Impact of Non-Esterified Fatty Acids during *In Vitro* Fertilisation on Bovine Preimplantation Embryo Development: Implications for Reproductive Medicine

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Abstract

Background: *In vitro* bovine models have shown that exposure to high NEFA concentrations (i.e., stearic acid [SA], palmitic acid [PA], oleic acid [OA]) impair oocyte competence and embryo quality. However, the effect of high NEFA exposure exclusively during the fertilisation process *per se* is less certain. The aim of this thesis was to investigate the effects of high NEFA (SA, PA, and OA) concentrations exclusively during *in vitro* fertilisation (IVF) on preimplantation embryo development and spermatozoa quality.

Methods: Bovine oocytes were *in vitro* matured, followed by IVF in physiological (control 1) and high NEFA concentrations. A second control group contained solvent. Zygotes were cultured for eight days (Day 0=IVF) and resultant preimplantation embryos examined. Spermatozoa were incubated for four hours under the same IVF conditions. Data were analysed with ANOVA.

Results: High NEFA exposure impaired fertilisation rate, cleavage, and blastocyst formation. Cell number in blastocysts was decreased, but reactive oxygen species production in zygotes was not affected. H3K27me3 was examined in early embryos, a repressive epigenetic mark that undergoes erasure from fertilisation to the time of embryonic genome activation to allow progression to the blastocyst stage. The expected lack of difference in H3K27me3 levels between 2- and 4-cell embryos was observed in the control groups. However, in the high-NEFA group levels in 2-cell embryos were still higher than in 4-cell embryos on day 2, suggesting that activation of developmentally important genes needed to progress to the blastocyst stage was potentially delayed, explaining partially the low blastocyst production. Spermatozoa exposed to high NEFA concentrations displayed damaged plasma membrane and acrosome, along with impaired mitochondrial membrane potential and increased DNA damage.

Conclusion: High NEFA concentrations can induce detrimental effects during the fertilisation process via both the oocyte and the spermatozoon resulting in impaired preimplantation embryo development.

Declaration

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Newcastle University and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications. I have read the University's current research ethics guidelines and accept responsibility for the conduct of the procedures in accordance with the University's Committee on the Use of animal tissues for Research purposes. I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

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Lists of Abbreviations

µM	Micromolar
3D	Three Dimensions
5mC	5-methylcytosine
ATP	Adenosine Triphosphate
AU	Arbitrary Units
BME	Basal Medium Eagle
BMI	Body Mass Index
BOECS	Bovine Oviductal Epithelial Cells
BSA	Bovine Serum Albumin
C1	Control 1
C2	Control 2
CDX2	Caudal-Type Homeobox 2
COCs	Cumulus Oocyte Complexes
dH_2O	Distilled Water
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DOHaD	Developmental Origins of Health and Disease
DPBS	Dulbecco's Phosphate Buffered Saline
e.g.	Exempli Gratia ("for example")
EED	Embryonic Ectoderm Development
EGA	Embryonic Genome Activation
EZH2	Enhancer of Zeste Homolog 2
FITC	Fluorescein Isothiocyanate

GH	Growth Hormone
H3K27me3	Histone H3 at Lysine 27 Trimethylation
hCG	Human Chorionic Gonadotrophin
hpf	Hours post-fertilisation
i.e.	Id Est (“that is”)
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IETS	International Embryo Transfer Society
IGF-1	Insulin-Like Growth Factor-1
ITS	Insulin, Transferrin, And Selenium
IU	International Unit
IVEC	*In Vitro* Embryo Culture
IVF	*In Vitro* fertilisation
IVM	*In Vitro* Maturation
JC-1	Carbocyanine Iodide 1
JMJD3	Jumonji Domain-Containing Protein 3
KDM6B	Lysine Demethylase 6B
LSD	Least Significant Difference
mEGF	Murine Epidermal Growth Factor
MEM	Minimum Essential Medium
mg	Milligram
ml	Millilitre
mM	Millimolar
mOsm	Osmotic concentration

mSOF	Modified Synthetic Oviductal Fluid
n	Number
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acids
nM	Nanomolar
OA	Oleic Acid
p.i.	Post Insemination
P70S6K	P70S6 kinase
PA	Palmitic Acid
PBS	Phosphate Buffered Saline
PcG	Polycomb Group
PCGF	Polycomb Group of RING Finger
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex
PSA	Pisum Sativum Agglutinin
PVP	Polyvinylpyrrolidone
RNAs	Ribonucleic Acids
ROS	Reactive Oxygen Species
SA	Stearic Acid
SCNT	Somatic Cell Nuclear Transfer
SEM	Standard Error of the Mean
siRNA	small interfering Ribonucleic Acid
SPSS	Statistical Package for the Social Sciences
SUZ12	Suppressor of Zeste 12

TCM	Tissue Culture Medium
TCN	Total Cell Number
TE	Trophectoderm
UPR	Unfolded Protein Response
ZP	Zona Pellucida
Δψm	Mitochondria Membrane Potential

Chapter 1: Introduction and Literature Review

1.1 Introduction

The mammalian preimplantation embryo represents a critical developmental stage in which cellular and molecular milestones not experienced by somatic cells take place, including embryonic genome activation, epigenetic reprogramming, and the establishment of founder cell lineages. The latter involves the formation of the blastocyst, a hollow fluid-filled sphere composed of the trophectoderm (TE) (a single external layer of cells) and the inner cell mass (ICM) (an internal cluster of cells), which contains the epiblast and the primitive endoderm. The epiblast will give rise to the foetus, and the trophectoderm and primitive endoderm will lead to the formation of extra-embryonic tissue (i.e., placenta and yolk sac) (Sun et al., 2016) (Figure 1.1). Environmental stressors (e.g., nutrition) during this developmental phase can impact pregnancy outcomes. Furthermore, they can also affect the developing foetus, and in turn, the health of the resultant offspring during postnatal life (i.e., developmental programming) (Fleming et al., 2015a, Fleming et al., 2015b).

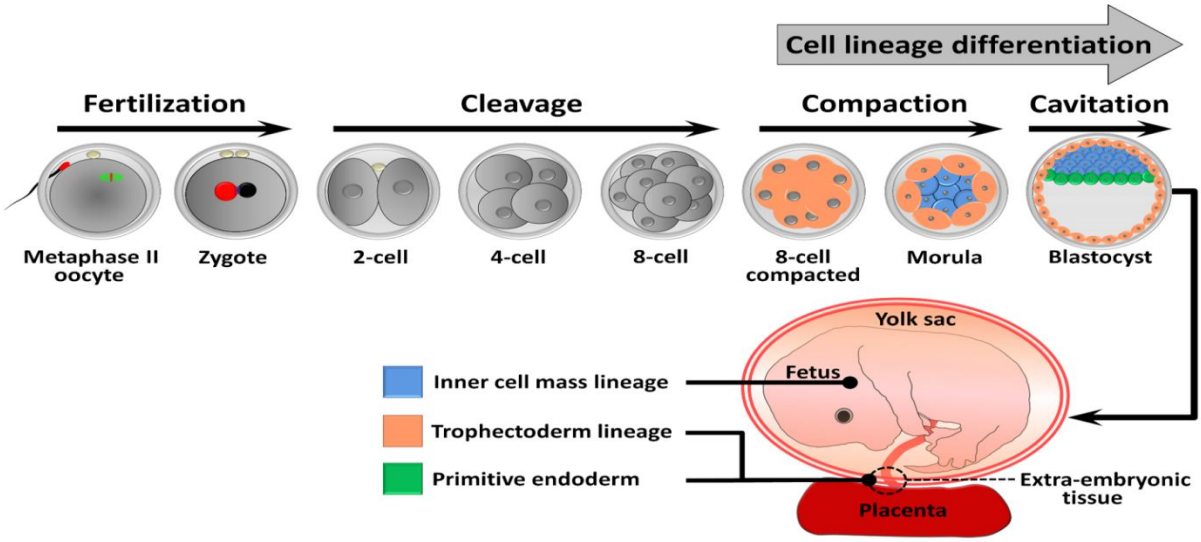


Figure 1-1. Depiction of the mammalian preimplantation period of a mouse model, from the zygote to the blastocyst stage (Sun et al., 2016). These events also take place in cattle and humans, but at different time points (e.g., the time of blastocyst formation is on day 5 post-fertilisation in humans and on day 7 post-fertilisation in cattle).

Nutrition is a major environmental factor essential for preimplantation embryo development. Number of studies have shown that nutritional disorders, such as obesity in humans and negative energy balance (NEB) in dairy cattle (usually present in high-production dairy cows during peak lactation), can impair fertility in both species (Leroy et al., 2015, Broughton and Moley, 2017). Several human epidemiological studies have demonstrated that disturbance of the endocrine signalling pathway in the hypothalamus-pituitary-ovary-axis by obesity can result in reproductive disorders (Zhang et al., 1984, Loughlin et al., 1985, Van Der Steeg et al., 2008, Lash and Armstrong, 2009). High consumption of fatty acids and carbohydrates have been associated with metabolic disorders such as hyperlipidaemia and diabetes, and are usually present in human obesity cases (Lash and Armstrong, 2009). The metabolic dysfunction can alter oocyte quality and the embryonic microenvironment, causing decreased fertility. Indeed, many fertility clinics providing assisted reproduction services showed a reduction in oocyte developmental competence *in vitro* (i.e., ability of an oocyte to reach the blastocyst stage) as well as higher spontaneous miscarriage rates (i.e., following natural conception) during early pregnancy in obese patients (Zaadstra et al., 1993, Fedorcsák et al., 2000, Metwally et al., 2007b).

On the other hand, in dairy cattle selected for high milk production a period of NEB usually occurs during early lactation (De Vries and Veerkamp, 2000, Macrae et al., 2019) that can decrease pregnancy rates following artificial insemination (Wathes et al., 2007). This period of NEB has been associated with a lack of dietary intake capacity to meet the increasing energy demands for high milk yield imposed by decades of genetic selection in the dairy industry (Velazquez, 2015). The detrimental effects of NEB on bovine reproductive function have been associated with the development of postpartum diseases including metritis, endometritis and retained placenta (Wathes et al., 2007, Cardoso et al., 2020), but a direct effect at the level of the ovary (Llewellyn et al., 2007), oviduct (Fenwick et al., 2008), and uterus (Wathes et al., 2009, Wathes et al., 2011) has also been demonstrated. Furthermore, oocyte quality and preimplantation embryo development can be also affected by NEB as shown by the lower production of preimplantation embryos suitable for embryo transfer in superovulated dairy cows with a low body condition score (Velazquez et al., 2005, Carvalho et al., 2014). A low body condition score is present during the period of NEB in dairy cattle (Meikle et al., 2004).

Several metabolic hormones and metabolites can be affected during human obesity and NEB in dairy cattle, but elevated levels of non-esterified fatty acids (NEFA) (also called free fatty acids) have been found to play a major role in the induction of subfertility in these two conditions (Van Hoeck et al., 2014, Valckx et al., 2014, Alves et al., 2015, Aardema et al., 2019, Baddela et al., 2020). As such, high concentrations of NEFA have been reported in blood circulation and in the ovarian follicle of obese women (Karpe et al., 2011, Valckx et al., 2014) and dairy cattle in NEB (Adewuyi et al., 2005, Leroy et al., 2005a). To analyse the direct effects of NEFA on oocyte developmental competence, an *in vitro* bovine model was developed by Leroy et al., (2005) where oocytes are exposed to high concentrations of stearic acid, palmitic acid and oleic acid. This *in vitro* model has revealed that high NEFA concentrations during oocyte maturation can caused a decrease in blastocyst formation (Leroy et al., 2005b). Moreover, the resultant blastocysts displayed a lower cell number, increased apoptosis, decreased glucose consumption, compromised oxidative metabolism, and alterations in both gene expression and amino acid turnover (Leroy et al., 2005b, Van Hoeck et al., 2013). Interestingly, a similar altered cellular and metabolic repertoire has been reported in human assisted reproduction, where a high concentration of NEFA in ovarian follicular fluid has been associated with poor oocyte quality (Jungheim et al., 2011) and oocytes derived from obese women showed a decreased capacity to reach the blastocyst stage, and the blastocysts produced displayed a low cell number, decreased glucose consumption, and compromised amino acid turnover (Leary et al., 2015). Furthermore, in an interspecies model, it was shown that addition of follicular fluid from obese women during *in vitro* maturation (IVM) of bovine oocytes resulted in a lower blastocyst production (Valckx et al., 2015).

In a complementary study of the high NEFA *in vitro* model developed by Leroy et al., (2005) in which high NEFA exposure is applied after oocyte maturation, i.e., during *in vitro* embryo culture (IVEC), it was found that bovine embryos exposed to high NEFA from the zygote stage onwards also caused a reduction in blastocyst formation along with down-regulation of genes related to embryonic cell growth, cell differentiation, and cell-cell interaction (Desmet et al., 2016). However, the extent to which early embryo development is affected by high NEFA exposure during the fertilisation process *per se* has been less studied. The only study available reported that performing bovine *in vitro* fertilisation under high NEFA decreased early embryonic cleavage but did not

affect total blastocyst formation, or their quality in terms of level of apoptosis, cell number and glucose metabolism (Desmet et al., 2018). It was also reported that sperm quality was partially affected by exposure to high NEFA levels where plasma membrane integrity, but not motility was clearly impaired (Desmet et al., 2018). Therefore, more research is needed to further elucidate the possible effects of high NEFA exposure during fertilisation. Hence, the overarching aim of this thesis is to elucidate the effects of high NEFA treatment exclusively during fertilisation on preimplantation embryo development and spermatozoa quality. Given the above-mentioned similarities between obesity in humans and NEB in cattle in regards to high NEFA levels and subfertility, and the parallels between cattle and human preimplantation embryo development (Ménézo and Hérubel, 2002, Adjaye et al., 2007, Santos et al., 2014), an *in vitro* production system of bovine preimplantation embryos is used in an effort to provide novel information of relevance for cattle reproduction that could also provide a conceptual model for human reproductive medicine.

1.2 What is the Preimplantation Period?

The mammalian preimplantation period starts when an oocyte is fertilised by a spermatozoon to form a zygote (1-cell stage embryo). After fertilisation, the zygote undergoes a series of mitotic divisions, increasing the number of cells (i.e., blastomeres), but without increasing the whole volume of the embryo (Fleming et al., 2015b). A few hours later, compaction takes place, which is considered the first morphogenetic progression, where blastomeres are assembled offering the embryo a more spherical shape and can therefore allocate more inner cells (Maître, 2017). This increases cell-cell contacts, thus blastomeres become apicobasally polarised as they form a domain of apical material in the middle of their contact-free surface (Ziomek and Johnson, 1980). Compaction takes place on day 4 post-fertilisation in humans (at the 8-16 cell stage) (Nikas et al., 1996, Iwata et al., 2014) and on day 5 post-fertilisation in cattle (at the 32-cell stage) (Van Soom et al., 1992). After compaction and polarisation, the embryo becomes a morula. The morula undergoes continuous asymmetric cleavage divisions until the formation of the blastocoel cavity (White et al., 2018). Cavitation permits full expansion of the blastocyst, a hollow fluid-filled sphere that composes distinct pluripotent cell lineages that are capable of initiating a developmental sequence independently (Condic, 2014), this is, the trophectoderm,

which is the single external layer of embryonic tissues, and the inner cell mass comprising the internal cluster of cells which contains epiblast and primitive endometrium lineage. The epiblast gives rise to the foetus, whilst the trophectoderm and primitive endoderm lead to the formation of extra-embryonic tissue (i.e., placenta and yolk sac) (Sun et al., 2016). The blastocyst hatches from the protective zona pellucida (ZP) and then implants into the uterus. (Figure 1.2).

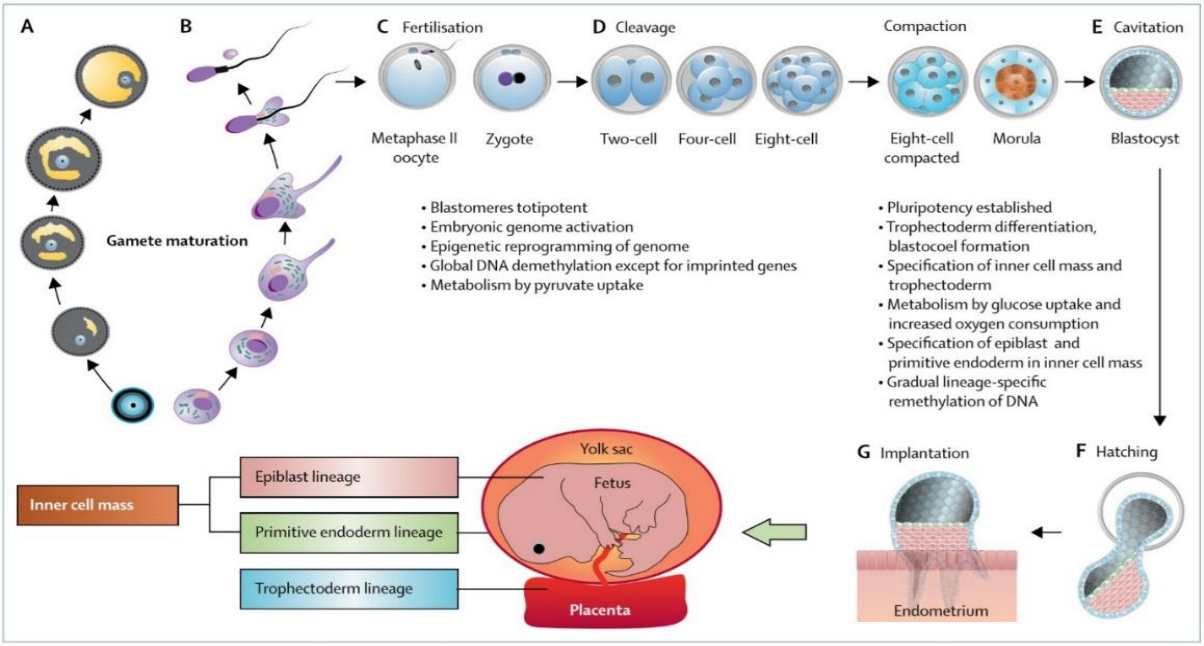


Figure 1-2. Developmental milestones taking place during the preimplantation period in mammals (Fleming et al., 2018). The diagram is based on the mouse model, but the events also take place in other species such as cattle and humans, but at different time points (e.g., the time of blastocyst formation is on day 5 post-fertilisation in humans and on day 7 post-fertilisation in cattle).

1.3 What is the Importance of the Preimplantation Period?

The mammalian preimplantation embryo represents a critical developmental stage, in which cellular and molecular milestones take place (Figure 1.2). Crucially, the few cells involved in this developmental stage are vulnerable to environmental stressors (e.g., nutrition) that can impair critical developmental features for early embryo development. In particular, these include embryonic genome activation, epigenetic reprogramming, the establishment of founder cell lineages, cell number, growth velocity, and metabolic homeostasis (e.g., mitochondrial activity); all of which have an impact on the potential for implantation (Feuer et al., 2017).

Furthermore, environmental stress that occurs within the earliest stages of development has not only significant effects on pregnancy outcomes but also in the resultant offspring during foetal development, which can result in the development of altered phenotypes (e.g., high blood pressure) during postnatal life (i.e., developmental programming) (Fleming et al., 2015b, Fleming et al., 2018). This notion is the base for the Developmental Origins of Health and Disease (DOHaD) concept. Indeed, nutritional imbalances during the preimplantation period may lead to changes in the early embryo with long-term consequences for post-natal development (Roseboom et al., 2011, Fleming et al., 2015a) (Figure 1.3).

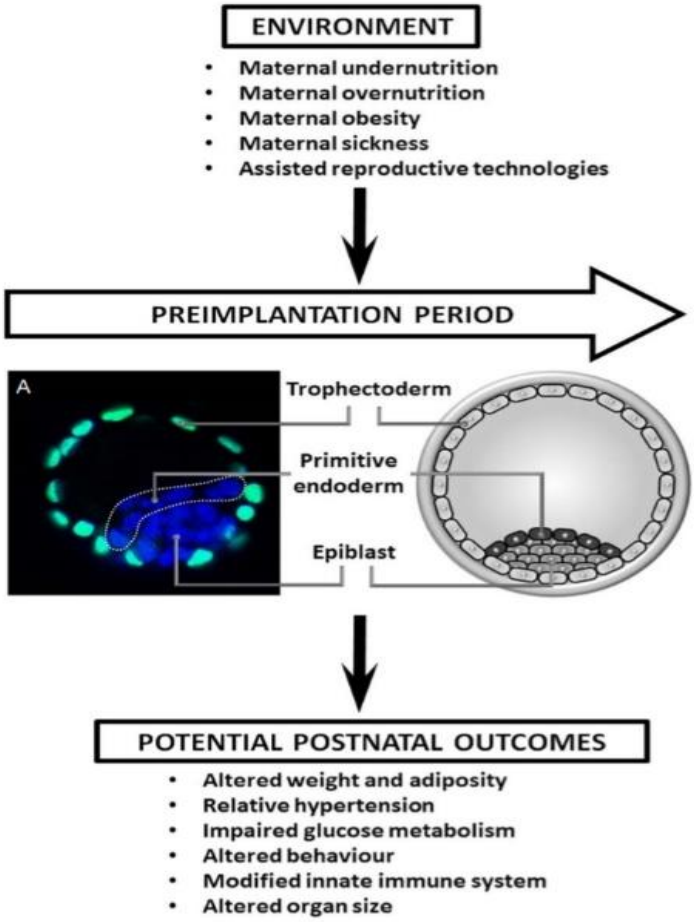


Figure 1-3. Environmental stressors during the preimplantation period and potential postnatal outcomes (Fleming et al., 2015a).

1.4 Metabolism of the Mammalian Preimplantation Embryo

1.4.1 Pyruvate and glucose requirements during the preimplantation period

In almost all species studied; the initial period of embryo cleavage, from fertilisation to formation of the morula, is considered a metabolically quiescent developmental phase. *In vitro* studies have showed that during this time oxygen consumption remains moderately low and the primary carbohydrate substrate exhausted from the culture environment is pyruvate (Leese, 1991, Leese et al., 1993, Leese, 2003). Pyruvate is frequently utilised at a constant rate during the cleavage stage, together with a carbon proportion (depending on the species) that appears in the culture medium as lactate with a production of metabolic energy (Leese, 2003). Pyruvate intake can be either from the glucose resulting from glycolysis or engage directly from the external environment. Pyruvate may also enter the tricarboxylic acid cycle and relies on oxygen for generating metabolic energy. Therefore, oxygen consumption is considered as a useful marker that indicates overall oxidative metabolic activity (Smith and Sturmey, 2013). In contrast, during the formation of the blastocyst, glucose is consumed at a rapid rate, and is characteristically elevated in almost all species studied (including human and cattle embryos) as a general pattern of “blastocyst glycolysis’’. The level of glucose consumption is accompanied by the rise in lactate release into the culture medium. Furthermore, throughout blastocyst formation oxygen consumption also increases, further supporting the generation of metabolic energy (Smith and Sturmey, 2013). However, glucose is not crucial for blastocyst formation as shown by the development of sheep (Thompson et al., 1992) and mouse (Leppens-Luisier et al., 2001) embryos to the blastocyst stage in the absence of glucose during embryo culture. Furthermore, excess of glucose during embryo culture is toxic for early embryo development (Moley et al., 1998, Fraser et al., 2007, Uhde et al., 2018).

1.4.2 Amino acid requirements during the preimplantation period

Amino acids, lipids (triglycerides and fatty acids) and vitamins such as folate, also play a crucial role to produce a viable embryo. Amino acids are essential components of the *in vitro* culture environment, and in addition to glucose, pyruvate and lactate may serve as an energy source and perform a number of other metabolic functions during preimplantation development (Sturmey et al., 2008). The turnover of amino acids (sum

of their depletion or accumulation into a culture medium droplet) has been associated with preimplantation embryo viability. For instance, the fluxes of alanine, arginine, glutamine, methionine and asparagine along with decreases in leucine may predict the ability of early human embryos to achieve the blastocyst stage (Houghton et al., 2002). Similar profiles were reported in another study with human embryos (Sturmey et al., 2009b). The profiles of amino acid turnover in human embryos has been also associated with the occurrence of aneuploidy (Picton et al., 2010) and with clinical pregnancy and live birth in human assisted reproduction (Brison et al., 2004). Moreover, in porcine and bovine embryos a positive correlation between amino acid turnover and DNA damage was reported (Sturmey et al., 2009a), indicating the pivotal role of amino acids for early embryo viability. Here is relevant to highlight that a low oxygen concentration (around 5%) is critical for blastocyst formation and research in mice embryos indicated that a low oxygen concentration promotes a better utilisation of amino acids (Wale and Gardner, 2012).

1.4.3 Fatty acid requirements during the preimplantation period

Kane (1979) was among the first to study the energy metabolism of early embryos through fatty acid beta-oxidation and the contribution made by endogenous triglyceride (Kane, 1979). This was studied in rabbit zygotes cultured in the presence of bovine serum albumin together with pyruvate and the addition of six long chain fatty acids (i.e., myristic, stearic, palmitic, oleic, linoleic, and arachidonic acid) and four short chain fatty acids (i.e., acetic, propionic, butyric and valeric acid). This research revealed that the addition of certain fatty acids to the culture medium were essential for growth to viable morulae, where all the long chain fatty acids, except arachidonic acid, supported embryo growth. Propionic and acetic acid was found to support embryo development but not butyric and valeric acid. These results suggested that fatty acids may act as energy sources for early embryo developmental (Kane, 1979).

Later research demonstrated that pig oocytes require fatty acid oxidation in order to develop (Sturmey and Leese, 2003). The authors evaluated the endogenous triglyceride content of single oocytes and embryos, and to calculate adenosine triphosphate (ATP) production, the consumption of oxygen and glucose, as well as the formation of lactate, were all measured. They observed a decrease in triglyceride levels throughout *in vitro* maturation but not during *in vitro* embryo development.

Moreover, throughout embryo development, oxygen consumption was limited, and at the blastocyst stage, glucose consumption and lactate generation were at their peak. These findings suggested that oocytes may use endogenous triglyceride as an energy source during *in vitro* maturation, and that oxidative phosphorylation produces the majority of the ATP produced during preimplantation embryo development (Sturmey and Leese, 2003).

Lipids produce energy (i.e., ATP) via beta-oxidation within mitochondria. Dunning et al., (2010) addressed the relevance of lipid metabolism in generating ATP for oocyte developmental competence and early embryo development in mice by measuring the rate of embryo growth following upregulation or inhibition of beta-oxidation with L-carnitine or etomoxir, respectively. Supplementation with L-carnitine during oocyte maturation enhanced beta-oxidation, improved developmental competence, and accelerated 2-cell cleavage in the absence of carbohydrate energy. On the other hand, inhibition of beta-oxidation during oocyte maturation or zygote cleavage hampered blastocyst development (Dunning et al., 2010). The above-discussed information indicates that fatty acids are an important source of energy for the development of oocytes and embryos. However, there is evidence indicating fatty acids can become toxic if gametes and embryos are exposed to supraphysiological levels.

1.5 Lipotoxicity Associated with Obesity and its Effect on Subfertility

Lipotoxicity results from the accumulation of lipids in non-adipose tissue, causing cellular dysfunction (Garbarino and Sturley, 2009), and with increasing rates of overweight and obesity being documented over the past few decades, especially in younger generations (Hales et al., 2020), many different aspects of fertility in obese women may be affected, resulting in anovulation, or decreased conception rates, increased miscarriage rates, and foetal abnormalities (Best et al., 2017). The lipotoxicity associated with overnutrition (usually resulting in obesity) can affect fertility at various levels, including oocyte developmental competence (Minge et al., 2008) and preimplantation embryo quality (Fedorcsák et al., 2000).

Body mass index (BMI) is a measure that uses height and weight to estimate in a relatively arbitrary way body fatness that then can be used to determine if an individual is obese or has a healthy weight. A BMI between 18-25 is considered a healthy weight,

while an individual with a BMI between 25-30 is considered overweight and with a BMI more than 30 is considered obese (Nuttall, 2015). Women with increasing BMI usually suffer from subfertility. For instance, a study with 3029 subfertile couples found that women with a BMI over 29 showed a lower probability of achieving spontaneous pregnancy (Van Der Steeg et al., 2008). However, the majority of studies examining the effect of obesity on reproductive success have focused on the success achieved following the application of assisted reproduction. As such IVF may aid in investigating oocytes, their fertilisation, and subsequent development into blastocysts before uterine transfer. An extensive study in Europe included 8457 obese women (BMI ≥27) which showed a reduction in the chance of live birth by a striking outcomes 33% during the first IVF cycle (Lintsen et al., 2005). Another study demonstrated a reduction in achieving pregnancy as BMI increases (Wang et al., 2000). The negative effects of lipotoxicity on the success of human IVF are primarily exerted on granulosa cells and cumulus-oocyte complexes, resulting in oocytes with decreased quality. Indeed, a significant reduction in oocyte quality was observed in women with a BMI ≥25 compared to those with BMI 20–25 (Wittemer et al., 2000). Similarly, fewer Metaphase II oocytes were retrieved from obese women (BMI>30) compared to those with a BMI of 20–30 (Carrell et al., 2001). A similar outcome has been reported in morbidly obese women (Dokras et al., 2006).

The fertilisation rate can also be affected, with some studies reporting up to 40% reduction in successful fertilisation in oocytes collected from women with BMI ≥25 compared to those with a BMI of 20–25 (Van Swieten et al., 2005). Another study showed a decreased fertilisation rate of 26% in obese women compared to those with moderate weight (Salha et al., 2001). A more recent study has confirmed the negative effect of obesity on fertilisation success in human IVF programs (Vural et al., 2015). The detrimental effect of obesity on fertilisation is backed up by research in animal models where obese mice (Wu et al., 2010) and cattle displayed an increased proportion of unfertilised oocytes (Velazquez et al., 2011a). Fertilisation failure in human IVF has been associated with oocytes presenting abnormal meiotic spindles and chromosomal malalignment (Machtinger et al., 2012). Morphologically abnormal meiotic spindles and misaligned chromosomes has been also reported in oocytes from obese mice that failed to achieve fertilisation (Luzzo et al., 2012). However, fertilisation *per se* should not be considered as a primary indicator of oocyte quality. The effects

of oocyte quality often become apparent during later developmental stages when it influences embryo quality. In this regard, a negative correlation has been reported between high maternal BMI and the first cell cleavage (Van Duijn et al., 2021).

Embryo quality based on number of blastomeres can also be decreased in women with a BMI>30 compared to those with a BMI of 20-30 (Carrell et al., 2001). More recently, it was reported that blastocyst from obese patients had a low number of cells in the trophectoderm and displayed reduced glucose consumption, along with altered amino acid turnover. The amino acid turnover was characterised by increased appearances in the culture medium of glutamate, aspartate, asparagine and tryptophan and increased depletion of serine and glutamine (Leary et al., 2015). Embryos from obese women also showed a reduced depletion of isoleucine and increased levels of endogenous triglyceride (Leary et al., 2015).

However, in some human IVF studies a high BMI did not affect embryo quality, but it reduced pregnancy rates (Zander‐Fox et al., 2012). This could be due to the subjective methodology used to determine oocyte and embryo quality in clinical settings (i.e., number of blastomeres in early embryonic stage, time of cleavage, level of expansion in blastocysts, etc.), which although non-invasive, their level of accuracy is usually low (Zander‐Fox et al., 2012). Still, the negative effects of obesity on IVF success have not been found in some studies (Table 1.1), which could also be related to variation in the BMI value used to determine obesity, sample size, and/or to the fact the several confounding factors cannot be controlled in human assisted reproductive studies (Velazquez et al., 2019). This highlights the relevance of using animal models, where experimental conditions can be better controlled.

Mice models are by far the most used animal model in reproductive biology. Indeed, rodent models have provided mechanistic data to understand how maternal obesity can affect not only pregnancy success but also offspring health during postnatal life (Velazquez, 2015, Zambrano et al., 2016, Velazquez et al., 2016, Fleming et al., 2018). However, although useful information has been reported with rodent models, other animal models are needed to better understand the effects of environmental factors on reproductive biology, especially when trying to create conceptual models of relevance for human reproductive medicine. In this regard, evidence indicates that cattle can be a good model for humans, as bovine embryos display similar

chromosome segregation errors in zygotes and recapitulate better events taking place during embryonic lineage specification (Daigneault et al., 2018, Gerri et al., 2020, Cavazza et al., 2021), making it a good model to investigate the effects of NEFA on early embryo development.

Table 1-1. Selected studies analysing the impact of human obesity on IVF outcomes.

Reference	Obese/total patients	BMI used for obesity	Oocyte number	Oocyte maturity	Fertilisation rate	Embryo quality	Implantation rate	Pregnancy rate
(Lewis et al., 1990)	36/368	>27.6	↓		↔			
(Crosignani et al., 1994)	44/111	>22	↓					
(Lashen et al., 1999)	76/333	>27.9	↔		↔		↔	↔
(Wittemer et al., 2000)	398/48	≥28	↓	↓				↔
(Wang et al., 2000)	421/3586	≥30						↓
(Fedorcsák et al., 2000)	79/383	≥25	↓		↔			↓
(Carrell et al., 2001)	34/247	≥30	↔	↓		↓		↓
(Loveland et al., 2001)	69/139	≥25	↔				↓	↓
(Salha et al., 2001)	50/100	≥26	↓		↓			↓

(Nichols et al., 2003)	372	≥28	↔				↓	↓
(Fedorcsák et al., 2004)	241/2660	≥30	↓		↔		↔	↓
(Spandorfer et al., 2004)	148/920	≥27	↓					↔
(Van Swieten et al., 2005)	29/162	≥30	↔		↓			↔
(Dechaud et al., 2006)	36/573	≥30	↔		↔		↔	↔
(Ku et al., 2006)	164	≥24	↔				↓	↓
(Dokras et al., 2006)	315/1293	≥30	↔	↓	↔			↔
(Metwally et al., 2007a)	72/426	≥30	↔		↔	↓		↔
(Esinler et al., 2008)	102/775	≥30	↓	↔	↔			↔
(Martinuzzi et al., 2008)	52/417	≥30	↔		↔			↔

(Matalliotakis et al., 2008)	138/278	24	↓		↓				↔
(Orvieto et al., 2009)	78/516	30	↓		↓				↓
(Robker et al., 2009)	32/96	≥30	↓		↔	↔			↔
(Bellver et al., 2010)	419/6500	≥30	↔	↔	↔	↔			↓
(Li et al., 2010)	1107/153	≥24	↔		↔				↔
(Zhang et al., 2010)	27/2628	30	↓		↓	↓			↔
(Depalo et al., 2011)	108/268	25	↔	↔	↔	↔	↓		↓
(Hill et al., 2011)	21/117	≥30	↔	↔	↔				↔
(Shah et al., 2011)	310/1721	≥30	↔	↔	↓	↔			↓
(Vilarino et al., 2011)	71/208	25	↔		↔	↔			↔
(Mirabi et al., 2017)	105/26	≥30	↔	↓		↓	↔		↔
(Zhang et al., 2019)	22043/1213	⩾27.5				↓	↔		↓
(Van Duijn et al., 2021)	268/43	≥30			↔	↔			↓

(↑) Increased; (↓) Decreased; (↔) No different, in overweight/obese compared to moderate weight women

1.6 Similarities between Human Obesity and Negative Energy Balance in Cattle

Evidence has shown that high-producing dairy cattle usually enter a status of NEB because of metabolic stress imposed by the high milk production. Interestingly, the effects of NEB in cattle are found to be similar as that in obese women and those with type II diabetes conditions (Van Hoeck et al., 2013). NEB and obesity ultimately lead to upregulated lipolysis and elevated serum NEFA concentrations and, as a result, jeopardise oocyte competence and embryo quality. Furthermore, it was demonstrated that human serum NEFA concentrations under obese conditions are very similar to the bovine serum NEFA concentrations during NEB (Table 1.2). Also, elevated plasma NEFA concentrations are reflected in the ovarian follicular microenvironment in both cattle and humans (Van Hoeck et al., 2013).

Table 1-2. Basal and lipolytic NEFA concentrations in bovine and human serum.

	Bovine serum	Human serum
Physiological NEFA concentrations	200µM (Meyer, 1983)	200–600µM (Wolever et al., 1995, Valckx et al., 2012)
Pathological lipolysis linked NEFA concentrations	400–1200µM (Meyer, 1983)	400–2000µM (Reaven et al., 1988, Valckx et al., 2012)
NEFA profile	27% stearic, 17% palmitic and 21% oleic acids (Van Hoeck et al., 2014)	10% stearic, 24% palmitic 38% oleic acids (Jungheim et al., 2011)

1.7 Negative Energy Balance and High Non-Esterified Fatty Acids in Cattle

In the last weeks before calving, nutrient demands for foetal growth increased in pregnant cattle (Sguizzato et al., 2020) along with a decreased voluntary feed intake associated with hormonal changes and lower capacity of the rumen due to the growing foetus (Leduc et al., 2021). This nutritional deficit is further exacerbated in dairy cows selected for high milk production, as they usually enter a period of negative energy balance during early lactation (Macrae et al., 2019). Decades of selection for milk production have produced modern dairy cows that are unable to cover their energy needs imposed by the high milk production, and in essence, the energy provided by their food intake is lower than their energy demands (Leduc et al., 2021).

