L-Carnitine Supplementation during IVM of Canine Oocytes on Their Nuclear Maturation and Development In Vitro. *Animals : an open access journal from MDPI,* 11**,** 581.

MONTJEAN, D., ENTEZAMI, F., LICHTBLAU, I., BELLOC, S., GURGAN, T. & MENEZO, Y. 2012. Carnitine content in the follicular fluid and expression of the enzymes involved in beta oxidation in oocytes and cumulus cells. *Journal of Assisted Reproduction and Genetics,* 29**,** 1221-1225.

MOON, R. T. 2005. Wnt/beta-catenin pathway. *Sci STKE,* 2005**,** cm1.

MOORE, K., RODRÍGUEZ-SALLABERRY, C. J., KRAMER, J. M., JOHNSON, S., WROCLAWSKA, E., GOICOA, S. & NIASARI-NASLAJI, A. 2007. In

vitro production of bovine embryos in medium supplemented with a serum replacer: effects on blastocyst development, cryotolerance and survival to term. *Theriogenology,* 68**,** 1316-25.

MORIS, N., ANLAS, K., VAN DEN BRINK, S. C., ALEMANY, A., SCHRÖDER, J., GHIMIRE, S., BALAYO, T., VAN OUDENAARDEN, A. & MARTINEZ ARIAS, A. 2020. An in vitro model of early anteroposterior organization during human development. *Nature,* 582**,** 410-415.

MORITA, Y., WADA-HIRAIKE, O., YANO, T., SHIRANE, A., HIRANO, M., HIRAIKE, H., KOYAMA, S., OISHI, H., YOSHINO, O., MIYAMOTO, Y., SONE, K., ODA, K., NAKAGAWA, S., TSUTSUI, K. & TAKETANI, Y. 2012. Resveratrol promotes expression of SIRT1 and StAR in rat ovarian granulosa cells: an implicative role of SIRT1 in the ovary. *Reproductive biology and endocrinology : RB&E,* 10**,** 14-14.

MORO, C. & LAFONTAN, M. 2012. Natriuretic peptides and cGMP signaling control of energy homeostasis. *American journal of physiology. Heart and circulatory physiology,* 304.

MUKHERJEE, A., MALIK, H., SAHA, A. P., DUBEY, A., SINGHAL, D. K., BOATENG, S., SAUGANDHIKA, S., KUMAR, S., DE, S., GUHA, S. K. & MALAKAR, D. 2014. Resveratrol treatment during goat oocytes maturation enhances developmental competence of parthenogenetic and hand-made cloned blastocysts by modulating intracellular glutathione level and embryonic gene expression. *J Assist Reprod Genet,* 31**,** 229-39.

MULAS, C., KALKAN, T., VON MEYENN, F., LEITCH, H. G., NICHOLS, J. & SMITH, A. 2019. Defined conditions for propagation and manipulation of mouse embryonic stem cells. *Development,* 146.

MURO, E., ATILLA-GOKCUMEN, G. E. & EGGERT, U. S. 2014. Lipids in cell biology: how can we understand them better? *Molecular Biology of the Cell,* 25**,** 1819-1823.

MUSICCO, C., CAPELLI, V., PESCE, V., TIMPERIO, A. M., CALVANI, M., MOSCONI, L., ZOLLA, L., CANTATORE, P. & GADALETA, M. N. 2009. Accumulation of overoxidized Peroxiredoxin III in aged rat liver mitochondria. *Biochim Biophys Acta,* 1787**,** 890-6.

NAGAI, T. 2001. The improvement of in vitro maturation systems for bovine and porcine oocytes. *Theriogenology,* 55**,** 1291-1301.

NAKAMURA, Y., TAMURA, H., TAKAYAMA, H. & KATO, H. 2003. Increased endogenous level of melatonin in preovulatory human follicles does not directly influence progesterone production. *Fertil Steril,* 80**,** 1012-6.

NICHOLS, J. & SMITH, A. 2009. Naive and primed pluripotent states. *Cell Stem Cell,* 4**,** 487-92.

NOSE, F., YAMAGUCHI, T., KATO, R., AIUCHI, T., OBAMA, T., HARA, S., YAMAMOTO, M. & ITABE, H. 2013. Crucial role of perilipin-3 (TIP47) in formation of lipid droplets and PGE2 production in HL-60-derived neutrophils. *PloS one,* 8**,** e71542-e71542.

NOZIK-GRAYCK, E., SULIMAN, H. B. & PIANTADOSI, C. A. 2005. Extracellular superoxide dismutase. *Int J Biochem Cell Biol,* 37**,** 2466-71.

ORDOÑEZ-LEON, E. A., MERCHANT, H., MEDRANO, A., KJELLAND, M. & ROMO, S. 2014. Lipid droplet analysis using in vitro bovine oocytes and embryos. *Reprod Domest Anim,* 49**,** 306-14.

ORTEGA, I. & DULEBA, A. J. 2015. Ovarian actions of resveratrol. *Ann N Y Acad Sci,* 1348**,** 86-96.

OZAWA, M., SAKATANI, M., HANKOWSKI, K. E., TERADA, N., DOBBS, K. B. & HANSEN, P. J. 2012. Importance of culture conditions during the morula-to-blastocyst period on capacity of inner cell-mass cells of

bovine blastocysts for establishment of self-renewing pluripotent cells. *Theriogenology,* 78**,** 1243-51.e1-2.
PABON, J. E., JR., FINDLEY, W. E. & GIBBONS, W. E. 1989. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil Steril,* 51**,** 896-900.
PACZKOWSKI, M., SCHOOLCRAFT, W. B. & KRISHER, R. L. 2014. Fatty acid metabolism during maturation affects glucose uptake and is essential to oocyte competence. 148**,** 429.
PACZKOWSKI, M., SILVA, E., SCHOOLCRAFT, W. B. & KRISHER, R. L. 2013. Comparative Importance of Fatty Acid Beta-Oxidation to Nuclear Maturation, Gene Expression, and Glucose Metabolism in Mouse, Bovine, and Porcine Cumulus Oocyte Complexes1. *Biology of Reproduction,* 88.
PAN, B. & LI, J. 2019. The art of oocyte meiotic arrest regulation. *Reprod Biol Endocrinol,* 17**,** 8.
PAPIS, K., POLESZCZUK, O., WENTA-MUCHALSKA, E. & MODLINSKI, J. A. 2007. Melatonin effect on bovine embryo development in vitro in relation to oxygen concentration. *J Pineal Res,* 43**,** 321-6.
PARIZA, M. W., PARK, Y. & COOK, M. E. 2001. The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res,* 40**,** 283-98.
PARK, T. Y., CHOI, K. H., LEE, D. K., OH, J. N., KIM, S. H. & LEE, C. K. 2018. Attempting to Convert Primed Porcine Embryonic Stem Cells into a Naive State Through the Overexpression of Reprogramming Factors. *Cell Reprogram,* 20**,** 289-300.
PAWLAK, P., MALYSZKA, N., SZCZERBAL, I. & KOLODZIEJSKI, P. 2020. Fatty acid induced lipolysis influences embryo development, gene expression and lipid droplet formation in the porcine cumulus cells†. *Biology of Reproduction*.
PEDERSEN, H. S., LØVENDAHL, P., LARSEN, K., MADSEN, L. B. & CALLESEN, H. 2016. Porcine oocyte mtDNA copy number is high or low depending on the donor. *Zygote,* 24**,** 617-623.
PEREIRA, R. M., BAPTISTA, M. C., VASQUES, M. I., HORTA, A. E., PORTUGAL, P. V., BESSA, R. J., SILVA, J. C., PEREIRA, M. S. & MARQUES, C. C. 2007. Cryosurvival of bovine blastocysts is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t,12c CLA). *Anim Reprod Sci,* 98**,** 293-301.
PESCE, V., NICASSIO, L., FRACASSO, F., MUSICCO, C., CANTATORE, P. & GADALETA, M. N. 2012. Acetyl-L-carnitine activates the peroxisome proliferator-activated receptor-γ coactivators PGC-1α/PGC-1β-dependent signaling cascade of mitochondrial biogenesis and decreases the oxidized peroxiredoxins content in old rat liver. *Rejuvenation Res,* 15**,** 136-9.
PHANIENDRA, A., JESTADI, D. B. & PERIYASAMY, L. 2015. Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J Clin Biochem,* 30**,** 11-26.
PHONGNIMITR, T., LIANG, Y., SRIRATTANA, K., PANYAWAI, K., SRIPUNYA, N., TREETAMPINICH, C. & PARNPAI, R. 2013. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. *Anim Sci J,* 84**,** 719-25.
PICTON, H., BRIGGS, D. & GOSDEN, R. 1998. The molecular basis of oocyte growth and development. *Mol Cell Endocrinol,* 145**,** 27-37.
PIKÓ, L. & MATSUMOTO, L. 1976. Number of mitochondria and some properties of mitochondrial DNA in the mouse egg. *Dev Biol,* 49**,** 1-10.
PODOLAK, A., WOCLAWEK-POTOCKA, I. & LUKASZUK, K. 2022. The Role of Mitochondria in Human Fertility and Early Embryo Development:

