Assessment of laser-assisted micromanipulation procedures in a commercial bovine *in vitro* production laboratory

Jonas Itilo Baroni

Thesis submitted for the degree of Master Research in Animal Science
July 2021

School of Bioscience
University of Nottingham

ABSTRACT:

Study Question:- What is the best (i.e., easiest, quickest and most effective) method for embryo biopsy in a commercial bovine laboratory? Also, can Laser Assisted Hatching (LAH) increase the number (proportion) of embryos that hatch following cryopreservation?

Summary answer:- Laser biopsy of trophectoderm (TE) cells is a safe and reliable procedure that can be incorporated in a commercial laboratory. Also, LAH advances the time of completion of hatching.

Background:- Embryo biopsy offers a means of retrieving cells for genomic evaluation and/or aneuploidy screening with a view to accelerating genetic selection and improving pregnancy rates following transfer in cattle. Laser biopsy is rarely used in commercial IVP laboratories because it is expensive, relatively time consuming and there are concerns that biopsy size may not yield sufficient DNA for analyses.

Study design:- *Experiment 1:* Biopsy of herniated and non-herniated Day 7 embryos was undertaken using either a microblade or laser assisted micromanipulation. Time taken for each biopsy, as well as ease of procedure, survivability and number of cells collected were recorded. *Experiment 2:* An extension of the first experiment, laser biopsy in herniated and non-herniated embryos was compared to non-biopsied embryos to establish merits of herniation and to assess survival rate following cryopreservation. *Experiment 3:* The timing and extent of hatching was assessed following laser-assisted induction of a hole in the zona pellucida on Day 7 relative to non-treated control embryos.

Results:- *Experiment 1:* Biopsy of herniated embryos were faster ($P<0.001$) and easier ($P=0.001$) than those where herniation was not induced independent of instrument (i.e., blade vs laser). Proportion of blastocysts that re-expanded after 3hours was greater in herniated embryos assigned to laser-assisted compared to other combinations ($P=0.031$). *Experiment 2:* Laser biopsy of herniated embryos was quicker ($P=0.05$) compared to laser biopsy in non-herniated embryos. Survival rate in biopsied embryos was similar to non-biopsied controls. *Experiment 3:* LAH increased the proportion of embryos hatching/hatched at 6h ($P=0.007$) and 30h ($P=0.048$), and that had completely hatched by 40h ($P=0.007$).

Limitations:- Further studies that analyse DNA (i.e., genotype, karyotype) from biopsies together with analyses of post-transfer embryo survival are required. Similarly, the benefits of LAH in terms of enhanced pregnancy rates following transfer need to be established.

Wider implications: - The transfer of high-genetic merit and karyotypically normal embryos has the potential to be of great benefit in cattle breeding. In order to facilitate this, embryo biopsy procedures need to be straightforward to learn, easy and rapid to apply, and have no detrimental effects on embryo survival following transfer. The benefits of routine LAH awaits studies to assess post-transfer embryo survival.

Acknowledgements

I would like to thank God in his magnitude to allow and provide. My wife Daniella and Daughter Isabella for love, comprehension of those days I was absent and to inspire me every day. I would like to express my gratitude to Professor Kevin Sinclair for his support and guidance. I am particularly grateful for Paragon Veterinary Group which supported the project. I would like to express my appreciation to the Embryologists for their willingness and for the help to achieve the goals of the project under commercial standards. I am grateful for my family, My Father, Mother and brother whom are always rooting for me and friends whom never fail to support me.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AH	Assisted Hatching
AI	Artificial Insemination
AFC	Antral Follicular Count
AMH	Anti-Mullarian Hormone
ART	Assisted Reproductive Technologies
BF's	Blastocyst fluids
BL	Blastocyst
BSA	Bovine Serum Albumin
CIDR	Controlled Internal Drug Release
COC's	Cumulus oophurus cells
DFR	Dominant Follicle Removal
EBV	Estimate Breeding Value
eCG	Equine Chorionic Gonadotropin
ET	Embryo Transfer
EXBL	Expanded Blastocyst
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
GEBV	Genetic Estimate Breeding Value
GMO	Genetic Modified Organism
HB	Hatching Blastocyst
HSP17B1	17β-hydroxysteroid dehydrogenase-2
ICSI	Intracytoplasmatic Sperm Injection
IFOT	Intrafollicular Oocyte Injection
IMC	Inner Mass Cells
IVC	In-vitro Culture
IVF	In-vitro Fertilisation
IVM	In-vitro Maturation
IVP	In-vitro Production
LB	Laser Biopsy
LD	Lipid Droplets
LH	Luteinizant Hormone
MOET	Multiple Ovulation Embryo Transfer
NGS	Next Generation Sequencing
OPU	Ovum Pick Up
P4	Progesterone
pFSH	Porcine Follicular Stimulant Hormone
PGF2	Prostaglandin F2 α
PGT-A	Pre implantation Genetic Testing Aneuploidy
PGT	Pre implantation Genetic Testing
PHE	Penicillamine hypotaurine and epinephrine
PMSG	Pregnant Mare Serum Gonadotropin
PR	Pregnancy Rate
PRID	Progesterone Releasing Intravaginal Device
RR	Recovery Rate
SCNT	Somatic cells nucleo transfer
SNP	Single Nucleotide Polymorphism
SS	Sexed Semen
TE	Trophectoderm cells
TZP	Tranzonal Projections
UDP	Glucose pyrophosphorylase-2
UFO	Unfertilised Ovum
ZP	Zona Pellucida
ZPH	Zona Pellucida hardening
ºC	Degree Celsius
18G	18 gauge
µl	Microgram/millilitre
µs	Microseconds
µm	Micrometre
mW	Megawatts
ms	Milliseconds
nm	Nanometre