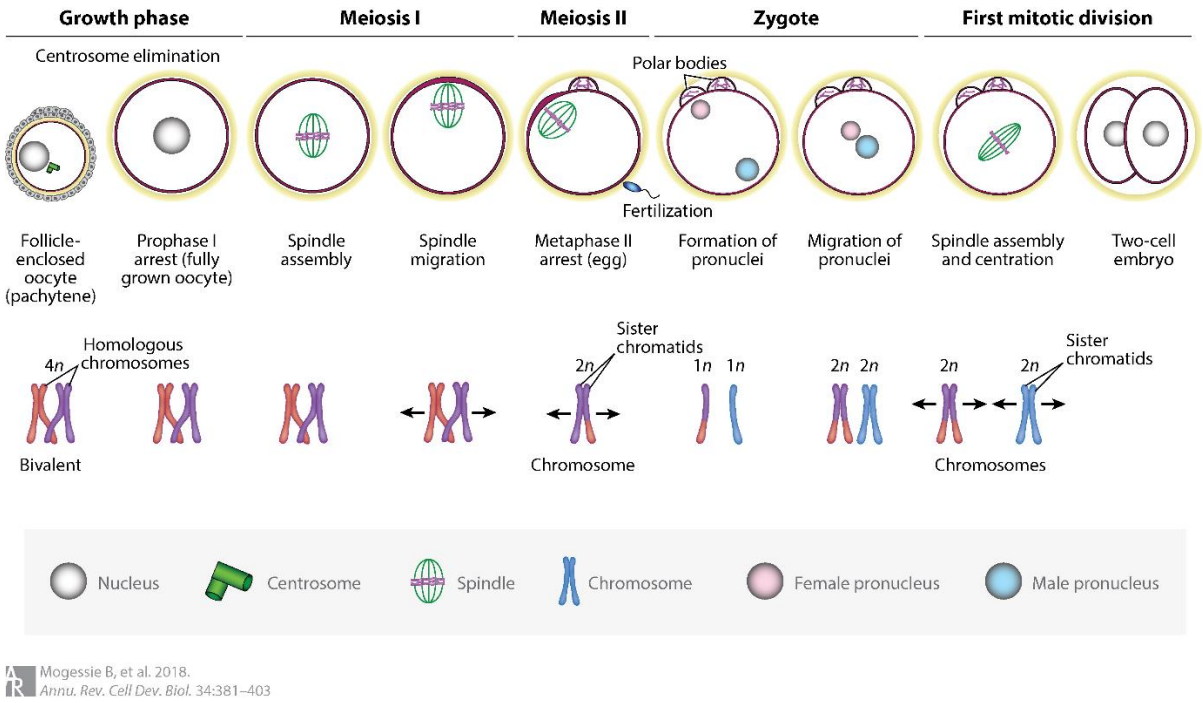
During NEB in cattle, insulin-like growth factor-1 (IGF-1) and insulin levels are decreased, and the low IGF-1 concentrations in turn increases (i.e., due to a less active negative feedback) growth hormone (GH) concentration. A combination of high GH and low insulin concentrations in circulation induces an enhanced hydrolysis of triglycerides in adipose tissue that in turn increases the generation free fatty acids in bloodstream (i.e., NEFA) (Leduc et al., 2021). The high NEFA concentration observed in dairy cows during early lactation has been associated with the development of metabolic diseases (e.g., ketosis) (Esposito et al., 2014, Sammad et al., 2022) and impaired reproductive performance (Ospina et al., 2010, Giuliodori et al., 2011).

To examine the potential effects of high NEFA concentrations on cattle reproduction, *in vitro* models have been used to determine the cellular and molecular changes that take place during high NEFA exposure. In the next sections *in vitro* studies examining the effects of high NEFA on gametes and preimplantation embryos will be discussed.

1.7.1 Effects of high NEFA concentration on oocyte maturation

Postnatal ovaries of mammalian species are set with fixed and nonrenewing pool of primordial follicles, which comprises a primordial oocyte arrested in the diplotene stage of prophase 1 (i.e., germinal vesicle) (Velazquez and Fleming, 2012). Primordial follicles remain in a quiescent state until activated to grow to form primary follicles, which further progress into preantral and antral stages. During the antral stages, the process of oocyte maturation takes place, which is an essential cellular event that allows the oocyte to achieve fertilisation and progression to early embryonic stages

(Scaramuzzi et al., 2011). Oocyte maturation begins with breakdown of the germinal vesicle and progresses to metaphase I and II, where it remains arrested until the time of fertilisation (Figure 1.4). Oocyte maturation involves redistribution of cytoplasmic organelles, cytoskeletal changes and transcription, storage and processing of maternal mRNA (Ferreira et al., 2009, Coticchio et al., 2015).



Mogessie B, et al. 2018.
Annu. Rev. Cell Dev. Biol. 34:381–403

Figure 1-4. Developmental stages during oocyte maturation, fertilisation and early embryonic cleavage (Mogessie et al., 2018).

In vitro oocyte maturation; coupled with *in vitro* fertilisation, and *in vitro* embryo culture, has been used since the 1980's to produce bovine preimplantation embryos (Sirard, 2018). These assisted reproductive techniques are currently use at commercial level worldwide to produce cattle offspring (Ferré et al., 2020) and involve the maturation of cumulus-oocyte complexes for 22-24 hours, followed by incubation with sperm usually for up to 19 hours, and the culture of resultant zygotes for 7-8 days (Figure 1.5).

A research group from Belgium (Leroy et al., 2005b) was the first to develop a physiological relevant *in vitro* model to study the effects of high NEFA concentrations during bovine oocyte maturation. The model was based on NEFA concentrations in ovarian follicular fluid of dairy cows experiencing NEB. To develop the *in vitro* model, ovarian follicular fluid was collected in live animals using transvaginal ultrasound

guided follicular aspiration on day 16 postpartum, representing the time of severe NEB, and compared to values on day 44 postpartum, the time where NEB is improving (Leroy et al., 2005b). NEFA concentrations were also measured in serum, and it was found that values in follicular fluid were lower than in serum (Figure 1.6). The study also revealed that the predominant NEFA in follicular fluid and serum were stearic acid, palmitic acid, and oleic acid (Leroy et al., 2005b).

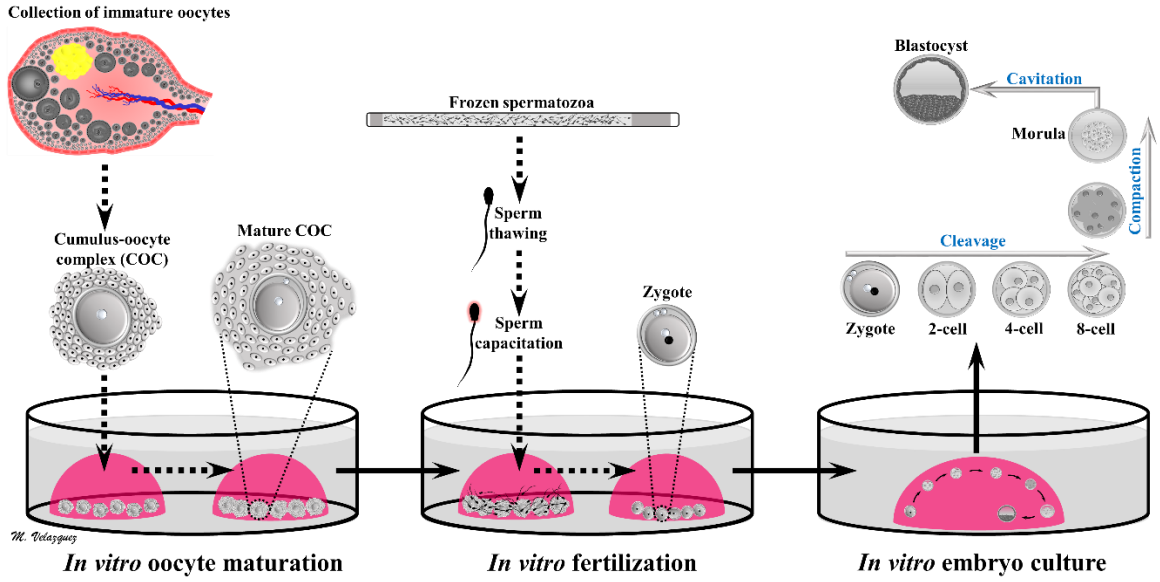


Figure 1-5. General steps in the *in vitro* production of bovine preimplantation embryos. Artwork courtesy of Dr Miguel Velazquez, Newcastle University.

To test the effect of high NEFA during oocyte maturation, a control group with basal NEFA concentrations was used, representing NEFA concentrations observed in follicular fluid on day 44 postpartum (Leroy et al., 2005b). The *in vitro* model revealed that oleic acid did not affect oocyte maturation and consequently there was no effect on the production of blastocysts. However, individual treatment with stearic or palmitic acid increased the number of apoptotic cells in cumulus-oocyte complexes and decreased the fertilisation rate. Nevertheless, a clear effect on blastocyst formation was only observed when cumulus-oocyte complexes were treated with stearic acid (Leroy et al., 2005b). The individual detrimental effects of stearic and palmitic acid were corroborated by a research group in the Netherlands (Aardema et al., 2011). The latter group also reported that oleic acid at a concentration of 250 µM was able to ameliorate the impaired blastocyst formation from oocytes exposed to high levels of stearic and palmitic acid (Aardema et al., 2011). In contrast, a previous study found

the oleic acid negatively affect oocyte maturation *in vitro* (Jorritsma et al., 2004). However, the latter study used oleic acid that was bound to bovine serum albumin (BSA), and it has been suggested that albumin can be detrimental during *in vitro* oocyte maturation (Leroy et al., 2005b). Also, oleic acid in the study of Jorritsma et al., (2004) was added to culture medium at very high concentrations not representative of NEFA values found *in vivo.* Furthermore, the authors used an undefined *in vitro* maturation medium that contained foetal calf serum, an undefined fatty acid source (Jorritsma et al., 2004). Optimal function of granulosa and cumulus cells is crucial for oocyte maturation, as these cells are responsible for endocrine and paracrine signalling (Bilodeau-Goeseels and Panich, 2002, Tanghe et al., 2002). Therefore, the toxic effect of NEFA on oocyte quality is partly exerted via cells surrounding the oocyte. Indeed, it has been reported that NEFA concentrations can impair bovine (Vanholder et al., 2005, Pedroza et al., 2022) and human (Mu et al., 2001) granulosa cell growth and function *in vitro*.

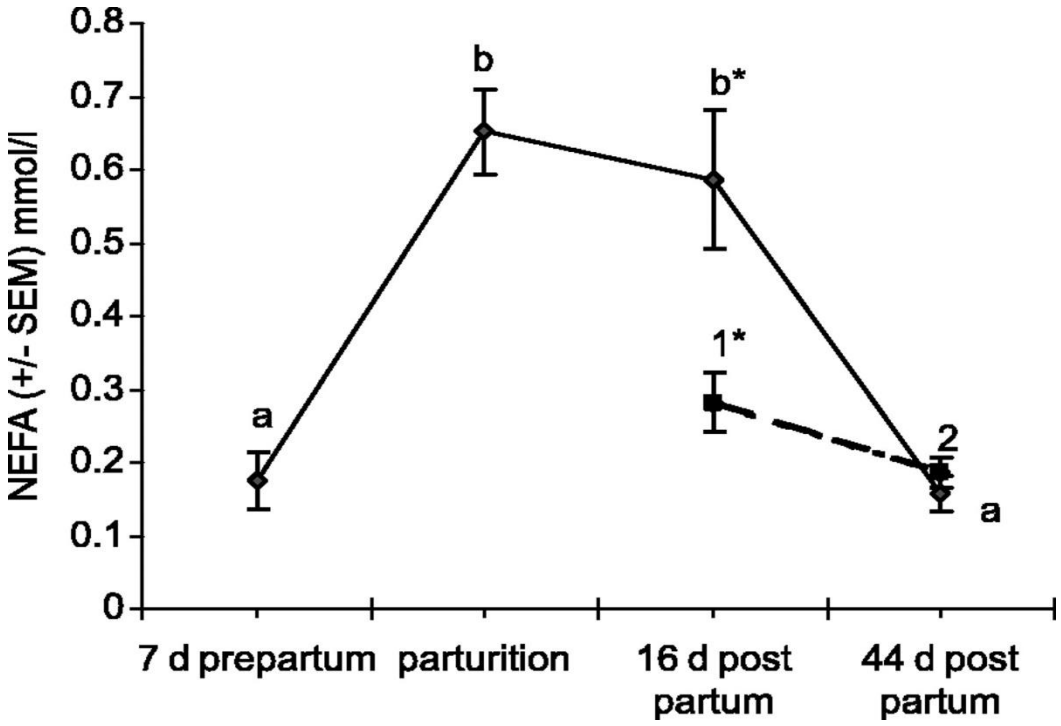


Figure 1-6. NEFA concentrations in bovine serum (black line) and in follicular fluid (dotted line) at different time points relative to parturition. Different letters (for serum) and numbers (follicular fluid) indicate significant differences between time points. The asterisk indicates a significant difference between serum and follicular fluid at the same time point (Leroy et al., 2005b).

Further *in vitro* studies revealed that addition of high levels of stearic or palmitic acid during oocyte maturation can decrease the cryotolerance of bovine blastocyst, whereas high oleic acid concentrations do not seem to affect freezing capacity of bovine embryos (Shehab-El-Deen et al., 2009). However, the detrimental effect of high NEFA during oocyte maturation on embryo cryotolerance was not replicated in a more recent experiment (Van Hoeck et al., 2015). A refined *in vitro* experiment of the initial model developed by Leroy et al., (2005) in which stearic, palmitic, and oleic acid were added together revealed that exposure of high NEFA during bovine oocyte maturation impaired the capacity of oocytes to reach the blastocyst stage, and the resultant blastocyst displayed a decreased number of cells, an increased level of apoptosis, and up-regulation of genes involved in DNA methylation, glucose transport (Van Hoeck et al., 2011) and fatty acid synthesis (Van Hoeck et al., 2013). Moreover, blastocyst from NEFA-exposed oocytes showed an enhanced amino acid metabolism characterised by elevated production and consumption of amino acids along with low consumption of oxygen (Van Hoeck et al., 2011). Interestingly, inhibition of mitochondrial fatty acid oxidation with β-mercaptoacetate in high NEFA-exposed oocytes restored their capacity to achieve the blastocyst stage, suggesting that mitochondrial fatty acid oxidation plays a significant role in the impaired developmental competence observed in bovine oocytes exposed to high levels of NEFA (Van Hoeck et al., 2013). Moreover, transcriptomic and whole-genome DNA methylation analyses carried out in blastocyst from oocytes exposed to high NEFA concentrations revealed that high NEFA can increase the expression of genes in lipid synthesis pathways (Van Hoeck et al., 2015) and altered methylation patterns in loci associated with cellular development, cell death and survival, amino acid metabolism and cellular growth and proliferation (Desmet et al., 2016).

With the same high NEFA concentrations used in the *in vitro* model from Leroy et al., (2005), research from Ireland reported that bovine oocytes exposed to high NEFA levels displayed aberrant DNA methylation of some maternal imprinted genes (O'Doherty et al., 2014). Correct methylation of imprinted genes is essential for normal embryonic and foetal development (Swales and Spears, 2005). Imprinted genes are genes that are monoallelically expressed in a parent-of-origin specific manner, this is, either from the maternal or paternal alleles through the process of genomic imprinting. Genomic imprinting can repress gene expression via DNA methylation. As such, if the

allele inherited from the mother is imprinted, it is turned off (i.e., the allele is methylated), and only the allele from the father remains active (i.e., unmethylated). On the other hand, if the paternal allele is imprinted, only the maternal allele is expressed (Swales and Spears, 2005, Tian, 2014). DNA methylation is an epigenetic event in which a methyl group is transferred (by DNA methyltransferases) to the fifth carbon of a cytosine residue, forming 5-methylcytosine (5mC) (Tian, 2014). However, parental imprinting involves other epigenetic mechanisms including histone modifications and noncoding RNA (Barlow and Bartolomei, 2014, Tian, 2014).

The Belgian high NEFA model (Leroy et al., 2005b) was also used by researchers in Australia, reporting that high NEFA during *in vitro* oocyte maturation can upregulate the expression of stress marker genes in the endoplasmic reticulum of bovine cumulus-oocyte complexes and decrease mitochondrial DNA copy number in oocytes and resultant blastocysts (Sutton-McDowall et al., 2016). These high NEFA effects were ameliorated by salubrinal (i.e., during *in vitro* oocyte maturation), an endoplasmic reticulum stress inhibitor. Nevertheless, the low mitochondrial DNA copy number did not seem to affect mitochondrial membrane potential (Sutton-McDowall et al., 2016). Interestingly, the decreased number of cells in blastocysts from oocytes exposed to high NEFA previously reported by the Belgian laboratory (Van Hoeck et al., 2011) was not replicated by this Australian research group (Sutton-McDowall et al., 2016). Detrimental effects of high NEFA (i.e., stearic, palmitic, and oleic acid) during *in vitro* oocyte maturation on blastocyst rates and blastocyst quality can also be ameliorated by adding alpha-linolenic acid, where beneficial effects are associated to decreased accumulation of reactive oxygen species in oocytes and preservation of cumulus cells viability by protecting their mitochondrial activity, preventing apoptosis and reducing their cellular and endoplasmic reticulum stress levels (Marei et al., 2017).

The inclusion of glucose in conjunction with the high NEFA model has been explored, since glucose microenvironment will be different between obesity (i.e., hyperglycaemia) and negative energy balance (i.e., hypoglycaemia). The available *in vitro* evidence indicates that, regardless of the level of glucose, high NEFA during oocyte maturation results in the production of blastocyst of reduced quality associated with high levels of apoptosis and impaired cell allocation (De Bie et al., 2017). Nevertheless, blastocyst metabolism and the transcriptome of metabolic and oxidative stress-related genes were not affected by glucose levels (De Bie et al., 2017).

In a related *in vitro* model of the high NEFA Belgian model where oocytes were only exposed to high palmitic acid concentrations, a proteomic analysis revealed that signalling pathways associated with cellular stress and metabolism were altered in cumulus cells (i.e., cells surrounding the oocyte) whereas in oocytes signalling pathways affected were related to pro-survival and adaptive mechanism including the P70S6 kinase (p70S6K) and unfolded protein response (UPR) pathways (Marei et al., 2019b). The p70S6K pathway is involved in the regulation of cell growth and survival (Carman and Wong, 2012) and the UPR pathway promotes cellular survival by promoting the recovery of endoplasmic reticulum and mitochondrial function during episodes of cellular stress (Hetz, 2012). A subsequent study with the same *in vitro* model found that high palmitic acid during oocyte maturation can impair blastocyst formation and quality, but interestingly, this detrimental effect of palmitic acid can be ameliorated by addition of a mitochondrial-targeted antioxidant (Mitoquinone) during embryo culture (Marei et al., 2019a). A similar effect was observed with Trolox, a vitamin E analogue with antioxidant properties, but the beneficial effect was most noticeable when oocytes were treated with Trolox before being exposed to high levels of palmitic acid (De Bie et al., 2021).

Recently an embryo transfer model was used to address the effect high NEFA exposure during post-hatching development. For this, *in vitro*-produced blastocyst derived from high palmitic-exposed oocytes were transferred to embryo recipients (non-lactating cows) and then embryos were recovered seven days after transfer (i.e., day 14 post-insemination [p.i.]) to study their developmental potential. Recovered day-14 embryos exposed to high levels of palmitic acid during oocyte maturation displayed features of slow growth as indicated by their decreased length and being in the spherical stage rather than on the tubular stage (Desmet et al., 2020). In cattle, a spherical blastocyst (7–8 days p.i.) develops into an ovoid embryo by Day 12 p.i., and then becomes tubular around day 14 p.i. (Machado et al., 2013, Van Leeuwen et al., 2015). The extra-embryonic tissue (i.e., trophectoderm cells) of embryos from high palmitic-exposed oocytes also consumed more pyruvate and less glucose, which was interpreted by the authors as an indication of impaired metabolic activity (Desmet et al., 2020). This interpretation was based on the known increase consumption of glucose that take place at the time the embryo reaches the state of compacted morula (Gardner and Wale, 2013). Moreover, this embryo transfer model also found that the

extra-embryonic tissue of embryos produced with oocytes exposed to high palmitic levels secreted less interferon tau (Desmet et al., 2020). Interferon tau is a type 1 interferon produced in peri-implantation ruminant embryos and serves as the major signal for maternal recognition of pregnancy in ruminant species (Forde and Lonergan, 2017).

Overall, the above discussed information gives strong support to the notion that high NEFA concentrations associated to lipotoxic conditions during oocyte maturation is detrimental for early pregnancy success in mammals.

1.7.2 Effects of high NEFA on preimplantation embryo development

The potential detrimental effects of high NEFA have been also analysed during the time of embryo development. Given that there is a correlation between the NEFA concentrations in oviductal fluid and plasma (Jordaens et al., 2017a), NEFA concentrations in plasma of cows experiencing negative energy balance were used in an *in vitro* model of high NEFA during preimplantation embryo development (Desmet et al., 2016). With this model it was found that exposure to high NEFA concentrations during the time of embryo culture resulted in a decreased blastocyst formation. The resultant blastocyst showed a decreased expression of genes associated with cell-cell interactions, cell growth, and cell differentiation. Furthermore, high NEFA-exposed embryos displayed altered methylation patterns in loci associated with apoptosis and mitochondria dysfunction (Desmet et al., 2016). Interestingly, *in vitro* exposure of zygotes to physiological levels of palmitic acid (25 μM) for five days resulted in the production of blastocysts that were less tolerant to cryopreservation, whereas addition of oleic acid at the same concentration improved the capacity of blastocyst to withstand cryopreservation. Furthermore, blastocysts that survive the freezing procedure showed an increased number of apoptotic cells when cultured with palmitic acid (Aardema et al., 2022).

During the preimplantation period human and bovine embryos undergo their first cellular divisions in the oviduct, entering the uterus at the stage of morula (Velazquez et al., 2010, Kim and Kim, 2017). The oviduct plays a pivotal role in oocyte pick-up following ovulation, sperm transport to the fertilisation site, and as provider of secretions (e.g., proteins, enzymes, growth factors and amino acids) essential for gamete function (e.g., sperm hyperactivation), the fertilisation process (i.e., sperm-

oocyte interaction) and early embryo development (Killian, 2004, Besenfelder et al., 2012, Kim and Kim, 2017, Pérez-Cerezales et al., 2018, Kölle et al., 2020, Saint-Dizier et al., 2020). Hence, *in vitro* models have been developed to investigate the potential effects of high NEFA on oviductal cells. Accordingly, bovine oviductal epithelial cells (BOECs) exposed to NEFA concentrations resembling cows in NEB showed decreased cell division during *in vitro* culture, and when sperm was co-incubated with high NEFA-exposed BOECs, the number of sperm bound to BOECs was also decreased (Jordaens et al., 2015). This *in vitro* model has also revealed that high NEFA exposure can increase permeability and impaired tight junctions of BOECS in a monolayer system (Jordaens et al., 2017b). Further analysis with this *in vitro* system also showed that co-culture of zygotes with pre-exposed BOECs to high NEFA levels resulted in the production of blastocysts with low cell number, with a decreased allocation of cells forming the inner cell mass (i.e., cells that will form the foetus) (Jordaens et al., 2020). Moreover, when exposure of high NEFA levels was carried out during co-culture of zygotes with BOECS, intracellular lipid content was increase in morulae resulting in decreased formation of blastocysts which displayed an increase in apoptosis mostly in the inner cell mass (Jordaens et al., 2020). Hence, high NEFA levels can directly affect the preimplantation embryo along with alterations in the physiology of the oviduct, creating a detrimental micro-environment for early embryo development.

1.7.3 Can high NEFA levels affect the fertilisation process?

The above-discussed information clearly indicates that exposure to high NEFA concentrations during oocyte maturation and preimplantation embryo development are detrimental for early embryo viability. However, the impact of high NEFA levels during fertilisation is less clear. Fertilisation takes place in the oviduct, and in addition to its important role in early embryonic development, the oviduct is also a complex mediator in spermatozoa selection, spermatozoa reservoir storage, spermatozoa motility regulation and spermatozoa guidance to the oocyte to achieve fertilisation (Holt and Fazeli, 2010, Coy et al., 2012, Ghersevich et al., 2015). During ovulation, spermatozoa detach from the oviductal epithelium through hyperactivation and capacitation and once they reach the oocyte, they undergo the acrosome reaction and

penetrate the cumulus cells and zona pellucida (Kölle, 2015). NEFA can reach the oviduct (Jordaens et al., 2017b), and hence could directly affect the spermatozoa.

In vitro studies have shown that NEFA, including stearic, palmitic and oleic acid can be incorporated and used by human and bovine spermatozoa (Neill and Masters, 1972, Abdel Aziz et al., 1983, Alvarez and Storey, 1995, Islam et al., 2021) and although fatty acids are essential for spermatozoa function (Van Tran et al., 2017), exposure of spermatozoa to high NEFA concentrations can be detrimental for male fertility (Collodel et al., 2020). For instance, concentrations of stearic acid in human spermatozoa were higher in patients with asthenozoospermia (i.e., low sperm motility) or oligospermia (i.e., low sperm count), whereas high levels of oleic acid were present in spermatozoa of patients presenting oligoasthenospermia (i.e., low sperm count and motility) or just oligospermia (Aksoy et al., 2006). Asthenoteratozoospermia (i.e., low sperm motility and abnormal sperm morphology) and oligoasthenoteratozoospermia (i.e., low sperm count and motility, and abnormal sperm morphology) have also been associated with high levels of stearic and oleic acid in human spermatozoa (Khosrowbeygi and Zarghami, 2007). In a more recent study, a negative correlation between oleic acid levels in human spermatozoa and sperm count was reported, confirming that high levels of oleic acid can affect spermatozoa number (Andersen et al., 2016). Similarly, high concentrations of palmitic acid in human spermatozoa have been associated with asthenozoospermia, asthenoteratozoospermia or oligoasthenoteratozoospermia (Tavilani et al., 2006, Khosrowbeygi and Zarghami, 2007). Furthermore, *in vitro* experiments have showed that stearic, palmitic and oleic acid can decrease human sperm motility in fertile sperm donors in a concentration dependent manner (Siegel et al., 1986).

To the best of the author's knowledge, there is no information in relation to the level of NEFA in bovine spermatozoa and quality outcomes. But *in vitro* studies indicate the exposure to NEFA may alter bovine spermatozoa quality. For example, it was reported that the addition of palmitic acid at low levels (i.e., 50 nM) can increase bovine sperm motility (Islam et al., 2021). In a previous study, *in vitro* exposure for four hours of bovine spermatozoa to high concentrations of stearic, palmitic and oleic acid at values known to be detrimental for *in vitro* development of preimplantation embryos (Desmet et al., 2016) resulted in the impairment of variables reflecting sperm motility (Desmet et al., 2018). The treatment also induced an increase in the percentage of

spermatozoa with damaged plasma membrane (Desmet et al., 2018). However, when IVF took place with spermatozoa that was pre-exposed to high NEFA no detrimental effect was observed on blastocyst formation and quality (Desmet et al., 2018). Nevertheless, exposing both oocytes and spermatozoa exclusively during IVF to high NEFA concentrations resulted in reduced fertilisation and cleavage rates, and an increased percentage of zygotes displaying polyspermy. Still, blastocyst formation was not affected by high NEFA exposure during IVF and resultant blastocysts showed an increase in cells of the trophectoderm that was not reflected in the total cell number of embryos (Desmet et al., 2018), suggesting perhaps a statistical artefact. Overall, this study showed that penetration of spermatozoa into oocytes and the first cell divisions can be impaired when fertilisation takes place under high concentrations of NEFA, but apparently with no effect on blastocyst production. And given that pre-exposed spermatozoa to high NEFA managed to achieve fertilisation, and blastocyst formation was not affected, the authors suggested that the detrimental effect of NEFA during fertilisation is via the oocyte and not the sperm (Desmet et al., 2018). However, it needs to be considered that in the Desmet et al., (2018) study the sperm was exposed to NEFA during a spermatozoa selection process (i.e., swim-up technique), hence the possibility exist that the majority of damaged spermatozoa was removed before IVF was performed. Furthermore, other relevant quality endpoints need to be tested, including sperm DNA damage, especially when considering that sperm with DNA damage can achieve fertilisation (Fatehi et al., 2006, Simon et al., 2010). Therefore, more research is needed to clarify the role of high NEFA during fertilisation.

1.8 Project Aim, Hypothesis, and Objectives

The above-discussed information indicates that a high NEFA microenvironment during oocyte maturation and preimplantation embryo development can be detrimental for early pregnancy success (i.e., formation of the blastocyst and its quality). However, the effects of high NEFA exclusively during fertilisation is less clear, with only one paper published after the start of the project for this thesis (i.e., Desmet et al., 2018). Hence, the overarching aim of this thesis is to elucidate the effects of high NEFA treatment exclusively during fertilisation on preimplantation embryo development and spermatozoa quality.

To achieve these aims, the following hypothesis will be tested:

H_1= Bovine oocytes and spermatozoa exposed to NEFA concentrations resembling a lipotoxic microenvironment during IVF will impair preimplantation embryo development and spermatozoa quality.

The following objectives will be pursued to test the above-mentioned hypothesis:

To determine the effects of high concentrations of stearic, palmitic and oleic acid exclusively during IVF on:

- Fertilisation success.
- Levels of reactive oxygen species in resultant zygotes.
- Blastocyst formation.
- Cell allocation in resultant blastocysts.
- Levels of an epigenetic mark during early cleavage.
- Spermatozoa DNA damage.
- Plasma and acrosomal membrane integrity of spermatozoa.
- Mitochondrial membrane potential of spermatozoa.

Chapter 2: Effects of Elevated Non-Esterified Fatty Acids Concentrations Exclusively during *In Vitro* Fertilisation on Levels of Reactive Oxygen Species in Bovine Zygotes

2.1 Introduction and Materials and Methods

Reactive oxygen species (ROS) is an umbrella term that makes references to a variety of molecules derived from the incomplete reduction of molecular oxygen during aerobic metabolism, which is produced by reduction-oxidation (redox) reactions or by electronic excitation (Sies and Jones, 2020). In mammalian cells, the major sites of ROS production include plasma membrane, peroxisome, mitochondrion, and endoplasmic reticulum (Zhang and Wong, 2021). ROS are usually classified as free radicals and non-radicals (Table 2.1). Free radicals are considered highly reactive due to the presence of unpaired electrons, and they remain in the subcellular location where they are produced unless they first become reduced. On the other hand, non-radical ROS are less active (i.e., do not have unpaired electrons), and are able to leave the subcellular site where they are formed and pass-through biological membranes. But still, they can undergo redox reactions resulting in the production of free radicals (Shields et al., 2021).

Table 2-1. Examples of free radical and non-radical ROS.

Free radical ROS	Non-radical ROS
Superoxide anion radical	Hydrogen peroxide
Hydroxyl radical	Organic hydroperoxides
Peroxyl radical	Singlet molecular oxygen
Alkoxyl radical	Electronically excited carbonyl
	Ozone
	Hypochlorous acid
	Hypobromous acid

The production of ROS is essential for cellular homeostasis, playing an important role in cell proliferation, immune cell activation, and promotion of autophagy, where low physiological generation of ROS seems to be inversely related with lifespan in several species, including mammals (Nathan and Cunningham-Bussel, 2013, Schieber and Navdeep, 2014, Checa and Aran, 2020, Shields et al., 2021). From a reproductive biology perspective, generation of physiological levels of ROS are required for oocyte maturation (Morado et al., 2009, Shkolnik et al., 2011), spermatozoa function (Aitken, 2017, Das and Roychoudhury, 2022) and preimplantation embryo development (Jamil et al., 2020, Hardy et al., 2021). However, when ROS production overwhelms ROS detoxification carried out by antioxidant systems (i.e., enzymatic and non-enzymatic antioxidants), oxidative stress takes place with detrimental consequences for cell survival (Mani, 2014, Checa and Aran, 2020). Accordingly, excess production of ROS can impair oocyte maturation, fertilisation and preimplantation embryo development, and treatment with some antioxidants can partially alleviate ROS damage during *in vitro* embryo production (Torres-Osorio et al., 2019, Jamil et al., 2020, Lin and Wang, 2020, Soto-Heras and Paramio, 2020, Hardy et al., 2021).

As previously discussed (i.e., chapter 1), lipotoxic microenvironments associated to high concentrations of NEFA can affect negatively oocyte maturation and preimplantation embryo development, and it is known that NEFA can modify intracellular ROS production (Schönfeld and Wojtczak, 2008). Indeed, *in vitro* exposure of bovine granulosa cells to elevated NEFA concentrations comprising a mix of oleic acid, linoleic acid, palmitic acid, palmitoleic acid, and stearic acid resulted in increased ROS levels in granulosa cells (Wang et al., 2020b). Bovine oocytes exposed to high levels of either linoleic acid (Marei et al., 2012), palmitic acid (Marei et al., 2019b) or a mix of stearic, palmitic and oleic acid (Marei et al., 2017) during *in vitro* oocyte maturation also showed an increase in ROS levels. Likewise, treatment of bovine oocytes with high levels of palmitic acid during *in vitro* oocyte maturation resulted in the production of early embryos (i.e., four or more cells 48 post IVF) with high levels of ROS (Marei et al., 2019a).

However, the effect of high NEFA exposure during fertilisation on the levels of ROS in resultant embryos is unknown. Hence, the aim of this experiment was to determine the influence of high levels of NEFA exclusively during IVF on levels of ROS in bovine zygotes.

2.2 Material and Methods

2.2.1 Handling of Glassware and Plasticware

Glassware (volumetric flasks, beakers, and bottles) designated for the preparation of embryo culture medium and stock solutions were washed and sterilised before use. Glassware was rinsed in distilled water (dH_20) then submerged in a solution of dH_20 with 1.0% (v/v) Distel™ overnight. Washed glassware were then removed from the Distel™ solution, drained and rinsed three times by immersion in dH_20. Rinsed glassware was let to dry and then wrapped with aluminium foil, labelled with autoclave tape, and sterilised in an oven at 180°C for four hours. Non-sterile plasticware including pipette tips, which were racked in appropriate boxes, and Eppendorf tubes, stored in glass jars or beakers, were sterilised in a bench top autoclave at 121°C for one hour. Autoclaved glassware and plasticware were transferred to a drying oven at 80°C for at least one hour to evaporate all moisture before use.

2.2.2 Collection of cumulus oocyte complexes (COCs)

Bovine ovaries were collected at a local abattoir (Burradon, Cramlington, UK) and kept between two reproductive track layers, to keep them warm, then they were transferred to the laboratory as soon as possible using a thermal bag. At the laboratory, ovaries were washed several times in a warm phosphate buffered saline (PBS, Sigma P4417) solution (38.5°C). Any visible follicles more than 10.0 mm in diameter were burst. Subsequently, ovaries were slashed using scalpels into a glass petri dish (150x25 mm) with warm oocyte recovery media (38.5°C) composed of serum-free tissue culture medium 199 (TCM-199, Sigma M2520) supplemented with 1.0% (w/v) bovine serum albumin (BSA, fatty acid free, Sigma A7030), 0.2 mM sodium pyruvate (Sigma P3662), 4.2 mM sodium bicarbonate (Sigma S57761), 50 mg/ml gentamycin sulphate (Sigma G3632), and 10 mM HEPES (Sigma 5310). The medium was produced with an osmolarity between 280–290 mOsm, a pH of 7.20, and sterilised by filtration (See Appendix 1 for detailed preparation of oocyte recovery medium). After the slashing process of all ovaries, the oocyte recovery medium was transferred to a glass beaker and allowed to set for 15 minutes at 38.5°C. Then the supernatant was aspirated to leave ~100 ml of oocyte recovery medium which was subsequently aliquoted in 15 ml conical tubes (Sarstedt Ltd 62.554.502). The precipitate (i.e., oocytes and cellular

debris) on each conical tube was aspirated and transferred into a searching plastic petri dish containing the same pre-warmed oocyte recovery medium. Oocyte searching was done under a stereomicroscope, and the petri dish was on top of a hot plate to keep the media warm during oocyte searching (Figure 2.1). Good quality (grade I) unexpanded cumulus oocyte complexes (COCs) with dark, homogenous ooplasm and at least five layers of compact cumulus cells were selected for *in vitro* maturation (Figure 2.2).

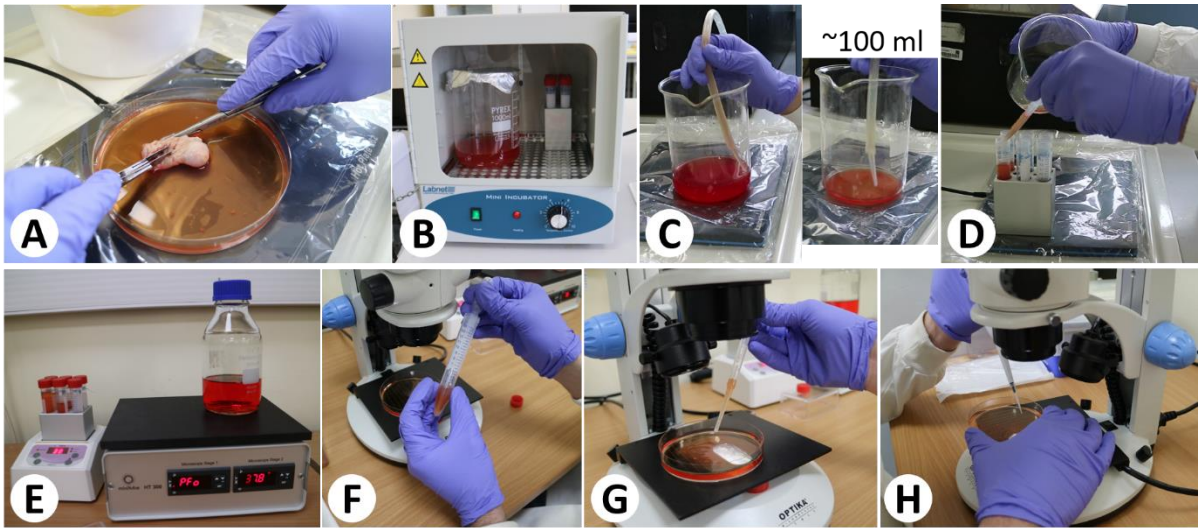


Figure 2-1. Extraction of oocytes from ovaries. **A**) Slashing procedure, where ovaries are held with a haemostatic forceps in collection medium and incisions are made with scalpels across the ovary avoiding the slashing of the corpus luteum. **B**) Collection medium is poured into a beaker to allow oocytes sediment at the bottom of the beaker. **C**) Collection medium is carefully aspirated to leave around 100 ml. **D**) Collection medium is allocated in conical tubes. **E**) Conical tubes are kept warm in block heater. **F**) Pellets are aspirated from the conical tube. **G**) Pellets are place in a petri dish containing oocyte collection medium. **H**) Searching of oocytes is done under a stereomicroscope.

2.2.3 *In vitro* maturation (IVM)

Selected COCs were matured in groups of 20–30 in a 4-well plate (Nunc®, Thermo Fisher 17982) containing serum-free maturation medium (500 µl/well without oil cover) composed of TCM199 supplemented with 1.0% (w/v) fatty-acid free BSA, 0.2 mM sodium pyruvate, 26 mM sodium bicarbonate, 50 mg/ml gentamycin sulphate, 20

ng/ml murine epidermal growth factor (mEGF, Sigma E4127), 5.0 IU human chorionic gonadotrophin (hCG, Chorulon®) and 10.0 IU pregnant mare serum gonadotrophin (PMSG-Intervet®). The osmolarity of the IVM medium was between 280–290 mOsm with a pH of 7.20 (see appendix 2 for detailed preparation of IVM medium). The medium was sterilised by filtration. COCs were *in vitro* matured for 22-24 hours in a humidified atmosphere with 5% CO_2**,** atmospheric O_2, and at 38.5°C.