What Can We Learn for Clinical Application of Assessing and Improving Mitochondrial DNA? *Cells,* 11.
POLI, G., LEONARDUZZI, G., BIASI, F. & CHIARPOTTO, E. 2004. Oxidative stress and cell signalling. *Curr Med Chem,* 11**,** 1163-82.
PRICE, C. A. 2016. Mechanisms of fibroblast growth factor signaling in the ovarian follicle. *Journal of Endocrinology,* 228**,** R31-R43.
QIANG, L., LIN, H. V., KIM-MULLER, J. Y., WELCH, C. L., GU, W. & ACCILI, D. 2011. Proatherogenic abnormalities of lipid metabolism in SirT1 transgenic mice are mediated through Creb deacetylation. *Cell Metab,* 14**,** 758-67.
RAMOS-IBEAS, P., GONZÁLEZ-BRUSI, L., USED, M. T., COCERO, M. J., MARIGORTA, P., ALBERIO, R. & BERMEJO-ÁLVAREZ, P. 2022. In vitro culture of ovine embryos up to early gastrulating stages. *Development,* 149.
RAMOS-IBEAS, P., LAMAS-TORANZO, I., MARTÍNEZ-MORO, Á., DE FRUTOS, C., QUIROGA, A. C., ZURITA, E. & BERMEJO-ÁLVAREZ, P. 2020. Embryonic disc formation following post-hatching bovine embryo development in vitro. *Reproduction,* 160**,** 579-589.
RAZZA, E. M., PEDERSEN, H. S., STROEBECH, L., FONTES, P. K., KADARMIDEEN, H. N., CALLESEN, H., PIHL, M., NOGUEIRA, M. F. G. & HYTTEL, P. 2019. Simulated physiological oocyte maturation has side effects on bovine oocytes and embryos. *Journal of assisted reproduction and genetics,* 36**,** 413-424.
RAZZA, E. M., SUDANO, M. J., FONTES, P. K., FRANCHI, F. F., BELAZ, K. R. A., SANTOS, P. H., CASTILHO, A. C. S., ROCHA, D. F. O., EBERLIN, M. N., MACHADO, M. F. & NOGUEIRA, M. F. G. 2018. Treatment with cyclic adenosine monophosphate modulators prior to in vitro maturation alters the lipid composition and transcript profile of bovine cumulus-oocyte complexes and blastocysts. *Reprod Fertil Dev,* 30**,** 1314-1328.
READER, K. L., COX, N. R., STANTON, J. A. & JUENGEL, J. L. 2015. Effects of acetyl-L-carnitine on lamb oocyte blastocyst rate, ultrastructure, and mitochondrial DNA copy number. *Theriogenology,* 83**,** 1484-92.
REBOUCHE, C. J. 2004a. Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Ann N Y Acad Sci,* 1033**,** 30-41.
REBOUCHE, C. J. 2004b. Kinetics, Pharmacokinetics, and Regulation of l-Carnitine and Acetyl-l-carnitine Metabolism. *Annals of the New York Academy of Sciences,* 1033**,** 30-41.
REIS, A., ROOKE, J. A., MCCALLUM, G. J., STAINES, M. E., EWEN, M., LOMAX, M. A. & MCEVOY, T. G. 2003. Consequences of exposure to serum, with or without vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced in vitro. *Reprod Fertil Dev,* 15**,** 275-84.
RG STURMEY, A. R., HJ LEESE AND TG MCEVOY 2009. Role of Fatty Acids in Energy Provision During Oocyte Maturation and Early

Embryo Development.
RIZOS, D., WARD, F., DUFFY, P., BOLAND, M. P. & LONERGAN, P. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development,* 61**,** 234-248.
ROACH, M., WANG, L., YANG, X. & TIAN, X. C. 2006. Bovine Embryonic Stem Cells. *Methods in Enzymology.* Academic Press.

ROBERTSON, I. & NELSON, R. 2010. Certification and identification of embryos. *Manual of The International Embryo Transfer Society. 4th ed, Champaign: International Embryo Transfer Society*, 86-105.

ROBINSON, R. S., PUSHPAKUMARA, P. G., CHENG, Z., PETERS, A. R., ABAYASEKARA, D. R. & WATHES, D. C. 2002. Effects of dietary polyunsaturated fatty acids on ovarian and uterine function in lactating dairy cows. *Reproduction,* 124**,** 119-31.

ROBKER, R. L., AKISON, L. K., BENNETT, B. D., THRUPP, P. N., CHURA, L. R., RUSSELL, D. L., LANE, M. & NORMAN, R. J. 2009. Obese Women Exhibit Differences in Ovarian Metabolites, Hormones, and Gene Expression Compared with Moderate-Weight Women. *The Journal of Clinical Endocrinology & Metabolism,* 94**,** 1533-1540.

ROCHA-FRIGONI, N. A., LEÃO, B. C., DALL'ACQUA, P. C. & MINGOTI, G. Z. 2016. Improving the cytoplasmic maturation of bovine oocytes matured in vitro with intracellular and/or extracellular antioxidants is not associated with increased rates of embryo development. *Theriogenology,* 86**,** 1897-905.

RODRIGUES-CUNHA, M. C., MESQUITA, L. G., BRESSAN, F., COLLADO, M. D., BALIEIRO, J. C., SCHWARZ, K. R., DE CASTRO, F. C., WATANABE, O. Y., WATANABE, Y. F., DE ALENCAR COELHO, L. & LEAL, C. L. 2016. Effects of melatonin during IVM in defined medium on oocyte meiosis, oxidative stress, and subsequent embryo development. *Theriogenology,* 86**,** 1685-94.

ROSTOVSKAYA, M., STIRPARO, G. G. & SMITH, A. 2019. Capacitation of human naïve pluripotent stem cells for multi-lineage differentiation. *Development,* 146.

ROURA, M., CATALA, M. G., SOTO-HERAS, S., HAMMAMI, S., IZQUIERDO, D., FOULADI-NASHTA, A. & PARAMIO, M. T. 2018. Linoleic (LA) and linolenic (ALA) acid concentrations in follicular fluid of prepubertal goats and their effect on oocyte in vitro maturation and embryo development. *Reprod Fertil Dev,* 30**,** 286-296.

ROY, S., GANDRA, D., SEGER, C., BISWAS, A., KUSHNIR, V. A., GLEICHER, N., KUMAR, T. R. & SEN, A. 2018. Oocyte-Derived Factors (GDF9 and BMP15) and FSH Regulate AMH Expression Via Modulation of H3K27AC in Granulosa Cells. *Endocrinology,* 159**,** 3433-3445.

SAEEDABADI, S., ABAZARI-KIA, A. H., RAJABI, H., PARIVAR, K. & SALEHI, M. 2018. Melatonin Improves The Developmental Competence of Goat Oocytes. *Int J Fertil Steril,* 12**,** 157-163.

SAGIRKAYA, H., MISIRLIOGLU, M., KAYA, A., FIRST, N. L., PARRISH, J. J. & MEMILI, E. 2007. Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Animal Reproduction Science,* 101**,** 225-240.

SALTIEL, A. R. & KAHN, C. R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature,* 414**,** 799-806.

SALUSTRI, A., CAMPAGNOLO, L., KLINGER, F. G. & CAMAIONI, A. 2019. Molecular organization and mechanical properties of the hyaluronan matrix surrounding the mammalian oocyte. *Matrix Biol,* 78-79**,** 11-23.

SANBUISSHO, A. & THRELFALL, W. R. 1990. The influence of serum and gonadotropins on in vitro maturation and fertilization of bovine oocytes. *Theriogenology,* 34**,** 341-8.

SANCHEZ-LAZO, L., BRISARD, D., ELIS, S., MAILLARD, V., UZBEKOV, R., LABAS, V., DESMARCHAIS, A., PAPILLIER, P., MONGET, P. & UZBEKOVA, S. 2014a. Fatty Acid Synthesis and Oxidation in Cumulus

Cells Support Oocyte Maturation in Bovine. *Molecular Endocrinology,* 28.
SANCHEZ-LAZO, L., BRISARD, D., ELIS, S., MAILLARD, V., UZBEKOV, R., LABAS, V., DESMARCHAIS, A., PAPILLIER, P., MONGET, P. & UZBEKOVA, S. 2014b. Fatty Acid Synthesis and Oxidation in Cumulus Cells Support Oocyte Maturation in Bovine. *Molecular Endocrinology,* 28**,** 1502-1521.
SANTIQUET, N. W., GREENE, A. F., BECKER, J., BARFIELD, J. P., SCHOOLCRAFT, W. B. & KRISHER, R. L. 2017. A pre-in vitro maturation medium containing cumulus oocyte complex ligand-receptor signaling molecules maintains meiotic arrest, supports the cumulus oocyte complex and improves oocyte developmental competence. *Mol Hum Reprod,* 23**,** 594-606.
SARAGUSTY, J. & ARAV, A. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction,* 141**,** 1-19.
SARAIVA, H., BATISTA, R., ALFRADIQUE, V. A. P., PINTO, P. H. N., RIBEIRO, L. S., OLIVEIRA, C. S., SOUZA-FABJAN, J. M. G., CAMARGO, L. S. A., FONSECA, J. F. & BRANDAO, F. Z. 2018. l-carnitine supplementation during vitrification or warming of in vivo-produced ovine embryos does not affect embryonic survival rates, but alters CrAT and PRDX1 expression. *Theriogenology,* 105**,** 150-157.
SATA, R., TSUJII, H., ABE, H., YAMASHITA, S. & HOSHI, H. 1999. Fatty Acid Composition of Bovine Embryos Cultured in Serum-Free and Serum-Containing Medium during Early Embryonic Development. *Journal of Reproduction and Development,* 45**,** 97-103.
SATOH, M. & KUROIWA, T. 1991. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. *Exp Cell Res,* 196**,** 137-40.
SAUER, H., WARTENBERG, M. & HESCHELER, J. 2001. Reactive Oxygen Species as Intracellular Messengers During Cell Growth and Differentiation. *Cellular Physiology and Biochemistry,* 11**,** 173-186.
SCHÖNFELD, P. & WOJTCZAK, L. 2016. Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. *J Lipid Res,* 57**,** 943-54.
SCOTT, R., 3RD, ZHANG, M. & SELI, E. 2018. Metabolism of the oocyte and the preimplantation embryo: implications for assisted reproduction. *Curr Opin Obstet Gynecol,* 30**,** 163-170.
SEIDEL, G. E., JR. 2006. Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology,* 65**,** 228-35.
SEJBAEK, C. S., HAGEMAN, I., PINBORG, A., HOUGAARD, C. O. & SCHMIDT, L. 2013. Incidence of depression and influence of depression on the number of treatment cycles and births in a national cohort of 42 880 women treated with ART. *Human Reproduction,* 28**,** 1100-1109.
SHAAKER, M., RAHIMIPOUR, A., NOURI, M., KHANAKI, K., DARABI, M., FARZADI, L., SHAHNAZI, V. & MEHDIZADEH, A. 2012. Fatty acid composition of human follicular fluid phospholipids and fertilization rate in assisted reproductive techniques. *Iran Biomed J,* 16**,** 162-8.
SHAFIEI, G., ALMASI, M., NIKZAD, H., MIYAN, J., MAHABADI, J. A. & MOSHKDANIAN, G. 2020. l-carnitine reduces the adverse effects of ROS and up-regulates the expression of implantation related genes in in vitro developed mouse embryos. *Theriogenology,* 145**,** 59-66.
SHAH, S. M., SAINI, N., ASHRAF, S., ZANDI, M., MANIK, R. S., SINGLA, S. K., PALTA, P. & CHAUHAN, M. S. 2015. Development,

Characterization, and Pluripotency Analysis of Buffalo (Bubalus bubalis) Embryonic Stem Cell Lines Derived from In Vitro-Fertilized, Hand-Guided Cloned, and Parthenogenetic Embryos. *Cell Reprogram,* 17**,** 306-22.