INTRODUCTION

Recent years have seen significant progress in assisted reproductive technologies (ART) in cattle. The combination of in vitro embryo production (IVP), sexed semen (SS) and genomic selection are now widely used in North and South America; and it has been increasing in Europe (**Figure 1.3**). Technological progress achieved during the past years in animal reproduction have resulted in the development of a variety of tools. Most of these have aimed to maximize the number of offspring from genetically superior animals and disseminate germplasm globally. Furthermore, ART allows for the exploitation of donors with sub-fertile conditions for safeguarding germplasm from endangered species and domestic breeds, and for reducing disease spread. Also, ART supports the selection of embryos for transfer based on the diagnosis of chromosomal errors or assistance to implantation which can improve pregnancy rates (PR). Several years of improvement has brought gains on the number of embryos produced in a Multiple Ovulation Embryo Transfer (MOET) and Ovum Pick-UP (OPU) application (**Appendix 1**) but a small progress is seen on PR.

Commented [j1]: Reordered Appendix 1 to show improvement over time

While the number of MOET produced embryos that are collected and transferred worldwide seems to have stabilized in recent years, the transfer of IVP embryos continues to grow at an average annual rate of 12%. This is despite the higher PR encountered from MOET embryos, typically around 45% vs 37% from an IVP (Hansen. 2020). MOET has contributed to improvements in our understanding of follicle wave dynamics (Adams. 1994) and synchronization of follicular wave emergency (Bó et al., 1995, 2002) improving the number of follicles and oocytes collected by OPU for IVP (**Appendix 1**). In 2019, the number of IVP embryos was in excess of one million worldwide, while the number of cattle embryos transferred was in the region of 800 K (IETS, newsletter Dec 2020). Bovine IVP can increase the selection of genetically superior animals and facilitates the international shipment of germplasm in an economically and environmentally sustainable manner, which also avoids the transportation of live animals. Currently it is possible to generate similar number of viable embryos from MOET per IVP cycle but, more importantly, many more cycles per donor can be undertaken per year (Boni. 2018), increasing the number of available embryos for transfer.

The implementation of embryo biopsies as an ART tool in livestock production facilitates this selection by allowing a few cells to be harvested from the pre-hatching embryo from which DNA can be extracted for genotyping. However, the technique is relatively slow and laborious, and so commercial uptake requires that it can be undertaken at scale without requiring a high degree of technical skill.

The current series of experiments reported in this thesis has two aims. Firstly, to identify the best approach for embryo biopsy in a commercial bovine IVP laboratory with the implementation of a faster and easy technique for non-experienced technicians and, secondly, for non-biopsied embryos, explore the benefits of laser assisted hatching (LAH) which could potentially improve pregnancy rates following embryo transfer (ET) and promote the uptake of OPU-ET in the UK bovine market.

1. CHAPTER 1. LITERATURE REVIEW

1.1 Overview of Cattle ART

It is estimated that the global human population will be around 9.7 billion people by 2050 (UN 2017). Humanity is already beginning to struggle with food production and supply which needs to increase by around 70% (FAO 2009) to meet demands whilst preserving wildlife and the environment. Dairy and beef cattle production represents a major environmental concern, yet there is an increasing demand for dairy and meat (**Figure 1.1**). Production must therefore increase but in a sustainable manner, with fewer, more efficient and productive animals. Genetic selection in cattle to date has focussed primarily on production traits such as milk yield and growth rates, with little emphasis on reproductive performance and environmental sustainability (Egger-Danner et al., 2015).

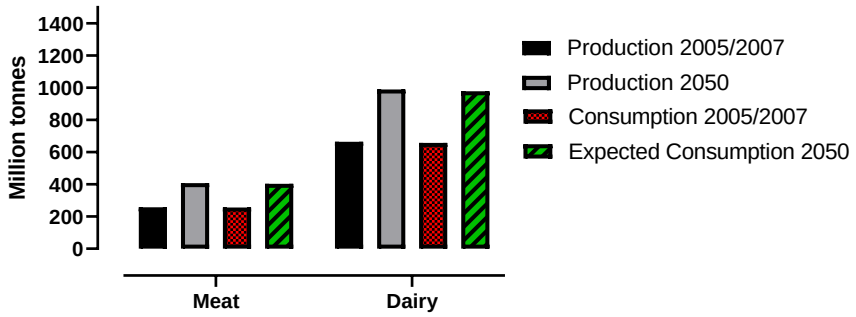

Figure 1.1: Expected production and consumption of meat and dairy products by 2050 (FAO world agriculture 2012 revision).