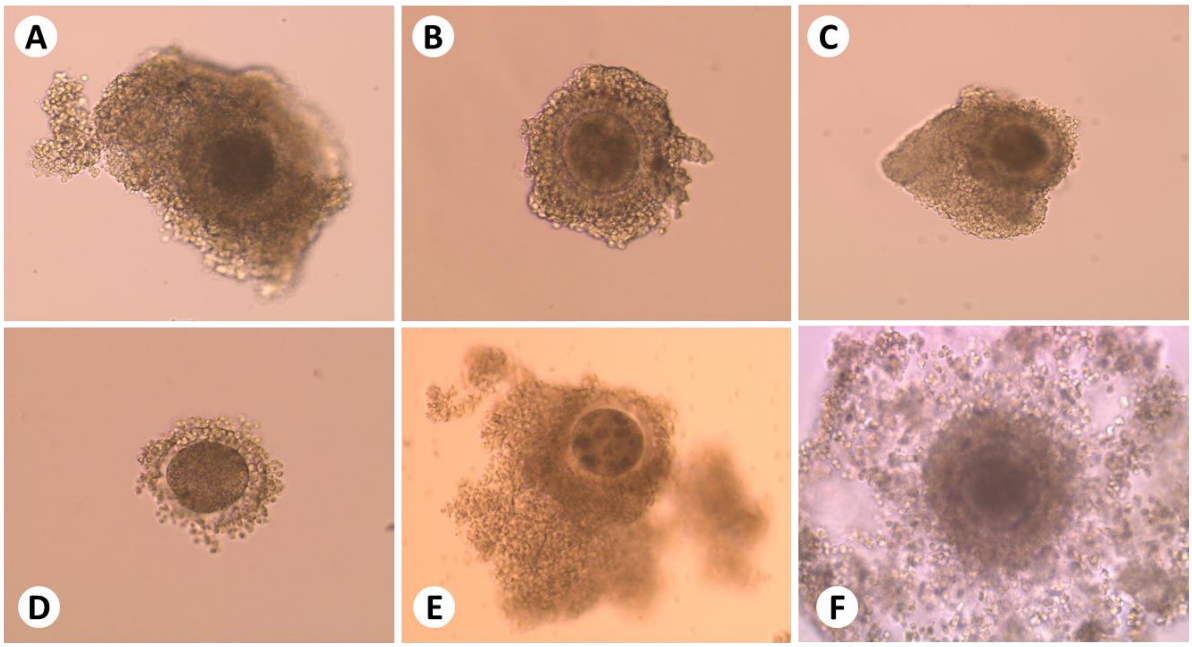


Figure 2-2. Classification of cumulus oocyte complexes. COCs are classified on overall cumulus cells and ooplasm appearance. **A**) Grade 1 COC with multiple (≥ 5 layers) layers of cumulus cells and homogenous ooplasm. **B**) Grade 2 COC with fewer than five layers of cumulus cells and homogenous ooplasm. **C**) Grade 3 COC with partial or incomplete covering of the oocyte with cumulus cells, but homogenous ooplasm. **D-F**) Grade 4 COCs which includes COCs with sparse cumulus cells, or near to being completely denuded (D), with decent covering of cumulus cell but irregular (i.e., granulated) ooplasm (E) or expanded cumulus (F). Grading based on Wood and Wildt, (1997) (Wood and Wildt, 1997) and Fouladi-Nashta et al. (2007) (Fouladi-Nashta et al., 2007), however, grading systems differ slightly between laboratories (reviewed in Aguila et al. 2020) (Aguila et al., 2020), especially for classification of COCs grade 3 and 4.

2.2.4 *In vitro* fertilisation (IVF)

After IVM, all COCs were co-incubated in groups of 20–30 with spermatozoa at a final concentration of $1x10^6$ sperm cells/ml in a 4-well plate (500 µl/well, without oil cover) containing fertilisation medium for 19 hours in a humidified atmosphere with 5% CO_2**,** atmospheric O_2, and at 38.5°C. The IVF medium was Fertilisation TALP (Tyrode's medium base, Albumin, Lactate and Pyruvate) known as Fert-TALP (Parrish, 2014). The medium consisted of 114 mM sodium chloride (Sigma S5886), 3.2 mM potassium chloride (Sigma P5405), 0.3 mM sodium phosphate monobasic dehydrate (Sigma 71500), 2.0 mM calcium chloride dehydrate (Sigma C7902), 0.5 mM magnesium chloride hexahydrate (Sigma M2393), 25 mM sodium bicarbonate (Sigma S5761), 0.2 mM sodium pyruvate (Sigma P3662), 10 mM sodium lactate (Sigma L4263), 0.01 µg/ml phenol red (Sigma P5530), 6.0 mg/ml fatty-acid free BSA (Sigma A7030), 20 mM D-penicillamine (Sigma P4875), 0.1 UI/ml heparin (Sigma H3149),10 µM hypotaurine (Sigma H1384), 1.0 µM epinephrine (Sigma H9892), 2.0 µM sodium metabisulfite (Sigma S9000) and 50 mg/ml gentamycin sulphate (Sigma G3632). Osmolarity of the IVF medium was between 280-290 mOsm with a pH of 7.40. The medium was sterilised by filtration (see appendix 3 for detailed preparation of IVF medium).

Semen straws from a commercial supplier were used for IVF. A semen straw from a bull of proven fertility for IVF (see appendix 4) was thawed in warm water (37°C) for one minute and immediately layered on top of 1.0 ml 90% BoviPure^{TM} (Nidacon International Laboratories AB). The sample was centrifuge (at 300 x g) for 10 minutes and the supernatant was carefully aspirated to leave approximately 50 µl of solution containing the sperm pellet. The solution was then quickly resuspended with 750 µl Fert-TALP without heparin, hypotaurine and epinephrine, and centrifuge for three minutes (at 400 x g) followed by removal of the supernatant, and resuspension with 750 µl of complete Fert-TALP (i.e., with heparin, hypotaurine, and epinephrine). A third centrifugation was applied for three minutes (at 400 x g), and the supernatant removed to leave approximately 50-75 µl of sperm solution. Sperm concentration was calculated with a haemocytometer (see appendix 5 for detailed processing of semen straws and sperm concentration calculation).

2.2.5 *In vitro* embryo culture (IVEC)

After 19 hours of co-incubation with spermatozoa, the presumptive zygotes were placed in a 1.5 ml Eppendorf tube containing 500 µl of oocyte recovery media and gently vortexed for four minutes to remove excess sperm and cumulus cells. Presumptive zygotes were then quickly wash three times (in 500 µl) in oocyte recovery media (to remove debris after vortexing) followed by three more washes in serum-free modified synthetic oviductal fluid (mSOF), which was the medium used for embryo culture. The mSOF medium contained 4.0% (w/v) fatty acid-free BSA (Sigma A7030), 108 mM sodium chloride (Sigma S5886), 7.2 mM potassium chloride (Sigma P5405), 1.2 mM potassium phosphate monobasic (Sigma P5655), 0.8 mM magnesium sulphate heptahydrate (Sigma M2643), 0.6 mM sodium lactate (Sigma L4263), 25 mM sodium bicarbonate (Sigma S5761), 0.026 mM phenol red (Sigma P5530), 0.73 mM sodium pyruvate (Sigma P3662), 1.78 mM calcium chloride dihydrate (Sigma C7902), 2.75 mM myo-inositol (Sigma 1.04507), 3.0% (v/v) basal medium eagle (BME) amino acid solution (50x) (Sigma B6766), 1.0% (v/v) minimum essential medium (MEM) amino acid solution (100x) (Sigma M7145), 0.4 mM glutamine (Sigma G6392) and 50 µg/ml gentamycin sulphate (Sigma G6392). The medium was prepared with osmolarity between 280-290 mOsm, a pH of 7.40, and sterilised by filtration before use. After washing, the presumptive zygotes were cultured for eight days (day of IVF= day 0) in groups of 20-30 in a 4-well plate containing mSOF medium (500 µl/well without oil cover) under a humidified atmosphere containing 5% CO_2, 5% O_2, and at 38.5°C. On day 8, the developmental stage of embryos was classified according to the guidelines of the International Embryo Transfer Society (Bó and Mapletoft, 2013) (IETS) (Figure 2.3). Cleavage rate was calculated on the number of zygotes culture and included any embryo with two cells or more on day 8. Degenerated embryos were calculated on the number of cleaved embryos and included any embryo with two cells or more, but not able to reach the compacted morula stage (Figure 2.3 B) on day 8 (see appendix 6 for detailed preparation of IVEC medium).

2.2.6 Preparation of non-esterified fatty acids (NEFA) treatments

Stearic acid (SA, Sigma S4751) and palmitic acid (PA, Sigma P0500) were dissolved in pre-warmed absolute ethanol for cell culture, while oleic acid (OA, Sigma O1257) was dissolved in pre-warmed nuclease-free water for molecular biology. NEFA stocks

were prepared at concentrations of 28 mM SA, 23 mM PA and 21 mM OA for physiological levels and 280 mM SA, 230 mM PA and 210 OA mM for pathophysiological levels. The stock solutions were vortexed for 15 minutes and sonicated for one hour at 35°C then added to the serum-free fertilisation medium containing FA-free BSA (see section 2.2.4 for fertilisation medium composition) as NEFA carrier (to improve NEFA solubility) to obtain the desired final concentration (see section 2.2.7 below). The fertilisation medium was then sonicated at 35°C for four hours and sterilised by filtration under aseptic conditions prior to IVF (see appendix 7 for detailed preparation of NEFA).

Figure 2-3. Preimplantation embryo classification carried out on day 8 (day of IVF=day 0) of embryo culture. **A**) Blastocyst of difference sizes. **B**) Morula. **C**) Blastocyst, note the inner cell mass (ICM), the blastocoel (fluid-filled cavity), and thickness of zona pellucida (ZP) similar to the morula in picture B. **D**) Expanded blastocyst, note the bigger blastocoel and thinner zona pellucida compared to the blastocyst in picture C. **E**) Hatching blastocyst, note the hatching site (white arrow). **F**) Hatched blastocyst, note the absence of the zona pellucida.

2.2.7 Experimental design

Since ethanol was used to dissolve two of the NEFA used in the experiments, first a comparison between fertilisation medium and fertilisation medium plus ethanol was carried out to determine if blastocyst formation is affected by the level of ethanol used

in the fertilisation medium. For the NEFA experiments, the NEFA concentrations used during IVF are based on the model developed by Desmet et al., (2018) where NEFA values observed in the serum of cows were considered to be present at the level of the oviduct, since previous research by the same group found a positive correlation between NEFA values in serum and oviductal fluid (Jordaens et al., 2017a). As such, a high NEFA group (High-NEFA) containing 280 µM SA, 230 µM PA and 210 µM OA (720 µM total NEFA) representing pathophysiological NEFA concentrations present in cows undergoing negative energy balance was compared to two control groups. One control group (Control-1) contained 28 µM SA, 23 µM PA and 21 µM OA (72 µM total NEFA) representing physiological NEFA concentrations observed in cows out of the phase of negative energy balance. The second control group contained fertilisation medium with 0.2% absolute ethanol equivalent to the same concentrations on the NEFA groups (Control 2) (Figure 2.4). A cycle of preimplantation embryo production including IVM, IVF and IVEC was considered a replicate.

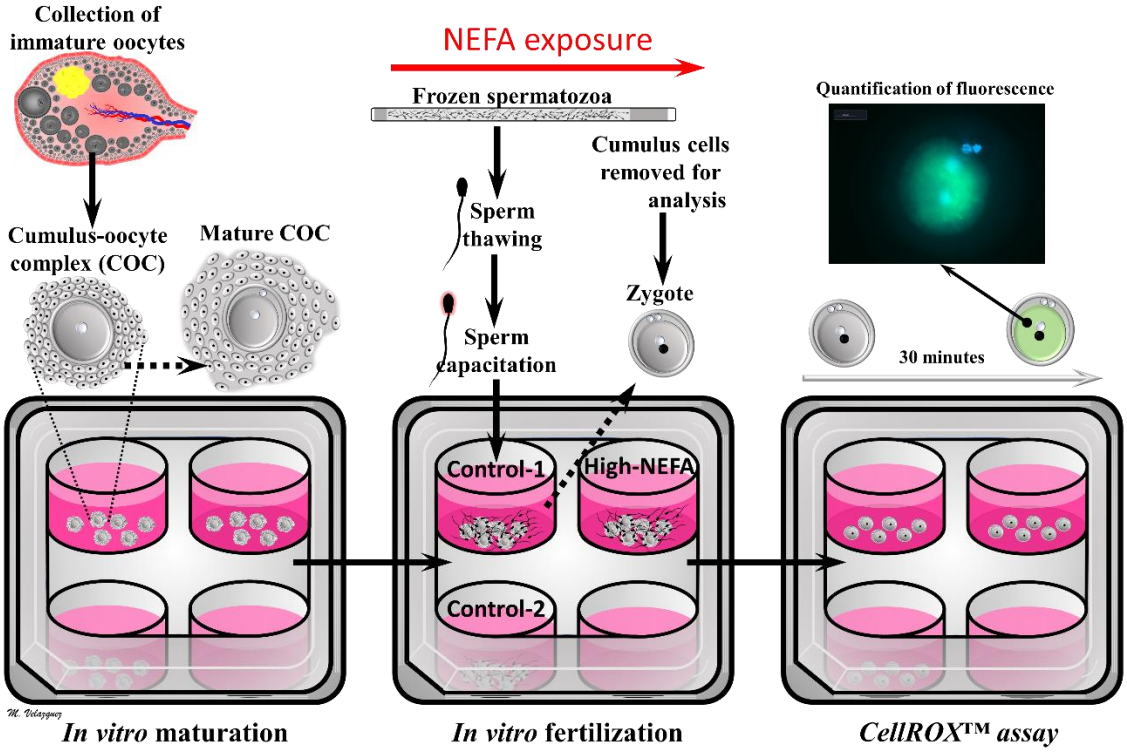


Figure 2-4. Graphical depiction of the experimental design used to test the effects of NEFA during IVF on ROS levels of resultant zygotes. Artwork credit: Dr Miguel Velazquez.

2.2.8 Analysis of reactive oxygen species (ROS) in zygotes

In this experiment, two mounting media and two cell nuclear dyes were tested to standardise the ROS quantification method. For this, zygotes were produced in standard IVM and IVF medium (i.e., without ethanol or NEFA) and subjected to the cellular assay to detect ROS (see below for a detailed description of ROS measurement). Glycerol (Sigma; G5516; n= 24) and vectashield (Vector Laboratories, H-1000-10; n= 21) antifade mounting media, and the fluorescent DNA-binding dyes Hoechst 33342 (10 µM) (n= 25) and 4′,6-diamidino-2-phenylindole (DAPI) (10 ng/ml) (n= 22) were tested. No major difference was found between mounting media, and glycerol was selected as it was easy to handle (i.e., lower viscosity). Hoechst 33342 showed the clearest presence of pronuclei in the images and was used as nuclear staining to determine the fertilisation status of presumptive zygotes subjected to ROS measurement (Figure 2.5).

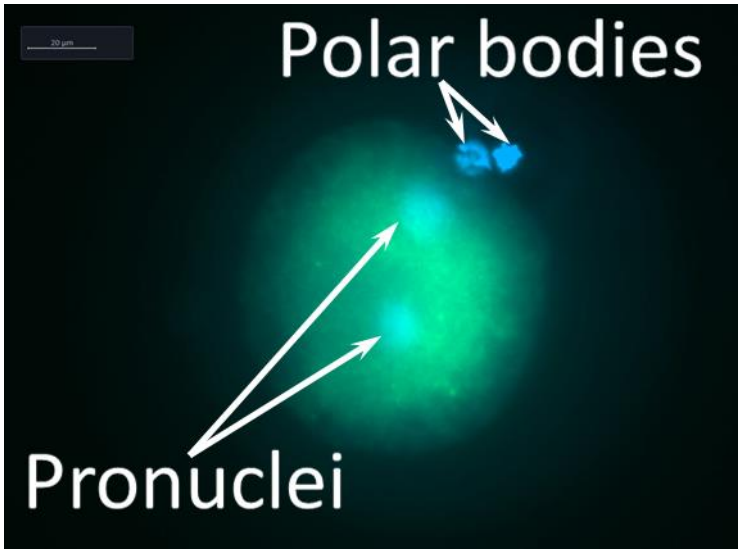


Figure 2-5. Fluorescence image of a day-1 bovine zygote with glycerol as mounting medium showing the presence of pronuclei and polar bodies stained with Hoechst 33342. The green area represents ROS expression. Bar= 20 µm.

For the NEFA experiment, presumptive zygotes (from 4 replicates) from the high-NEFA (n= 129) and control (Control-1, n= 128; Control-2, n= 98) groups were subjected to ROS measurement. After cleaning of the presumptive zygotes (see section 2.2.5), they were cultured in groups of 20-30 in a 4-well plate containing mSOF medium (500 µl/well) in the presence of CellROX™ Green (Thermo Fisher Scientific, C10444) at a concentration of 5.0 µM (Ortega et al., 2016) and Hoechst 33342 at a

concentration of 10.0 µM for 30 minutes in a humified atmosphere, under 5% CO_2, 5% O_2 and at 38.5°C. Afterwards, presumptive zygotes were washed three times (five minutes each wash) in 0.1% polyvinylpyrrolidone (PVP) in PBS to remove mSOF culture media and subsequently fixed with 4.0% formaldehyde in PBS for 15 minutes at room temperature (20°C). Zygotes were then washed three times (five minutes each) in 0.1% PVP in PBS to remove fixation medium. Finally, presumptive zygotes were mounted onto a glass microscope slide with double enforcement rings in a small drop of glycerol medium (~5.0 µl), cover slipped, sealed with nail varnish and kept in a fridge until analysis of fluorescence intensity. Florescence intensity of ROS levels and number of nuclei were performed as soon as possible after staining (i.e., same day). Digital photographs of presumptive zygotes were obtained in a darkened room with an epifluorescence microscope (Zeiss Axio Imager, Carl Zeiss Optics Co., Ltd, Oberkochen, Germany) equipped with a digital camera (Zeiss 105) using a 40X objective lens with DAPI filter (excitation 360–370 nm, emission 420–460 nm; for Hoechst fluorescence) and Alexa 488 filter (excitation 460–490, emission 500–520 nm for CellROX™ Green fluorescence) (Figure 2.6).

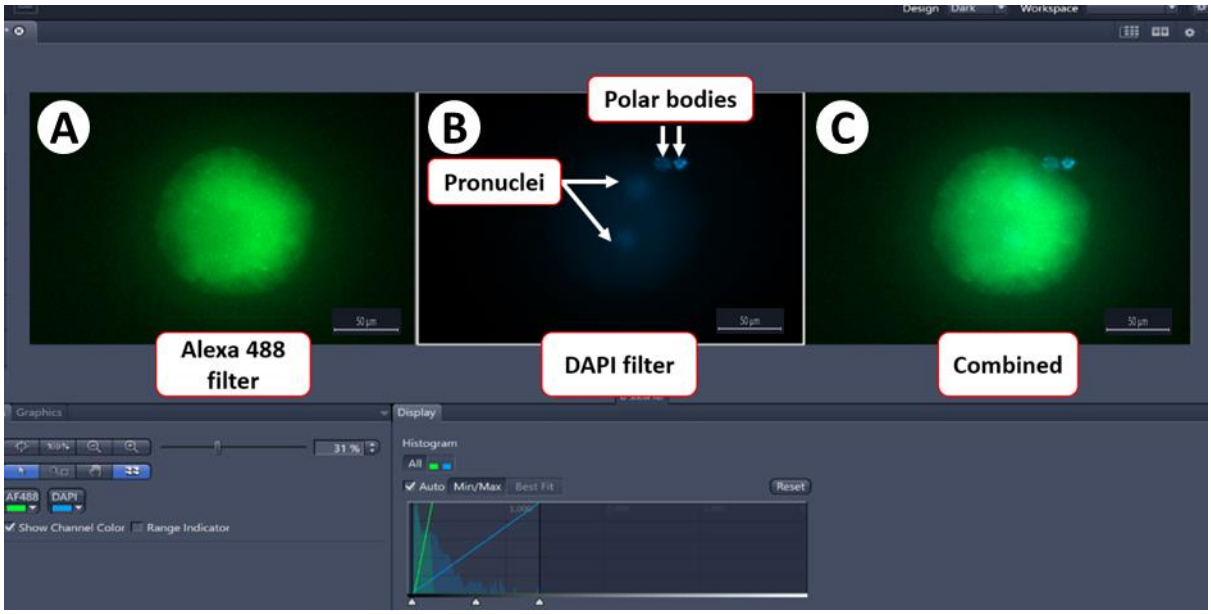


Figure 2-6. Image acquisition with the ZEN microscopy software. **A)** Alexa fluorescence filter (green) showing fluorescence intensity indicative of reactive oxygen species levels. **B)** DAPI filter (blue) showing DNA staining. **C)** A merged image for both channels. Bars= 50 µm.

To quantify ROS levels in a standard area in all zygotes (to control for oocyte size), a circular area of 2500 µm² was drawn with the Zeiss software in the middle of the zygote, and an identical area was also drawn in the black background (Figure 2.7 A). The fluorescence intensity was then measured by subtracting the black background intensity from the green fluorescence intensity. Fertilisation status was also determined (Figure 2.7 B-D). Overlapping or out-of-focus nuclei in the digital photographs were identified by manual focus scanning of the samples under the microscope at the time of imaging. The same settings were used for all presumptive zygotes.

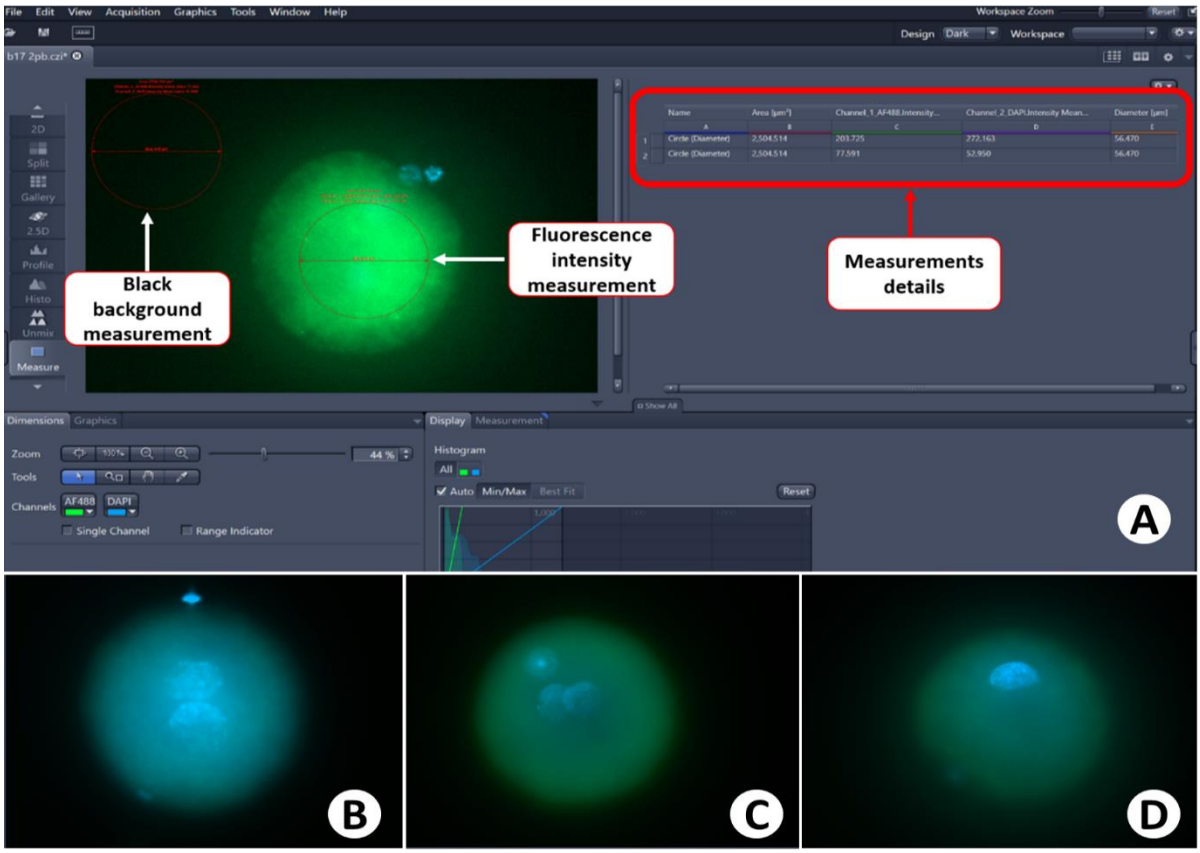


Figure 2-7. Image analysis with the ZEN microscopy software and classification of fertilisation status in bovine zygotes. **A)** ZEN software showing the areas of measurement for fluorescence intensity and black background. **B)** Monospermic bovine zygote, note presence of two pronuclei. **C)** Polyspermic bovine zygote, note the presence of three pronuclei. **D)** Unfertilised bovine oocyte, note the presence of only one pronucleus. Bars= 50 µm.

2.2.9 Statistical analysis

Statistical analysis was performed using the statistical package for the social sciences (SPSS) 26 software (IBM). The Shapiro-Wilk test was used to test if the variables analysed had a normal distribution. Differences between groups were identified using analysis of variance (ANOVA) or T-test, with percentage data arcsine transformed before analysis. A P-Value less than 0.05 was considered statistically significant. Post-hoc comparisons were done with the least significant difference (LSD) method. Data was reported as mean ± standard error of the mean (SEM) unless otherwise indicated.

2.3 Results

The inclusion of ethanol in fertilisation medium did not alter degeneration rate (Ethanol-free= 39.88±4.05%, ethanol= 42.01±2.88%), cleavage (Ethanol-free= 54.28±2.47%, ethanol= 48.22±2.38%), morula formation (Ethanol-free= 10.30±0.75%, ethanol= 8.16±0.95%) and blastocyst production (Ethanol-free= 22.0±1.5%, ethanol= 19.96±1.81%) when compared to fertilisation medium without ethanol (Figure 2.8).

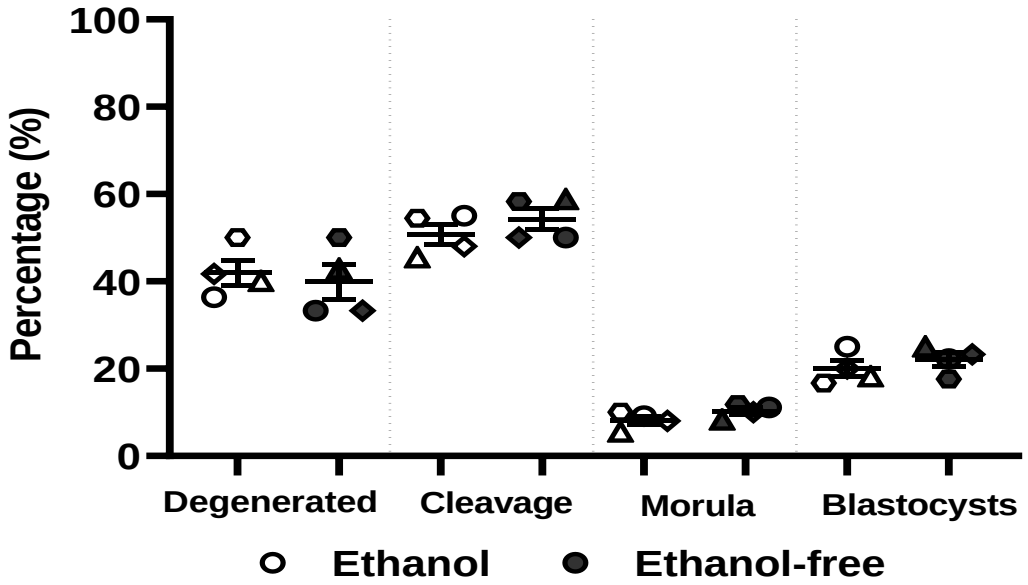


Figure 2-8. Effect of supplementing fertilisation medium with ethanol (i.e., 0.2% ethanol used as solvent for NEFA) on *in vitro* preimplantation embryo production in cattle. Presumptive zygotes (Ethanol-free n=77, ethanol n=74) were cultured in 4 replicates. No significant difference between the treatment groups was observed for all embryo production variables (P>0.05). ◯= 1st, ⬡= 2nd, △= 3rd and ◇ = 4th cycle.

The percentage of monospermic fertilisation was significantly decreased in the high NEFA group (28.32±2.82%) when compared to control-1 (51.41±6.86%, P= 0.026) and control-2 (59.16±6.04%, P= 0.006). However, there was no significant difference in polyspermy rate between all treatment groups (Control-1= 15.84±5.09%, Control-2= 15.42±4.75%, high-NEFA= 10.74±4.18%). The percentage of unfertilised oocytes was also decreased in the high-NEFA group (60.94±6.76%) in comparison to controls (Control-1= 32.75±6.53%, P= 0.017; Control-2= 25.42±7.72 %, P= 0.006) (Figure 2.9).

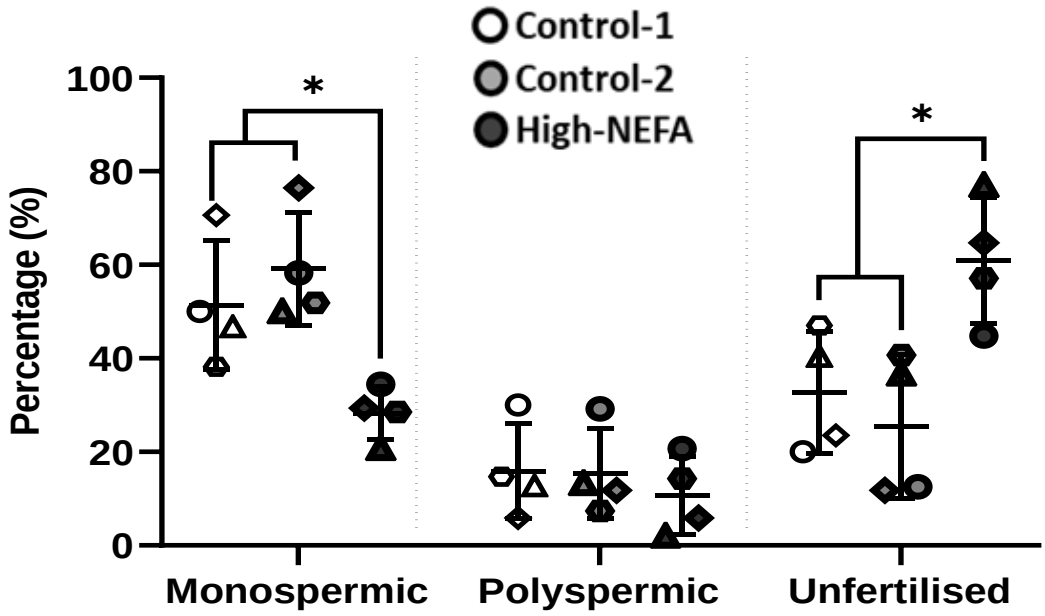


Figure 2-9. Effect of high-NEFA during IVF on fertilisation variables. The asterisk indicates a significant difference between groups (P<0.05). ◯ = 1st, ⬡ = 2nd, △ = 3rd and ◇ = 4th cycle.

Analysis of fluorescence intensity (arbitrary units) associated to ROS levels indicated that high-NEFA zygotes (861.78±79.80, n= 51) did not increase their ROS production when compared to control groups (Control-1= 911.08±71.20, n= 62; Control-2= 968.53±108.35, n= 52) (Figure 2.10). A second analysis was carried out within groups, to compared ROS levels between fertilised (monospermic and polyspermic zygotes) and unfertilised oocytes. No effect according to fertilisation status was detected in controls (Control-1= fertilised: 671.01±52.57, n= 83, unfertilised: 619.76±48.84, n= 45; Control-2= fertilised: 615.73±55.95, n=69, unfertilised: 780.47±77.0, n= 29) and the high-NEFA group (fertilised: 640.58±42.15, n= 63, unfertilised: 667.71±46.54, n= 66), indicating that the fertilisation status did not affect ROS levels in the *in vitro* model used in this experiment (Figure 2.11).

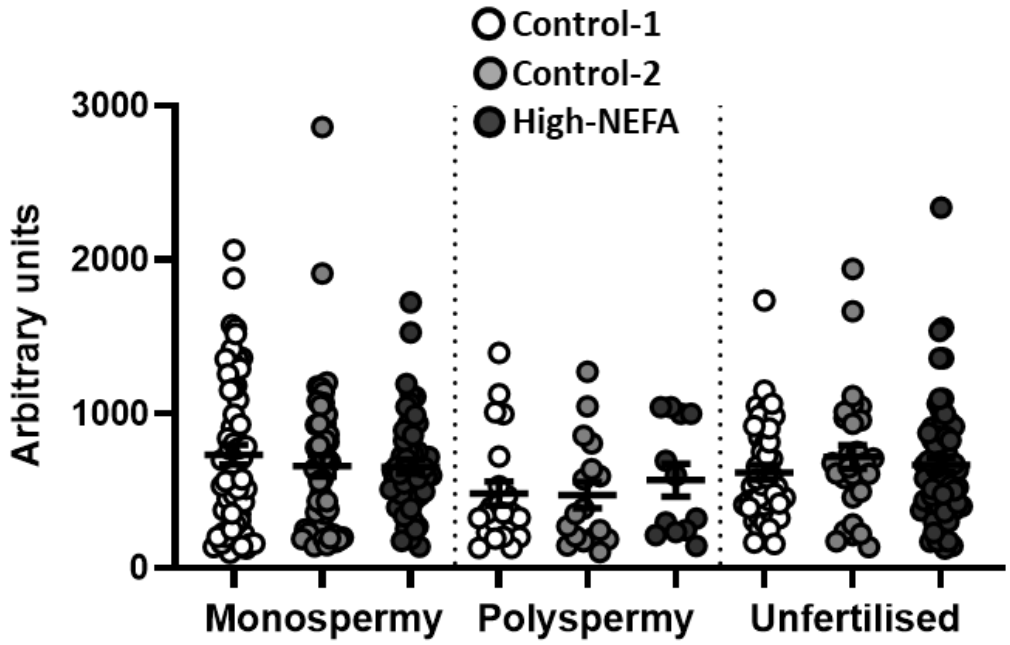


Figure 2-10. ROS fluorescence intensity in zygotes developed under high NEFA exposure during fertilisation. No significant differences in ROS production were observed between groups (P>0.05).

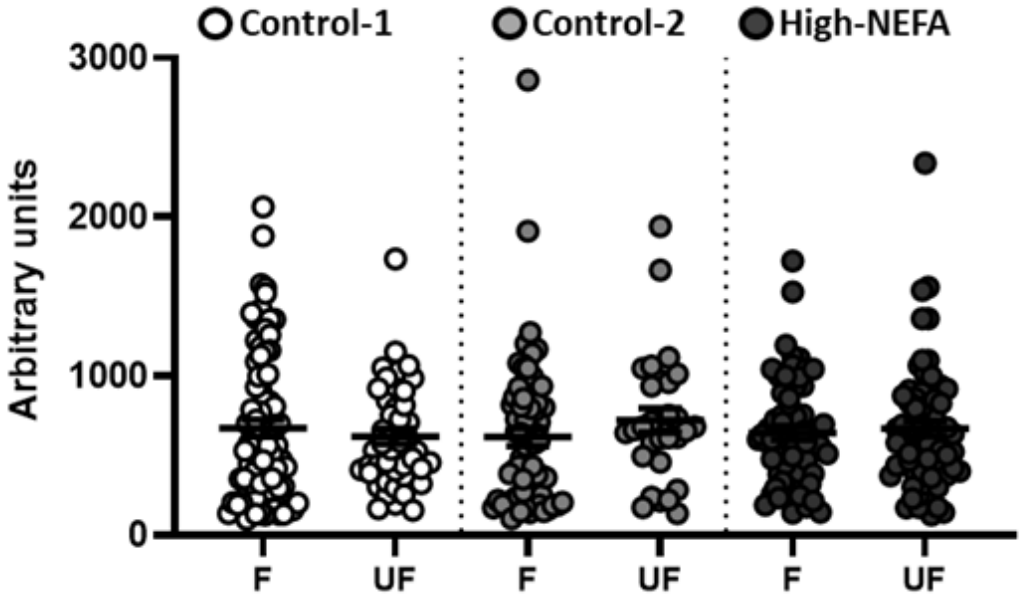


Figure 2-11. ROS fluorescence intensity in zygotes within groups in relation to fertilisation status. (F= fertilised, UF= unfertilised). No significant differences in ROS production were observed according to fertilisation on any experimental group (P>0.05)

2.4 Discussion

It is well documented that mammalian oocyte quality and developmental competence (i.e., ability to form a blastocyst after fertilisation) is impaired when maturation takes place under high NEFA concentrations (Marei and Leroy, 2021, Shi and Sirard, 2022). However, the potential detrimental effects of high levels of NEFA during the fertilisation process has been less studied, with only one paper published in the specialised literature (Desmet et al., 2018) after the beginning of this thesis project.

The ethanol used as solvent for the NEFA used in this experiment did not affect blastocyst production when compared with standard fertilisation medium (i.e., medium without NEFA or ethanol). This allowed to reduce the number of control groups used in the experiments. As such, for experimental comparisons one of the controls contain ethanol, and another control group contained normal physiological NEFA concentrations (i.e., cows not experiencing negative energy balance). The level of ethanol used in the present experiment (i.e., 0.2%) is lower than the 0.3% reported to be detrimental for preimplantation embryo development in cattle (Avery and Greve, 2000). However, in other studies ethanol at 0.27%, 0.3% or 0.53% did not negatively affect blastocyst formation (Shehab-El-Deen et al., 2009, Novaes et al., 2021). Furthermore, the ethanol level used in the present experiment is also lower than the 0.45% ethanol level used in a similar NEFA model as solvent, and with no detrimental effects for blastocyst formation (Desmet et al., 2018).

The impaired fertilisation rate in response to high NEFA exposure during IVF found in the present experiment was also reported by Desmet et al., (2018). However, in the same study the level of polyspermy was reported to be increased by high NEFA during IVF (Desmet et al., 2018), which contrast with the results of the present experiment where polyspermy levels remain unchanged after the NEFA challenge. From this perspective, this indicates that mechanisms that avoid polyspermy during fertilisation in cattle does not seem to be affected by exposure to high NEFA concentrations as previously suggested (Desmet et al., 2018). Interestingly, Desmet et al., (2018) reported that polyspermy was only observed in oocytes exposed to NEFA, regardless of the concentration. The 0% of polyspermy in the control group containing solvent reported by Desmet et al., (2018) is the exception rather than the rule as polyspermy is a common abnormality during *in vitro* embryo production in mammals with an

incidence from 3% to 30% in humans, and from 5% to 45% in cattle (Coy and Avilés, 2010, Gutiérrez-Añez et al., 2021).