SHARMA, A., BADDELA, V. S., ROETTGEN, V., VERNUNFT, A., VIERGUTZ, T., DANNENBERGER, D., HAMMON, H. M., SCHOEN, J. & VANSELOW, J. 2020. Effects of Dietary Fatty Acids on Bovine Oocyte Competence and Granulosa Cells. *Frontiers in Endocrinology,* 11.

SHI, J. M., TIAN, X. Z., ZHOU, G. B., WANG, L., GAO, C., ZHU, S. E., ZENG, S. M., TIAN, J. H. & LIU, G. S. 2009. Melatonin exists in porcine follicular fluid and improves in vitro maturation and parthenogenetic development of porcine oocytes. *J Pineal Res,* 47**,** 318-23.

SHIMANO, H. & SATO, R. 2017. SREBP-regulated lipid metabolism: convergent physiology - divergent pathophysiology. *Nat Rev Endocrinol,* 13**,** 710-730.

SILVA, J. & SMITH, A. 2008. Capturing pluripotency. *Cell,* 132**,** 532-536.

SIMUNOVIC, M. & BRIVANLOU, A. H. 2017. Embryoids, organoids and gastruloids: new approaches to understanding embryogenesis. *Development (Cambridge, England),* 144**,** 976-985.

SINCLAIR, K. D. & WATKINS, A. J. 2013. Parental diet, pregnancy outcomes and offspring health: metabolic determinants in developing oocytes and embryos. *Reproduction, Fertility and Development,* 26**,** 99-114.

SINGH, P., AMIN, M., KELLER, E., SIMERMAN, A., AGUILERA, P., BRITON-JONES, C., HILL, D. L., ABBOTT, D. H., CHAZENBALK, G. & DUMESIC, D. A. 2013. A novel approach to quantifying ovarian cell lipid content and lipid accumulation in vitro by confocal microscopy in lean women undergoing ovarian stimulation for in vitro fertilization (IVF). *Journal of assisted reproduction and genetics,* 30**,** 733-740.

SOMFAI, T., KANEDA, M., AKAGI, S., WATANABE, S., HARAGUCHI, S., MIZUTANI, E., DANG-NGUYEN, T. Q., GESHI, M., KIKUCHI, K. & NAGAI, T. 2011. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod Fertil Dev,* 23**,** 912-20.

SOTO, D. A. & ROSS, P. J. 2016. Pluripotent stem cells and livestock genetic engineering. *Transgenic Res,* 25**,** 289-306.

SOVERNIGO, T. C., ADONA, P. R., MONZANI, P. S., GUEMRA, S., BARROS, F., LOPES, F. G. & LEAL, C. 2017. Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. *Reprod Domest Anim,* 52**,** 561-569.

SPICER, L. J., ALPIZAR, E. & ECHTERNKAMP, S. E. 1993. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. *J Anim Sci,* 71**,** 1232-41.

SPICER, L. J. & STEWART, R. E. 1996. Interactions among Basic Fibroblast Growth Factor, Epidermal Growth Factor, Insulin, and Insulin-Like Growth Factor-I (IGF-I) on Cell Numbers and Steroidogenesis of Bovine Thecal Cells: Role of IGF-I Receptors1. *Biology of Reproduction,* 54**,** 255-263.

SPRINGER, C., ZAKHARTCHENKO, V., WOLF, E. & SIMMET, K. 2021. Hypoblast Formation in Bovine Embryos Does Not Depend on NANOG. *Cells,* 10**,** 2232.

ST-PIERRE, J., GRÉGOIRE, J.-C. & VAILLANCOURT, C. 2017. A simple method to assess group difference in RT-qPCR reference gene

selection using GeNorm: The case of the placental sex. *Scientific Reports,* 7**,** 16923.

ST JOHN, J. C., FACUCHO-OLIVEIRA, J., JIANG, Y., KELLY, R. & SALAH, R. 2010. Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. *Hum Reprod Update,* 16**,** 488-509.

STACHELSCHEID, H., WULF-GOLDENBERG, A., ECKERT, K., JENSEN, J., EDSBAGGE, J., BJÖRQUIST, P., RIVERO, M., STREHL, R., JOZEFCZUK, J., PRIGIONE, A., ADJAYE, J., URBANIAK, T., BUSSMANN, P., ZEILINGER, K. & GERLACH, J. C. 2013. Teratoma formation of human embryonic stem cells in three-dimensional perfusion culture bioreactors. *Journal of Tissue Engineering and Regenerative Medicine,* 7**,** 729-741.

STEFANELLO, J. R., BARRETA, M. H., PORCIUNCULA, P. M., ARRUDA, J. N., OLIVEIRA, J. F., OLIVEIRA, M. A. & GONÇALVES, P. B. 2006. Effect of angiotensin II with follicle cells and insulin-like growth factor-I or insulin on bovine oocyte maturation and embryo development. *Theriogenology,* 66**,** 2068-76.

STOECKLEIN, K. S., ORTEGA, M. S., SPATE, L. D., MURPHY, C. N. & PRATHER, R. S. 2021. Improved cryopreservation of in vitro produced bovine embryos using FGF2, LIF, and IGF1. *PloS one,* 16**,** e0243727-e0243727.

STOJKOVIC, M., MACHADO, S. A., STOJKOVIC, P., ZAKHARTCHENKO, V., HUTZLER, P., GONÇALVES, P. B. & WOLF, E. 2001. Mitochondrial Distribution and Adenosine Triphosphate Content of Bovine Oocytes Before and After In Vitro Maturation: Correlation with Morphological Criteria and Developmental Capacity After In Vitro Fertilization and Culture1. *Biology of Reproduction,* 64**,** 904-909.

STRAUSS, J. F., MODI, B. & MCALLISTER, J. M. 2014. Chapter 18 - Defects in Ovarian Steroid Hormone Biosynthesis. *In:* ULLOA-AGUIRRE, A. & CONN, P. M. (eds.) *Cellular Endocrinology in Health and Disease.* Boston: Academic Press.

STRINGFELLOW D, G. M. M. O. T. I. E. T. S., 4TH EDN. CHAMPAIGN, IL: INTERNATIONAL EMBRYO TRANSFER SOCIETY, 2010 2010. Manual of the International Embryo Transfer Society, 4th edn. Champaign, IL: International Embryo Transfer Society, 2010.

STURMEY, R., REIS, A., LEESE, H. & MCEVOY, T. 2009. Role of Fatty Acids in Energy Provision During Oocyte Maturation and Early Embryo Development. *Reproduction in Domestic Animals,* 44**,** 50-58.

STURMEY, R. G. & LEESE, H. J. 2003. Energy metabolism in pig oocytes and early embryos. *Reproduction,* 126**,** 197-204.

SUDANO, M. J., RASCADO, T. D., TATA, A., BELAZ, K. R., SANTOS, V. G., VALENTE, R. S., MESQUITA, F. S., FERREIRA, C. R., ARAÚJO, J. P., EBERLIN, M. N. & LANDIM-ALVARENGA, F. D. 2016. Lipidome signatures in early bovine embryo development. *Theriogenology,* 86**,** 472-484.e1.

SUDANO, M. J., SANTOS, V. G., TATA, A., FERREIRA, C. R., PASCHOAL, D. M., MACHADO, R., BURATINI, J., EBERLIN, M. N. & LANDIM-ALVARENGA, F. D. C. 2012. Phosphatidylcholine and Sphingomyelin Profiles Vary in Bos taurus indicus and Bos taurus taurus In Vitro- and In Vivo-Produced Blastocysts1. *Biology of Reproduction,* 87.

SUGIURA, K. & EPPIG, J. J. 2005. Society for Reproductive Biology Founders Lecture 2005. Control of metabolic cooperativity between oocytes and their companion granulosa cells by mouse oocytes. *Reproduction, Fertility and Development,* 17**,** 667-674.

SUN, G. W., KOBAYASHI, H., SUZUKI, M., KANAYAMA, N. & TERAO, T. 2002. Link protein as an enhancer of cumulus cell-oocyte complex expansion. *Mol Reprod Dev,* 63**,** 223-31.

SUTTON-MCDOWALL, M., FEIL, D., ROBKER, R., THOMPSON, J. & DUNNING, K. 2012. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. *Theriogenology,* 77**,** 1632-41.

SUTTON-MCDOWALL, M. L., GILCHRIST, R. B. & THOMPSON, J. G. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. 139**,** 685.

SUTTON-MCDOWALL, M. L., WU, L. L. Y., PURDEY, M., ABELL, A. D., GOLDYS, E. M., MACMILLAN, K. L., THOMPSON, J. G. & ROBKER, R. L. 2016. Nonesterified Fatty Acid-Induced Endoplasmic Reticulum Stress in Cattle Cumulus Oocyte Complexes Alters Cell Metabolism and Developmental Competence1. *Biology of Reproduction,* 94.

SUTTON, M. L., GILCHRIST, R. B. & THOMPSON , J. G. 2003. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus–oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update,* 9**,** 35-48.

SZTALRYD, C. & BRASAEMLE, D. L. 2017. The perilipin family of lipid droplet proteins: Gatekeepers of intracellular lipolysis. *Biochimica et biophysica acta. Molecular and cell biology of lipids,* 1862**,** 1221-1232.

TABOR, D. E., KIM, J. B., SPIEGELMAN, B. M. & EDWARDS, P. A. 1998. Transcriptional activation of the stearoyl-CoA desaturase 2 gene by sterol regulatory element-binding protein/adipocyte determination and differentiation factor 1. *J Biol Chem,* 273**,** 22052-8.

TAKAHASHI, M., KEICHO, K., TAKAHASHI, H., OGAWA, H., SCHULTZ, R. M. & OKANO, A. 2000. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology,* 54**,** 137-45.

TAM, P. P., LOEBEL, D. A. & TANAKA, S. S. 2006. Building the mouse gastrula: signals, asymmetry and lineages. *Curr Opin Genet Dev,* 16**,** 419-25.

TAMURA, H., NAKAMURA, Y., KORKMAZ, A., MANCHESTER, L. C., TAN, D. X., SUGINO, N. & REITER, R. J. 2009. Melatonin and the ovary: physiological and pathophysiological implications. *Fertil Steril,* 92**,** 328-43.

TANGHE, S., VAN SOOM, A., NAUWYNCK, H., CORYN, M. & DE KRUIF, A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev,* 61**,** 414-24.

TATEMOTO, H., OOTAKI, K., SHIGETA, K. & MUTO, N. 2001. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during in vitro maturation. *Biol Reprod,* 65**,** 1800-6.