The question remains on what could affect the ability of oocytes to get fertilised or the potential of spermatozoa to achieve fertilisation. Previous research indicated that zygotes formed under high exposure to NEFA during IVF did not show an increase in lipid content, indicating that lipids accumulated during fertilisation in a high NEFA microenvironment do not seem to affect the capacity of the oocyte to achieve fertilisation (Desmet et al., 2018). But given that cumulus cells can select spermatozoa with an increased chance of achieving fertilisation in *in vitro* settings (Franken and Bastiaan, 2009, Wang et al., 2018) and that cumulus cells can be damaged by high NEFA exposure (Mu et al., 2001, Vanholder et al., 2005, Marei et al., 2017, Pedroza et al., 2022), it is reasonable to propose that fertilisation rate in the present experiment could have been affected via impaired cumulus cell activity. Nevertheless, studies analysing the capacity of cumulus cells to select spermatozoa under high NEFA concentrations are not available to the best of the author's knowledge.

On the other hand, the possible detrimental effect of NEFA-damaged spermatozoa should also be considered. A previous study reported that the percentage of spermatozoa with intact plasma membrane was decreased during exposure to high NEFA concentrations for four hours (Desmet et al., 2018), which agrees with the findings in chapter 5 of this thesis, where the effects of high NEFA on sperm quality were analysed. Indeed, the spermatozoa plasma membrane plays a significant role in physiological changes necessary for the spermatozoa to achieve fertilisation including sperm motility, capacitation, and acrosome reaction (Leahy and Gadella, 2011, Gautier and Aurich, 2021).

In this experiment the effects of high NEFA during IVF were also investigated to determine if levels of ROS are altered in resultant zygotes. This is the first time this has been analysed and the results indicated that exposing bovine oocytes and sperm to high NEFA concentration during the fertilisation process do not seem to affect ROS levels in the resultant monospermic zygotes. Research conducted in cattle showed that during co-incubation of matured oocytes and spermatozoa for 24 hours (i.e., during IVF), there were significant peaks of ROS production at 7, 19 and 24 hours, coinciding with energy demanding events such as pronuclear formation and first

mitotic division in fertilised oocytes (Morado et al., 2013). Indeed, peaks of oxygen consumption with a concomitant increase in ROS production were also observed during *in vitro* oocyte fertilisation in cattle (Lopes et al., 2010). The increase in ROS production has been also reported in murine (Nasr-Esfahani and Johnson, 1991) and *Xenopus* zygotes (Han et al., 2018). To the best of the author's knowledge there is no information available on this topic in humans (i.e., ROS production during the fertilisation process). Hence, since there is a peak in ROS production in zygotes analysed 24 hours after IVF (Lopes et al., 2010, Morado et al., 2013), the possibility exists that this physiological increase in ROS production masked differences in ROS expression in zygotes associated with the level of NEFA exposure in the present experiment. Previous research indicated that oocyte maturation under high NEFA levels can increase ROS levels in resultant early embryos with four or more cells (Marei et al., 2019a). This might be related to the fact that development after the zygote stage, during the cleavage stages, is characterised by relatively constant levels of ROS production (Dalvit et al., 2005, Hardy et al., 2021) and by the low levels of O_2 the embryo is exposed to during *in vitro* culture (see below for discussion of O_2 influence on ROS production in preimplantation embryos). Hence subtle increases in ROS production associated to stressors might be better detected during preimplantation embryo development than during zygote formation. The results of this experiment and the available evidence regarding ROS production during the formation of 1-cell embryos (Lopes et al., 2010, Morado et al., 2013) will suggest that zygotes might not be a suitable developmental stage to analysed oxidative stress in response to stressors during fertilisation.

Here is important to mention that the impact of NEFA on the production of ROS in reproductive cells seems to be contradictory. For instance, Marei et al. (2017) reported that exposure of oocytes during *in vitro* maturation to a mix of stearic, palmitic, and oleic acid resulted in high production of ROS in oocytes, but in another study bovine oocytes matured under the same NEFA mix (including concentrations) showed a reduction in ROS levels (Sutton-McDowall et al., 2016). The reasons behind these contrasting results are unknown but it could be related to the cell assay used to detect ROS levels. Sutton-McDowall et al., (2016) used peroxyfluor-1, which can detect changes in hydrogen peroxide (Miller et al., 2005), whereas Marei et al., (2017) used 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), which can detect not only

hydrogen peroxide, but also hydroxyl radical and peroxyl radical (Gomes et al., 2005). The ROS detection kit used in the present study was CellROX™ Green which can detect hydroxyl radical and superoxide anion (McBee et al., 2017). CellROX™ Green has been used in bovine zygotes, where an increase in ROS production was detected when zygotes were exposed after IVF to heat stress (Ortega et al., 2016) or aflatoxins (Jiang et al., 2019). However, to the best of the author's knowledge there are no studies analysing stressors exclusively during IVF with concomitant measurement of ROS production in resultant zygotes.

Also relevant is the role of O_2 level during *in vitro* culture as it is known it can affect ROS production in the preimplantation embryo, including zygotes. As such atmospheric O_2 (21%) induces more ROS production in bovine zygotes than 5% O_2 (Ortega et al., 2016). Hence, since IVF took place under atmospheric O_2 in the present experiment, this could have further masked any possible effects of NEFA on ROS production in zygotes. The use of atmospheric O_2 during fertilisation is standard practice in cattle IVF as blastocyst formation is reduced when IVF takes place at 5% O_2 (Bermejo-Álvarez et al., 2010). On the other hand, 5% O_2 is the standard practice for *in vitro* culture of preimplantation embryos in cattle as it enhances blastocyst production (Harvey, 2007).

In conclusion, the novel results reported in this chapter suggest that exposure to high NEFA concentrations during fertilisation does not seem to affect the level of polyspermy and the level of ROS production in resultant monospermic bovine zygotes is not affected.

Chapter 3: Effects of Elevated Non-Esterified Fatty Acids Concentrations during *In Vitro* Bovine Fertilisation on Preimplantation Embryo Development and Cell Lineage Allocation of Resultant Blastocyst

3.1 Introduction

The first observable differentiation event during mammalian preimplantation embryo development occurs once the early embryo starts forming the blastocyst when the inner cell mass (ICM) and the trophectoderm (TE) segregate (Sakurai et al., 2016). The ICM contain pluripotent cells located within the inner surface of the TE that give rise to foetal structures, while the TE is composed of a single layer of polarised cells surrounding the ICM (Polar TE) and blastocoel (Mural TE) that develops into the placenta (Carreiro et al., 2022, Rossant and Tam, 2022). The expression of several transcription factors is required in the differentiation of ICM and TE, with some of them displaying ICM or TE cell-specific expression during blastocyst formation that allows them to be used as cell lineage markers during preimplantation embryo development (Piliszek et al., 2016, Pérez-Gómez et al., 2021, Aguila et al., 2022) (Figure 3.1).

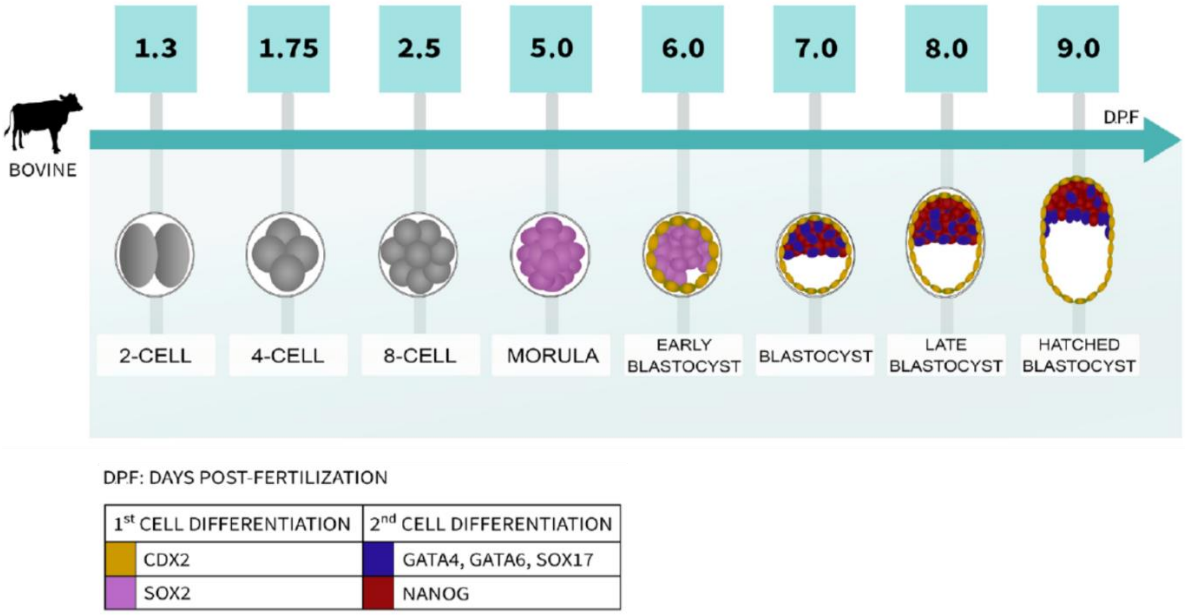


Figure 3-1. Temporal expression of major transcription factors during bovine preimplantation embryo development. Adapted from (Carreiro et al., 2022).

For example, the transcription factor caudal-type homeobox 2 (CDX2) is expressed exclusively on the TE of bovine blastocysts (Goissis and Cibelli, 2014). In cattle, CDX2 is not essential for blastocyst formation (Goissis and Cibelli, 2014, Sakurai et al., 2016) but it participates in the maintenance of the TE epithelial barrier (Goissis and Cibelli, 2014). Cell lineage markers can be used to assess preimplantation embryo viability as some of them are expressed in specific locations at the blastocyst stage, providing number of cells allocated to the ICM and TE (Pérez-Gómez et al., 2021, Aguila et al., 2022). Indeed, several studies have used immunofluorescence to detect protein expression of CDX2 in blastocysts to be able to quantify cell number in the TE, and the ICM by counting CDX2-negative blastomeres stained with fluorescent DNA-binding dyes such as Hoechst 33342 and DAPI. Cell number increases during blastocyst formation (Leidenfrost et al., 2011) and has been positively correlated with oxygen consumption in bovine blastocysts, where blastocysts that managed to hatch display a higher oxygen consumption (Sugimura et al., 2012). The allocation of cells to the ICM and TE is also important to consider during preimplantation embryo development as aberrant cell allocation at the blastocyst stage has been associated with early pregnancy loss (Velazquez et al., 2011b). As such, CDX2 staining for cell number quantification has been applied to examine the quality of bovine blastocysts in experimental exposure to various elements known to affect bovine early embryo development including phospholipids (Yu et al., 2021), serum (Soto-Moreno et al., 2021), cytokines (Sang et al., 2020, Wooldridge and Ealy, 2019, Wooldridge and Ealy, 2021) and NEFA (Desmet et al., 2018).

Although it is known that the oocyte and the embryo are both affected by high NEFA levels (Leroy et al., 2017, Marei and Leroy, 2021, Shi and Sirard, 2022), the extent to which bovine early embryo development is affected by high NEFA exposure during the fertilisation process *per se* has been less studied. The only study available on this topic did not find a clear effect of NEFA during IVF on blastocyst formation (Desmet et al., 2018). In the latter study, culture medium during embryo culture was supplemented with insulin, transferrin, and selenium (ITS), which is known to increase blastocyst production in several species, including cattle (Wydooghe et al., 2014). Hence, this could have masked the possible detrimental effects of high NEFA exposure during IVF. Indeed, a recent study indicated that bovine zygotes derived from oocytes *in vitro* matured under high NEFA concentrations, but cultured in medium supplemented with

ITS, managed to achieve blastocyst formation to levels observed in controls (Smits et al., 2020).

Therefore, the aim of this experiment was to investigate the effects of high NEFA during IVF on bovine preimplantation embryo production and cell lineage allocation in resultant blastocysts using a simple chemically semi-defined *in vitro* culture medium (Velazquez et al., 2011b).

3.2 Material and Methods

3.2.1 Collection of cumulus oocyte complexes (COCs)

For handling of glassware and plasticware please see section 2.2.1. For detailed description of collection of COCs see section 2.2.2. Briefly, ovaries collected from a local abattoir were slashed in oocyte recovery medium to obtain immature oocytes. Then good quality (grade I) unexpanded COCs with homogenous ooplasm and at least five layers of compact cumulus cells were selected for *in vitro* maturation.

3.2.2 *In vitro* maturation (IVM)

For detailed description of IVM please see section 2.2.3. Briefly, COCs were matured in groups of 20–30 in a 4-well plate containing serum-free maturation medium (500 µl/well without oil cover) for 22-24 hours in a humidified atmosphere with 5% CO_2, atmospheric O_2, and at 38.5°C.

3.2.3 *In vitro* fertilisation (IVF)

Commercial semen straws were used for IVF. For detailed processing of semen straws and description of IVF please see section 2.2.4. Briefly, matured COCs were co-incubated in groups of 20–30 with spermatozoa at a final concentration of $1x10^6$ sperm cells/ml in a 4-well plate (500 µl/well, without oil cover) containing fertilisation medium. IVF took place for 19 hours in a humidified atmosphere with 5% CO_2, atmospheric O_2, and at 38.5°C.

3.2.4 *In vitro* embryo culture (IVEC)

For detailed description of IVEC please see section 2.2.5. Briefly, presumptive zygotes were cultured for eight days (day of IVF= day 0) in groups of 20-30 in a 4-well plate

containing mSOF medium (500 µl/well without oil cover) under a humidified atmosphere containing 5% CO_2, 5% O_2, and at 38.5°C. On day 8, the developmental stage of embryos was classified according to the guidelines of the IETS (see section 2.5. for details). A cycle of preimplantation embryo production including IVM, IVF and IVEC was considered a replicate. (Figure 3.2).

3.2.5 Experimental design

For preparation of NEFA treatments please see section 2.2.6. NEFA exposure during fertilisation was based on the model developed by Desmet et al. (2018) (see explanation in section 2.2.7). The following groups were tested:

● High NEFA group (High-NEFA) containing 280 µM SA, 230 µM PA and 210 µM OA (720 µM total NEFA).

● Control group 1 (Control-1) containing 28 µM SA, 23 µM PA and 21 µM (72 µM total NEFA).

● Control group 2 (Control-2) containing 0.2% absolute ethanol equivalent to the same concentrations on the NEFA groups.

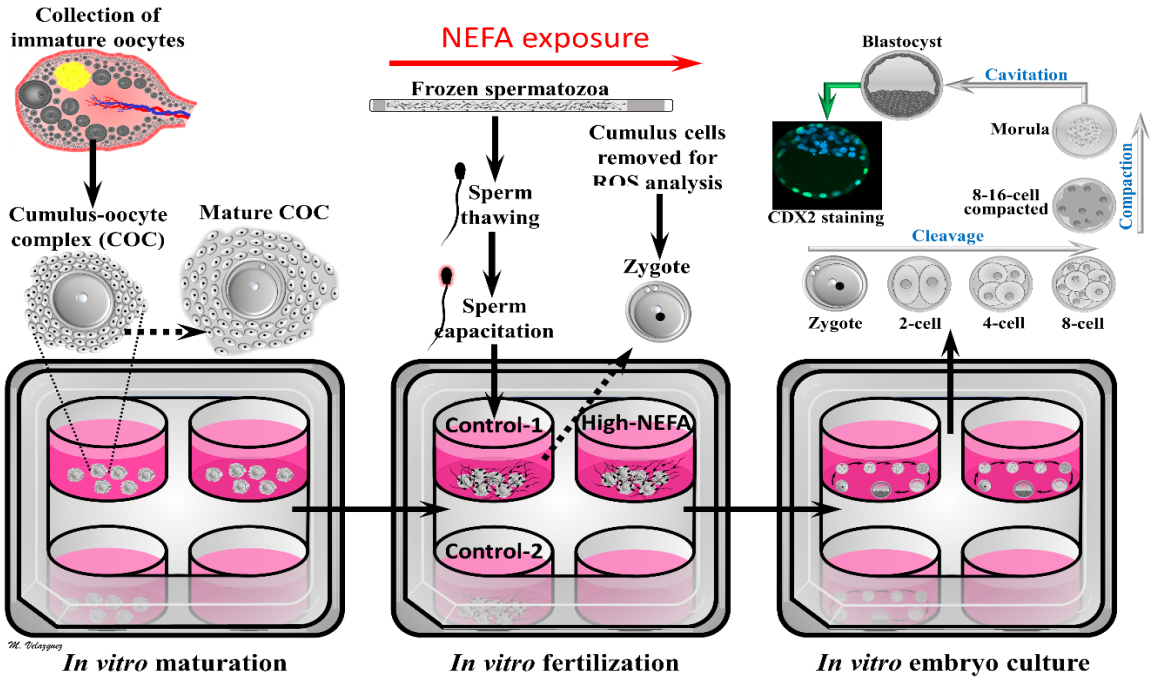


Figure 3-2. Graphical depiction of the experimental design used to test the effects of NEFA during IVF on preimplantation embryo development in cattle. Artwork credit: Dr Miguel Velazquez.

3.2.6 CDX2 immunostaining

The primary and secondary antibody were tested in dilutions of 1:50 and 1:200, and 1:200 and 1:500, respectively. A dilution of 1:200 for the primary and 1:500 for the secondary antibody worked well and it was used for the CDX2 staining protocol. The protocol started with washing (to remove culture medium) of day-8 zona-intact blastocysts in 0.1% (w/v) polyvinylpyrrolidone (PVP) (Sigma; P0930) in PBS (Sigma; P4417) with subsequently fixing in 4.0% (v/v) formaldehyde/PBS (Sigma; F8775) for 15 minutes at room temperature. Washing consisted in moving embryos three times (five minutes each) from one well (i.e., four-well dishes) to another containing washing solutions. Fixing solution was then removed by washing embryos in 0.1% (v/v) tween (Sigma; P9416) in PBS (tween/PBS), and after washing, blastocysts were permeabilised and blocked in a solution containing 3.0% (w/v) FA-free BSA (Sigma: A7030) and 0.5% (v/v) triton (Sigma; 648464) in PBS for one hour at a room temperature. After blocking and permeabilisation, blastocysts were washed in tween/PBS followed by incubation with a ready to use anti-CDX2 primary antibody (Abcam ab227201) at a 1:200 dilution in tween/PBS overnight at 4.0°C, in the dark. Next day, blastocysts were washed in tween/PBS and incubated with goat anti-rabbit secondary antibody (Thermo Fisher; R37116) in a 1:500 dilution in tween/PBS for 30 minutes at room temperature in the dark. Antibody solution was then removed by washing in tween/PBS and blastocysts were incubated with DAPI (Sigma; D9542, 10 ng/ml) in tween/PBS for 30 minutes at room temperature in the dark. After a final wash, blastocysts were mounted on a microscope slide within double reinforcement rings in a small drop of Citifluor (Electron Microscopy Sciences, AF1, 17970) anti-fading medium (~5.0 µl), coverslipped, sealed with nail varnish, and stored at 4.0°C in the dark until analysis (i.e., embryos were analysed same day of mounting them on microscope slides). Negative controls were treated as described above except that exposure to the anti-CDX2 primary antibody was omitted (Figure 3.3).

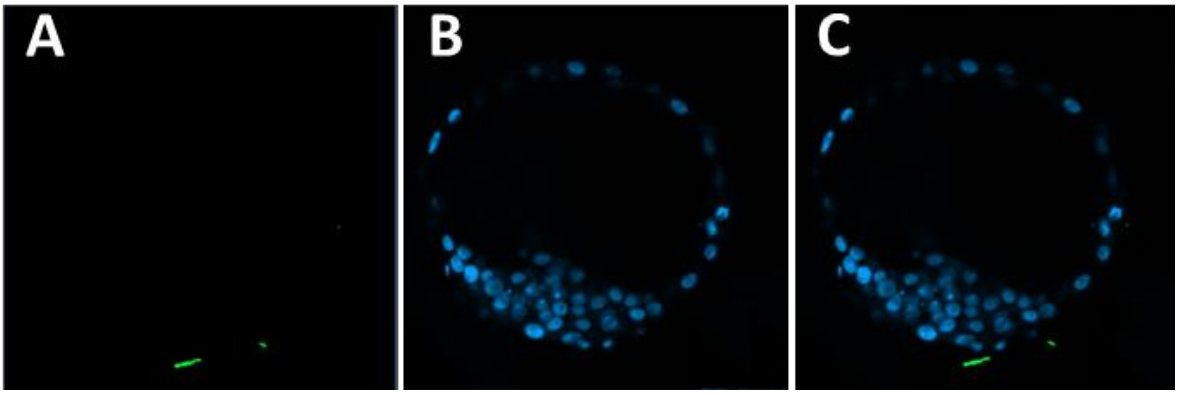


Figure 3-3. Negative control for CDX2. **A)** Green channel for visualisation of CDX2 fluorescence, note the absence of blastomeres displaying fluorescence. **B)** Blue channel showing DAPI staining of all blastomeres. **C)** Merged image showing both channels. Bars= 50 µm.

Embryos were examined in a dark room with a confocal laser scanning microscope equipped with a Zeiss LSM 800 Airyscan microscope (Carl Zeiss Optics Co, Ltd, Oberkochen, Germany) running the Zen 3.1 software and a Plan-Neofluar 25/0.8 objective lens. A solid-state laser was used to detect DAPI (405 nm excitation) and Alexa AF488 (488 nm excitation) using GaAsP detectors and a 400-650 nm bandpass, switching sequentially between channels. Optical sections were taken at 1.00 µm intervals across the whole embryo using a 37 µm pinhole (equivalent to a 5.80 µm optical section). Cell number in blastocysts (Control-1, n= 14; Control-2, n= 12; High-NEFA, n= 8) was obtained manually using the IMARIS software (Oxford Instruments, United Kingdom). The software allows three-dimensional (3D) visualisation of the entire preimplantation embryo (Figure 3.4) and provides specific channel visualisation to quantify CDX2 positive cells representing TE cells (Figure 3.5), and DAPI stained cells (Figure 3.6). The IMARIS software also allows 3D rotation of images, which facilitate the counting of cells at different angles of the embryo. This provides a thorough examination of each blastocyst. ICM was calculated by subtracting CDX2 cell number to the total cell number (i.e., DAPI stained cells) on each embryo.

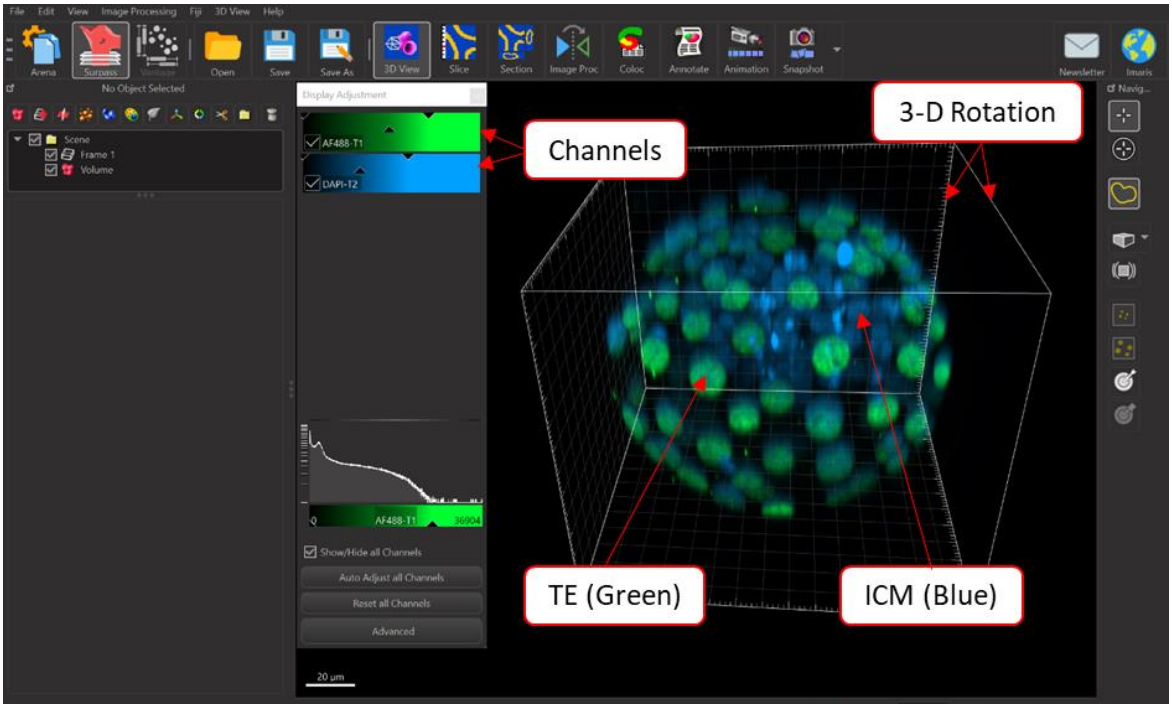


Figure 3-4. 3D blastocyst image in the IMARIS software. The combined image shows CDX2 positive cells in green, representing TE cells, and DAPI stained cells in blue representing the ICM. The 3D rotation feature allows an in-depth view of the blastomeres in each embryo from different angles. Bars= 20 µm.

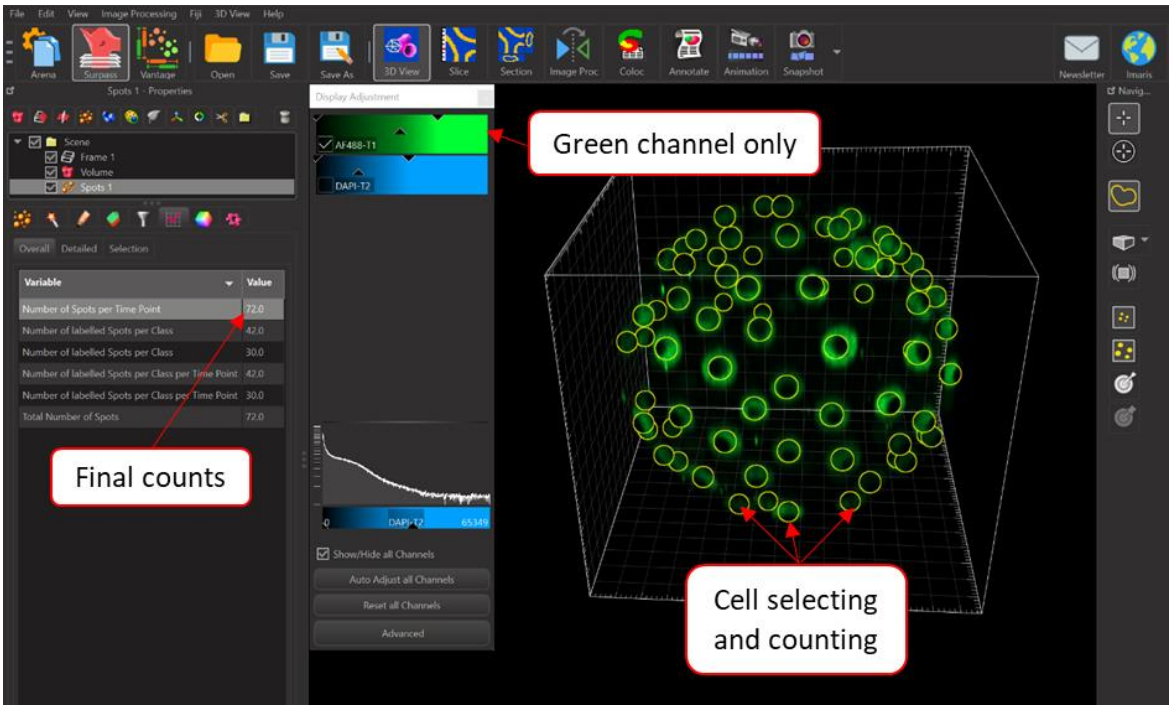


Figure 3-5. Blastocyst image in the IMARIS software displaying the green channel to quantify CDX2 positive cells. Bars= 20 µm.

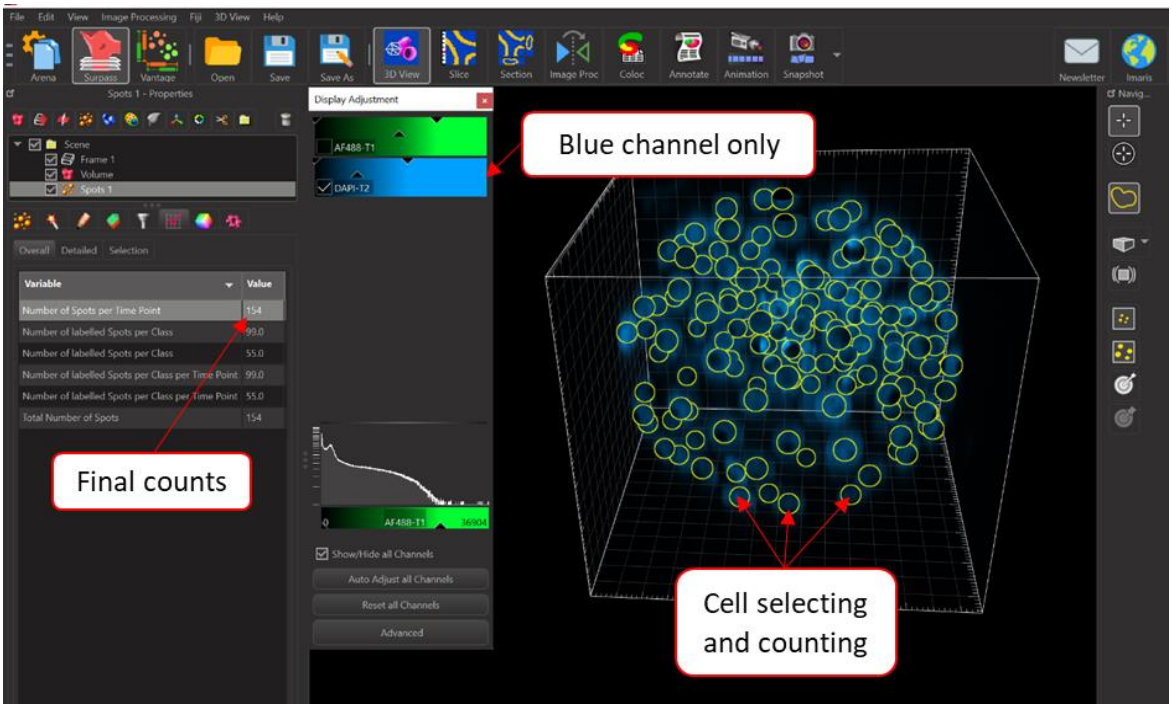


Figure 3.6. Blastocyst image in the IMARIS software displaying the blue channel to quantify DAPI stained cells, the total cell number for each embryo. The 3D rotation feature was applied for thorough counting. Bars= 20 µm.

3.2.7 Statistical analysis

Statistical analysis was performed using SPSS 26 software (IBM). The Shapiro-Wilk test was used to test if the variables analysed had a normal distribution. Differences between groups were identified using analysis of variance (ANOVA) with percentage data arcsine transformed before analysis. Post-hoc comparisons were done with the least significant difference (LSD) method. A P-Value less than 0.05 was considered statistically significant. Data was reported as mean ± standard error of the mean (SEM) unless otherwise indicated.

3.3 Results

3.3.1 *In vitro* embryo development following NEFA exposure during IVF

Cleavage rate was decreased when IVF took place under high NEFA concentrations compared to the control groups (High-NEFA= 46.16±2.95%, Control-1= 64.97±5.06%, C2= 68.96±1.33%, P= 0.006). The rate of embryo degeneration was higher in the high-NEFA group (66.52±3.73%) compared to control-1 (42.44±2.49%, P= 0.046) and

control-2 (49.35±7.28%) but the difference with the latter did not reach statistical significance (P= 0.052). Morula formation was higher in control groups (Control-1, 10.69±1.18%, P= 0.033; Control-2, 10.42±2.39%, P= 0.014) when compared to the high-NEFA group (4.68±0.20%). Similarly, blastocyst formation was decreased in the high-NEFA group (9.36±0.40%, P<0.001) in comparison with Control-1 (25.49±4.08%) and Control-2 (23.54±4.64%). There were no significant differences between the control groups in any of the embryo production variables analysed (Figure 3.7).

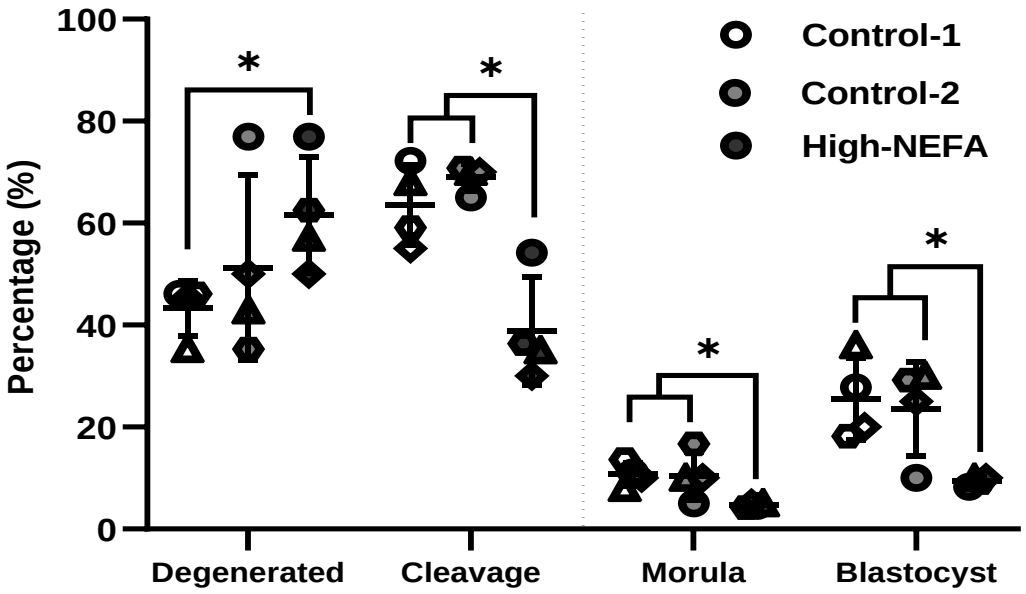


Figure 3-6. Effect of high exposure to NEFA concentrations exclusively during IVF on i*n vitro* embryo production in cattle. Presumptive zygotes cultured in 4 replicates: Control-1 (n= 85), Control-2 (n= 84), High-NEFA (n= 86). The asterisk indicates a significant difference between groups (P<0.05).◯= 1st,⎔= 2nd,△= 3rd and◇= 4th cycle.

3.3.2 Cell allocation in blastocyst derived from high NEFA exposure during IVF

The number of cells in the TE of blastocyst derived from high NEFA exposure (41.3±4.1) was lower in comparison to control-1 (90.2±5.9, P= 0.001); and control-2 (96.7±6.4, P<0.001). Cell number in the ICM of blastocysts from the high-NEFA group (26.0±2.2) was also lower when compared to control groups (Control-1= 35.0±2.4; Control-2= 36.0±3.5, P<0.001). This resulted in a decreased total cell number (TCN) in high NEFA-derived blastocyst (67.3±5.6) compared to controls (Control-1= 125.2±6.6; Control-2= 132.3±8.4, P<0.001) (Figure 3.8).

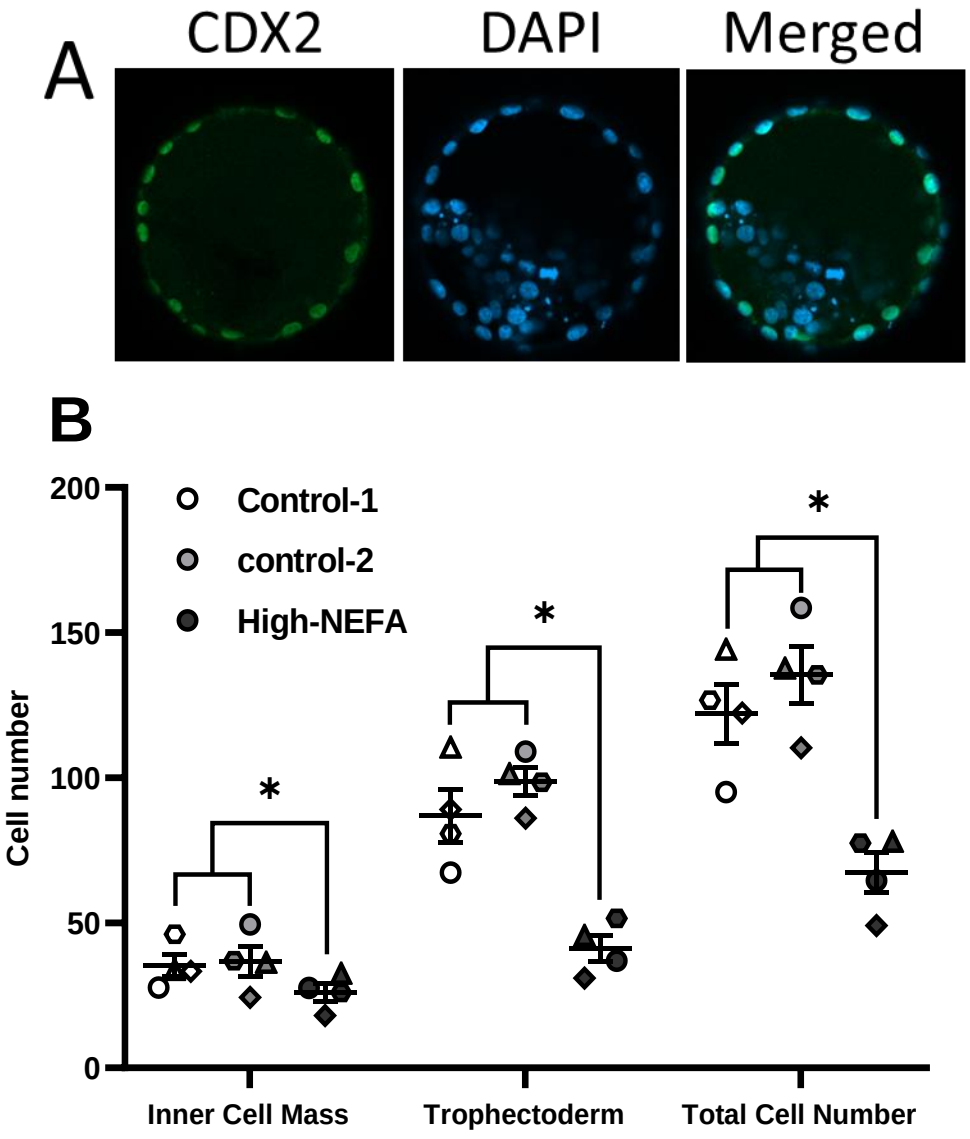


Figure 3-7. Effect of high NEFA exposure exclusively during IVF on cell number of resultant bovine blastocysts. **A**) Mid-plane confocal section showing CDX2 and DAPI staining in a bovine blastocyst. **B**) Cell number in trophectoderm and inner cell mass of blastocysts. The asterisk indicates a significant difference between groups (P<0.05). Bars= 50 µm. ◯= 1st,⬡= 2nd,△= 3rd and◇= 4th cycle.