TATONE, C., DI EMIDIO, G., VITTI, M., DI CARLO, M., SANTINI, S., JR., D'ALESSANDRO, A. M., FALONE, S. & AMICARELLI, F. 2015. Sirtuin Functions in Female Fertility: Possible Role in Oxidative Stress and Aging. *Oxidative medicine and cellular longevity,* 2015**,** 659687-659687.

THOMPSON, J. G. 2000. In vitro culture and embryo metabolism of cattle and sheep embryos - a decade of achievement. *Anim Reprod Sci,* 60-61**,** 263-75.

THUNDATHIL, J., FILION, F. & SMITH, L. C. 2005. Molecular control of mitochondrial function in preimplantation mouse embryos. *Mol Reprod Dev,* 71**,** 405-13.

TIAN, H., LIU, K., ZHANG, Y., QI, Q., WANG, C., GUAN, H., YAN, F. & HOU, J. 2019. Adult follicular fluid supplementation during in vitro maturation improves the developmental competence of prepubertal lamb oocytes. *Theriogenology,* 130**,** 157-162.

TIAN, X., WANG, F., HE, C., ZHANG, L., TAN, D., REITER, R. J., XU, J., JI, P. & LIU, G. 2014. Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. *Journal of Pineal Research,* 57**,** 239-247.

TIAN, X., WANG, F., ZHANG, L., HE, C., JI, P., WANG, J., ZHANG, Z., LV, D., ABULIZI, W., WANG, X., LIAN, Z. & LIU, G. 2017. Beneficial Effects of Melatonin on the In Vitro Maturation of Sheep Oocytes and Its Relation to Melatonin Receptors. *Int J Mol Sci,* 18.

TIEGS, A. W., SUN, L., PATOUNAKIS, G. & SCOTT, R. T., JR 2019. Worth the wait? Day 7 blastocysts have lower euploidy rates but similar sustained implantation rates as Day 5 and Day 6 blastocysts. *Human Reproduction,* 34**,** 1632-1639.

TOMEK, W. & SMILJAKOVIC, T. 2005. Activation of Akt (protein kinase B) stimulates metaphase I to metaphase II transition in bovine oocytes. *Reproduction,* 130**,** 423-30.

TORRES, V., HAMDI, M., MILLÁN DE LA BLANCA, M. G., URREGO, R., ECHEVERRI, J., LÓPEZ-HERRERA, A., RIZOS, D., GUTIÉRREZ-ADÁN, A. & SÁNCHEZ-CALABUIG, M. J. 2018. Resveratrol-cyclodextrin complex affects the expression of genes associated with lipid metabolism in bovine in vitro produced embryos. *Reprod Domest Anim,* 53**,** 850-858.

TRIPATHI, A., KUMAR, K. V. & CHAUBE, S. K. 2010. Meiotic cell cycle arrest in mammalian oocytes. *J Cell Physiol,* 223**,** 592-600.

TRUONG, T. & GARDNER, D. K. 2017. Antioxidants improve IVF outcome and subsequent embryo development in the mouse. *Hum Reprod,* 32**,** 2404-2413.

TRUONG, T. T., SOH, Y. M. & GARDNER, D. K. 2016. Antioxidants improve mouse preimplantation embryo development and viability. *Human Reproduction,* 31**,** 1445-1454.

TSUJII, H., KHANDOKER, M. A. M. Y. & HAMANO, K.-I. 2001. Lipid in Mammalian Embryo Development. *Journal of Mammalian Ova Research,* 18**,** 73-80.

TURNER, D. A., GIRGIN, M., ALONSO-CRISOSTOMO, L., TRIVEDI, V., BAILLIE-JOHNSON, P., GLODOWSKI, C. R., HAYWARD, P. C., COLLIGNON, J., GUSTAVSEN, C., SERUP, P., STEVENTON, B., P LUTOLF, M. & ARIAS, A. M. 2017. Anteroposterior polarity and elongation in the absence of extra-embryonic tissues and of spatially localised signalling in gastruloids: mammalian embryonic organoids. *Development (Cambridge, England),* 144**,** 3894-3906.

TUTT, D. A. R., SILVESTRI, G., SERRANO-ALBAL, M., SIMMONS, R. J., KWONG, W. Y., GUVEN-ATES, G., CANEDO-RIBEIRO, C., LABRECQUE, R., BLONDIN, P., HANDYSIDE, A. H., GRIFFIN, D. K. & SINCLAIR, K. D. 2021. Analysis of bovine blastocysts indicates ovarian stimulation does not induce chromosome errors, nor discordance between inner-cell mass and trophectoderm lineages. *Theriogenology,* 161**,** 108-119.

URRUTIA, N., YING, Y. & HARVATINE, K. J. 2017. The effect of conjugated linoleic acid, acetate, and their interaction on adipose tissue lipid metabolism in nonlactating cows. *J Dairy Sci,* 100**,** 5058-5067.

VALLIER, L., REYNOLDS, D. & PEDERSEN, R. A. 2004. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev Biol,* 275**,** 403-21.
VAN BLERKOM, J. 2009. Mitochondria in early mammalian development. *Semin Cell Dev Biol,* 20**,** 354-64.
VAN BLERKOM, J. 2011. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion,* 11**,** 797-813.
VAN HOECK, V., BOLS, P. E., BINELLI, M. & LEROY, J. L. 2014. Reduced oocyte and embryo quality in response to elevated non-esterified fatty acid concentrations: a possible pathway to subfertility? *Anim Reprod Sci,* 149**,** 19-29.
VAN HOECK, V., STURMEY, R. G., BERMEJO-ALVAREZ, P., RIZOS, D., GUTIERREZ-ADAN, A., LEESE, H. J., BOLS, P. E. J. & LEROY, J. L. M. R. 2011. Elevated Non-Esterified Fatty Acid Concentrations during Bovine Oocyte Maturation Compromise Early Embryo Physiology. *PLOS ONE,* 6**,** e23183.
VAN LEEUWEN, J., BERG, D. & PFEFFER, P. 2015. Morphological and gene expression changes in cattle embryos from hatched blastocyst to early gastrulation stages after transfer of in vitro produced embryos. *PLoS ONE,* 10**,** e0129787.
VAN WINKLE, L. J. 2001. Amino Acid Transport Regulation and Early Embryo Development. *Biology of Reproduction,* 64**,** 1-12.
VANELLA, A., RUSSO, A., ACQUAVIVA, R., CAMPISI, A., DI GIACOMO, C., SORRENTI, V. & BARCELLONA, M. L. 2000. L -propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector. *Cell Biol Toxicol,* 16**,** 99-104.
VASHISTHA, V. K. & BHUSHAN, R. 2015. Bioanalysis and enantioseparation of dl-carnitine in human plasma by the derivatization approach. *Bioanalysis,* 7**,** 2477-2488.
VAZ, F. M. & WANDERS, R. J. A. 2002. Carnitine biosynthesis in mammals. *The Biochemical journal,* 361**,** 417-429.
VÉLEZ, L. M., ABRUZZESE, G. A. & MOTTA, A. B. 2013. The biology of the peroxisome proliferator-activated receptor system in the female reproductive tract. *Curr Pharm Des,* 19**,** 4641-6.
WAI, T., TEOLI, D. & SHOUBRIDGE, E. A. 2008. The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. *Nat Genet,* 40**,** 1484-8.
WALTHER, T. C. & JR., R. V. F. 2012. Lipid Droplets and Cellular Lipid Metabolism. *Annual Review of Biochemistry,* 81**,** 687-714.
WANG, F., TIAN, X., ZHANG, L., HE, C., JI, P., LI, Y., TAN, D. & LIU, G. 2014a. Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after in vitro fertilization. *Fertil Steril,* 101**,** 577-86.
WANG, F., TIAN, X., ZHANG, L., HE, C., JI, P., LI, Y., TAN, D. & LIU, G. 2014b. Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after in vitro fertilization. *Fertility and Sterility,* 101**,** 577-586.e1.
WATANABE, K., UENO, M., KAMIYA, D., NISHIYAMA, A., MATSUMURA, M., WATAYA, T., TAKAHASHI, J. B., NISHIKAWA, S., NISHIKAWA, S.-I., MUGURUMA, K. & SASAI, Y. 2007. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology,* 25**,** 681-686.

WATT, M. J., STEINBERG, G. R., CHEN, Z. P., KEMP, B. E. & FEBBRAIO, M. A. 2006. Fatty acids stimulate AMP-activated protein kinase and enhance fatty acid oxidation in L6 myotubes. *J Physiol,* 574**,** 139-47.

WEINBERGER, L., AYYASH, M., NOVERSHTERN, N. & HANNA, J. H. 2016. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. *Nature Reviews Molecular Cell Biology,* 17**,** 155-169.

WETTERAU, L. A., MOORE, M. G., LEE, K.-W., SHIM, M. L. & COHEN, P. 1999. Novel Aspects of the Insulin-like Growth Factor Binding Proteins. *Molecular Genetics and Metabolism,* 68**,** 161-181.

WIGGLESWORTH, K., LEE, K.-B., EMORI, C., SUGIURA, K. & EPPIG, J. J. 2015. Transcriptomic Diversification of Developing Cumulus and Mural Granulosa Cells in Mouse Ovarian Follicles1. *Biology of Reproduction,* 92.

WONNACOTT, K. E., KWONG, W. Y., HUGHES, J., SALTER, A. M., LEA, R. G., GARNSWORTHY, P. C. & SINCLAIR, K. D. 2010. Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction,* 139**,** 57-69.

WU, B. & ZAN, L. 2012. Enhance beef cattle improvement by embryo biotechnologies. *Reprod Domest Anim,* 47**,** 865-71.

WU, D., CHEUNG, Q. C.-K., WEN, L. & LI, J. 2006. A Growth-Maturation System That Enhances the Meiotic and Developmental Competence of Porcine Oocytes Isolated from Small Follicles1. *Biology of Reproduction,* 75**,** 547-554.

WU, G., LV, C., MEMON, S., LIANG, J., ZHAO, X., OUYANG, Y., WANG, S., JIANG, Y., SHAO, Q., HONG, Q. & QUAN, G. 2020. The presence of synthetic polymers in the maturation medium affects the cryotolerance and developmental capacity after parthenogenic activation of vitrified goat oocytes. *Cryobiology,* 93**,** 84-90.

WU, G. Q., JIA, B. Y., LI, J. J., FU, X. W., ZHOU, G. B., HOU, Y. P. & ZHU, S. E. 2011. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. *Theriogenology,* 76**,** 785-93.

WU, L. L., RUSSELL, D. L., NORMAN, R. J. & ROBKER, R. L. 2012. Endoplasmic Reticulum (ER) Stress in Cumulus-Oocyte Complexes Impairs Pentraxin-3 Secretion, Mitochondrial Membrane Potential (ΔΨm), and Embryo Development. *Molecular Endocrinology,* 26**,** 562-573.

WU, M.-L., TSAI, K.-L., WANG, S.-M., WU, J.-C., WANG, B.-S. & LEE, Y.-T. 1996. Mechanism of Hydrogen Peroxide and Hydroxyl Free Radical–Induced Intracellular Acidification in Cultured Rat Cardiac Myoblasts. *Circulation Research,* 78**,** 564-572.

XIAO, Y., AMARAL, T. F., ROSS, P. J., SOTO, D. A., DIFFENDERFER, K. E., PANKONIN, A. R., JEENSUK, S., TRÍBULO, P. & HANSEN, P. J. 2021. Importance of WNT-dependent signaling for derivation and maintenance of primed pluripotent bovine embryonic stem cells†. *Biol Reprod,* 105**,** 52-63.

XIONG, X., LAN, D., LI, J., LIN, Y. & LI, M. 2018. Selenium supplementation during in vitro maturation enhances meiosis and developmental capacity of yak oocytes. *Anim Sci J,* 89**,** 298-306.

XU, H.-Y., GENG, S.-S., LI, T.-T., FU, Q., LU, S.-S., LIANG, X.-W., LU, Y.-Q., ZHANG, M., YANG, X.-G. & LU, K.-H. 2019. Maturation of buffalo oocytes in vitro with acetyl-l-carnitine improves cryotolerance due to changes in mitochondrial function and the membrane lipid profile. *Reproduction, Fertility and Development,* 31**,** 386-394.

XU, H.-Y., YANG, X.-G., LU, S.-S., LIANG, X.-W., LU, Y.-Q., ZHANG, M. & LU, K.-H. 2018a. Treatment with acetyl-l-carnitine during in vitro maturation of buffalo oocytes improves oocyte quality and subsequent embryonic development. *Theriogenology,* 118**,** 80-89.

XU, H., JIA, C., CHENG, W., ZHANG, T., TAO, R., MA, Y., SI, L., XU, Y. & LI, J. 2020. The Effect of L-Carnitine Additive During In Vitro Maturation on the Vitrification of Pig Oocytes. *Cell Reprogram,* 22**,** 198-207.

XU, H. E., LAMBERT, M. H., MONTANA, V. G., PLUNKET, K. D., MOORE, L. B., COLLINS, J. L., OPLINGER, J. A., KLIEWER, S. A., GAMPE, R. T., JR., MCKEE, D. D., MOORE, J. T. & WILLSON, T. M. 2001. Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A,* 98**,** 13919-24.

XU, H. Y., YANG, X. G., LU, S. S., LIANG, X. W., LU, Y. Q., ZHANG, M. & LU, K. H. 2018b. Treatment with acetyl-l-carnitine during in vitro maturation of buffalo oocytes improves oocyte quality and subsequent embryonic development. *Theriogenology,* 118**,** 80-89.

XU, Z., ROBITAILLE, A. M., BERNDT, J. D., DAVIDSON, K. C., FISCHER, K. A., MATHIEU, J., POTTER, J. C., RUOHOLA-BAKER, H. & MOON, R. T. 2016. Wnt/β-catenin signaling promotes self-renewal and inhibits the primed state transition in naïve human embryonic stem cells. *Proc Natl Acad Sci U S A,* 113**,** E6382-e6390.

YANG, X., WU, L. L., CHURA, L. R., LIANG, X., LANE, M., NORMAN, R. J. & ROBKER, R. L. 2012. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertil Steril,* 97**,** 1438-43.

YAZAKI, T., HIRADATE, Y., HOSHINO, Y., TANEMURA, K. & SATO, E. 2013. L-carnitine improves hydrogen peroxide-induced impairment of nuclear maturation in porcine oocytes. *Animal Science Journal,* 84**,** 395-402.

YENUGANTI, V. R., VIERGUTZ, T. & VANSELOW, J. 2016. Oleic acid induces specific alterations in the morphology, gene expression and steroid hormone production of cultured bovine granulosa cells. *General and comparative endocrinology,* 232**,** 134-144.

YING, Q. L., WRAY, J., NICHOLS, J., BATLLE-MORERA, L., DOBLE, B., WOODGETT, J., COHEN, P. & SMITH, A. 2008. The ground state of embryonic stem cell self-renewal. *Nature,* 453**,** 519-23.

YOON, M. 2009. The role of PPARalpha in lipid metabolism and obesity: focusing on the effects of estrogen on PPARalpha actions. *Pharmacol Res,* 60**,** 151-9.

YOU, J., LEE, J., HYUN, S. H. & LEE, E. 2012. L-carnitine treatment during oocyte maturation improves in vitro development of cloned pig embryos by influencing intracellular glutathione synthesis and embryonic gene expression. *Theriogenology,* 78**,** 235-43.

YOUNG, L. E., SINCLAIR, K. D. & WILMUT, I. 1998. Large offspring syndrome in cattle and sheep. *Rev Reprod,* 3**,** 155-63.

YUAN, Y., SPATE, L. D., REDEL, B. K., TIAN, Y., ZHOU, J., PRATHER, R. S. & ROBERTS, R. M. 2017. Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation. *Proc Natl Acad Sci U S A,* 114**,** E5796-e5804.

ZARE, Z., MASTERI FARAHANI, R., SALEHI, M., PIRYAEI, A., GHAFFARI NOVIN, M., FADAEI FATHABADI, F., MOHAMMADI, M. & DEHGHANI-MOHAMMADABADI, M. 2015. Effect of L-carnitine supplementation on maturation and early embryo development of

immature mouse oocytes selected by brilliant cresyle blue staining. *Journal of Assisted Reproduction and Genetics,* 32**,** 635-643.

ZEGERS-HOCHSCHILD, F., ADAMSON, G. D., DE MOUZON, J., ISHIHARA, O., MANSOUR, R., NYGREN, K., SULLIVAN, E., VAN DER POEL, S., ICMART, O. B. O. & WHO 2009. The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009†. *Human Reproduction,* 24**,** 2683-2687.

ZELEZNIK, A. J., SAXENA, D. & LITTLE-IHRIG, L. 2003. Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. *Endocrinology,* 144**,** 3985-94.

ZENG, H. T., REN, Z., YEUNG, W. S., SHU, Y. M., XU, Y. W., ZHUANG, G. L. & LIANG, X. Y. 2007. Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in in vitro matured human oocytes. *Hum Reprod,* 22**,** 1681-6.

ZHANG, J. Y., WU, Y., ZHAO, S., LIU, Z. X., ZENG, S. M. & ZHANG, G. X. 2015a. Lysosomes are involved in induction of steroidogenic acute regulatory protein (StAR) gene expression and progesterone synthesis through low-density lipoprotein in cultured bovine granulosa cells. *Theriogenology,* 84**,** 811-7.

ZHANG, K. & EALY, A. D. 2012. Supplementing fibroblast growth factor 2 during bovine oocyte <i>in vitro</i> maturation promotes subsequent embryonic development. *Open Journal of Animal Sciences,* Vol.02No.02**,** 8.

ZHANG, L., HAN, L., MA, R., HOU, X., YU, Y., SUN, S., XU, Y., SCHEDL, T., MOLEY, K. H. & WANG, Q. 2015b. Sirt3 prevents maternal obesity-associated oxidative stress and meiotic defects in mouse oocytes. *Cell Cycle,* 14**,** 2959-2968.

ZHANG, M., SU, Y. Q., SUGIURA, K., XIA, G. & EPPIG, J. J. 2010. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science,* 330**,** 366-9.

ZHANG, S. & CUI, W. 2014. Sox2, a key factor in the regulation of pluripotency and neural differentiation. *World journal of stem cells,* 6**,** 305-311.

ZHAO, F., ZHAO, W., REN, S., FU, Y., FANG, X., WANG, X. & LI, B. 2014. Roles of SIRT1 in granulosa cell apoptosis during the process of follicular atresia in porcine ovary. *Anim Reprod Sci,* 151**,** 34-41.

ZHOU, L., LIM, Q.-E., WAN, G. & TOO, H.-P. 2010. Normalization with genes encoding ribosomal proteins but not GAPDH provides an accurate quantification of gene expressions in neuronal differentiation of PC12 cells. *BMC genomics,* 11**,** 75-75.

ZHOU, P., BAUMGARTEN, S. C., WU, Y., BENNETT, J., WINSTON, N., HIRSHFELD-CYTRON, J. & STOCCO, C. 2013. IGF-I signaling is essential for FSH stimulation of AKT and steroidogenic genes in granulosa cells. *Mol Endocrinol,* 27**,** 511-23.

ZOLINI, A. M., CARRASCAL-TRIANA, E., RUIZ DE KING, A., HANSEN, P. J., ALVES TORRES, C. A. & BLOCK, J. 2019. Effect of addition of l-carnitine to media for oocyte maturation and embryo culture on development and cryotolerance of bovine embryos produced in vitro. *Theriogenology,* 133**,** 135-143.

APPENDICES

I. MEDIA RECIPES FOR IN VITRO PRODUCTION

1. Ovary collection medium- Phosphate buffered saline (PBS)

Component	g/l	M
NaCl	8	0.1369
KCl	0.2	0.0027
Na_2HPO_4	1.44	0.0101
KH_2PO_4	0.24	0.0018

pH adjusted to 7.4 and sterilised by autoclaving.

2. Oocyte search and collection medium

Component	For 20ml	For 50ml
TCM 199 (HEPES)	20ml	50ml
FCS	1ml	2.5ml
L-Glutamine (100mM)	200µl	500µl
Pen/Strep	200µl	500µl

Warmed at 37ºC at least for 1 hour before use.

3. Oocyte maturation medium

Component	Amount
TCM199 (NaHCO3)	9ml
FCS	1ml
L-Glutamine (100mM)	100µl
Na pyruvate (20mM)	100µl
Pen/Strep	100µl
LH/FSH (5IU/ml)	40µl

pH adjusted to 7.3-7.4 with NaOH and HCl and mOsmo adjusted to 290-300 with H_2O and NaCl. Freshly prepared on a day before use and equilibrated in 5% CO_2 in air overnight. Sterilised by filtration through a 0.22 µm Millipore filter.