The ratio of TE to ICM was also decreased in the high-NEFA group (1.6±0.2), when compared to control-1 (2.7±0.2 P<0.001) and control-2 (2.9±0.3, P<0.001). The proportion of cells allocated to the ICM (ICM/TCN) was increased in high NEFA-derived blastocyst (39.1±2.2%) in comparison with controls (Control-1= 28.2±1.7%, Control-2= 26.9±1.8%, P<0.001). Cell allocation variables did not differ between the control groups (Figure 3.9).

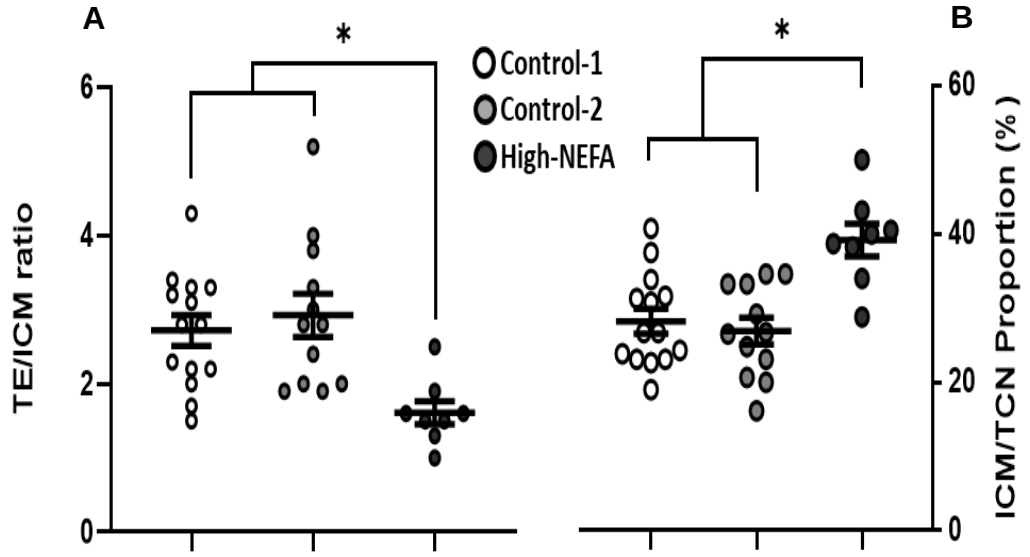


Figure 3-8. Effect of high NEFA exposure exclusively during IVF on cell allocation of resultant blastocysts. **A)** TE/ICM ratio. **B)** ICM/TCN proportion. The asterisk indicates a significant difference between groups (P<0.05).

3.4 Discussion

A previous study reported that although embryo cleavage was decreased when IVF took place under high NEFA concentrations, blastocyst production was not affected (Desmet et al., 2018). In contrast, a decrease in both embryo cleavage and blastocyst formation was found in the present experiment. These contrasting results could be related to the type of culture medium used during embryo development. Desmet et al. (2018) supplemented their embryo culture medium with insulin, transferrin, and selenium (ITS), and supplementation with ITS during embryo culture can increase blastocyst production in cattle, and it is used in combination with BSA as substitute for serum in embryo culture medium (Wydooghe et al., 2014). Hence, since ITS can facilitate blastocyst formation, the possibility exists that detrimental effects as result of NEFA exposure during IVF on blastocyst formation could have been undetected due to beneficial effect of ITS. Indeed, supplementation of ITS during embryo culture can rescue compromised oocyte competence, where bovine zygotes derived from oocytes *in vitro* matured under high NEFA concentrations (which is known to impair oocyte competence), but cultured in medium supplemented with ITS, can achieve blastocyst formation to levels observed in controls (Smits et al., 2020).

In this experiment blastocyst production was 23-25% for control groups and 9% for the High-NEFA group, whereas in the previous paper using the same NEFA model blastocyst production was 28-36% for control groups and 27% for high-NEFA (Desmet et al., 2018). As discussed above, this higher blastocyst production can be attributed to the supplementation of ITS during embryo development. As such the basic synthetic oviductal fluid (SOF) supplemented with BSA used in this thesis project is considered by some as medium with “no supportive conditions” (Smits et al., 2020). However, here it is important to highlight that the previous experiment analysing blastocyst formation from oocytes exposed to high NEFA levels found that supplementation of embryo culture medium with ITS promoted the formation of blastocyst from compromised oocytes that would have not achieved the blastocyst stage in the absence of ITS. Furthermore, embryo quality was impaired as indicated by the high level of apoptosis and altered pyruvate consumption detected in the resultant blastocysts. This indicates that ITS can promote blastocyst formation but not quality of such blastocysts derived from oocytes exposed to a high NEFA microenvironment during maturation (Smits et al., 2020). It remains to be determined whether this scenario will also be present in oocytes exposed to high NEFA concentrations during fertilisation in relation to the current project. Nevertheless, the blastocyst production in control groups in the present experiment was within the expected range, as normally 20-40% of presumptive zygotes cultured will reach the blastocyst stage in *in vitro* embryo production systems in cattle (Ferré et al., 2020). From this point of view the embryo production system used in this experiment was suitable to address NEFA effects on blastocysts formation and quality.

The question remains on the cause for the low blastocyst production observed in the present experiment. This could be related partially to the lower fertilisation rate found in the high-NEFA group reported in chapter 2 (see section 2.3.) and a previous study (Desmet et al., 2018). Although the levels of ROS production do not seem to be affected following NEFA exposure during IVF (see chapter 2, section 2.3), it is clear that the developmental competence of zygotes was affected. The capacity of the mammalian zygote to achieve the blastocyst stage is determined to a great extent by a correct embryonic genome activation (EGA), this is, when embryonic development stops relying on maternally ribonucleic acids (RNAs) and proteins initially stored in the oocyte, and the embryo becomes transcriptionally active (Paonessa et al., 2021). EGA

starts with low levels of transcription (i.e., minor EGA) follow by a significant increase in transcription (i.e., major EGA). In humans, major EGA occurs around the 4- and 8-cell stage (Braude et al., 1988, Vassena et al., 2011, Leng et al., 2019) but signs of embryonic transcription has been detected at the 1-cell stage (Asami et al., 2022). In cattle, the major EGA also takes place around the 4- and 8-cell stage (Kues et al., 2008, Graf et al., 2014, Jiang et al., 2014). Correct EGA relies on epigenetic changes taking place during preimplantation embryo development, where changes in structure and function of chromatin modify the transcriptional landscape in blastomeres, but without changes in the DNA sequence (O'Neill, 2015). Hence analysis of epigenetic markers can provide insights into the epigenetic mechanism involved in embryo demise. As such, in chapter 4, evidence is provided that alteration in the expression profile of a relevant epigenetic marker (H3K27me3) is associated with the poor blastocyst formation observed in this experiment (see section 4.4 in chapter 4 for discussion).

Another important element to be considered here is the damage via spermatozoa. It is known the exposure to high concentrations of NEFA during IVF can induce damage to the cell membrane which will decrease the chances of fertilisation (Desmet et al. 2018, chapter 2). However, in chapter 5 of this thesis the effects on spermatozoa DNA damage were examined, providing evidence that high NEFA concentrations can increase the percentage of spermatozoa with DNA damage. This is in turn could also affect the ability of resultant zygotes to achieve the blastocyst stage (see discussion in section 5.4 of chapter 5).

In the present experiment the total number of cells in blastocyst was decreased, which was related to low cell number in both the TE and ICM and resulted in a low TE/ICM ratio. This contrast with the high TE/ICM ratio associated with a high number of cells exclusively in the TE reported in a previous paper of high NEFA exposure at the time of IVF (Desmet et al., 2018). This difference could be attributed to the use of ITS in the latter study as mSOF medium supplemented with BSA and ITS can increase blastocyst cell number when compared to mSOF medium supplemented only with BSA (i.e., culture medium used in this thesis project) (Wydooghe et al., 2014). Here it is relevant to mention that the increase in TE cells was not accompanied with an increase in the total cell count (which should be expected), and ICM cell number was not affected in previous research addressing the effects of NEFA exposure during IVF

(Desmet et al. 2018). Albeit speculative, this could be related to the methodology used to count cells as in the study from Desmet et al. (2018) images were processed with a fluorescence microscope allowing only a two-dimensional visualisation of embryos whereas in the present study a 3D visualisation was possible with confocal microscopy which allows detailed exploration on blastocysts, especially in the cells of the ICM.

The low cell number in blastocysts from the high-NEFA group in the present study indicates impaired embryo quality as cell number in preimplantation embryos can influence developmental potential. For instance, in embryo transfer studies it has been found that *in vivo*-produced embryos achieved higher pregnancy rates than *in vitro*-produced embryos, reviewed in Ealy et al., (2019) (Ealy et al., 2019), and this enhanced capacity to achieve pregnancy has been associated with embryo cell number, where a higher cell number is present in *in vivo*-produced blastocysts (Knijn et al., 2003). Similarly, embryo transfer studies in mice show that blastocysts with a low number of blastomeres had a decreased potential to maintain pregnancy (Tam, 1988, Hishinuma et al., 1996). Another finding of the present experiment was an increased allocation of cells to the ICM compartment in relation to the total number of blastomeres (i.e., ICM/TCN proportion) in high-NEFA blastocysts. *In vivo* (Van Soom et al., 1997, Koo et al., 2002, Rho et al., 2007) and *in vitro* (Velazquez et al., 2011b) data have shown that an ICM/TCN of between 20% to 40% can be considered within the normal range. Bovine blastocysts with an ICM/TCN proportion of more than 40% have been associated with impaired blastocyst quality in models of excessive *in vitro* exposure to growth factors (i.e., insulin-like growth factor-1) (Velazquez et al., 2011b) and in embryos produced via somatic cell nuclear transfer (SCNT) (Koo et al., 2002, Amarnath et al., 2004, Li et al., 2004). The altered cell allocation in SCNT blastocysts have been linked to pregnancy loss in the first trimester following embryo transfer (Koo et al., 2002). This will be associated to a decreased number of TE cells that could result in failed implantation or a foetus with impaired placental function that would increase the risk of pregnancy loss (Velazquez et al., 2011b). In the present experiment embryos with an ICM/TCN proportion higher than 40% was only observed in the NEFA groups, with 7% (1/14) in control-1 (physiological NEFA levels) and 37% (3/8) in the high-NEFA group. Hence, it is reasonable to suggest that the lower quality of embryos derived from high exposure to NEFA levels during the fertilisation process will have the potential to jeopardise pregnancy, which could account for the lower

pregnancy rates usually found in cows in negative energy balance (Garverick et al., 2013). Nevertheless, an embryo transfer study will be needed to test this hypothesis.

In conclusion, under the conditions of the present *in vitro* experiment, exposure to high NEFA levels exclusively during the fertilisation process can be detrimental for blastocyst formation, and an altered cell allocation is present in resultant blastocysts that could potentially disrupt pregnancy.

Chapter 4: Effects of Elevated Non-Esterified Fatty Acids Concentrations during *In Vitro* Fertilisation on Histone H3 Lysine 27 Trimethylation during Early Development of Bovine Embryos

4.1 Introduction

Gametogenesis is a biological process in which germ cells go through a series of mitotic and meiotic divisions to finally differentiate into spermatozoa and oocytes (Larose et al., 2019). Then, during the fertilisation process, the union of gametes leads to the formation of the zygote, an individual totipotent cell with the capacity to generate all the cells of the body, and importantly, it also has the ability to organize them in a specific temporal and spatial sequence that ultimately leads to the development of a fertile adult individual (Condic, 2014). This totipotency is lost in the first cleavage divisions of the preimplantation embryo, around the time the embryo reaches the 4-cell stage (Mitalipov and Wolf, 2009). The acquisition of totipotency requires extensive epigenetic remodelling of the parental chromatin in the single-cell zygote (Clift and Schuh, 2013, Ross and Sampaio, 2018, Larose et al., 2019). In simple terms, epigenetic remodelling makes reference to molecular modifications that affect the transcription and readout of DNA without altering the DNA sequence (Canovas and Ross, 2016). The epigenetic remodelling continues during the first divisions of the zygote, and this is critical for a correct embryonic genome activation (EGA), which in turn determines to a great extent the progression to the blastocyst stage (Ross and Canovas, 2016, Ross and Sampaio, 2018). EGA occurs when embryonic development stops relying on maternally RNAs and proteins initially stored in the oocyte, and the embryo becomes transcriptionally active (Paonessa et al., 2021). In general, EGA involves a minor and a major wave of transcription. In humans, major EGA occurs around the 4- and 8-cell stage (Braude et al., 1988, Vassena et al., 2011, Leng et al., 2019) but signs of embryonic transcription have been detected at the 1-cell stage (Asami et al., 2022). In cattle, the major EGA also takes place around the 4- and 8-cell stage (Kues et al., 2008, Graf et al., 2014, Jiang et al., 2014).

There are several epigenetic mechanisms, including DNA methylation, post-translational modifications of histones, non-coding RNAs and chromatin conformation changes (Ross and Sampaio, 2018, Zhu et al., 2021). Of these, DNA methylation and

post-translational histone modifications have been studied the most, where DNA methylation is generally associated with silencing of gene transcription, and histone modifications can be associated with either transcriptional activation or repression (Bogliotti and Ross, 2012). Among the histone modifications taking place during the preimplantation period, demethylation of trimethylated histone 3 at lysine 27 (H3K27me3) is considered an important epigenetic change during early embryo development in mammals (Gao et al., 2010, Bogliotti and Ross, 2012, Liu et al., 2016, Zheng et al., 2016, Xia et al., 2019, Chen et al., 2021, Zhou et al., 2022). During formation of the zygote at the time of fertilisation H3K27me3 is a histone mark present in the maternal genome and basically absent in the paternal chromatin (Canovas and Ross, 2016) (Figure 4.1).

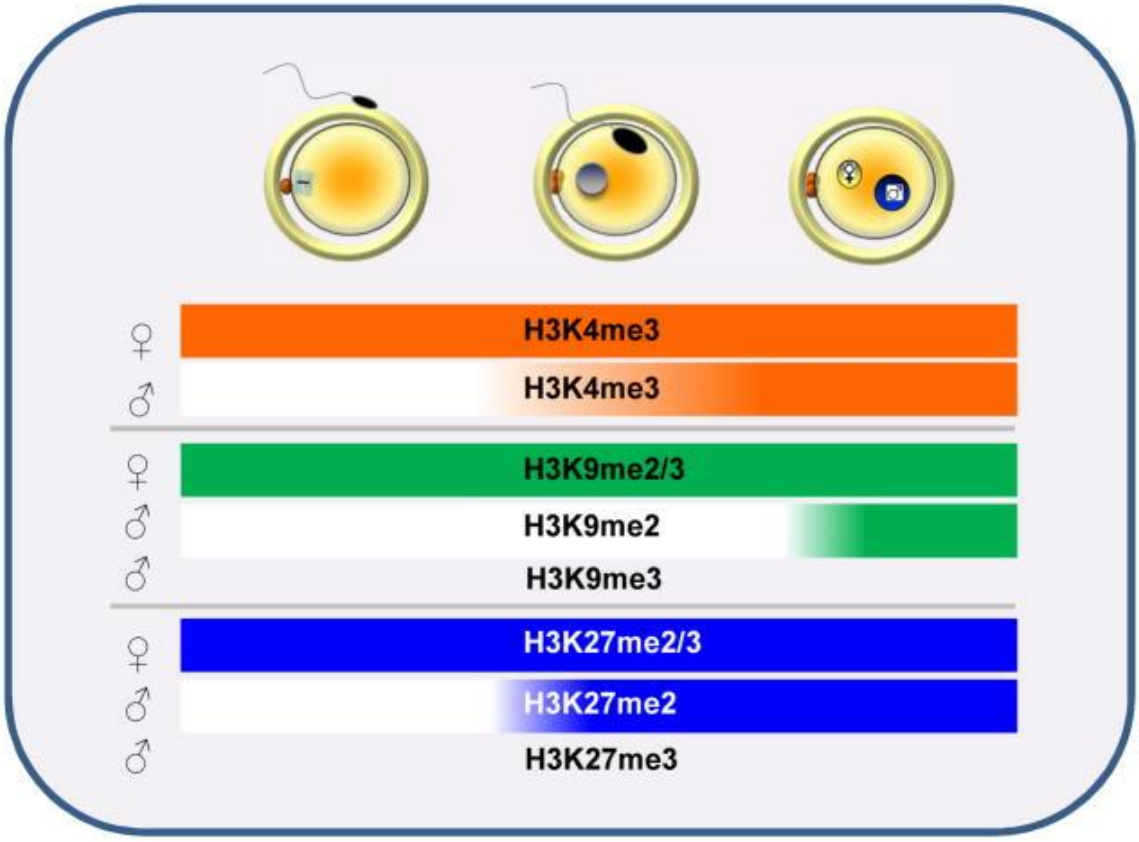


Figure 4-1. Dynamics of H3K27me3 and other histone marks during formation of the mammalian zygote (Canovas and Ross, 2016).

H3K27me3 is a repressive epigenetic mark, with its methylation being regulated by polycomb group (PcG) proteins that form two main functional complexes; the polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC1 and PRC2 complexes

have core components that interact with other accessory proteins. The core components in PRC1 include the E3 ubiquitin ligase Ring1B and one polycomb group of ring finger (PCGF). PRC2 has three core components: enhancer of zeste homolog 2 (EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 (SUZ12). PRC2 main function is to generate the di-/trimethylated form of lysine 27 on histone H3 (H3K27me2/3) (Aranda et al., 2015) (Figure 4.2).

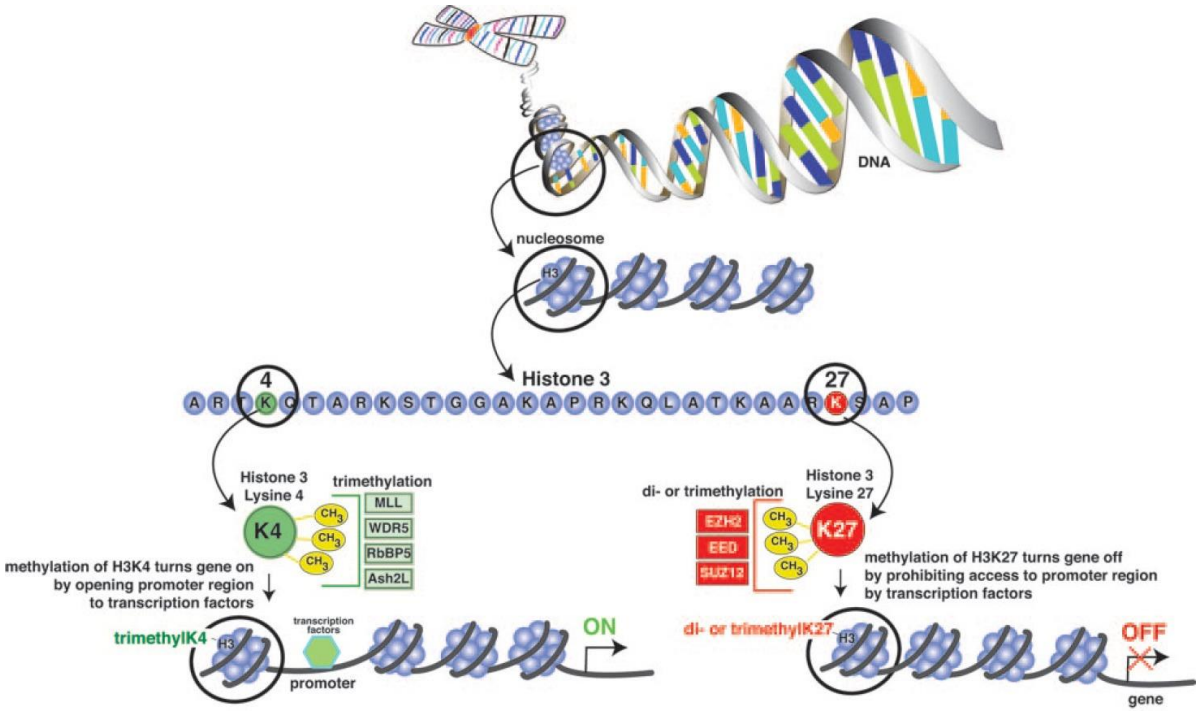


Figure 4-2. Graphical depiction of the role of PRC2 core components in the generation of the di-/trimethylated form of lysine 27 on histone H3 (H3K27me2/3). Methylation of H3K27, keeps the chromatin in a conformation that stops transcription factors from reaching the promoter, resulting in gene silencing. An example of activation of gene expression is also provided on the bottom left hand side of the figure, where trimethylation of lysine 4 on histone H3 (H3K4me3) changes the conformation of the chromatin allowing access of transcription factors to the promoter, resulting in activation of transcription (Wen et al., 2008).

Levels of H3K27me3 in bovine embryos decrease during the first embryo divisions reaching a very low level at the 8-cell stage followed by a re-establishment of expression by the blastocyst stage (Ross et al., 2008, Breton et al., 2010, Zhou et al., 2019). Similarly, in human embryos H3K27me3 expression becomes nearly absent in 8-cell embryos, regaining expression during the morula-blastocyst transition (Zhang

et al., 2012, Saha et al., 2013, Xia et al., 2019, Lu et al., 2021). This is in contrast with mice, where the H3K27me3 mark is maintained until the 8-cell stage, followed by a decline at the morula stage, and re-establishment of expression at the blastocyst stage (Zhang et al., 2009, Saha et al., 2013). Gene knockdown studies using small interfering RNA (siRNA) have shown that demethylation of H3K27me3 is under the control of lysine demethylase 6B (KDM6B, also known as Jumonji Domain-Containing Protein 3 [JMJD3]) in bovine preimplantation embryos (Canovas et al., 2012, Chung et al., 2017). Gene knockdown technology using siRNA relies on the natural molecular mechanism of RNA interference, which evolved as defence mechanism to protect the genome against invasion of genetic elements such as viruses (Hu et al., 2020). As such, siRNA induces gene-specific RNA degradation resulting in downregulation of gene expression (Hu et al., 2020). Accordingly, MII oocytes injected with KDM6B siRNA resulted in the production of 8-cell embryos with very low expression of KDM6B but higher levels of H3K27me3 when compared to control embryos, demonstrating that expression of KDM6B is necessary for the erasure of H3K27me3 by the time the bovine embryo reaches the 8-cell stage (Canovas et al., 2012, Chung et al., 2017). This sustained expression of H3K27me3 in 8-cell embryos also resulted in a decreased ability to reach the blastocyst stage (Canovas et al., 2012, Chung et al., 2017).

The above-discussed information indicates that a decline in H3K27me3 levels during the time of EGA seems to be required for activation of developmentally important genes needed to proceed to the blastocyst stage (Bogliotti and Ross, 2012, Chung et al., 2017). Hence, analysis of epigenetic markers during early embryo divisions can provide insights into the epigenetic mechanism involved in embryo demise. Given the decreased blastocyst production indicated in chapter 3 after exposure to high NEFA during IVF, the objective of this chapter was to examine levels of H3K27me3 in early embryos derived from zygotes formed under a high NEFA microenvironment (i.e., during IVF). It was hypothesised that high NEFA levels during IVF delay the decrease in H3K27me3 levels from 2-cell to 4-cell embryos at 48 hpf.

4.2 Material and Methods

4.2.1 Collection of cumulus oocyte complexes (COCs)

For handling of glassware and plasticware please see section 2.2.1. A detailed description of collection of COCs is available in see section 2.2.2. Briefly, ovaries were collected from a local abattoir, once in the lab they were slashed in oocyte recovery medium to obtain immature oocytes. Then good quality (grade I) unexpanded COCs with homogenous ooplasm and at least five layers of compact cumulus cells were selected for *in vitro* maturation (see figure 2.2. in chapter 2 for classification of COCs).

4.2.2 *In vitro* maturation (IVM)

A detailed description of the IVM procedure is available in chapter 2 (please see section 2.2.3). Briefly, COCs were matured in groups of 20–30 in a 4-well plate containing serum-free maturation medium (500 µl/well without oil cover) for 22-24 hours in a humidified atmosphere with 5% CO_2, atmospheric O_2, and at 38.5°C.

4.2.3 *In vitro* fertilisation (IVF)

Commercial semen straws were used for IVF. For detailed processing of semen straws and description of IVF please see section 2.2.4. Briefly, matured COCs were co-incubated in groups of 20–30 with spermatozoa at a final concentration of $1x10^6$ sperm cells/ml in a 4-well plate (500 µl/well, without oil cover) containing fertilisation medium. IVF took place for 19 hours in a humidified atmosphere with 5% CO_2, atmospheric O_2, and at 38.5°C.

4.2.4 *In vitro* embryo culture (IVEC)

For handling of presumptive zygotes after IVF and composition of medium see section 2.2.5 in chapter 2. After IVF presumptive zygotes (Control-1 n=139, Control-2 n=154, High-NEFA n=183, in five replicates) were cultured for two days (day of IVF= day 0) in groups of 20-30 in a 4-well plate containing mSOF medium (500 µl/well without oil cover) under a humidified atmosphere containing 5% CO_2, 5% O_2, and at 38.5°C. On day two (i.e., 48 hours post-fertilisation), 2- and 4-cell embryos were collected and process for H3K27me3 fluorescence staining. A cycle of preimplantation embryo production including IVM, IVF and IVEC was considered a replicate. (Figure 4.3).

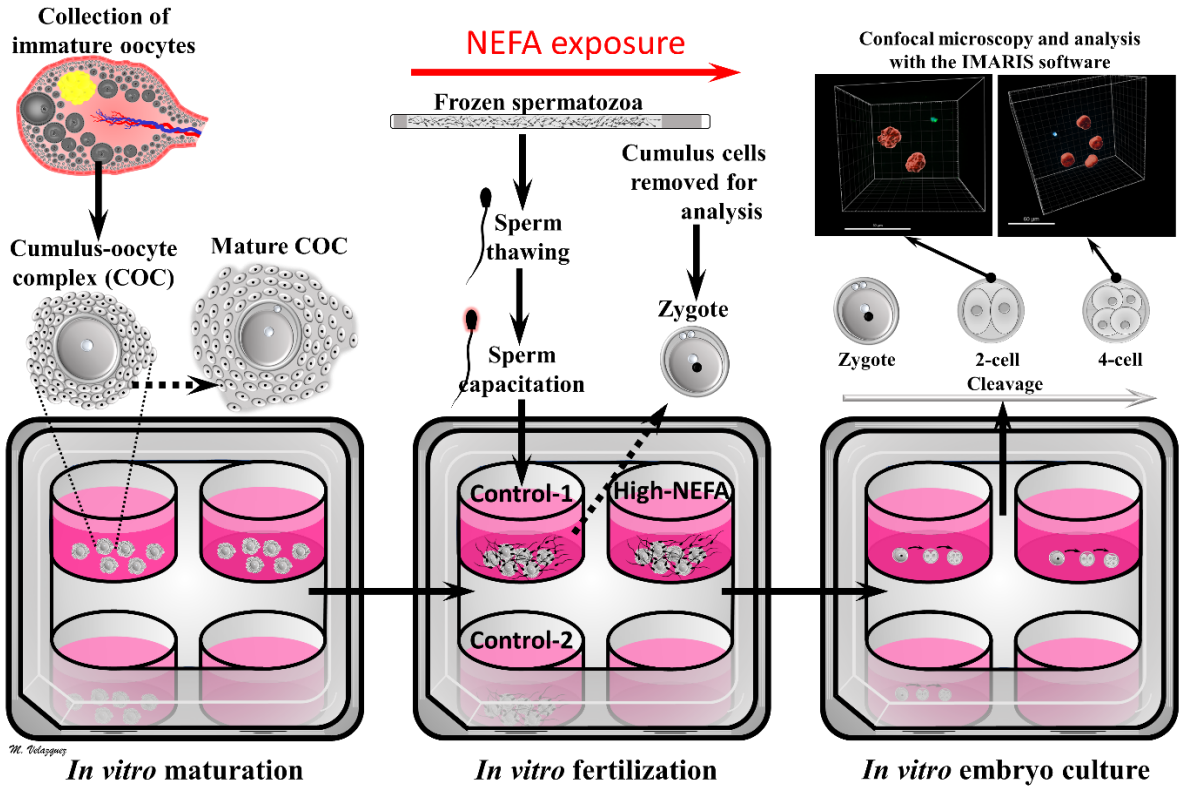


Figure 4-3. Graphical depiction of the experimental design used to test the effects of NEFA during IVF on H3K27me3 levels in 2- and 4-cell embryos. Artwork credit: Dr Miguel Velazquez.

4.2.5 Experimental design

For preparation of NEFA treatments please see section 2.2.6 in chapter 2. NEFA exposure during IVF was based on the model developed by Desmet et al. (2018) (see detailed explanation in section 2.2.7, in chapter 2). Three groups were tested, where a high-NEFA group containing 280 µM SA, 230 µM PA and 210 µM OA representing pathophysiological NEFA levels was compared to two control groups. The first control (Control-1) contained 28 µM SA, 23 µM PA and 21 µM OA representing normal physiological NEFA levels. A second control group (Control-2) contained 0.2% absolute ethanol equivalent to the same concentrations on the NEFA groups.

4.2.6 Immunofluorescence assay for H3K27me3

The primary and secondary antibodies were tested in dilutions of 1:50 and 1:100, and 1:100 and 1:200, respectively. A dilution of 1:50 for the primary and 1:100 for the secondary antibody worked well and it was used for the H3K27me3 staining protocol.

After IVEC, embryos (2- and 4-cell) were washed (i.e., moving embryos from one well to another three times in a 4-well dish, five minutes on each well) in 0.1% PVP/PBS and subsequently fixed with 4.0% formaldehyde/PBS for 15 minutes at room temperature. Embryos were then washed in 0.1% tween/PBS followed by permeabilisation in 1.0% triton/PBS for 30 minutes at room temperature. Permeabilisation solution was removed by washing embryos in tween/PBS and embryos were then blocked in 10% goat serum (Sigma; G9023) for two hours at room temperature, followed by washing in tween/PBS. Then, embryos were incubated with anti-H3K27me3 primary antibody (Abcam; ab6002) (Canovas et al., 2012) at a 1:50 dilution in tween/PBS, in the dark, and the 4-well plate containing the embryos was placed on a microplate shaker overnight at 4.0°C. Next morning embryos were washed (5 times, 10 minutes each at room temperature) with 0.1% tween/PBS and then incubated with goat anti-mouse secondary antibody (Abcam; ab205719) at a dilution of 1:100 in tween/PBS, in the dark, and the 4-well plate containing the embryos was placed on a microplate shaker for 30 minutes at room temperature. This was followed by five washes (10 minutes each) with 0.1% tween/PBS. Next, embryos were incubated for 30 minutes at room temperature with DAPI (10 ng/ml) followed by five washes (10 minutes each) with 0.1% tween/PBS. Embryos were then mounted on a glass slide with double reinforcement rings in a small drop of citifluor anti-fading medium (~5.0 µl), coverslipped, sealed with nail varnish, and stored at 4.0°C in the dark until analysis (i.e., embryos were analysed same day of mounting them on microscope slides). Negative controls were treated as described above except that exposure to the anti-H3K27me3 primary antibody was omitted (Figure 4.4). 1-cell structures (i.e., non-cleaved) were stained with DAPI and images were analysed with an epifluorescence microscope to determine fertilisation status (see methodology details in section 2.2.8 of chapter 2).

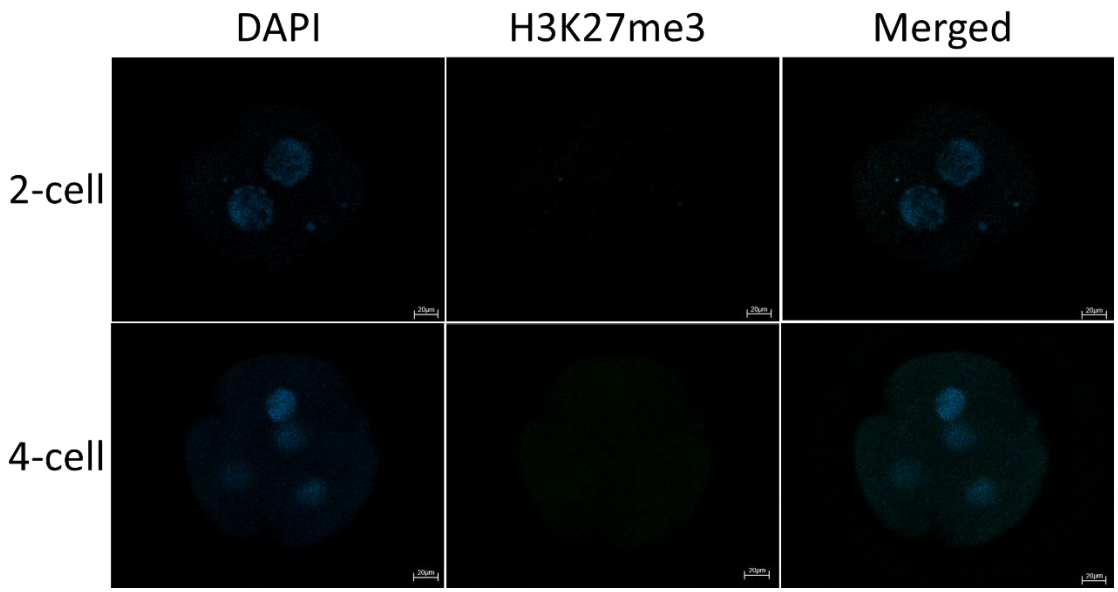


Figure 4-4. Negative controls for H3K27me3. Primary antibody for HE3K27 was omitted during the immunofluorescence protocol. Bars = 20.

Embryos were examined in a dark room with a confocal laser scanning microscope equipped with a Zeiss LSM 800 Airyscan microscope (Carl Zeiss Optics Co, Ltd, Oberkochen, Germany) running the Zen 3.1 software and a Plan-Apochromat 20/0.8 objective lens. A solid-state laser was used to detect DAPI (405 nm excitation) and Alexa AF488 (488 nm excitation) using GaAsP detectors and a 400-650 nm bandpass, switching sequentially between channels. Optical sections were taken at 7.59 µm intervals across the whole embryo using a 100 µm pinhole (equivalent to a 15.20 µm optical section). The fluorescence intensity for H3K27me3 was measured in the IMARIS software. In the 3D mode, the diameter of an individual nuclei in the blue channel (DAPI) was indicated in order to delineate nuclei on each embryo (Figure 4.5). Then, in the surface tool mode, fluorescence intensity in arbitrary units was provided by the software for each delineated nucleus. The fluorescence in the green channel was used to determine levels of H3K27me3 (Figure 4.6). The mean fluorescence value from the total number of blastomeres on each embryo was used for statistical analysis.

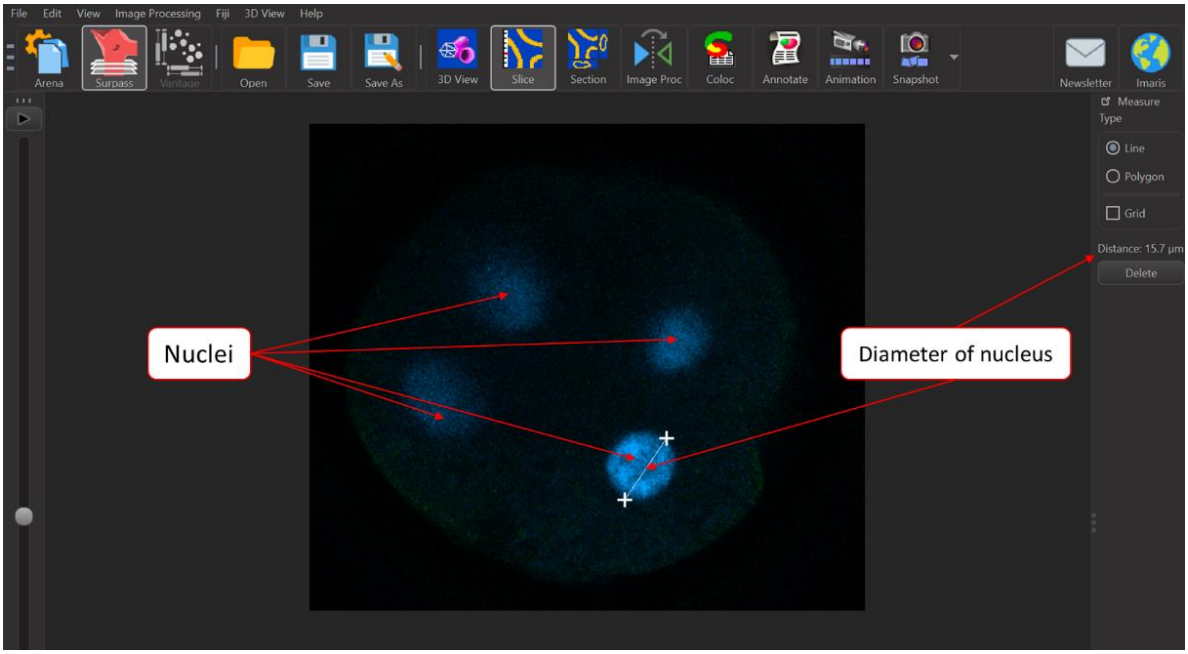


Figure 4-5. IMARIS software showing the measurement of the diameter of a nucleus in a 4-cell embryo in the blue channel (DAPI). The same procedure was done for 2-cell embryos. Bars = 20 µm.