4. TALP based medium

a. TALP base

Reagent	For 50ml	For 100ml	For 500ml	Stock
H_2O	40 ml	80 ml	401 ml	
$NaHCO_3$	5 ml	10 ml	50 ml	250mM
NaCl	2.17 ml	4.34 ml	21.7 ml	2.3M
$MgCl_2$	550 µl	1.1 ml	5.5 ml	100mM
CaCl2	500 µl	1 ml	5 ml	200mM
NaH_2PO_4	500 µl	1 ml	5 ml	34mM
KCl	160 µl	0.32 ml	1.6 ml	1M
Na-Lactate	152 µl	0.304 ml	1.519 ml	As 60% syrup

Made freshly every week.

b. TALP based medium preparation

Reagent	Sperm-TALP (Capacitation)	Hepes-TALP (Oocyte wash)	IVF-TALP (Fertilisation)	Stock

BSA	60 mg	30 mg	60 mg	
TL base	10 ml	10 ml	10 ml	1x
Pen-strep	100 µl	100 µl	100 µl	10000 U/ml
pyruvate	52 µl	100 µl	100 µl	20 mM
HEPES	100 µl	250 µl	0	1 M
Caffeine	0	0	100 µl	200 mM
heparin	0	0	10 µl	1500 U/ml
Hypotaurine Epinephrine	0	0	10 µl	0.5 mg/ml
pH	7.4	7.3	7.4	
Osmolarity (mOsm)	295-305	275-285	290-300	

Fertilization medium equilibrated in 5% CO_2 in air overnight and sterilised by filtration through a 0.22 µm Millipore filter.

5. Synthetic Oviduct Fluid medium (Stoecklein et al.) based medium

a. SOF base

Reagent	**For 50 ml**	**For 100 ml**	**Stock**
H_2O	46 ml	92 ml	
NaCl	2.38 ml	4.76 ml	2.3 M
$CaCl_2.H_2O$	600 µl	1.2 ml	200 mM
KCl	360 µl	720 µl	1 M
KH_2PO_4	300 µl	600 µl	200 mM
$MgSO_4$	175 µl	350 µl	200 mM
Tri-sodium citrate	100 µl	200 µl	200 mM
Phenol red	100 µl	200 µl	0.5% solution
Glucose	50 µl	100 µl	200 mM
Na-Lactate	38 µl	75.7 µl	As 60% syrup

Made freshly every week.

b. SOF base medium preparation

Reagent	Culture	Holding
BSA	30mg	30mg
SOF base	10ml	10ml
$NaHCO_3$ (250mM stock)	1ml	150ul
BME amino acids (50x)	400 µl	100 µl
MEM amino acids (100x)	100 µl	50 µl
Myo-inositol (50 mg/ml)	100 µl	-
Na pyruvate (20 mM)	100 µl	100 µl
L-glutamine (100 mM)	100 µl	100 µl
Pen/Strep	100 µl	100 µl
HEPES	-	250 µl
pH	7.4	7.4
mOsm	270-290	270-290

6. Acetyl-L-Carnitine preparation

Top stocks (100 mM ALC) were prepared in TCM199 for IVM and in SOF base for IVC. Stocks were aliquoted and frozen in -20ºC. Reagents were increased by 10% while medium was prepared.

100mM ALC
(0.2397 g/10 ml TCM199 or SOF base)

↓

200 µl top stock + 1.8 ml base
(10 mM ALC-base)

↓

200 µl 10 mM ALC-base +1.8 ml media
(1mM)

↓

200 µl (10 mM ALC-base + 1.8 ml base) 1 mM ALC-base +1.8 ml media
(0.1 mM)

↓

200 µl (1 mM ALC-base + 1.8 ml base) 0.1 mM ALC-base +1.8 ml media
(0.01 mM)

7. Ingredients added to *in vitro* modified maturation media

Ingredients and their stock concentrations	Solvents	Aliquot concentration	Amount added to 20 ml media	Final Concentrations in the media
IAI (0.88 µg/µl)	Keep stock as received	-	220 µl	1 µg/ml
IGF1 (0.5 mg/ml)	Dilute 1:10 with water	50 µg/ml	4 µl	10 ng/ml
FGF2 (0.5 mg/ml)	Dilute 1:200 with water	2.5 µg/ml	4 µl	0.5 ng/ml
Insulin (10 mg/ml)	**Dilute 1:100 (100ug/ml); further dilute 1:10 mg with 1% Acetic acid	10 mg/ml	4 µl	2 ng/ml
Selenium (50 mg/ml)	Dilute 1:100 (500 ng/ml); further dilute 1:10	50 ng/ml	4 µl	10 ng/ml
Transferrin (10 mg/ml)	Keep stock as received	-	20 µl	10 µg/ml
Ascorbic acid (10 mg/ml)	Keep stock as received	-	80 µl	40 µg/ml
L-cysteine (25 mg/ml)	Keep stock as received	-	71 µl	90 µg/ml
Beta-mercaptoethanol (14.3M)	Dilute 1:14	1 M	2 µl	0.1mM
*Glycine	Keep stock as received	-	9 mg	450 µg/ml
*Palmitic acid (500x)	128 mg in 5ml absolute ethanol	100 mM	10 µl	50 µM
*Stearic acid (500X)	71 mg in 5 ml absolute ethanol	50 mM	10 µl	25 µM
*Oleic Acid (500x)	212 mg in 5 ml absolute ethanol	150 mM	10 µl	75 µM
*Linoleic acid (500x)	70 mg in 2.5 ml absolute ethanol	100 mM	10 µl	40 µM
*Linolenic acid (500x)	69 mg in 2.5 ml absolute ethanol	100 mM	10 µl	40 µM

***** They were added directly to maturation media on day of OPU and/or DFR.

** Insulin which comes as a lyophilised powder needs to be dissolved in a 1% acetic acid solution. Therefore, 100 µl acetic acid needs to be added to 9.9 ml of water to make a 1% acetic acid solution

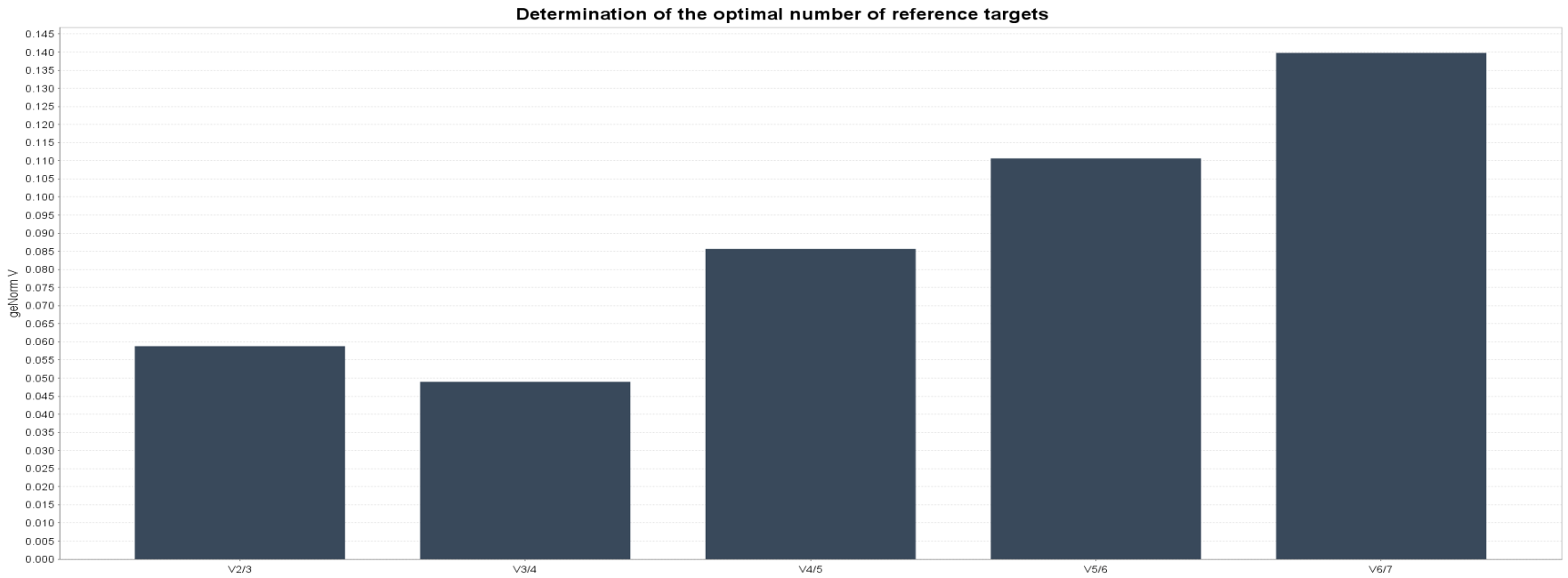
8. Fatty acid preparation for conjugation with BSA

Fatty Acids	1 M (mg/ml)	Added amount to 5 ml absolute ethanol	Aliquot Conc.	Final Conc.	Amount ordered
Palmitic	256.43	32.05 mg	25 mM	25 µM	10 g - Powder
Stearic	284.48	21.34 mg	15 mM	15 µM	1 g - Powder
Oleic	282.47	21.19 mg	15 mM	15 µM	1 g - Liquid
Linoleic	280.5	7.01 mg	5 mM	5 µM	1 g - Liquid
Linolenic	278.43	6.96 mg	5 mM	5 µM	500 mg - Liquid

To make ethanol stocks – add FA to absolute ethanol and warm to 38 °C to dissolve and vortex.