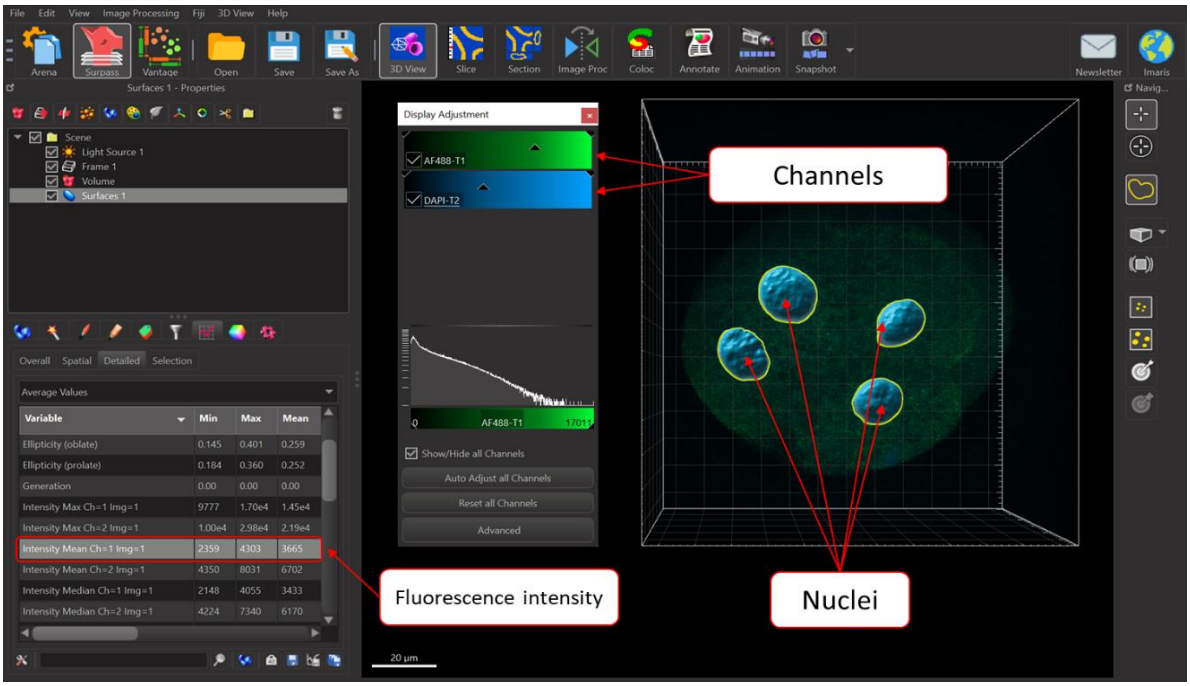


Figure 4-6. IMARIS software showing delineated nuclei in a 4-cell embryo. Fluorescence intensity in the green channel was used to quantify H3K27me3 levels. The same procedure was done for 2-cell embryos. Bars = 20 µm.

4.2.7 Statistical analysis

Statistical analysis was performed using SPSS 26 software (IBM). The Shapiro-Wilk test was used to test if the variables analysed had a normal distribution. Data that were not normally distributed was Log10 transformed before analysis. Differences between groups were identified using either analysis of variance (ANOVA) or T-test, with percentage data arcsine transformed before analysis. Post-hoc comparisons after ANOVE were done with the least significant difference (LSD) method. A P-Value less than 0.05 was considered statistically significant. Data was reported as mean ± standard error of the mean (SEM) unless otherwise indicated.

4.3 Results

4.3.1 Fertilisation and cleavage rate

Fertilisation rate (calculated on cleaved embryos and non-cleaved monospermic zygotes) was significantly decreased in the presence on high NEFA levels (47.54±2.15%) in comparison with control groups (Contro-1= 78.14±0.93%, Control-2= 80.06±1.71%, P<0.001). Similarly, the capacity of zygotes to achieve cleavage was lower in the high-NEFA group (29.8±2.6%) than in the control groups (Control-1= 57.1±7.4%; Control-2= 59.1±7.1%, P= 0.009) (Figure 4.7).

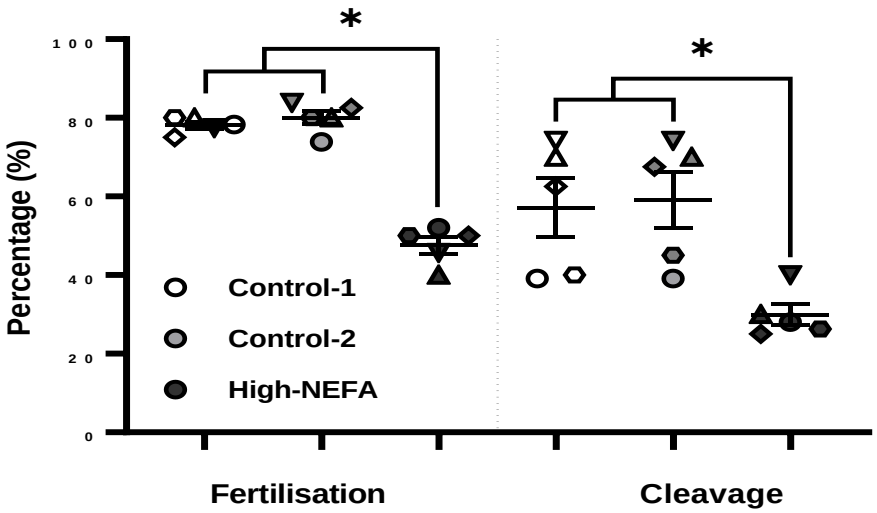


Figure 4-7. Fertilisation and cleave rate following NEFA exposure during IVF. The asterisk indicates a significant difference between groups (P<0.05). ◯ = 1st, ⬡ = 2nd △ = 3rd, ◇ = 4th and ▽ = 5th cycle.

4.3.2 H3K27me3 levels in 2- and 4-cell embryos

Analysis of H3K27me3 levels (Log10 transformed data) between experimental groups indicated that H3K27me3 expression (i.e., arbitrary units) was not affected in 2-cell (Control-1, mean= 5242.6±363.5, n =31; Control-2, mean= 5833.3±432.5, n=29; High-NEFA, mean=6073.1±432.5, n= 29 or 4-cell (Control-1, mean= 5840.1±450.0. n= 23; Control-2, mean= 6011.4±512.2, n= 32; High-NEFA, mean= 5475.2±504.1, n= 13) embryos exposed to high NEFA concentrations (Figure 4.8).

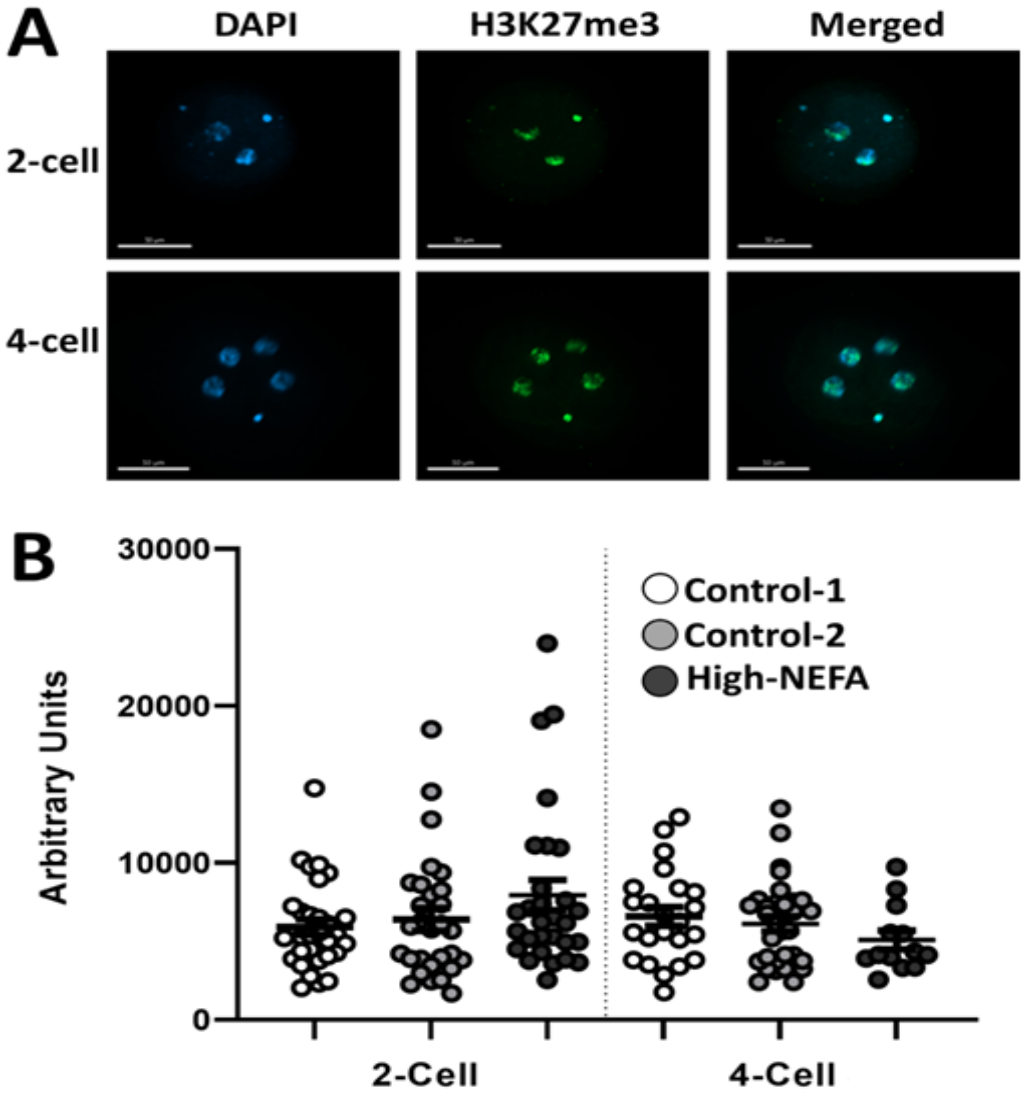


Figure 4-8. Levels of H3K27me3 in early embryos following NEFA exposure during IVF. **A**) Representative mid-plane confocal sections showing H3K27me3 and DAPI staining in 2- and 4-cell embryos. Bars = 20 µm. **B**) Analysis H3K27me3 levels between groups.

However, comparison of H3K27me3 levels between 2- and 4-cell embryos within each experimental group revealed that 2-cell embryos displayed a higher H3K27me3 expression than 4-cell embryos in the high NEFA group (P=0.038). This difference in H3K27m3 levels between developmental stages was not observed in control groups (Figure 4.9).

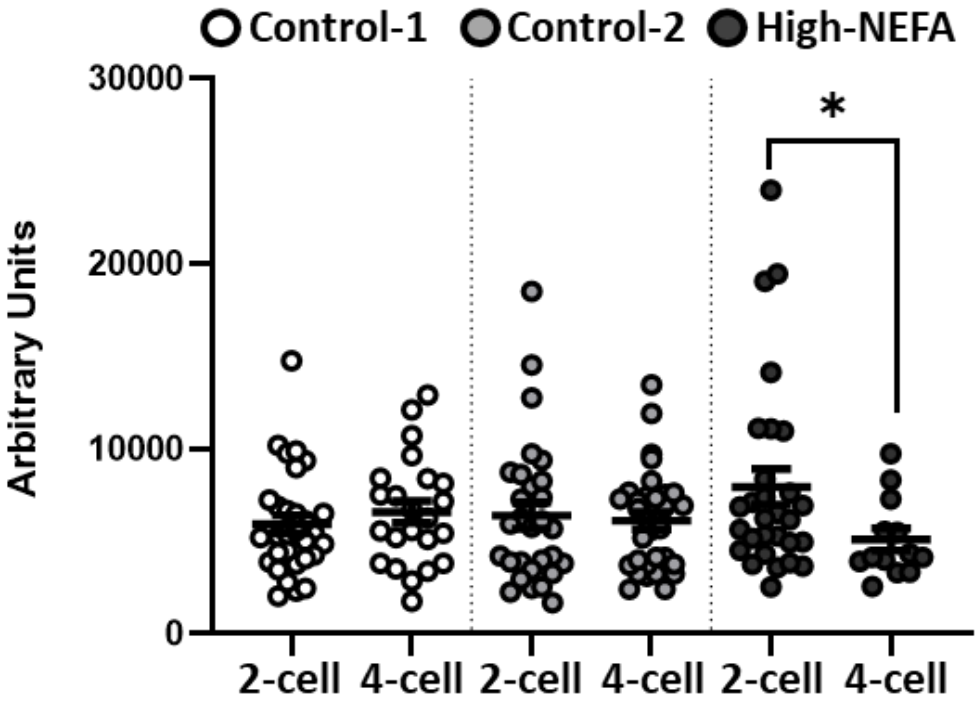


Figure 4-9. Levels of H3K27me3 in 2- and 4-cell embryos within each experimental group following NEFA exposure during IVF. The asterisk indicates a significant difference between groups (P<0.05).

4.4 Discussion

It has been shown that exposure to high NEFA concentrations during the time of *oocyte maturation and preimplantation embryo development in cattle*, can alter the epigenetic landscape of the resultant blastocyst (Desmet et al., 2016). However, changes in epigenetic marks after exposure to high NEFA concentrations exclusively during the fertilisation process have not been examined. In this experiment it was found that levels of H3K27me3, a relevant epigenetic mark during preimplantation development, was not different between the groups analysed. However, within groups,

the expected profile expression of H3K27me3 in 2- and 4-cell embryos 48 hours post-fertilisation (hpf) was not observed following exposure to high NEFA concentrations.

Previous *in vitro* research showed that H3K27me3 levels start decreasing at the 1-cell stage, reaching a nadir around the 8-cell stage in cattle (Ross et al., 2008, Breton et al., 2010, Zhou et al., 2019). It was also shown that this decrease in H3K27me3 is not the result of dilution of methyl marks during cleavage, but rather a cell division-independent process carried out by the catalytic activity of KDM6B, a histone demethylase specific for H3K27me3 (Ross et al., 2008, Chung et al., 2017). As such, 2-cell bovine embryos collected 33 hpf displayed higher H3K27me3 levels than 4-cell embryos collected 44 hpf (Canovas et al., 2012). Similarly, parthenogenetic bovine embryos at the 2-cell stage examined 32 hours post-activation (HPA) also have a higher H3K27me3 levels than 4-cell embryos examined 46 hpf. However, when cell cycle was reversibly blocked with aphidicolin (a reversible DNA replication inhibitor that blocks the progression of cleavage division) at 32 HPA in 2-cell parthenogenetic embryos, resultant 2-cell embryos examined 46 HPA did not differ in H3K27me3 levels when compared to untreated 4-cell embryos at the same time point (Canovas et al., 2012). This indicates that 2-cell embryos with late cleavage should have similar levels to 4-cell embryos examined at 46 hpf in this study. Indeed, it was recently reported that 2- and 4-cell bovine embryos examined at 40 hpf did not display differences in H3K27me3 levels (Ispada and Milazzotto, 2022). Time-lapse studies in cattle have shown that the second embryo cleavage can appear around 34-36 hpf (Somfai et al., 2010), but it can take up to 80 hpf (Beck, 2014).

In the control groups, as expected, there was no difference in H3K27me3 levels between 2- and 4-cell embryos at 48 hpf. However, in the high NEFA group, the 2-cell embryos had higher H3K27me3 levels than the 4-cell embryos at 48 hpf. This suggests that the erasure of H3K27me3 during the 2- to 4-cell transition is slower after exposure to high NEFA concentrations, and given that the decrease of H3K27me3 taking place around the time of EGA is necessary for reactivation of pluripotency-related genes silenced during gametogenesis (Bogliotti and Ross, 2012), the possibility exist that activation of key developmental genes could be delayed in embryos experiencing a high NEFA microenvironment during fertilisation, which partially explain the decreased blastocyst formation observed in chapter 3. Indeed,

undisturbed and timely gene expression is essential to ensure successful development of the mammalian preimplantation process carried out embryo (Paonessa et al., 2021).

Altered methylation levels of H3K27me3 has been associated with the lower embryo quality usually seen in bovine clone embryos when compared to IVF embryos, where levels of H3K27me3 were higher from the 2- to the 8-cell stage in embryos produced by somatic cell nuclear transfer (SCNT) (Zhou et al., 2019). A similar scenario has been reported in mouse (Bai et al., 2018, Wang et al., 2020a) and pig (Xie et al., 2016, Liu et al., 2021) SCNT embryos. An increase in H3K27me3 levels have been also observed at the blastocyst stage in bovine embryos subjected to cryopreservation, suggesting that repression of gene transcription could be associated with the lower pregnancy rate observed in cryopreserved embryos in comparison to non-cryopreserved counterparts (Maldonado et al., 2015). In rabbits, multiple superovulation can cause a decrease in preimplantation embryo production associated to increased H3K27m3 in 4-cell embryos and blastocysts (Tang et al., 2019). Furthermore, dysregulated expression of H3K27m3 at the preimplantation stage can have long-term consequences as shown by the post-implantation defects and abnormal placentas associated to a loss of H3K27me3 imprinting in mouse SCNT blastocysts (Matoba et al., 2018). This evidence and the results of the present experiment support the notion that analysis of H3K27me3 during the preimplantation period can provide insights into the epigenetic mechanism involved in embryo demise.

In vitro (Malmgren et al., 2013, Kumar et al., 2016) and *in vivo* (Zhang et al., 2018) models of lipotoxicity in mice associated to obesity have shown that NEFA can alter H3K27me3 enrichment in somatic cells resulting in impaired expression of genes associated with lipid metabolism, cell senescence, insulin signalling, gluconeogenesis and apoptosis. Moreover, mice foetuses derived from dams fed a high-fat diet showed impaired differentiation of embryonic osteogenic calvarial cells into mature osteoblasts. This impairment was linked with higher enrichment levels of H3K27me3 in several genes, especially on the SATB2 gene (SATB2: Special AT-rich sequence-binding protein 2), a critical transcription factor required for osteoblast differentiation (Chen et al., 2020). Experiments in mice have also shown that fasting, which increases NEFA levels (Il'Yasova et al., 2010), can also altered the expression of H3K27me3 in somatic cells, affecting the regulation of genes involved in mitochondrial β-oxidation (Seok et al., 2018). This evidence illustrates the influence that both undernutrition and

overnutrition can exert on H3K27me expression and that this can be mediated via NEFA.

However, there is lack of studies addressing this interrelationship in preimplantation embryos in models of lipotoxicity. To the best of the author's knowledge, the present experiment is the only one available showing that high NEFA concentrations can affect the expected decrease in H3K27me3 levels in bovine preimplantation embryos. Nevertheless, the lack of difference in H3K27m3 levels between the treatment groups suggests that the altered demethylation of H3K27 associated to high NEFA exposure during IVF is subtle, at least in the present *in vitro* NEFA model. Future experiments should test other histones marks known to play an important role during preimplantation embryo development (Wu et al., 2011, Carvalheira et al., 2019) to better characterise epigenetic modifications during NEFA exposure. Also, it has been reported that male bovine blastocysts express a higher global amount of H3K27m3 in cells of the trophectoderm than female embryos (Carvalheira et al., 2019). This sex difference will be relevant to explore in future *in vitro* NEFA toxicity models.

In conclusion, under the conditions of the present experiment, the expected decrease in H3K27me3 between the first and second cleavage is delayed when the fertilisation process takes place under high NEFA levels. This in turn could delayed the activation of developmentally important genes needed to progress to the blastocyst stage, explaining partially the low blastocyst production observed in this *in vitro* model of high NEFA exposure.

Chapter 5: Effects of High Non-Esterified Fatty Acids on Spermatozoa Quality in Cattle

5.1 Introduction

The mature mammalian spermatozoon is a highly specialised cell that is produced in the testes after germ stem cells go through numerous mitotic and meiotic divisions to generate the haploid spermatozoa. The production of functional spermatozoa involves the process of spermatogenesis within the seminiferous tubules located in the testes, which are comprised of myoid cells, Sertoli cells and different germ cell stages (Staub and Johnson, 2018). Spermatogenesis starts with spermatogonial stem cells (i.e., spermatogonia) located in the basal lamina of seminiferous tubules undergoing a series of mitotic divisions (i.e., spermatocytogenesis) to produce primary spermatocytes. Primary spermatocytes move towards the lumen of the seminiferous tubules to undergo the first meiotic division to form haploid secondary spermatocytes. These secondary spermatocytes experience a second meiotic division and become round spermatids. In the final stages of spermatogenesis, round spermatids go through the process of spermiogenesis where they differentiate into elongated spermatids and finally into mature spermatids. During spermiogenesis, the condensation of the spermatid DNA takes place along with the formation of the spermatozoa head (including acrosome biogenesis), midpiece (containing mitochondria) and a tail for forwarding propulsion (Allais-Bonnet and Pailhoux, 2014, Staub and Johnson, 2018, Foot and Kumar, 2021) (Figure 5.1).

Mature spermatids are then released into the lumen of the seminiferous tubules via a process called spermiation. Once spermatids are released from Sertoli cells into the seminiferous tubule lumen they are considered spermatozoa, and the remainder of the spermatid cytoplasm (i.e., residual body) are phagocytosed by Sertoli cells (O'Donnell et al., 2011). Spermatozoa then acquire functional maturity during their migration through the different regions of the epididymis, allowing them to develop progressive motility and the capacity to achieve fertilisation. The three major sections in the epididymis that provide essential proteins for spermatozoa maturation are the caput (head), corpus (body) and cauda (tail). The maturational events include modifications to the proteins on the sperm surface, and most of them take place in the

caput and corpus, whereas the main function of the cauda is to store functional spermatozoa (Cornwall, 2009, Dacheux et al., 2012) (Figure 5.1).

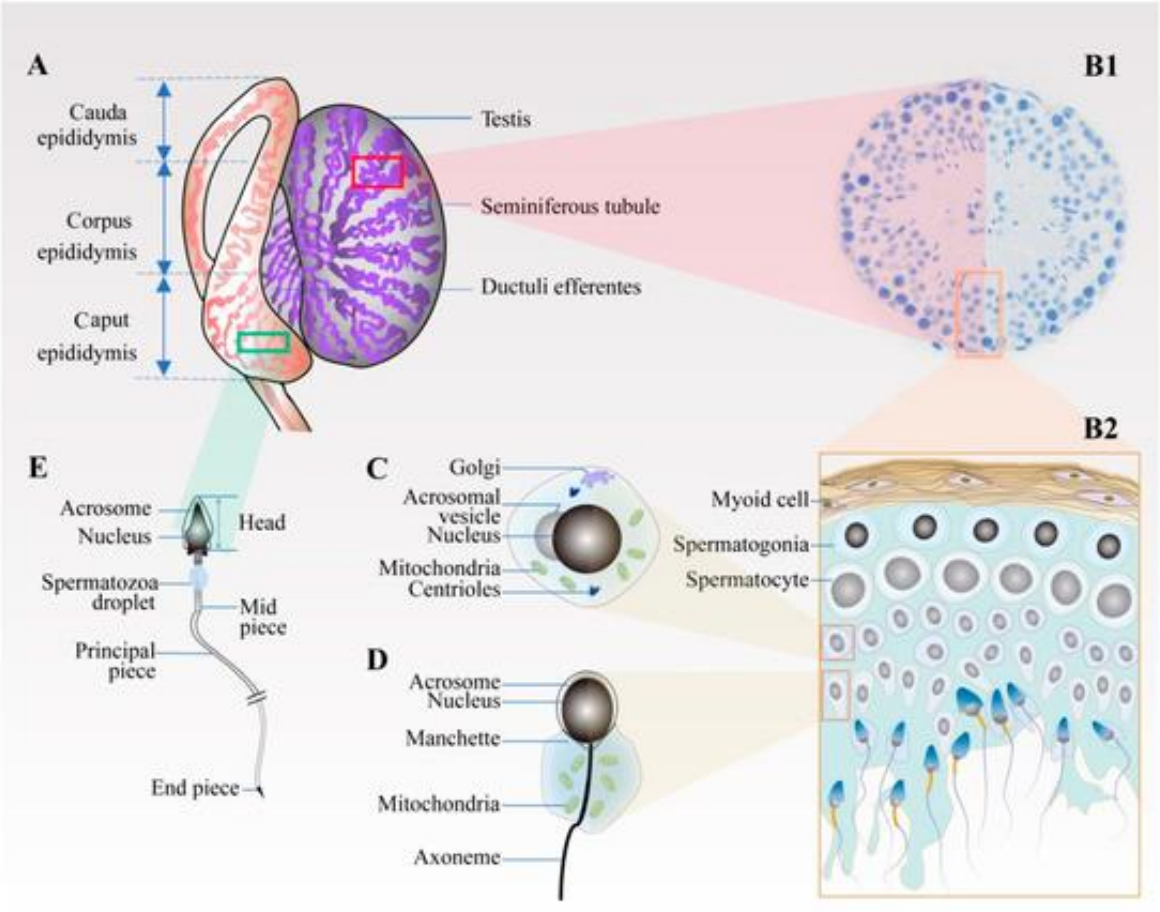


Figure 5-1. Formation and maturation of spermatozoa in mammals. **A**) Spermatogenesis takes place in the seminiferous tubules located in the testis. **B1–B2**) In the epithelium of the seminiferous tubules primordial germs cells go through a series of developmental stages until they finally become spermatozoa. **C–E**) Major components of round spermatids **(C)**, elongated spermatids **(D)** and matured spermatozoa in the epididymis **(E)** (Li et al., 2021).

Once spermatozoa are deposited in female reproductive tract via natural mating (vaginal deposition in both humans and cattle) or artificial insemination (usually intrauterine insemination in both humans and cattle) they need to travel to the site of fertilisation in the oviduct (i.e., fallopian tube). Research done in cattle and humans have shown that spermatozoa can reach the oviducts within the first hour after artificial insemination (Suarez and Pacey, 2005) and once spermatozoa pass through the uterotubal junction a so-called sperm reservoir is formed in the initial segment of the

oviduct (i.e., in the isthmus) (Pérez-Cerezales et al., 2017, Kölle, 2022). During spermatozoa migration through the reproductive tract a selection process takes place including inside the oviduct, where spermatozoa with high DNA integrity that manage to cross the first selection barriers (e.g., vaginal pH, cervical mucus) is selected and capacitated. Capacitation enable spermatozoa acquire hyperactive motility that allows them to detach from the oviduct and move towards the site of fertilisation using thermotaxis (swimming up a temperature gradient) and chemotaxis (swimming up a concentration gradient of chemoattractant) (Pérez-Cerezales et al., 2017) (Figure 5.2).

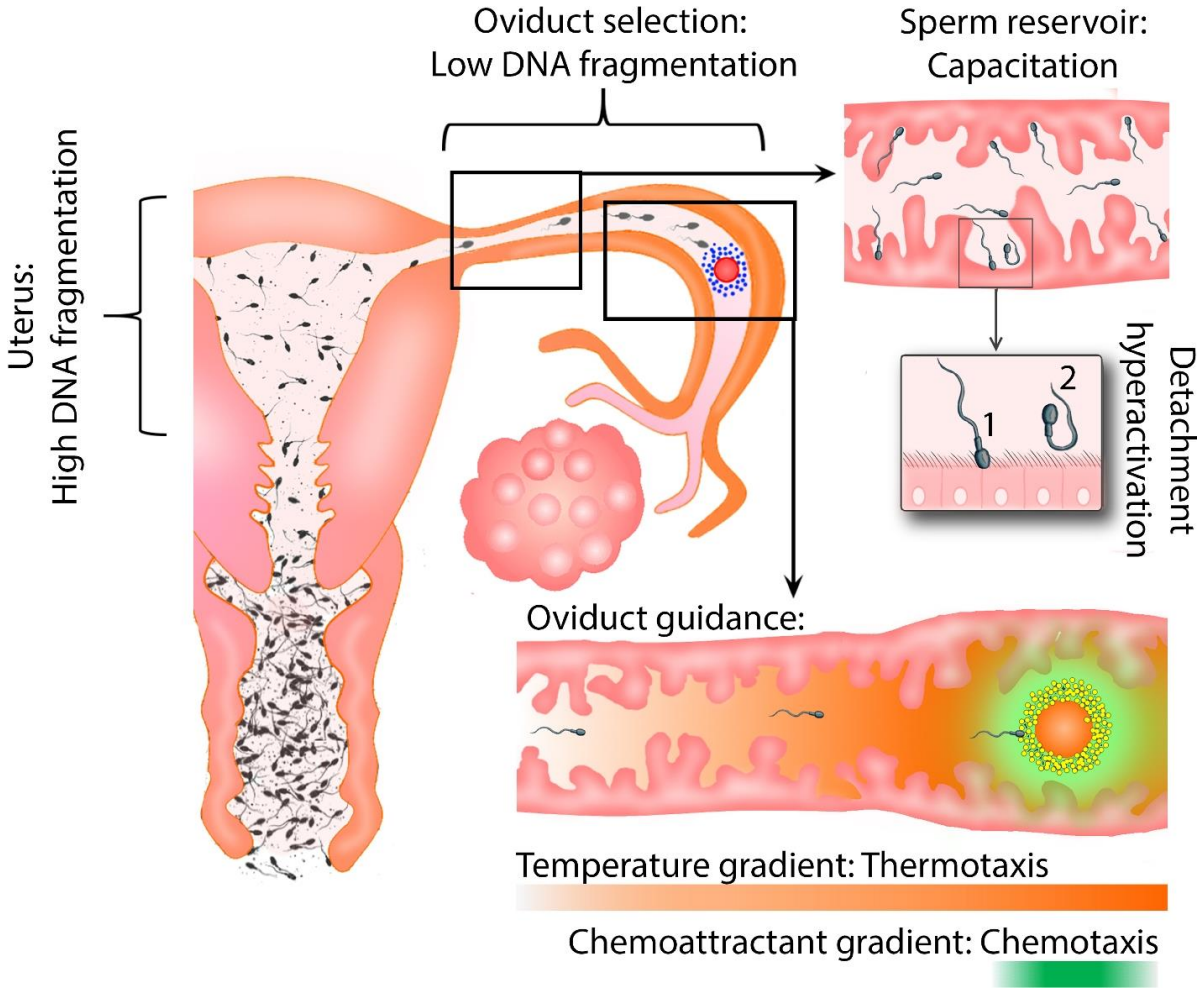


Figure 5-2. Major physiological events taken place during transit of spermatozoa in the reproductive tract towards the site of fertilisation in the oviduct in mammals. Reproductive tract is representative of humans, but the physiological events (e.g., capacitation of spermatozoa in the sperm reservoir) depicted also take place in other mammals (e.g., mice and cattle) (Pérez-Cerezales et al., 2017).

Thus, the spermatozoa will be exposed to the maternal microenvironment before fertilisation and during the fertilisation process, and conditions in the reproductive tract reflect the metabolic status of the mother (Velazquez, 2015). Therefore, given that high NEFA exposure can affect oocyte developmental competence (Marei and Leroy, 2021, Shi and Sirard, 2022), it is also important to determine the potential effects of NEFA exposure on spermatozoa around the time of fertilisation in lipotoxic conditions. A previous *in vitro* study found that spermatozoa exposed to high NEFA concentrations displayed impaired motility and increased damage in the plasma membrane (Desmet et al., 2018). However, other relevant quality endpoints indicating spermatozoa quality were not examined, and this is needed to better understand how spermatozoa is affected when exposed to lipotoxicity conditions in the maternal microenvironment around the time of fertilisation. Hence, the aim of this experiment was to assess acrosome integrity, mitochondrial membrane potential, plasma membrane integrity, and DNA damage of spermatozoa exposed to high NEFA concentrations in *in vitro* conditions.

5.2 Materials and Methods

5.2.1 Preparation of spermatozoa for NEFA exposure

For handling of glassware, plasticware, preparation IVF medium, processing of semen straws and preparation of NEFA solutions please refer to sections 2.2.1., 2.2.4, and 2.2.6). Sperm straws were thawed and processed to obtain a concentration of concentration of 25×10^6 sperm/ml (for fluorescent assay, 1 bull) or 20×10^6 sperm/ml (for DNA fragmentation assay, 2 bulls) in Fert-Talp medium. A volume of 500 µl in 4-well plate (500 µl/well, without oil cover) was used to exposed spermatozoa to NEFA under IVF conditions (i.e., at 38.5°C in a humidified atmosphere with 5% CO_2, and atmospheric O_2).

5.2.2 Experimental design

Spermatozoa were exposed to NEFA using the same concentrations used in previous chapters. Briefly, a high-NEFA group representing pathophysiological NEFA levels (280 µM SA, 230 µM PA and 210 µM OA) was compared to a control group (i.e., Control-1) representing normal physiological NEFA levels (28 µM SA, 23 µM PA and 21 µM OA). A second control group (Control-2) contained 0.2% absolute ethanol

equivalent to the same concentrations on the NEFA groups. The rationale behind these NEFA concentrations is explained in section 2.2.7. Spermatozoa was exposed to NEFA for four hours, followed by analyses of acrosome and plasma membrane integrity, mitochondrial membrane potential, and DNA fragmentation. A cycle of thawing, NEFA exposure and spermatozoa analyses was considered a replicate (Figure 5.3).

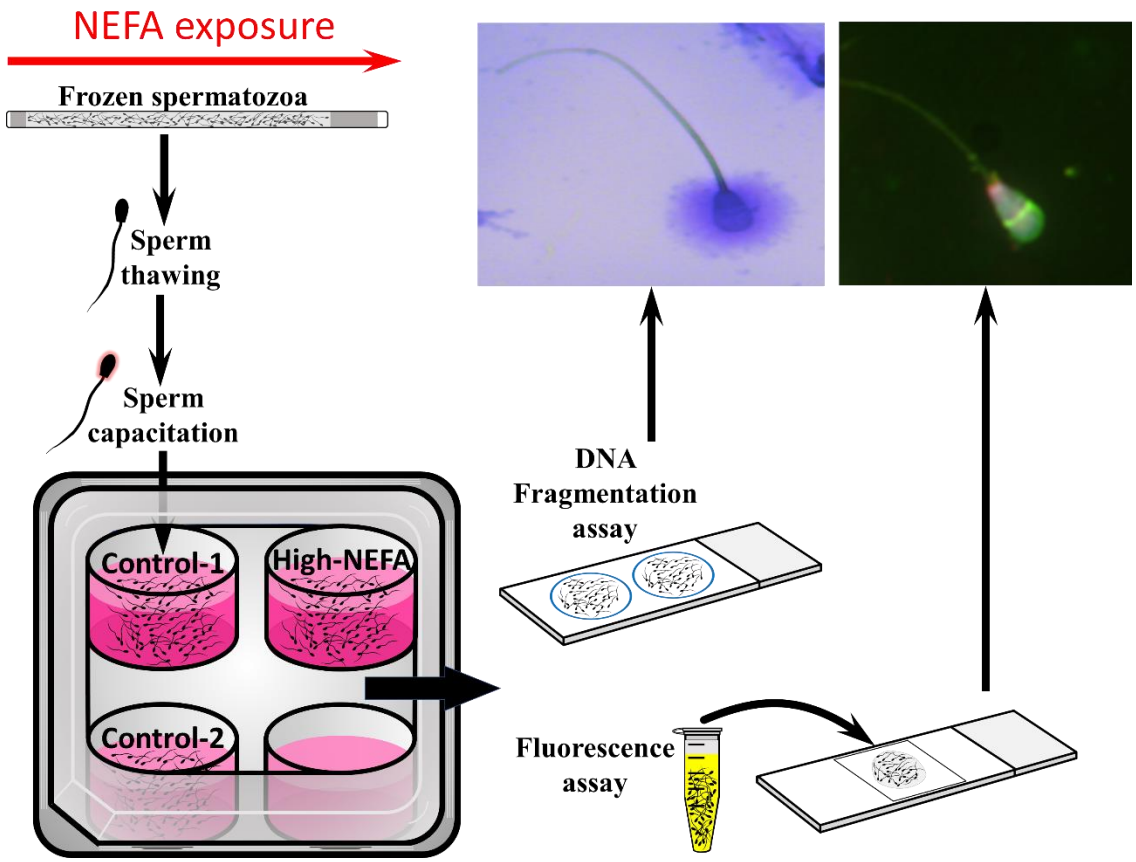


Figure 5-3. Graphical depiction of the experimental design used to test the effects of NEFA of spermatozoa quality. Artwork credit: Dr Miguel Velazquez.

5.2.3 Fluorescence assay for evaluation of spermatozoa quality

Fluorescence assays were used to evaluate simultaneously acrosome integrity, mitochondrial membrane potential and plasma membrane integrity in spermatozoa. The protocol used was based on the technique developed by Celeghini et al. (2007) with some modifications. Staining probes used to examine spermatozoa were as follow:

● Bisbenzimide Hoechst H33342 (Sigma; B2261). A stock solution of 25 mg H33342/ml (50 mM) in dimethyl sulphoxide (DMSO) (Sigma; D8418) was used to prepare a H33342 working solution of 40 µg/ml (72 µM) in dulbecco`s phosphate buffered saline (DPBS) (Sigma; D5652) (Celeghini et al., 2007). This working solution was stored in 10 µl aliquots at -20°C in the dark. For the purpose of this protocol, H33342 was used to identify spermatozoa with undamaged plasma membrane as the dye is cell permeant and is often used to stain DNA in living cells (or fixed cells) (Bucevičius et al., 2018).

● DRAQ7™ (Biostatus; DR70250). A working solution of 0.3 µM in DPBS was prepared and stored in 10 µl aliquots at 4°C in dark (Chan et al., 2017). For the purpose of this protocol, DRAQ7 was used to identify spermatozoa with damaged plasma membrane as the dye does not enter cells with intact plasma membrane integrity (Wlodkowic et al., 2013).

● Lectin from Pisum Sativum (Pisum sativum agglutinin [PSA]), labelled with fluorescein isothiocyanate (FITC) (Sigma; L0770, PSA, FITC conjugate). A working solution was prepared containing 1 mg FITC-PSA/ml (20 µM) in DPBS and stored in 75 µl aliquots at 4°C in the dark. FITC-PSA was used to determine acrosomal status as PSA binds acrosome-reacted spermatozoa. For the purpose of this protocol, a spermatozoon showing the acrosomal region totally or partly marked with FITC-PSA was considered to be acrosome-reacted (Sánchez et al., 2021), and an indication of damaged acrosome (Celeghini et al., 2007) as the spermatozoa were not fixed (i.e., permeabilised) and the acrosome reaction was not induced for analysis.

● 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) (Thermo Fisher; T3168). A stock solution of 1.53 mM JC-1 in DMSO was used to prepare a working solution of 153 µM JC-1 in DMSO that was stored in 10 µl aliquots at -20°C in the dark. The JC-1 probe allows detection of changes in mitochondria membrane potential (Δψm) by changes in the colour of the dye, turning from green (i.e., low Δψm) to red-orange (Δψm) as polarisation of the mitochondrial membrane increases (Cossarizza et al., 1993, Garner et al., 1997).