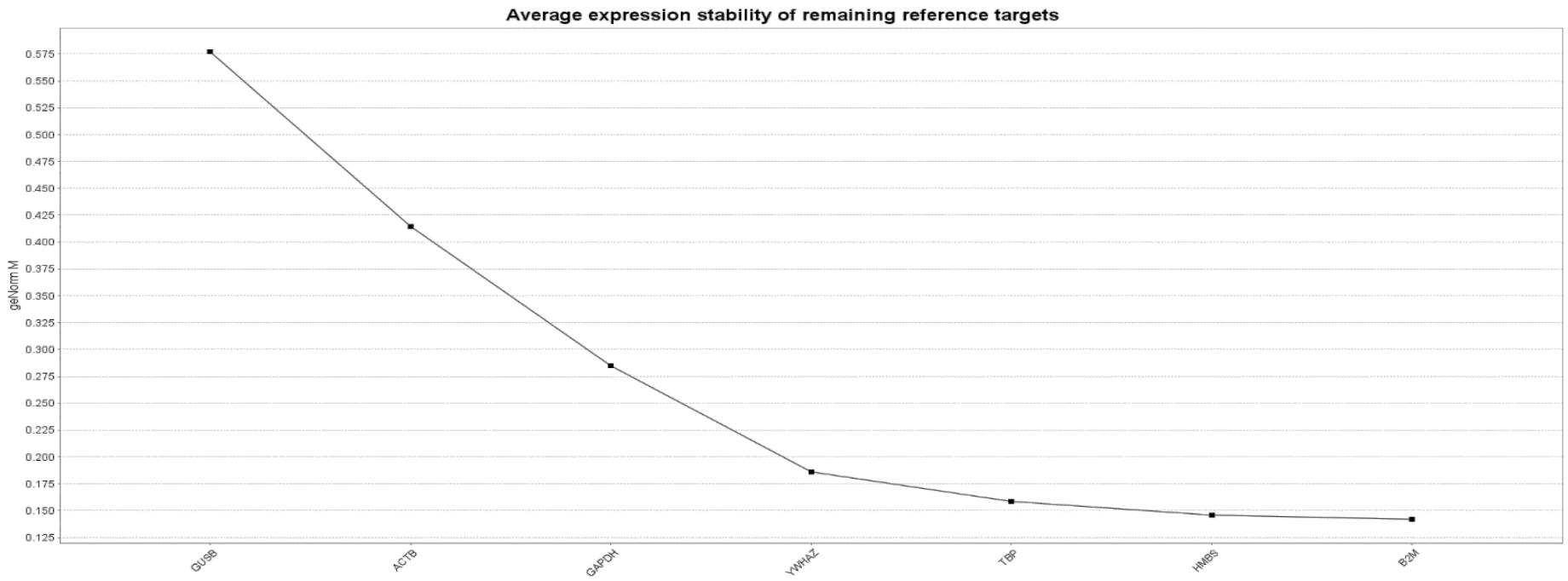
II. Reference gene selection using GeNorm

1. Determination of the optimal number of reference genes



GeNorm analysis was run on 2 samples (cumulus cells exposed to 0 mM ALC and 1 mM ALC) using 7 candidate reference genes. Optimal reference target is 5 (geNorm V<0.15).

2. Average expression stability of reference genes



GeNorm analysis was run on 2 samples (cumulus cells exposed to 0 mM ALC and 1 mM ALC) using 7 candidate reference genes. High reference target stability (average geNorm M≤0.5).

III. GC-MS using FALOWSM6 program

Inlet	Mode		Gas: Helium	
Injection volume	2 µl			
Split ratio	1:25			
Split flow	33.2 ml/min			
Heater temperature	260°C			
pressure	43.1 psi			
Total flow	36.3 ml/min			
Column				
mode	Constant flow			
Pressure	43.1 psi			
Flow	1.3 ml/min			
Average velocity	24 cm/sec			
Oven				
Oven ramp	°C/min	Next °C	Hold min	Run time
Initial		140	0	
Ramp1	4	240	25	50

IV. Immunocytochemistry

1. Primary Antibodies

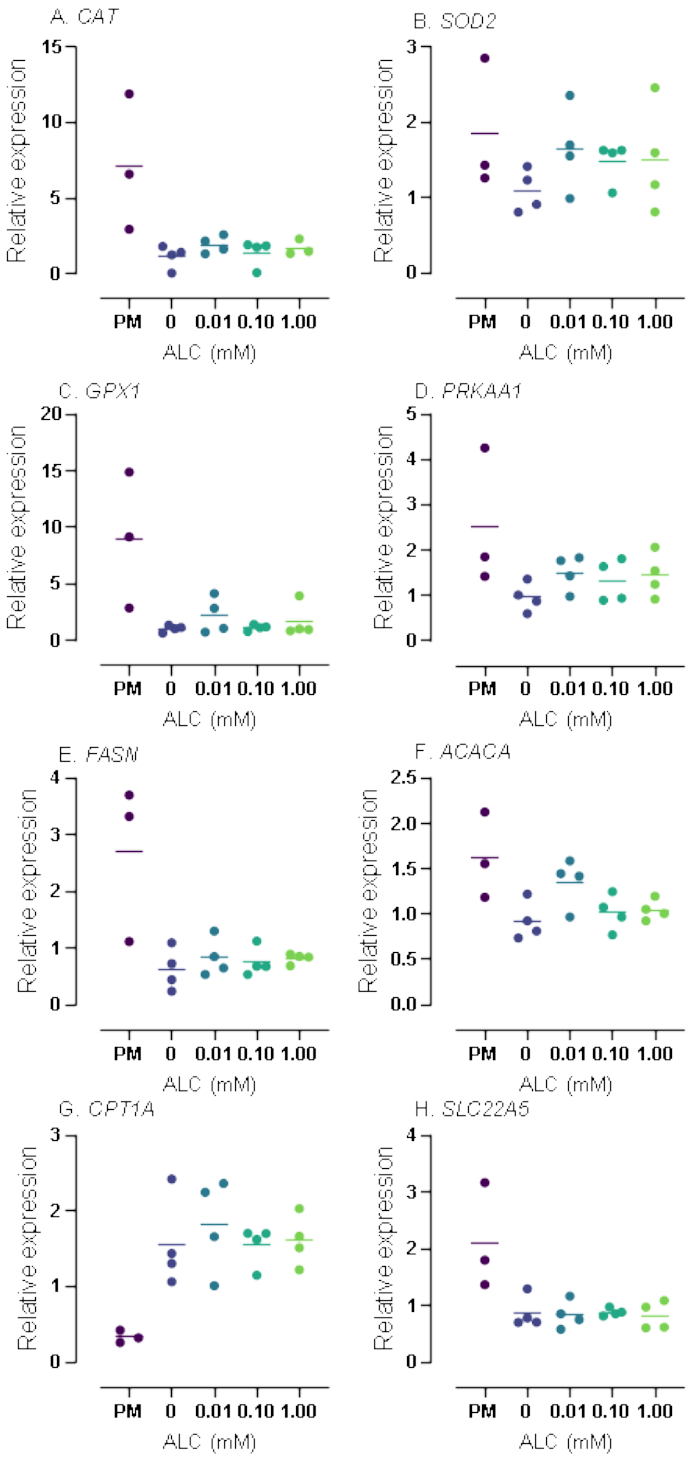
Antibodies	**Description**	**Company**	**Concentration**
Nanog	Rabbit polyclonal	Peprotech 500-P236	1:500
OCT4	Goat polyclonal	Santa Cruz SC8628	1:1000
SOX2	Rat monoclonal	Invitrogen 14-9811-82	1:5000
SSEA-4	Mouse monoclonal	Santa Cruz SC-21704	1:50
FOXA2	Goat polyclonal	Novusbio NB100-1263	1:100
TRA-1-60	Mouse monoclonal	Santa Cruz SC-21705	1:50
TRA-1-81	Mouse monoclonal	Santa Cruz SC-21706	1:50
SOX17	Rabbit monoclonal	Abcam ab224637	1:500
T (Brachyury)	Goat polyclonal	R&D AF2085	1:500

2. Secondary Antibodies

Antibodies	Description	Company	Concentration
Donkey anti-Rat	Cy3	Jackson 712-165-150	1:500
Donkey anti-Rabbit	488	Invitrogen A31572	1:500
Donkey anti-Mouse	Alexa 647	Invitrogen A-31571	1:500
Donkey anti-Goat	Alexa 647	Invitrogen A21447	1:500