All staining probes and plasticware were warmed up prior to use (at 37°C). Following NEFA exposure in 500 µl of Fert-Talp medium (see above, section 5.2.1.), 150 µl were aspirated and transferred into a 0.5 ml Eppendorf tube, where 2 µl of H33342 (72 µM)

were added, and the resultant sperm suspension incubated for 10 minutes at 37°C. The sperm suspension was then centrifuged (100x g for one minute) with the supernatant then removed, leaving 60-70 µl of sperm solution. Then 100 µl Fert-Talp medium was added, plus 3 µl of DRAQ7™ (0.3 µM), 2 µl of JC-1 (153 µM) and 50 µl of FITC-PSA (1 mg/ml = 20 µM), followed by incubation at 37°C for at least eight minutes. The sperm suspension was then centrifuged (100x g for one minute) and the supernatant removed to leave 60-70 µl of sperm suspension to which 40 µl of Fert-Talp medium were added. From this final sperm suspension an 8 µl drop was placed on a microscope slide, coverslipped, and evaluated immediately with a widefield fluorescence microscope (Zeiss AxioImager, Carl Zeiss Optics, Germany) equipped with a digital camera (Zeiss Axiocam 105) magnification 63x oil objective lens with a multi-bandpass filter for visualisation of Hoechst 33342 (excitation 352 nm and emission 454 nm), DRAQ7 (excitation 598 nm and emission 697 nm), FITC-PSA (excitation 498 nm and emission 518 nm), and JC-1 (excitation 499/593 nm and emission 595 nm). Four replicates were applied for each treatment group, and two hundred sperm cells per replicate were examined and classified manually using the Ziess ZEN microscopy software (Figure 5.4).

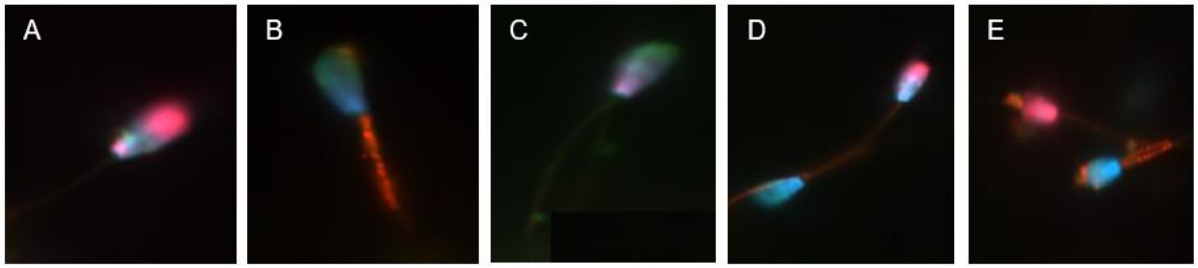


Figure 5-4. Fluorescence microscopy images of sperm cells stained with multiple fluorescent probes. **(A)** Damaged plasma membrane, intact acrosome and low mitochondrial membrane potential cell. **(B)** Intact plasma membrane, damaged acrosome and high mitochondrial membrane potential cell. **(C)** Damaged plasma membrane, damaged acrosome and low mitochondrial membrane potential cell, **(D)** Upper sperm: damaged plasma membrane, intact acrosome and high mitochondrial membrane potential cell. Lower sperm: intact plasma membrane, damaged acrosome and high mitochondrial membrane potential cell. **(E)** Upper sperm: damaged plasma membrane, intact acrosome and high mitochondrial membrane potential cell. Lower sperm: intact plasma membrane, damaged acrosome and high mitochondrial membrane potential.

5.2.4 Assessment of DNA fragmentation in spermatozoa

Sperm DNA damage was assessed with the sperm chromatin dispersion assay (Fernández et al., 2018). A commercial kit (Halosperm G2® kit, Halotech DNA, S.L., Madrid, Spain) previously used in bovine spermatozoa (Komsky-Elbaz et al., 2018) was applied to NEFA treated spermatozoa according to the manufacturer's instructions. The agarose screw tube provided in the kit was placed in a water bath at 95-100°C for five minutes to fully melt the agarose, then the agarose was then divided in 100 µl aliquots in 0.5 Eppendorf tubes. One tube was used per group and the rest of the tubes were kept at 4°C for future use. The tubes to be used were kept at 37°C for five minutes, and then 50 µl of the 500 µl sperm suspension used for NEFA exposure (see section 5.2.1) was added to the tube and mixed gently with a pipette, making sure no bubbles were formed. Then an 8 µl drop of the sperm/agarose suspension was immediately placed in the well of the coated microscope slides provided in the kit (two wells per slide) and coverslipped. The slides were then placed on a pre-cold glass plate and moved to a refrigerator to keep the slides at 4°C for five minutes to allow solidification of the agarose containing the spermatozoa. Then, out of the refrigerator, coverslips were removed by sliding them gently off the slides. For the rest of the protocol, the procedure was done at room temperature (around 20°C), slides were kept horizontally at all times, and solutions, water, and ethanol were removed by gently tilting of the slides, without shaking them. Slides were placed in an elevated position inside a petri dish and solution 1 of the kit was added (i.e., the denaturant agent) and let to incubate for seven minutes, making sure it covered entirely the wells in the slides. Solution 1 was then removed and solution 2 (i.e., lysis solution) was added, making sure wells were immersed in the solution. After 20 minutes of incubation, solution 2 was removed and slides where washed with distilled water (i.e., were let for five minutes with abundant water). Water was then removed and samples were dehydrated with a two-step exposure to ethanol (two minutes each), first with 70% and then with 100%. Following removal of ethanol and air drying of samples, a 7 to 10-minutes incubation with solution 3 (i.e., eosine staining solution) was done, making sure the solution covered completely the wells. Solution 3 was removed and solution 4 (i.e., thiazine staining solution) was applied in the same way as solution 3. After removal of solution 4, slides were air-dried and wells were cover with coverslips and analysed with bright-field microscopy using a 40x objective.

Spermatozoa with a big or medium-sized halo were considered normal, without fragmented DNA, whereas spermatozoa with a small or no halo and exhibiting degeneration were considered to have fragmented DNA (Figure 5.5). Approximately 150 spermatozoa were counted per slide-well. Six replicates were performed to examine DNA fragmentation. For the positive control, (i.e., all spermatozoa display a halo), all protocol steps were applied except adding the denaturant agent (solution 1) (Figure 5.6.A), while for the negative control. (i.e., all spermatozoa do not show a halo), only the lysis solution (solution 2) was omitted (Figure 5.6.B).

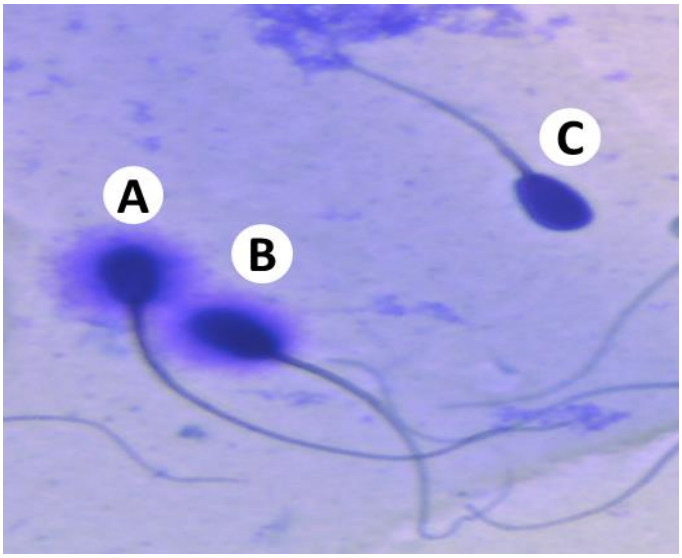


Figure 5-5. Classification of spermatozoa in the DNA fragmentation assay. Spermatozoa with a big or medium-sized **(A, B)** halo were considered normal, without fragmented DNA. Spermatozoa with a small or no halo **(C)** and exhibiting degeneration were considered to have fragmented DNA.

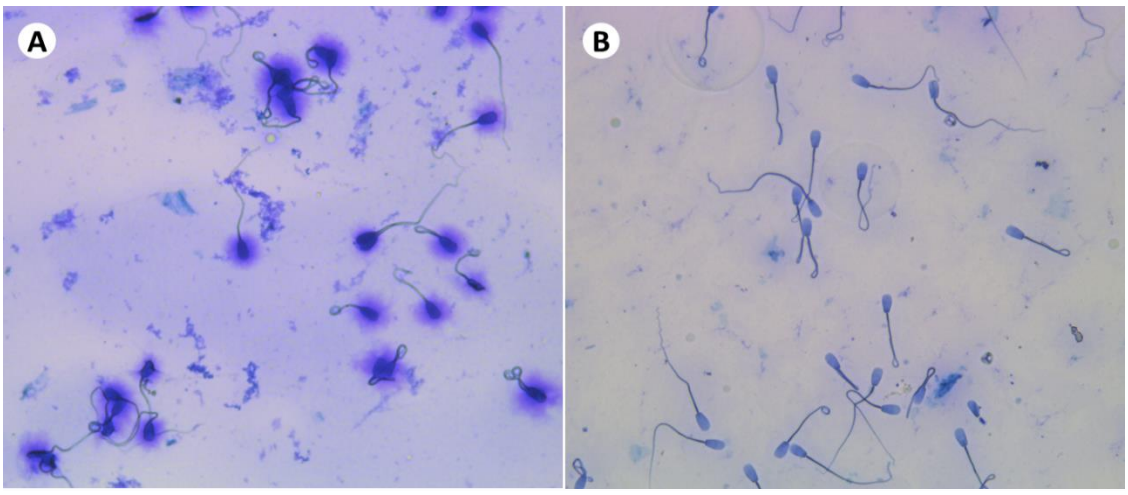


Figure 5-6. Positive **(A)** and negative **(B)** controls for the DNA fragmentation assay.

5.2.5 Statistical analysis

Statistical analysis was performed using SPSS 26 software (IBM). The Shapiro-Wilk test was used to test if the variables analysed had a normal distribution. Differences between groups were identified using either analysis of variance (ANOVA) or T-test, with percentage data arcsine transformed before analysis. Post-hoc comparisons after ANOVA were done with the least significant difference (LSD) method. A P-Value less than 0.05 was considered statistically significant. Data was reported as mean ± standard error of the mean (SEM).

5.3 Results

The fluorescence assay revealed that the percentage of spermatozoa with intact acrosome was higher in the control groups compared with high-NEFA (Control-1= 64.50±6.34%, Control-2= 69.13±4.08%, High-NEFA= 49.75±6.14%), but it reached statistical significance only when compared to control-2 (P= 0.037, Control-1 vs High-NEFA P= 0.096) (Figure 5.7).

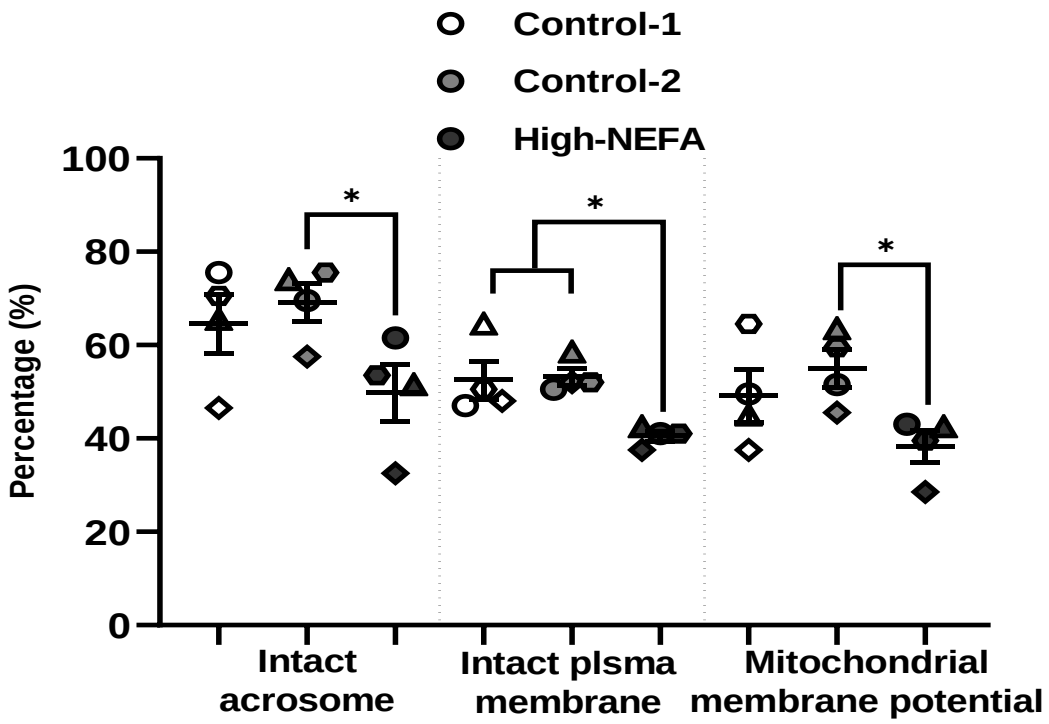


Figure 5-7. Effect of NEFA exposure on the percentage of spermatozoa with intact acrosome and plasma membrane, and high mitochondrial membrane potential.◯= 1st, ⬡ = 2nd, △= 3rd and ◇= 4th cycle.

Likewise, the percentage of spermatozoa with high mitochondrial membrane potential was decreased following exposure to high concentrations of NEFA (Control-1= 49.12±5.69%, Control-2= 55.00±4.03%, High-NEFA= 38.37 ± 3.38%), but a significant difference was only observed when compared to control-2 (P= 0.027, Control-1 vs High-NEFA P= 0.124). The high-NEFA group showed a higher plasma membrane damage (59.50±1.06%) compared to controls (Control-1= 47.50±4.07% P= 0.01, Control-2= 46.75±1.79% P= 0.008). The control groups did not have any significant differences in any of the variables analysed in the fluorescence assay (Figure 5.7).

In the DNA fragmentation assay, the two bulls tested did not differ in any of the groups (Figure 5.8) and data from both bulls was used to analyses DNA damage after exposure to NEFA. The percentage spermatozoa showing DNA damage was increased in the high-NEFA group (20.00±0.64%) compared to controls (Control-1= 8.90±0.71%, Control-2= 6.82 ± 0.78%, P<0.001) (Figure 5.9).

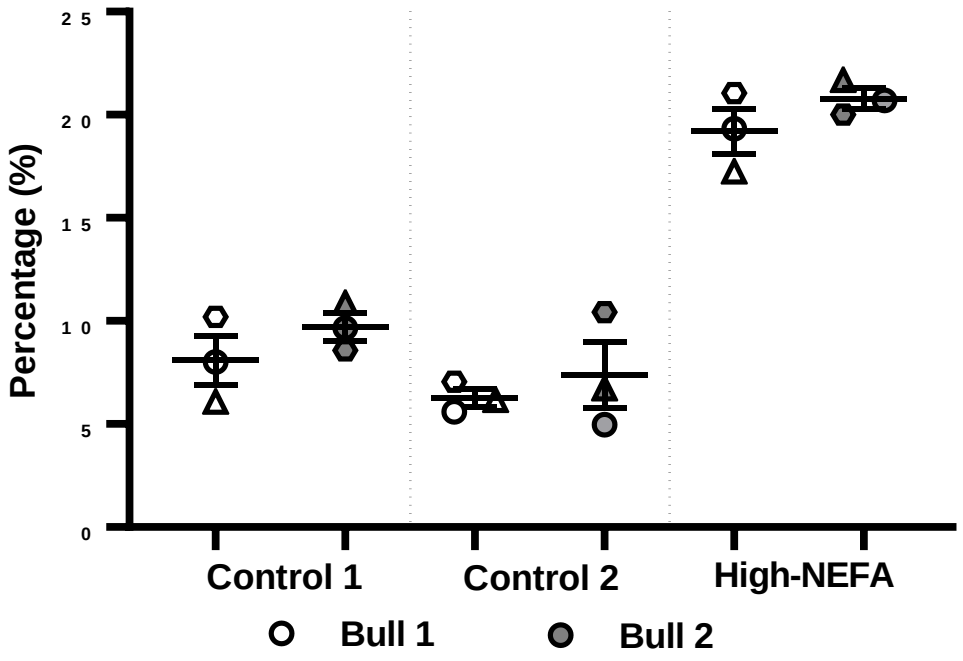


Figure 5-8. Percentage of fragmented (damaged) sperm DNA in different bulls. There was no difference between the bulls analysed. ◯ = 1st, ⬡ = 2nd, and △ = 3rd cycle.

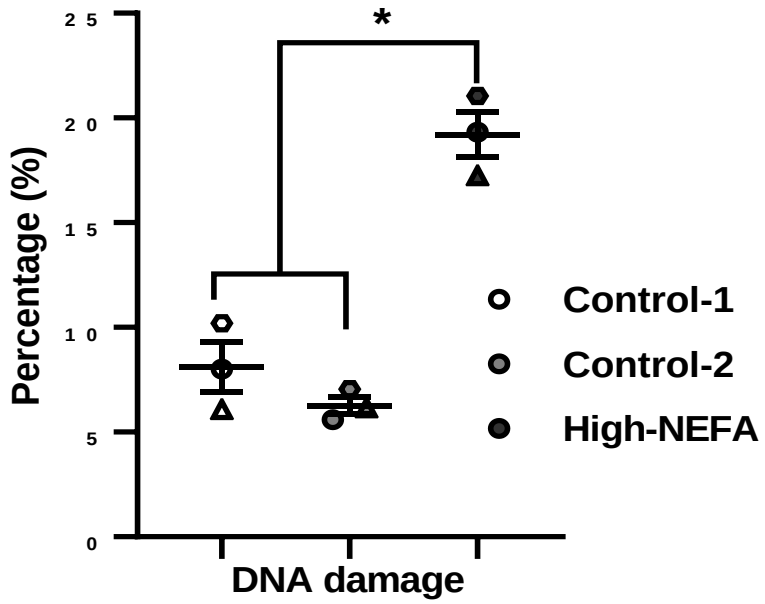


Figure 5-9. Effect of NEFA exposure on spermatozoa DNA damage. High NEFA sperm exposure increased the percentage of spermatozoa displaying DNA fragmentation. ◯ = 1st, ⬡ = 2nd, and △ = 3rd cycle.

5.4 Discussion

Given the detrimental effect of high NEFA exposure on fertilisation rate, embryo cleavage, and blastocyst formation in previous chapters of the present dissertation, in this study, the effects of high NEFA concentrations on sperm quality were investigated in order to determine the potential effects of NEFA exposure on spermatozoa around the time of fertilisation in lipotoxic conditions. The data from this experiment indicate that high NEFA concentrations can disrupt the acrosome and plasma membrane as well as the mitochondrial membrane potential in spermatozoa. Furthermore, an increased in sperm DNA damage following high NEFA exposure was also observed.

Nevertheless, the negative effect on the acrosome membrane and mitochondrial membrane potential was significant only when the high-NEFA group was compared with the control solvent (i.e., Control-2). The lack of difference between the NEFA groups, albeit speculative, suggests that even physiological concentrations of NEFA might be able to affect the acrosome membrane and mitochondrial potential of spermatozoa. Exposure to a range of incremental concentrations of NEFA might clarify this suggestion in future experiments.

The decreased proportion of spermatozoa with intact acrosome following exposure to high NEFA concentrations contrasts with a previous study that reported that high NEFA exposure *in vitro* did not alter the percentage of bovine spermatozoa with reacted acrosome (Desmet et al., 2018). This difference could be due to the lack of heparin in the medium used to exposed spermatozoa to NEFA in the previous study (Desmet et al., 2018). Heparin can induce capacitation of spermatozoa, rather than acrosome reaction, at least in cattle (Parrish, 2014), and spermatozoa capacitation is a prerequisite for acrosome reaction (De Lamirande et al., 1997, Vadnais et al., 2007, Stival et al., 2016). Albeit speculative, perhaps the lack of spermatozoa capacitation precluded the detection of changes in acrosome reaction. A low proportion of spermatozoa (less than 10%) with acrosome reaction will be expected in medium without heparin (Parrish et al., 1988), however, no information regarding the percentage of acrosome-reacted sperm was reported in the previous study analysing the effects of NEFA exposure on spermatozoa quality (Desmet et al., 2018).

In vitro incubation of spermatozoa in capacitating medium (e.g., containing heparin) will always results in the induction of the acrosome reaction in a low percentage of spermatozoa defined as spontaneous acrosome-reacted sperm (Jaiswal, 1999). In cattle, around 10-30% spermatozoa can display spontaneous acrosome reaction after four hours of *in vitro* incubation in capacitating medium (Parrish et al., 1988, Fernández et al., 2018, Komsky-Elbaz et al., 2018), which is similar to the percentage observed in the control groups of the present experiment. *In vitro* studies in mice (Bleil and Wassarman, 1983), cattle (Florman and First, 1988a, Florman and First, 1988b), and humans (including incubation of human sperm with transgenic mouse oocytes expressing human zona pellucida proteins) (Oehninger, 2003, Gupta, 2021) have provided evidence indicating that the acrosome reaction is triggered when the spermatozoon gets in contact with the zona pellucida; and hence, spermatozoa with spontaneous acrosome reaction (i.e., before getting in contact with the oocyte) will have an impaired ability to bind the zona pellucida and fertilise the oocyte (Abou-haila and Tulsiani, 2009). From this perspective, the increased percentage acrosome-reacted spermatozoa in the high-NEFA group could result in a decreased fertilisation rate as found in chapter 2 and 4. The role of the zona pellucida as the trigger of the acrosome reaction is a matter of current debate where although it is clear that the acrosome reaction is essential for fertilisation (Siu et al., 2021, Yanagimachi, 2022) it

was found that the acrosome reaction can take place before the spermatozoa reach the zona pellucida, and these acrosome-reacted spermatozoa can achieve fertilisation in mice (Jin et al., 2011). Moreover, murine acrosome-reacted spermatozoa recovered from the perivitelline space are capable of passing the zona pellucida for a second time and produce live offspring (Inoue et al., 2011) challenging the notion that mouse spermatozoa with spontaneous acrosome reaction possess a decreased fertilising capacity (Florman and Storey, 1982). Nevertheless, in human IVF programs it has been showed that a high rate of spontaneous acrosome reaction is associated with a low fertilisation rate (El-Ghobashy and West, 2003, Wiser et al., 2014), supporting the author's suggestion of a detrimental effect of high NEFA on fertilisation rates via increased proportion of spontaneous acrosome-reacted spermatozoa.

A previous study reported that the percentage of bovine spermatozoa with intact plasma membrane was decreased during exposure to high NEFA concentrations for four hours (Desmet et al., 2018), which agrees with the findings of the present experiment. Damage to the spermatozoa plasma membrane could have contributed to the low fertilisation rate (and hence decreased blastocyst production) resulting after high NEFA exposure (see chapters 2-4). The spermatozoa plasma membrane plays a significant role in physiological changes necessary for the spermatozoa to achieve fertilisation including sperm motility, capacitation, and acrosome reaction (Leahy and Gadella, 2011, Gautier and Aurich, 2021). Indeed, previous research indicated that bovine spermatozoa with damaged plasma membrane displayed a lower motility (Krishnan et al., 2016, Yániz et al., 2017), which relate to the finding of Desmet et al. (2018), where spermatozoa exposed to high NEFA concentrations showed increased damage to the plasma membrane and impaired motility. The low mitochondrial membrane potential found in this chapter could also affect motility as studies in humans have shown that spermatozoa with low mitochondrial membrane potential had decreased sperm motility, possibly associated with lower production of ATP (Marchetti et al., 2002, Agnihotri et al., 2016, Alamo et al., 2020). Similar findings have been reported in cattle (Krishnan et al., 2016, Madeja et al., 2021). Unfortunately, in the present experiment motility was not analysed as our lab does not have a computer-assisted sperm analysis (CASA) system (Amann and Waberski, 2014). From a reproductive performance point of view, the alterations in the acrosome, plasma membrane and mitochondrial membrane potential of spermatozoa could impair the

establishment of pregnancy. Indeed, in cattle it was found that artificial insemination with semen batches with a high percentage of spermatozoa displaying intact plasma membrane, intact acrosome, and high mitochondrial membrane potential resulted in higher pregnancy rates compared with semen batches with a low proportion of spermatozoa with these sperm integrity variables (Oliveira et al. 2014).

In a previous study blastocyst formation was not affected in pre-exposed spermatozoa to high NEFA even though plasma membrane and motility were impaired, which led the authors to suggest that in a high NEFA microenvironment the fertilisation process may not be affected via the spermatozoa but rather via the oocyte (Desmet et al., 2018). Nevertheless, in the Desmet et al. (2018) study the swim-up technique was used while the spermatozoa were exposed to NEFA, which is in essence a spermatozoa selection technique. Hence the possibility exists that non-motile spermatozoa was removed before IVF was performed, masking potential detrimental effects associated with impaired plasma membrane and low sperm motility found in that study. But it is important to recognise that it is challenging to analyse the potential of NEFA-exposed spermatozoa to achieve fertilisation in a normal experimental IVF setting as the sperm suspension exposed to NEFA needs to be washed to remove NEFA before incubating spermatozoa with non-treated oocytes, and during the washing procedure damaged spermatozoa (in this case associated to NEFA exposure) could be removed. Nevertheless, an intracytoplasmic sperm injection (ICSI) model could help to clarify this issue, especially in relation to DNA damage found in the present study. The increase DNA damage found in the high-NEFA group could partially explain the low blastocyst formation rate found in this group (see chapter 3) as bovine spermatozoa with DNA damage can achieve fertilisation and cleavage but the capacity to develop to the blastocyst stage is significantly decreased (Fatehi et al., 2006). Similarly, in human IVF programs sperm samples with high percentage of DNA damage can form preimplantation embryos, but pregnancy rates are decreased following embryo transfer (Henkel et al., 2004, Simon et al., 2014).

In conclusion, under the conditions of this experiment, exposing bovine spermatozoa to high NEFA concentrations results in premature acrosome reaction, reduction of mitochondrial membrane potential, and damage to the plasma membrane that can decrease the ability of spermatozoa to achieve fertilisation. High NEFA exposure also

can result in loss of DNA integrity which in turn, could jeopardise the capacity of the resultant zygote to develop to the blastocyst stage.

Chapter 6: General Discussion

6.1 The Main Aim of the Research

The present thesis project was developed to elucidate the effects of high concentrations of NEFA during the fertilisation process, an essential physiological event for successful reproduction. High NEFA concentrations associated to nutritional disorders, such as obesity in humans and NEB in dairy cows (often seen in high-yielding cows during peak lactation), can impair fertility in both species (Leroy et al., 2015, Broughton and Moley, 2017), where several studies have indicated that a high NEFA microenvironment during oocyte maturation and preimplantation embryo development can be detrimental for early pregnancy success (i.e., formation of the blastocyst and its quality) (Van Hoeck et al., 2014, Valckx et al., 2014, Alves et al., 2015, Aardema et al., 2019, Baddela et al., 2020). However, it is unclear how far early embryonic development is affected by elevated NEFA exposure exclusively during fertilisation or what the possible impacts may be on sperm quality, with only one paper published in the specialised literature (Desmet et al., 2018) after the beginning of this thesis project. Since there are similarities between obesity in humans and NEB in cattle regarding high NEFA levels and subfertility (Leroy et al., 2015, Broughton and Moley, 2017), and between the development of preimplantation embryos in cattle and humans (Ménézo and Hérubel, 2002, Adjaye et al., 2007, Santos et al., 2014), an *in vitro* production system for bovine preimplantation embryos was used with the aim of generating new information relevant to bovine reproduction but which may also provide a conceptual model for human reproductive medicine. Three groups were compared (for details of the development of the *in vitro* NEFA model please see sections 1.7.3 and 2.2.7 in chapters 1 and 2, respectively), with a high-NEFA group representing pathophysiological NEFA levels, and two control groups, one representing normal physiological NEFA levels, and a second one containing 0.2% absolute ethanol (solvent use to dilute NEFA) equivalent to the same concentrations on the NEFA groups.

6.2 The Main Outcomes of the Research

Partial results from this thesis have been presented at national and international conferences (Appendix 8). In the first experimental trial (i.e., Chapter 2) the effects of high NEFA during IVF on ROS levels in resultant zygotes were investigated for the first time, and it was observed that ROS levels in zygotes were not affected by high exposure to NEFA. This could be related to the normal peak in ROS production present in zygotes (Lopes et al., 2010, Morado et al., 2013) masking any increase in ROS production associated with NEFA exposure (See full discussion in section 2.4, in chapter 2). It was also confirmed that fertilisation rate was decreased as previously reported (Desmet et al., 2018), but the level of polyspermy was not altered in the present research, which contrasted with the results from Desmet et al. (2018) where polyspermy was observed in the NEFA groups but not in the control group containing solvent. Here it is important to highlight that the 0% polyspermy reported by Desmet et al. (2018) is the exception rather than the rule as polyspermy is a common feature present in *in vitro* embryo production models with an incidence of from 3% to 30% in humans, and from 5% to 45% in cattle (Coy and Avilés, 2010, Gutiérrez-Añez et al., 2021). Hence, the present research suggests that successful fertilisation is impaired by high NEFA exposure, but it is not related with increased polyspermy as previously suggested (Desmet et al., 2018) (See full discussion in section 2.4, in chapter 2). Nevertheless, the lack of effect of high NEFA concentrations on the induction of polyspermy needs to be corroborated in future studies.

In the second experimental trial (i.e., Chapter 3), the effect of high NEFA during IVF on preimplantation embryo development and quality of resultant blastocyst were analysed. This second experimental trial revealed that a high NEFA exposure during the fertilisation process led to a decreased blastocyst formation, and the number of cells was also decreased in these blastocysts, in both the ICM and TE. These results are in contrast with a previous study where blastocyst production was not affected and cell number was increased only in the TE following exposure to high NEFA concentrations during IVF (Desmet et al., 2018). This differences could be due to the culture media used, as Desmet et al. (2018) added insulin, transferrin, and selenium (ITS) in their embryo culture medium, a supplement known to increase blastocyst production in cattle (Wydooghe et al., 2014). ITS supplementation can also promote blastocyst formation from compromised oocytes associated to high NEFA exposure,

but the resultant embryos displayed impaired quality (Smits et al., 2020). Hence, the inclusion of ITS could have masked any detrimental effects of high NEFA in the previous study (Desmet et al., 2018). (See full discussion in section 3.4, in chapter 3). The contrasting results regarding cell allocation in resultant blastocyst may be also related to the supplementation with ITS as this supplement is capable of increasing blastocyst cell number (Wydooghe et al., 2014). Another factor to consider is the way embryos were analysed, as in the experiment of this thesis confocal microscopy was used along with 3D visualisation allowing detailed exploration on blastocyst, especially in the cells of the ICM, whereas in the previous study (Desmet et al., 2018) blastocyst were processed with a fluorescence microscope allowing only a two-dimensional visualisation of embryos. Importantly, the basic synthetic oviductal fluid supplemented with BSA used in this thesis project provided a blastocyst production (23-35%) within the expected range (20-40%) (Ferré et al., 2020), and therefore, the embryo production system used in this thesis project was suitable to address NEFA effects on blastocysts formation and quality. Nevertheless, this discrepancy between studies highlights the importance of the *in vitro* system used in reproductive research and emphasizes the relevance of testing similar hypothesis among laboratories.

In the third experimental trial (i.e., Chapter 4), it was provided novel information on the expression of H3K27me3 in 2- and 4-cell embryos 48 hpf after exposure to high NEFA concentrations during IVF. H3K27me3 is an epigenetic mark which levels start declining at the 1-cell stage, reaching a nadir around the 8-cell stage (around the time of EGA), followed by a re-establishment of expression by the blastocyst stage in cattle (Ross et al., 2008, Breton et al., 2010, Zhou et al., 2019). The current consensus is that the fall in H3K27me3 levels during the time of EGA is required for the reactivation of pluripotency-related genes that are silenced during gametogenesis and needed to proceed to the blastocyst stage (Bogliotti and Ross, 2012, Chung et al., 2017). The decrease in H3K27me3 is not the result of dilution of methyl marks during cleavage, but rather a cell division-independent process (Ross et al., 2008, Chung et al., 2017). As such, 2-cell embryos with late cleavage should have similar levels to 4-cell embryos examined at 48 hpf (See full discussion in section 4.4, in chapter 4). Indeed, a recent study did not find differences in H3K27me3 levels in 2- and 4-cell bovine embryos examined at 40 hpf (Ispada and Milazzotto, 2022). The analysis of the present thesis revealed that H3K27me3 expression between experimental groups was not affected

in any of the embryonic stages following exposure to high NEFA concentrations exclusively during IVF. However, the expected lack of difference in H3K27me3 levels between 2- and 4-cell embryos within each group was observed in the control groups but not in the high-NEFA group. In the latter group, levels of H3K27me3 in 2-cell embryos were still higher than in 4-cell embryos at 48 hpf, suggesting that activation of developmentally important genes needed to progress to the blastocyst stage was delayed, possibly explaining the low blastocyst production observed in this *in vitro* model of high NEFA exposure.

In the final experimental trial (i.e., Chapter 5) sperm quality was evaluated following exposure to high NEFA concentrations. The experiment reveals that high NEFA treatment increased the percentage of spermatozoa with plasma membrane damage as previously reported (Desmet et al., 2018). High NEFA also decrease the proportion of spermatozoa showing an intact acrosome, which contrast with a previous study where no effect on acrosome reaction was observed following NEFA treatment (Desmet et al., 2018). This could have been due to the lack of heparin in the medium used in the previous study (Desmet et al., 2018) as heparin induces capacitation of spermatozoa (Parrish, 2014), a prerequisite for acrosome reaction (De Lamirande et al., 1997, Vadnais et al., 2007, Stival et al., 2016). Hence, the lack of spermatozoa capacitation might have precluded the detection of changes in acrosome reaction in the study by Desmet et al. (2018) (See full discussion in section 5.4, in chapter 5). The high-NEFA group also showed a decreased in the percentage of spermatozoa with high mitochondrial potential along with an increased proportion of spermatozoa displaying DNA fragmentation, a novel finding that further illustrate the detrimental effects of high NEFA concentrations of spermatozoa quality.

Putting it all together, the results of this thesis suggest that when fertilisation takes place under a lipotoxic microenvironment of high NEFA the ability of the spermatozoon to reach (i.e., due to low mitochondrial membrane potential that in turn will decrease motility), attach, and penetrate the oocyte (i.e., due to the detrimental effects on the plasma membrane and the acrosome) is compromised resulting in fertilisation failure. When affected spermatozoa manage to fertilise the oocyte, the resultant zygote can manage to cleave but cannot proceed in many cases to the blastocyst stage (i.e., due to DNA damage in spermatozoa). Furthermore, the capacity of zygotes to proceed to the blastocyst stage is further jeopardised by delayed activation of developmentally

important genes at the time of EGA as shown by the altered expression of H3K27me3 observed in the high-NEFA group. This latter effect of high NEFA exposure will be at the level of the oocyte as H3K27me3 is a histone mark present in the maternal genome and basically absent in the paternal chromatin (Ross and Canovas, 2016). Hence, the present research indicates that high NEFA concentrations can induce detrimental effects during the fertilisation process via both the oocyte and the spermatozoon.

6.3 Potential Implications for Reproductive Human Medicine

Although the results from experimental animal models cannot be directly extrapolated to humans, they are valuable to develop conceptual models that can be useful to try to improve the efficacy of human assisted reproduction. From this perspective, the results of this thesis project suggest that high NEFA concentrations usually observed in obese patients undergoing assisted reproductive technologies (ART) could affect fertilisation or preimplantation embryo development jeopardising the chances of a successful early pregnancy. This will be particularly relevant in situations where intrauterine artificial insemination is performed as the spermatozoa will be exposed to a high-NEFA microenvironment that will impair their fertilising potential. Another possible scenario will be when using gamete intrafallopian transfer (GIFT, both oocyte and sperm are transfer into the oviduct via laparoscopy), especially when using oocytes and spermatozoa from donors (Kovacs and King, 1994). Compared to other ART, GIFT is rarely performed (Chiamchanya and Pruksananonda, 2019, Chiware et al., 2021), usually in cases of anatomical malformations or diseases of the reproductive tract (Lodhi et al., 2004, Lee and Lie, 2012). Nevertheless, it is relevant to provide information that could provide insights into the factors affecting the efficacy of any ART, regardless of their popularity. Hence, in the case of GIFT, placing oocytes (3-4 oocytes are usually transferred) and spermatozoa from healthy donors into the oviduct of an obese patient (i.e., with high NEFA concentrations) will not only impair the capability of spermatozoa to achieve fertilisation but also the ability of the oocyte to reach the blastocyst stage if fertilisation occurs, reducing the changes of an early successful pregnancy. Therefore, strategies to ameliorate a lipotoxic microenvironment during fertilisation might increase the efficacy of these ART.

6.4 Limitations and Future Research

In any research project, it is important to indicate the limitations of the research so that data can be put in perspective. As such, it has to be considered that NEFA concentrations in the oviducts of cattle undergoing NEB are not available, and the concentrations used in the present project and previous study (Desmet et al., 2018) rely on the assumption that NEFA values in peripheral circulation reflect accurately NEFA concentrations in the oviduct (i.e., where fertilisation takes place). However, it is known that concentrations of blood components (e.g., metabolites, metabolic hormones, etc.) are not always correlated with concentrations in the oviductal and uterine luminal fluid (Velazquez, 2015). Future efforts should be focussed on determining the profile of constituents (including NEFA) in oviductal and uterine luminal fluid during nutritional challenges (e.g., cows in NEB) using undiluted samples. However, sampling of undiluted samples (especially in the oviduct) is challenging to obtain and new techniques need to be develop to resolve this technical issue (Velazquez et al., 2010). Another limitation in this project is that the capacity of NEFA-exposed spermatozoa to produce blastocysts was not tested, but this should be tested in future experiments using ICSI rather than IVF settings (See rationale for this suggestion in section 5.4. in chapter 5) in conjunction with artificial insemination in live heifers using sperm intrafallopian transfer (SIFT) (Grossfeld et al., 2011) as this will allow the use of small amounts of *in vitro*-exposed sperm. Indeed, the lack of a complementary *in vivo* model is another limitation of this project. In future studies transfer of blastocysts could also be performed to elucidate the potential of *in vitro* embryos derived from a high NEFA microenvironment during fertilisation to achieve implantation and full-term delivery. Zygotes derived from the present high NEFA model could also be transferred to the oviduct and then recovered at different developmental stages to analysed epigenetic marks during early divisions and the quality of result blastocyst developed *in vivo*. However, although these *in vivo* models are feasible (Besenfelder et al., 2012), they are expensive and required skilled staff. Another future line of research should focus of the development of therapies to reverse the negative effects of high NEFA concentrations. Indeed, a previous study showed that β-mercaptoacetate can ameliorate the detrimental effects of high NEFA exposure during on oocyte maturation (Van Hoeck et al., 2013).

6.5 General Conclusions

The results of this thesis indicate that high concentrations of NEFA at the time of fertilisation can impair oocyte and spermatozoon quality, resulting in either fertilisation failure or embryo demise. Taking into consideration previous research on oocyte maturation and early embryo development, under natural conditions, high NEFA concentrations will also directly affect oocyte developmental competence (i.e., before fertilisation) and preimplantation embryo quality (i.e., after fertilisation). Consequently, this cumulative detrimental effects of high NEFA exposure on these developmental milestones will play a significant role in the infertility observed in cattle experiencing NEB and probably in obese individuals as well.

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Appendices

Appendix 1: Preparation of oocyte recovery medium

1. Ovary wash solution

Media	Code No.	Store temp.	M.W. Da	Conc. (g/1L H_2O)	Mass
Phosphate Buffer Saline $Cl_2H_3K_2Na_3O_8P_2$	Sigma P4417	20.0°C	411.04		5 tablets/1000ml prewarmed dH_2O

2. Oocyte recovery TCM-Air (stable in room atmosphere)

Media	Code No.	Store temp.	M.W.	Conc. (g/500ml)	Mass
Sodium Bicarbonate* $NaHCO_3$	Sigma S5761	20.0°C	84.01	4.16mM	0.175g
TCM-199	Sigma M2520	4.0°C			7.35g
Gentamycin Sulfate $C_{60}H_{125}N_{15}O_{25}S$	Sigma G3632	4.0°C	516.6	50mg/ml	0.025g
Sodium Pyruvate $C_3H_3NaO_3$	Sigma P3662	4.0°C	110.04	0.20mM	0.011g
HEPES Stock	Sigma 5310-OP	4.0°C	238.30	10mM	37.50ml
	Sigma H3784		260.29	10mM	
H_2O	Sigma W3500	20.0°C	18.02		~462.50ml
Sodium Hydroxide NaOH (pH:7.20)	Sigma S2770	4.0°C	40.00		Until reach pH:7.20
Bovine Serum Albumin** $C_{123}H_{193}N_{35}O_{37}$	Sigma A7030	4.0°C	66463	15mM (1.0%)	0.50g (500mg)

*****Dissolve separately.

******Add after pH adjusted to pH 7.20 (Osmolarity: 280-290 mOsm) with NaOH. Filter and store at 4 °C for 2 weeks.

HEPES Stock	Code No.	Store temp.	M.W. Da	Conc. (g/150ml)	Mass
HEPES Free Acid $C_8H_{18}N_2O_4S$	Sigma 5310-OP	20.0°C	238.30	125.89mM	4.500g (30mg/ml)
HEPES Sodium Salt $C_8H_{17}N_2NaO_4S$	Sigma H3784	20.0°C	260.29	124.86mM	4.875g (32.5mg/ml)

(Osmolarity: 375 mOsm). Filter and store at 4 °C for 4 weeks.

Appendix 2: Preparation of *in vitro* maturation (IVM) medium

Media	Code No.	Store	M.W. Da	Conc. (g/100ml)	Mass
Sodium Bicarbonate * $NaHCO_3$	Sigma S5761	20.0°C	84.01	26.187mM	0.22g
TCM-199	Sigma M2520	4.0°C			1.47g
Gentamycin Sulfate $C_{60}H_{125}N_{15}O_{25}S$	Sigma G3632	4.0°C	516.6	50mg/ml	0.005g
Sodium Pyruvate $C_3H_3NaO_3$	Sigma P3662	4.0°C	110.04	0.2mM	0.0022g
H_2O	Sigma W3500	20.0°C			≈100ml
Sodium Hydroxide NaOH (pH:7.40)	Sigma S2770	4.0°C	40.00		Until reach pH:7.40
Bovine Serum Albumin ** $C_{123}H_{193}N_{35}O_{37}$	Sigma A7030	4.0°C	66463	15mM (1.0%)	0.10g (100mg)

*****Dissolve separately.

**Add after pH is adjusted to pH 7.40 (stir for approx. 1 hour and in an open beaker, pH will adjust automatically) (Osmolarity: 285-290 mOsm). Filter and store at 4 °C for 2 weeks.

IVM Hormones to be added to IVM medium

1. Pregnant Mare Serum Gonadotrophin (PMSG) Equine chorionic gonadotropin (eCG) (Chorulon®).

10.00 IU Pregnant Mare Serum Gonadotropin (PMSG) Equine chorionic gonadotropin (eCG).

Store as 250µl PMSG at -20°C kept for 1 year.

2. Human Chorionic Gonadotrophin (hCG) (Chorulon®).

5.00 IU human chorionic gonadotropin (hCG) dissolved in 1.0ml solvent.

Store as 100µl hCG aliquots at -20°C kept for 1 year.

3. Epidermal Growth Factor (EGF) (Sigma E4127).

Stock 1 (1.0% BSA): 1.0g BSA + 99ml PBS.

Stock 2 (EGF primary): 1.0ml of stock 1 + 0.1mg of EGF (one vial) *=100,000ng/1000µl =10ng/µl.*

Stock 3 (EGF diluent): 1.0ml of stock 2 + 19ml of stock 1 *=100,000ng/20,000µl =5ng/µl.*

1µl→5ng, so: 4µl→20ng, add 16µl from EGF diluent stock aliquots to 4ml IVM Culture media = (20ng/ml).

Store as 400µl aliquots (total of 50 Aliquots) at -20 °C kept for 1 year.

In vitro maturation (IVM) final preparation for culturing.

- IVM medium + Hormones.
- (3.724ml TCM-Culture + 200µl PMSG + 66.4µl hCG + 16µl EGF) = 4.0ml as 500µl/well (filled with dH_2O in between wells).
- Wash and IVM culture 4-well plates prepared inside the hood, equilibrated at 38.5 °C & 20% CO_2 for at least 1 hour.

Appendix 3: Preparation of in vitro fertilisation (IVF) medium

1. IVF Tyrode's Albumin Lactate Pyruvate (FERT-TALP) stock solution

Media	Code No.	Store temp.	M.W.	Conc. (g/250ml)	Mass
Sodium Bicarbonate* $NaHCO_3$	Sigma S5761	20.0°C	84.01	25mM	0.525g
Sodium Chloride NaCl	Sigma S5886	20.0°C	58.44	114mM	1.665g
Potassium chloride KCl	Sigma P5405	20.0°C	74.55	3.20mM	0.060g
Sodium Phosphate Monobasic Dehydrate $NaH_2PO_4•2H_2O$	Sigma 71500	20.0°C	156.01	0.30mM	0.0102g
Calcium Chloride Dihydrate $CaCl_2•2H_2O$	Sigma C7902	20.0°C	147.01	2.00mM	0.0735g
Magnesium Chloride Hexahydrate $MgCl_2•6H_2O$	Sigma M2393	20.0°C	203.30	0.50mM	0.012g
Phenol-Red Sodium $C_{19}H_{13}NaO_5S$	Sigma P5530	20.0°C	376.36	0.01 µg/ml	0.0025g
D-Penicillamine $C_5H_{11}NO_2S$	Sigma P4875	4.0°C	149.21	20mM	0.0007g
H_2O	Sigma W3500	20.0°C	18.02		≈250ml
Sodium Lactate** $NaC_3H_5O_3$	Sigma L4263	4.0°C	112.06	10mM	0.465g

*****Dissolve separately.

**Add as the last reagent. pH 7.40 (Osmolarity: 280-290 mOsm) *Dissolve separately. Filter, store at 4°C for 3 months.

2. Albumin-Pyruvate-Gentamycin (APG) stock solution.

Media	Code No.	Store temp.	M.W. Da	Conc.	Mass
Bovine Serum Albumin $C_{123}H_{193}N_{35}O_{37}$	Sigma A9647	4.0°C	66463	6.0mg/ml	2.4g/40ml TALP
Gentamycin Sulfate $C_{60}H_{125}N_{15}O_{25}S$	Sigma G3632	4.0°C	516.6	50mg/ml	0.02g/0.4ml H_2O
Sodium Pyruvate $C_3H_3NaO_3$	Sigma P3662	4.0°C	110.4	0.2mM	0.02g/1ml TALP

- Transfer Gentamycin-solution (50mg/ml) completely to TALP + BSA solution (60mg/ml).
- Add 280µl of sodium pyruvate solution to TALP + BSA + Gentamycin solution (5.6mg/ml).
- Store as 1ml aliquots (110mg/ml + 5.6mg/ml= 116.6mg/ml) at –20°C for 1 year.

3. Hypotaurine-Heparin-Epinephrine (HHE) stock solution

Media	Code No.	Store temp.	M.W. Da	Conc.	Mass
1.Hypotaurine $C_2H_7NO_2S$	Sigma H1384	20.0°C	109.15	10mM	0.00218g/20ml H_2O
2.Heparin $C_{12}H_{19}NO_{20}S_3$	Sigma H3149	20.0°C	6000	0.1 UI/ml	0.0052g/20 ml H_2O
Sodium Lactate**$NaC_3H_5O_3$	Sigma L4263	4.0°C	112.06	10mM	0.165g
Sodium Metabisulfite	Sigma S9000	20.0°C	190.11	2.0µM	0.05g

$Na_2S_2O_5$					
H_2O	Sigma W3500	20.0°C	18.02		50ml
Hydrochloric Acid HCL (pH:4.0)	Sigma H9892	4.0°C	36.46		~
3. Epinephrine $C_9H_{13}NO_3$	Sigma E4250	4.0°C	183.20	1.0µM	0.0023g

Final HHE working solution

Media			Volume
Hyptaurine + Epinephrine Solution	1.Hypotaurine solution	Previously prepared	20ml (11mg/20ml)
	3.Epinephrine solution	Previously prepared	8.0ml (217.3mg/8ml)
	H_2O	Ready to use	+52ml (80ml)
FILTER			
HHE Solution	(1&3) Hypotaurine + Epinephrine	Previously prepared	40ml (17.5mg/40ml
	2.Heparin solution	Previously prepared	20ml (5.20mg/20ml)
	Total volume (ml)		60ml (22.4mg/60ml)

Filter and store as 120µl aliquots at -20 °C for 1 year.

***In vitro* fertilisation (IVF) final preparation for culturing**

- Mix 9.0ml of FERT-TALP stock (in the fridge) with 1000µl of APG (in the freezer).
- Mix each 2.0ml of FERT-TALP + APG with 100µl of HHE (in the freezer).
- Prepare IVF 4-well plate (filled with dH_2O in between wells).
- Wash and IVF culture 4-well plate prepared in the hood, equilibrated at 38.5 °C & 20% CO_2 for at least 1 hour.

Appendix 4: Proven fertility of different bulls for IVF

	Blastocyst formation rate		
Bull No.	Bull 1	Bull 2	Bull 3
AVG	19.67	19.09	18.28
STD	1.36	1.72	1.67
SEM	0.78	0.99	0.96

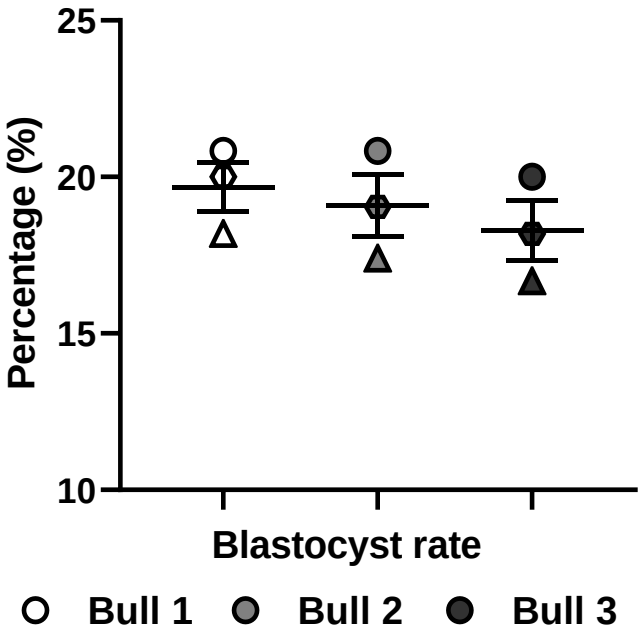


Figure. Proven fertility of different bulls for IVF. No significant difference between the bulls was observed for the blastocyst production (P>0.05) (3 replicates).

Appendix 5: Preparation of sperm straws

- Thaw a straw at 37.0°C for 1 minute.
- Layer sperm over 1.0ml 90% BoviPure (Nidacon) (900µl B.Pure + 100µl B.Diluent) in 1.5ml Eppendorf tube.
- Centrifuge at 300x g for 10 minutes.
- Remove supernatant, leave approximately 50µl and be careful not to disturb the pellet.
- Wash pellet with 750µl of FERT-TALP + APG, centrifuge at 400x g for 3 minutes.
- Remove supernatant, leave approximately 50µl and be careful not to disturb the pellet.
- Wash pellet with 750µl of FERT-TALP + APG + HHE, centrifuge at 400x g for 3 minutes.
- Remove supernatant, leave 50-75µl and be careful not to disturb the sperm pellet.
- Calculate sperm concentration (leave the tube containing the sperm cells open in the incubator).
- For a dilution factor of 1/50 (2.0µl sperm, 98µl dH_2O in 1.0ml Eppendorf tube); add 10µl to each side of the chamber.
- Count the complete chamber and use formula: Conc.(start) X Vol.(start)=Conc. (final)X Vol. (final).
- Example: 50 sperm were counted in the complete chamber; (50x50)/0.1=25,000. The 25000 are the total sperm/µl. but not all are alive (Approx. 90% alive); so multiply per 0.90 = 22,500 sperm/µl. The final volume is 500µl/well; so, to add 1.0 million sperm/well: (500x1.000=500.000)/22.500= 22.22µl to be added/well.
- Incubate in humified air for 19h at 39°C & 20% CO_2.

Appendix 6: Preparation of *in vitro* embryo culture (IVEC) medium

1. Stock solutions for Synthetic Oviductal Fluid (SOF)

I. SOF – stock A (10x)

Media	Code No.	Store temp.	M.W. Da	Conc. (g/50ml)	Mass
Sodium chloride NaCl	Sigma S5886	20.0°C	58.44	108mM	3.145g
Potassium chloride KCl	Sigma P5405	20.0°C	74.55	7.20mM	0.267g
Potassium phosphate monobasic KH_2PO_4	Sigma P5655	4.0°C	136.09	1.20mM	0.081g
Magnesium sulfate Heptahydrate $MgSO_4 \cdot 7H_2O$	Sigma M2643	20.0°C	246.47	0.80mM	0.091g
H_2O	Sigma W3500	20.0°C	18.02		49.2ml
Sodium lactate** $NaC_3H_5O_3$	Sigma L4263	4.0°C	112.06	0.6mM	300µl

******Add as the last reagent. Filter and store at 4°C for 4 weeks.

II. SOF – stock B (10x)

Media	Code No.	Store temp.	M.W. Da	Conc. (g/50ml)	Mass
Sodium bicarbonate $NaHCO_3$	Sigma S5761	20.0°C	84.01	25mM	1.050g
Phenol-Red sodium salt $C_{19}H_{13}NaO_5S$	Sigma P5530	20.0°C	376.36	0.026mM	0.005g
H_2O	Sigma W3500	20.0°C	18.02		50ml

Filter and store at 4°C for 4 weeks.

III. SOF – stock C (100x)

Media	Code No.	Store temp.	M.W. Da	Conc. (g/5ml)	Mass
Soduim Pyruvate $C_3H_3NaO_3$	Sigma P3662	4.0°C	110.04	0.73mM	0.04g
H_2O	Sigma W3500	20.0°C	18.02		5.0ml

Filter and store at 4°C for 4 weeks.

IV. SOF – stock D (100x)

Media	Code No.	Store temp.	M.W. Da	Conc. (g/5ml)	Mass
Calcium chloride dihydrate $CaCl_2x2H_2O$	Sigma C7902	20.0°C	147.01	1.78mM	0.131g
H_2O	Sigma W3500	20.0°C	18.02		5.0ml

Filter and store at 4°C for 4 weeks.

1. Glutamine stock solution

Media	Code No.	Store temp.	M.W. Da	Conc. (g/10ml)	Mass
Glutamine $C_5H_{10}N_2O_3$	Sigma G6392	-20.0°C	146.14	0.40mM	0.292g
H_2O	Sigma W3500	20.0°C	18.02		10.0ml

Dissolve 0.292g Glutamine in 10ml dH_2O, Store as 100µl aliquots at -20°C for 1 year.

2. Final IVC SOF media

Media	Code No.	Store temp.	M.W. Da	Conc. (g/50ml)	Mass
Moy-Inositol $C_6H_{12}O_6$	Sigma 1.04507	20.0°C	180.16	2.75mM	0.025g
Gentamycin Sulfate $C_{60}H_{125}N_{15}O_{25}S$	Sigma G3632	4.0°C		50µg/ml	0.0025g
H_2O	Sigma W3500	20.0°C	18.02		37.0ml
Glutamine $C_5H_{10}N_2O_3$	Sigma G6392	-20.0°C	146.14	0.40mM	50µl (0.05mg)
SOF stock A	Prepared	4.0°C			5.0ml
SOF stock B	Prepared	4.0°C			5.0ml
SOF stock C	Prepared	4.0°C			500µl
SOF stock D	Prepared	4.0°C			500µl
Minimum Non Essential Medium Amino Acid Solution MEM (100×)	Sigma M7145	4.0°C	124.57	10µl/ml (1.0%)	500µl
Basal Medium Eagle Amino Acid Solution BME (50×)	Sigma B6766	4.0°C		30µl/ml (3.0%)	1.50ml
Bovine Serum Albumin $C_{123}H_{193}N_{35}O_{37}$	Sigma A7030	4.0°C	66463	60mM (4.0%)	0.20g (200mg)

pH 7.40 (Osmolarity: 280-290 mOsm). Filter and store at 4°C for 2 weeks.

In vitro embryo culture (IVEC) final preparation for culturing

- Wash 4-well plate: 500µl SOF culture medium.
- IVC culture 4-well plate: 500µl SOF culture medium (filled with dH_2O in between well).
- Wash and IVC culture 4-well plate prepared in the hood, equilibrated at 39°C & 5% CO_2 for at least 1 hour.
- 0.5ml TCM-Air in 1.5ml Eppendorf tube.
- Transfer presumptive zygotes to the tube and vortex at 1200r/m for 4 min.
- Transfer zygotes carefully to a small petri dish (rinse tube repeatedly with TCM-Air).
- Remove remaining cumulus cells using a pipette.
- Wash zygotes in wash wells.
- Place up to 30 zygote /well in IVEC culture 4-well plate.
- Incubate in humified air at 39°C, 5% O_2 & 5% CO_2 for desired cell stage.

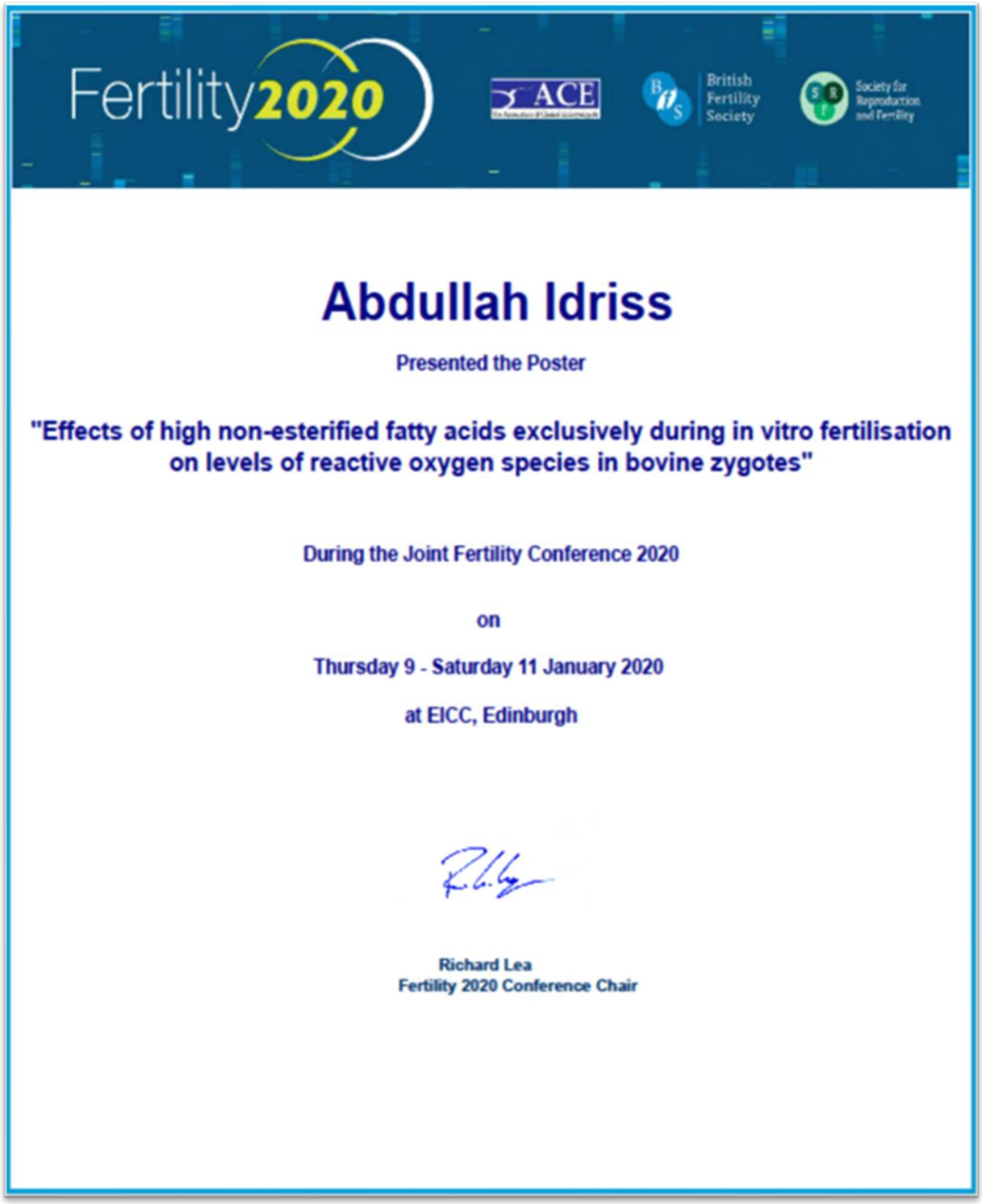
Appendix 7: Fatty acids preparation

Media	Code No.	Store temp.	M.W. Da
Stearic Acid (SA) $C_{18}H_{36}O_2$	Sigma S4751	4.0°C	284.48
Palmitic Acid (PA) $C_{16}H_{32}O_2$	Sigma P0500	4.0°C	256.42
Oleic Acid (OA) water-soluble C_2H_5OH	Sigma O1257	-20.0°C	282.5
Thioglycolic Acid (TA) $HSCH_2COOH$	Sigma T3758	-20.0°C	92.12
Ethanol 100% for cell culture	Sigma 51976	4.0°C	46.07
H2O	Sigma W3500	20.0°C	18.02

BASAL	F.W.	Mass (g)	Volume	Basal Stock	Basal Media
SA	284.48	0.024	3ml Ethan	28mM	28µM (**1**µl SA Stock in **1**ml Media)
PA	256.40	0.018	3ml Ethan	23mM	23µM (**1**µl PA Stock in**1**ml Media)
OA	282.47	0.018	3ml H2O	21mM	23µM (**1**µl OA Stock in**1**ml Media)
Total				72mM	72µM

NEFA IVF	F.W.	Mass (g)	Volume	IVF Stock	IVF Media
SA	284.48	0.237	3ml Ethan	280mM	280µM (**1**µl SA Stock/**1**ml Media)
PA	256.40	0.177	3ml Ethan	230mM	230µM (**1**µl PA Stock/**1**ml Media)
OA	282.47	0.177	3ml H2O	210mM	210µM (**1**µl OA Stock/**1**ml Media)
Total				720mM	720µM

Appendix 8: Posters certificates



Fertility2020

ACE

British Fertility Society

Society for Reproduction and Fertility

Abdullah Idriss

Presented the Poster

"Effects of high non-esterified fatty acids exclusively during in vitro fertilisation on levels of reactive oxygen species in bovine zygotes"

During the Joint Fertility Conference 2020

on

Thursday 9 - Saturday 11 January 2020

at EICC, Edinburgh

Richard Lea
Fertility 2020 Conference Chair

eshre

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PEOPLE
MOVING SCIENCE

37th Virtual Annual Meeting of ESHRE
26 June - 1 July 2021

This is to certify that

Idriss, Abdullah - United Kingdom

gave a poster presentation with title:

" Effects of high non-esterified fatty acids exclusively during bovine in vitro fertilization on cell lineage allocation of blastocysts"

during the **37th virtual Annual Meeting of the European Society of Human Reproduction and Embryology** from 26 June to 1 July 2021.

Cristina Magli
Chairman of ESHRE

T165

IMPACT OF EXPOSURE TO HIGH NON-ESTERIFIED FATTY ACIDS DURING IN VITRO FERTILIZATION ON TRIMETHYLATION OF HISTONE H3 AT LYSINE 27 IN BOVINE EMBRYOS

A. Idriss [3], E. Okello [3], R. Sturmey [1], M.A. Velazquez [2]

[1]Center for Atherothrombosis and Metabolic Research, Hull-York Medical School, University of Hull, UK

[2]School of Natural and Environmental Sciences, Newcastle University, UK

[3]Translational And Clinical Research Institute, Newcastle University, UK

BACKGROUND-AIM

Exposure to elevated levels of non-esterified fatty acids (NEFA) such as steric acid (SA), palmitic acid (PA]) and oleic acid (OA) during in vitro oocyte maturation (IVM) and embryo culture can impair preimplantation embryo development in cattle. However, the effect of high NEFA during the fertilisation process per se have been less studied. In the present study histone H3 at lysine 27 trimethylation (H3K27me3) was analysed in bovine embryos exposed to high NEFA during in vitro fertilisation (IVF). H3K27me3 is a repressive epigenetic mark that undergoes gradual erasure from fertilisation to the time of embryonic genome activation (EGA) to allow a correct EGA and progression to the blastocyst stage (Epigenetics 2012;7:976-81).

METHODS

After IVM, IVF took place under different NEFA levels representing physiological (Control-1[C1], 28µM SA, 23µM PA, 21µM OA) and pathophysiological (High-NEFA, 280µM SA, 230µM PA, 210µM OA, levels found in cows experiencing negative energy balance) concentrations. A second control (C2) group contained solvent. Presumptive zygotes were then culture, and resultant 2- and 4-cell embryos were collected on day 2 (IVF=day 0) to examine H3K27me3 protein expression by immunofluorescence and confocal microscopy. Fluorescence levels were quantified with the Imaris software. Data were analysed (SPSS statistical software) by ANOVA and T-test (mean±SEM), with percentage data arcsine transformed before analysis.

RESULTS

Cleavage rate was decreased in the high-NEFA group compared to controls (C1=57.1±7.4%, C2=59.1±7.1%, High-NEFA=29.8±2.6%, P=0.012). H3K27me3 levels (Log10 transformed data) in 2- (C1; n=31, C2; n=29, High-NEFA; n=29) and 4-cell (C1; n=23, C2; n=32, High-NEFA; n=13) embryos was not different between the groups. There was no difference in H3K27me3 expression between 2-cell and 4-cell embryos in the control groups. However, in the high-NEFA group, 2-cell embryos displayed a higher H3K27me3 expression than 4-cell embryos (P=0.038).

CONCLUSIONS

Our data suggest that activation of key developmental genes may be delayed in embryos experiencing a high NEFA microenvironment during fertilization, which partially explain the decreased blastocyst formation previously observed in this model of high NEFA exposure (ESHRE meeting 2021, P-176).

CERTIFICATE FOR POSTER PRESENTATION

To whom it may concern
Undersigned, hereby certifies that

Idriss, Abdullah - United Kingdom

presented a poster at the ESHRE Hybrid 38th Annual Meeting (**3 – 6 July 2022**) titled:

Exposure to high concentrations of non-esterified fatty acids impairs bovine sperm quality

organised online on https://www.eshre.eu/ESHRE2022 and in person in Milan, Italy.

Professor Dr Carlos Calhaz-Jorge

ESHRE Chair

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Appendix 9: Ethical approval

Faculty of Medical Sciences
Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH

27th March 2018

STRICTLY PRIVATE AND CONFIDENTIAL

Mr Abdullah Idriss
School of Natural and Environmental Sciences
Agriculture Building
Newcastle University
Newcastle upon Tyne
NE1 7RU

Dear Mr. Idriss,

REGISTRATION OF UNLICENCED WORK
AWERB Project ID No: ID 667
Targeting the preimplantation period to protect offspring from maternal lipotoxicity

Thank you very much for submitting your project proposal for ethical approval. The Animal Welfare and Ethical Review Body are happy that this work should go ahead, and we will keep a record of this approval on file.

A condition of this approval is that you will report any adverse effects suffered by animals to the AWERB and stop the work associated with the adverse effects until advice is received from the Chair of the committee. Adverse effects should be reported via email.

Yours sincerely

Mr Paul Dearden
Licencing and Regulatory Administrator
Secretary to the Animal Welfare and Ethical Review Body
Comparative Biology Centre
Faculty of Medical Sciences
Newcastle University
☎ (0191) 208 6188
Paul.Dearden@ncl.ac.uk

Appendix 10: Summary of all experimental cycles

Ethanol vs Ethanol-free

Ethanol																	
Matured COCs for IVF	Presumptive zygotes cultured	Unfertilised No.	Unfertilised rate (%)	Polyspermy No.	Polyspermy rate (%)	Degeneration rate (%)	Cleavage No. (2cell to <morula)	Total cleavage No. (all)	Total cleavage rate (%)	Morula No.	Morula rate (%)	Early blastocyst	Mid blastocyst	Expanded blastocyst	Hatching Blastocysts No	Hatched Blastocysts No	Total Blastocyst No.	Total Blastocyst rate (%)
20	20	7	35.00	2	10.00	36.36	4	11	55.00	2	10.0	1	1	1	1	1	5	25.00
18	18	8	44.44	2	11.11	50.00	4	8	44.44	1	5.56	1	1	1	0	0	3	16.67
11	11	5	45.45	1	9.09	40.00	2	5	45.45	1	9.09	0	1	1	0	0	2	18.18
25	25	11	44.00	2	8.00	41.67	5	12	48.00	2	8.00	1	1	2	1	0	5	20.00

Ethanol-free																	
Matured COCs for IVF	Presumptive zygotes cultured	Unfertilised No.	Unfertilised rate (%)	Polyspermy No.	Polyspermy rate (%)	Degeneration rate (%)	Cleavage No. (2cell to <morrula)	Total cleavage No. (all)	Total cleavge rate (%)	Morula No.	Morula rate (%)	Early blastocyst	Mid blastocyst	Expanded blastocyst	Hatching Blastocysts No	Hatched Blastocysts No	Total Blastocysts No.	Blastocyst rate (%)
18	18	6	33.33	3	16.67	33.33	3	9	50.0	2	11.1	0	1	2	0	1	4	22.22
17	17	5	29.41	2	11.76	50.00	5	10	58.8	2	11.7	1	1	1	0	0	3	17.65
12	12	4	33.33	1	8.33	42.86	3	7	58.3	1	8.33	0	1	1	0	1	3	25.00
30	30	11	36.67	4	13.33	33.33	5	15	50.00	3	10.00	1	1	3	1	1	7	23.33

Fertilisation status Reactive Oxygen Species (ROS)

Replicate No	Treatment	total oocyte no.	monospermic no	monospermic rate	monospermic/100	polyspermy no	polyspermic rate	polyspermic/100	unfertilised no	unfertilised rate	unfertilised/100
1	**control 1**	30	15	50.000	0.500	9	30.000	0.300	6	20.000	0.200
1	**control 2**	24	14	58.333	0.583	7	29.167	0.292	3	12.500	0.125
1	**high NEFA**	29	10	34.483	0.345	6	20.690	0.207	13	44.828	0.448
2	**control 1**	34	13	38.235	0.382	5	14.706	0.147	16	47.059	0.471
2	**control 2**	27	14	51.852	0.519	2	7.407	0.074	11	40.741	0.407
2	**high NEFA**	35	10	28.571	0.286	5	14.286	0.143	20	57.143	0.571
3	**control 1**	47	22	46.809	0.468	6	12.766	0.128	19	40.426	0.404
3	**control 2**	30	15	50.000	0.500	4	13.333	0.133	11	36.667	0.367
3	**high NEFA**	48	10	20.833	0.208	1	2.083	0.021	37	77.083	0.771
4	**control 1**	17	12	70.588	0.706	1	5.882	0.059	4	23.529	0.235
4	**control 2**	17	13	76.471	0.765	2	11.765	0.118	2	11.765	0.118
4	**high NEFA**	17	5	29.412	0.294	1	5.882	0.059	11	64.706	0.647

Cell allocation CDX2

BASAL								
Presumptive zygotes cultured No	Degeneration rate (%)	Cleavage No	Total Cleaved No	Cleavege rate (%)	Morula No	Morula rate (%)	Blast No	Blast rate (%)
18	46.154	6	13	72.222	2	11.111	5	27.778
22	46.154	6	13	59.091	3	13.636	4	18.182
25	35.294	6	17	68.000	2	8.000	9	36
20	45.455	5	11	55.000	2	10.000	4	20.000

SOLVENT								
Presumptive zygotes cultured No	Degeneration rate	Cleavage No	Total Cleaved No	Cleavege rate (%)	Morula No	Morula rate (%)	Blast No	Blast rate NEFA (%)
20	76.9231	10	13	65.000	1	5.000	2	10.000
24	35.2941	6	17	70.833	4	16.667	7	29.167
20	42.8571	6	14	70.000	2	10.000	6	30.000
20	50	7	14	70.000	2	10.000	5	25.000

High NEFA								
Presumptive zygotes cultured No	Degeneration rate	Cleavage No	Total Cleaved No	Cleavege rate (%)	Morula No	Morula rate (%)	Blast No	Blast rate NEFA (%)
24	76.923	10	13	54.167	1	4.1667	2	8.333
22	62.500	5	8	36.364	1	4.5455	2	9.091
20	62.500	5	8	40.000	1	5.0000	2	10.000
20	57.143	4	7	35.000	1	5.0000	2	10.000

Fertilisation status for H3K27me3

Control 1 (BASAL)											
Matured COCs for IVF	Presumptive zygotes cultured	Unfertilised	Polyspermy	2-Pronuclei	2-cell	4-cell	> 4-cell	Total fertilised	Total Cleaved No	Fertilisation Rate (%)	Cleavege rate (%)
23	23	5	2	7	5	4	0	18	9	78.26	39.13
25	25	5	3	7	5	5	0	20	10	80.00	40.00
20	20	4	1	1	6	8	0	16	14	80.00	70.00
40	40	10	2	3	11	9	5	30	25	75.00	62.50
31	31	6	0	1	9	9	5	24	23	77.42	74.19

Control 2 (SOLVENT)											
Matured COCs for IVF	Presumptive zygotes cultured No	Unfertilised	Polyspermy	2-Pronuclei	2-cell	4-cell	> 4-cell	Total Fertilised	Total Cleaved No	Fertilisation Rate (%)	Cleavege rate (%)
23	23	6	2	6	4	5	0	17	9	73.91	39.13
40	40	8	4	10	8	10	0	32	18	80.00	45.00
20	20	4	1	1	5	9	0	16	14	80.00	70.00
40	40	9	3	3	11	10	6	33	27	82.50	67.50
31	31	6	2	1	10	9	4	26	23	83.87	74.19

High NEFA											
Matured COCs for IVF	Presumptive zygotes cultured No	unfertilised	Polyspermy	2-Pronuclei	2-cell	4-cell	> 4-cell	Total Fertilised	Total Cleaved No	Fertilisation Rate (%)	Cleavege rate (%)
50	50	24	8	4	10	4	0	26	14	52.00	28.00
38	38	19	6	3	6	4	0	19	10	50.00	26.32
20	20	12	1	1	4	2	0	8	6	40.00	30.00
40	40	22	5	5	6	4	0	20	10	50.00	25.00
35	35	19	1	1	9	5	0	16	14	45.71	40.00

Sperm DNA damage between the two bulls

		Control 1 Basal		Control 2 Solvent		High NEFA	
	Rep. No.	Normal%	Degenerated%	Normal%	Degenerated%	Normal%	Degenerated%
	1	92.00	8.00	94.41	5.59	80.67	19.33
	2	89.80	10.20	92.95	7.05	78.95	21.05
Bull 1	3	93.92	6.08	93.84	6.16	82.76	17.24
	4	90.35	9.65	95.04	4.96	79.31	20.69
	5	91.43	8.57	89.58	10.42	80.00	20.00
Bull 2	6	89.13	10.87	93.29	6.71	78.33	21.67

Sperm integrity

	Control1 (Basal)							Control2 (Solvent)							High NEFA					
	Acrosome		Membrane		Mitochondria			Acrosome		Membrane		Mitochondria			Acrosome		Membrane		Mitochondria	
Replicate	Intact	Damage	Intact	Damage	High	Low	Replicate	Intact	Damage	Intact	Damage	High	Low	Replicate	Intact	Damage	Intact	Damage	High	Low
1	151	49	94	106	99	101	1	139	61	101	99	103	97	1	123	77	82	118	86	114
2	141	59	101	99	129	71	2	151	49	104	96	119	81	2	107	93	82	118	79	121
3	131	69	129	71	90	110	3	148	52	117	83	127	73	3	103	97	85	115	85	115
4	93	107	96	104	75	125	4	115	85	104	96	91	109	4	65	135	75	125	57	143

Bulls' fertility comparison

Bull 1 (Golden Bar)						
Presumptive zygotes cultured No	Not fertilised	Polyspermy	Cleavage (2cell to <morula)	Morula No	Blast No	Blast rate (%)
22	7	3	6	2	4	18.18
20	6	2	7	1	4	20.00
24	9	4	5	1	5	20.83

Bull 3 (Rusty)						
Presumptive zygotes cultured No	Not fertilised	Polyspermy	Cleavage (2cell to >morula)	Morula No	Blast No	Blast rate (%)
25	8	4	6	2	5	20.00
18	5	2	7	1	3	16.67
22	5	3	9	1	4	18.18

Bull 2 (Chicago)						
Presumptive zygotes cultured No	Not fertilised	Polyspermy	Cleavage (2cell to >morula)	Morula No	Blast No	Blast rate (%)
23	6	3	8	2	4	17.391
24	7	4	7	1	5	20.833
21	5	2	9	1	4	19.048