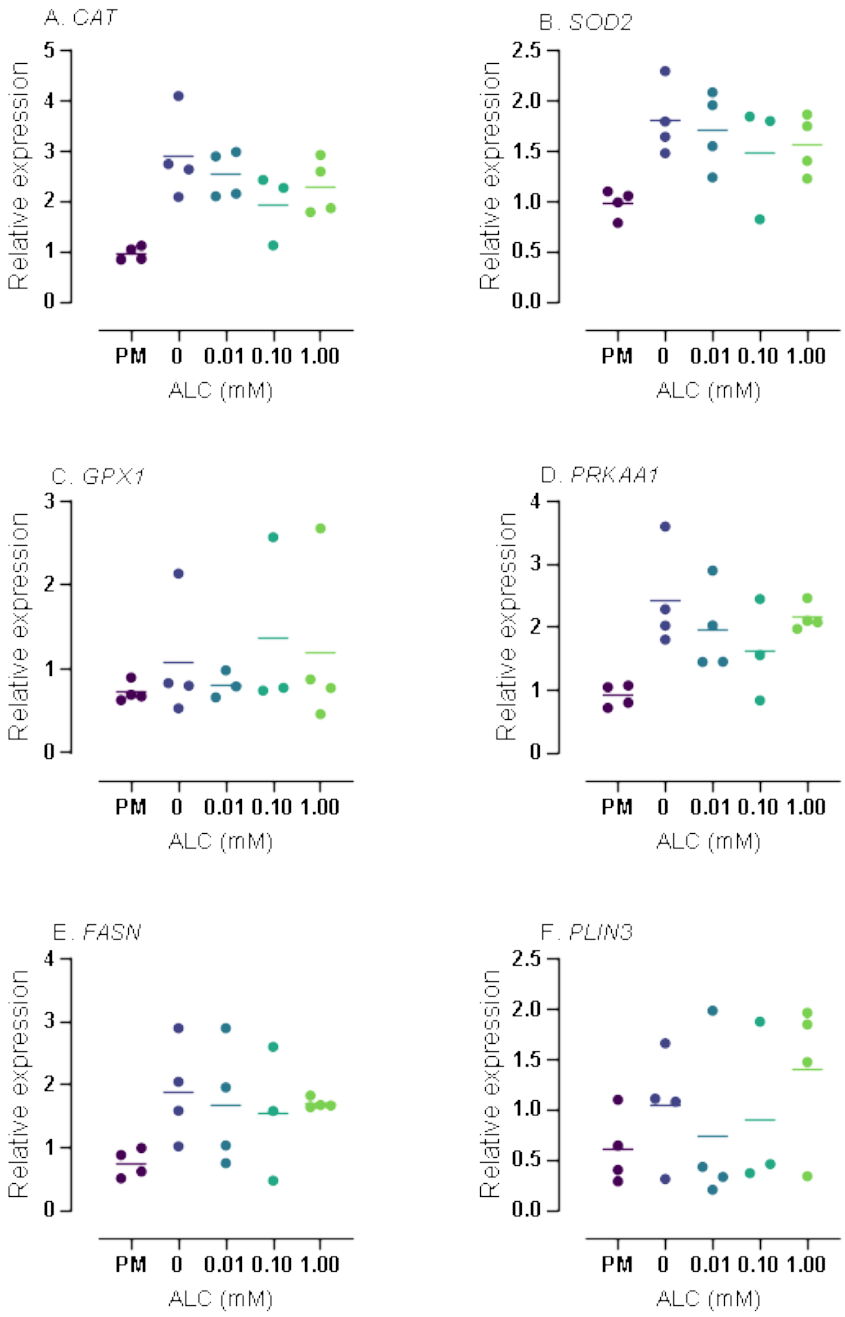
V. Raw data scatter plots

1. Cumulus cells gene expression (Figure 2.11,12 and 13)



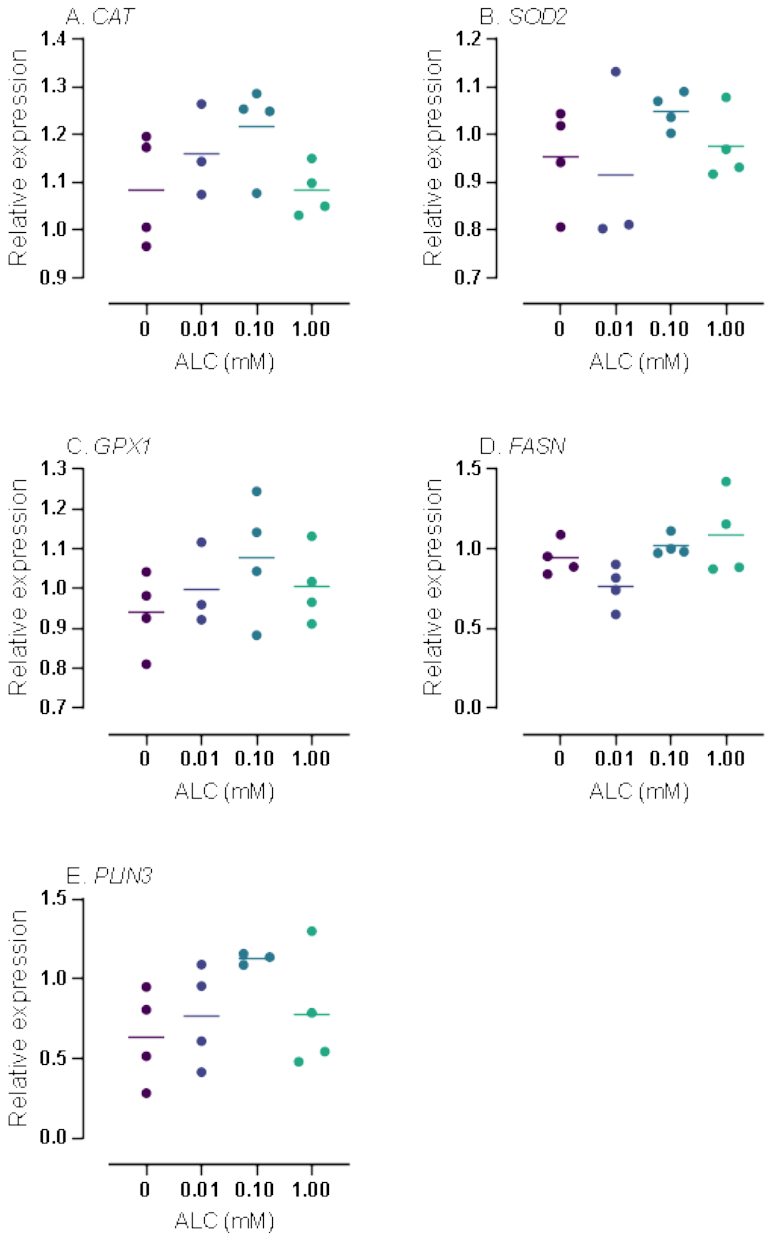
Gene expression in cumulus cells before and following the maturation with 0, 0.01, 0.1 and 1 mM ALC. PM; pre-maturation.

2. Oocyte gene expression (Figure 2.14)



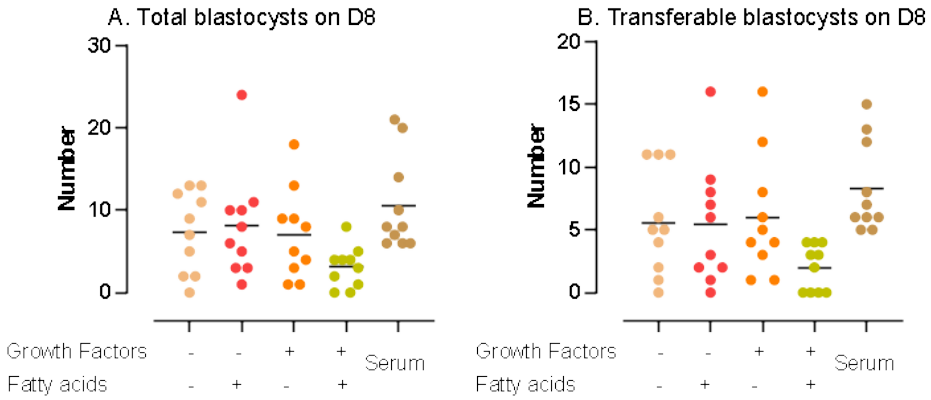
Gene expression in oocytes before and following the maturation with 0, 0.01, 0.1 and 1 mM ALC. PM; pre-maturation

3. Blastocysts gene expression (Figure 2.15)



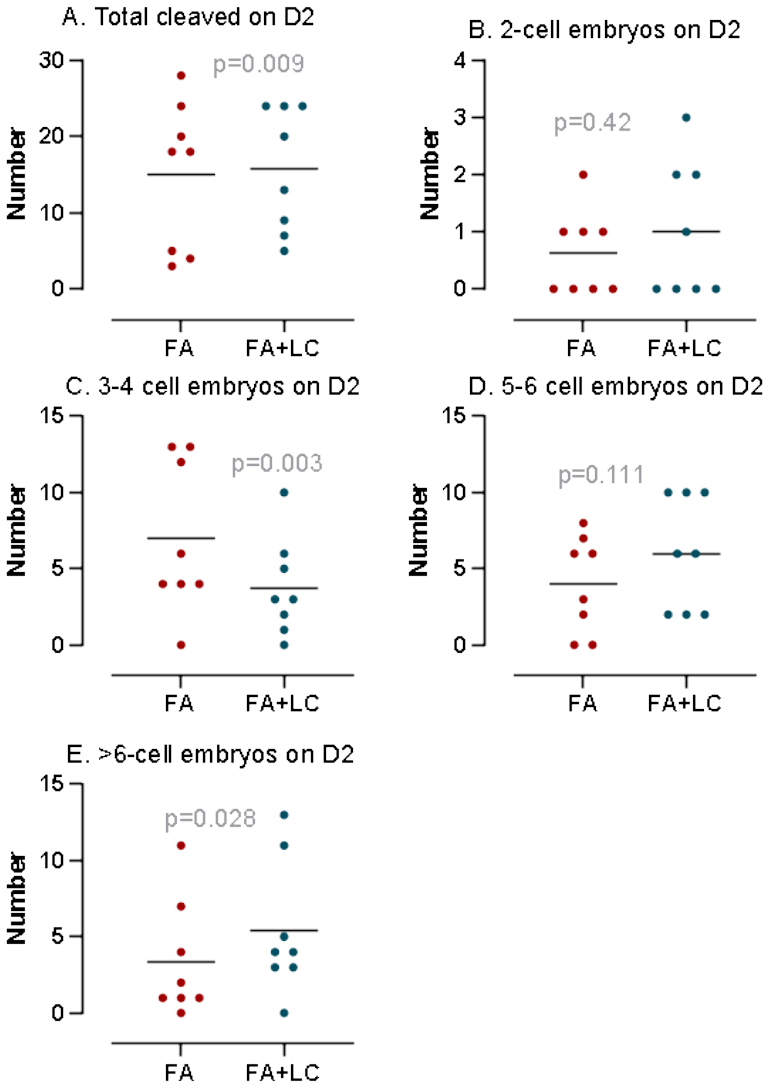
Gene expression on D8 blastocysts following the zygotes were cultured with 0, 0.01, 0.1 and 1 mM ALC.

4. Embryo Development (Figure 3.10)



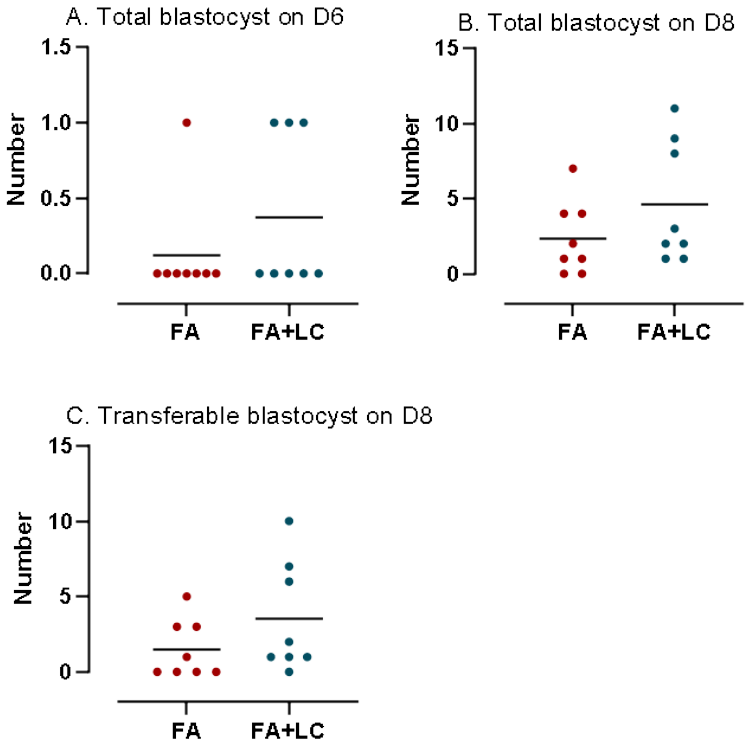
Day 8 blastocyst development following oocyte maturation in PVP based media supplemented with combinations of growth factors and fatty acids; '+' indicates the supplement exists in the media and '-' is for absence of supplements. Maturation in the presence of serum served as a positive control. Letters indicate statistically similarities and differences between treatment groups. Transferrable blastocysts refers to IETS Stages 7, 8 and 9.

5. Day 2 embryo development following insemination (Figure 4.1)



Day 2 embryo development following insemination. FA = Fatty acids, FA+LC = FA and L-carnitine.

6. Embryo Development (Figure 4.2)



D8 total and transferable (IETS Stage 7 to 9, Grades 1 and 2) blastocysts distribution per cycle. FA = Fatty acids, FA+LC = FA and L-carnitine.