ART's represent the best means by which to achieve higher cattle efficiency through genetic selection. Contemporary methodologies integrate genomic evaluations which can be undertaken from Day 7 embryo biopsies (Peippo et al., 2007). This has the advantage of increasing selection intensity for desired traits (e.g. milk yield, health and longevity traits) whilst reducing the generation interval (i.e. selection decisions can be made prior to embryo transfer); thus increasing response to selection. Such technologies also facilitate gene banking (cryopreserved and stored gametes and embryos) and biosecure national and international dissemination of superior genetics in the form of genomically evaluated embryos

(Blodin. 2018, Morrel and Myer. 2017). Artificial Insemination (AI) was the first biotechnology successfully established by a Russian called Milovanov who described a practical procedure to AI sheep and cattle in 1938 (Foote. 2010). This technology has had a huge impact worldwide; a total of 110 million first inseminations were reported in a huge survey in 1999 (Thibier et al., 2002) demonstrating that AI was the main technology to accelerate genetic dissemination. Twenty years later this procedure became increasingly accepted among the public enabling the introduction of new biotechnologies focused on genetic progress such as SS, IVP, gamete/embryo cryopreservation, ET, nuclear transfer, somatic cell nuclear transfer (SCNT), Intracytoplasmatic Sperm Injection (ICSI) and generation of genetically modified organism (GMO) where some of these technologies are still in progress to demonstrate livestock efficiency gain in a more sustainable production.

1.2 *In Vivo* Derived Embryos (MOET)

ET has been used for many years for genetic selection in individual herds, not only for its ability to disseminate superior genes in the herd but also to increase pregnancy rates in unfavourable conditions such as during heat stress (Ambrose et al., 1999; Baruselli et al., 2011) or for repeat breeders. Non-surgical embryo recovery procedures in cattle were introduced in the 1970s (Elsden et al., 1976, Greve et al., 1977), followed by non-surgical transfer during the early 1980s (Sreenan. 1978, Tervit et al., 1980), and these techniques have largely been used since then. In the last 18 years, a wide variety of flushing techniques were practiced with apparently comparable success rates; however the number of embryos recovered per flush has not increased and the average remains at around 6 embryos (IETS newsletter 2001-2019). A total of 12,807,347 transferable embryos were recovered from 2,009,629 flushes worldwide with a slight increase in the recovery average after 2008 (IETS newsletter 2001-2019). This amounts to an average of 640 K embryos produced annually. Granleese et al. (2015), analysed several breeding programs where the use of MOET could be compared to AI when selecting animals for superior genetics. They found that is possible to increase over 38% the genetic gain in dairy cattle herd by using MOET. In another study using a MOET program, two embryo transfers per cow per year elevated the rate of genetic

improvement around 30% when compared to AI with selected sires (Nicholas et al., 1983).

The great advance in MOET centres on approaches to visualise and regulate follicle development in order to increase the number of ovulated follicles. Initially, Pregnant Mare Serum Gonadotropin (PMSG) was largely used to stimulate donors and induce the ovulation of follicles. This complex gonadotropic hormone was normally given on days 9-14 of the oestrous cycle followed a prostaglandin ($PGF_{2\alpha}$) injection 48 h later (Greve. 1976; 1977). However, the use of PMSG was associated with a high number of non-ovulated follicles due to its long half-life; approximately 5 days in peripheral circulation. Callesen et al. (1992) used an antiserum for PMSG in order to reduce this effect but did not succeed in increasing embryo recovery and quality. Follicle stimulating hormone (FSH) became popular thereafter when Looney et al. (1988) reported the successful stimulation of ovaries without exogenous luteinizing hormone (LH). However, its short half-life required lots of injections over a short period of time. In addition to FSH, equine chorionic gonadotropin (eCG) and $PGF_{2\alpha}$, Porgesterone (P4) devices such as controlled internal drug release (CIDR) (Eazi-breed™ Pfizer®, Zoetis, US), and progesterone releasing intravaginal device (PRID) (Ceva®, France) are now used to increase donor performance before embryo collection. These devices help to increase plasma P4 levels and maintain follicles under low pulsatile release of LH from the anterior pituitary (Mackmillan and Peternson. 1993; Roche et al., 1981); thereby suppressing oestrus and ovulation while follicles continue to grow (Mackmillan et al., 1991). After almost 50 years of development in MOET programs, many protocols exist, tailored to a certain extent to individual breeds and countries (Bo and Mapletoft. 2014). In fact, the use of hormonal synchronization programs facilitates herd management routines and can fix physiological issues related to postpartum anoestrus and low heat detection efficiency.

Although effective and widely used in the past decades, *In vivo* derived embryos from MOET no longer constitute the majority of embryo production worldwide. According to IETS figures from 2020, *in vivo* derived embryos now represents 24% of cattle embryos produced globally, falling from 623,997 to 319,961 declining by over 48% since 2013 (IETS newsletter), this reduction has been succeeded by the exponential growth of OPU-IVP (**Figure 1.2**). Pregnancy rates from MOET continue to be superior to that of IVP embryos (Ferraz. 2016).

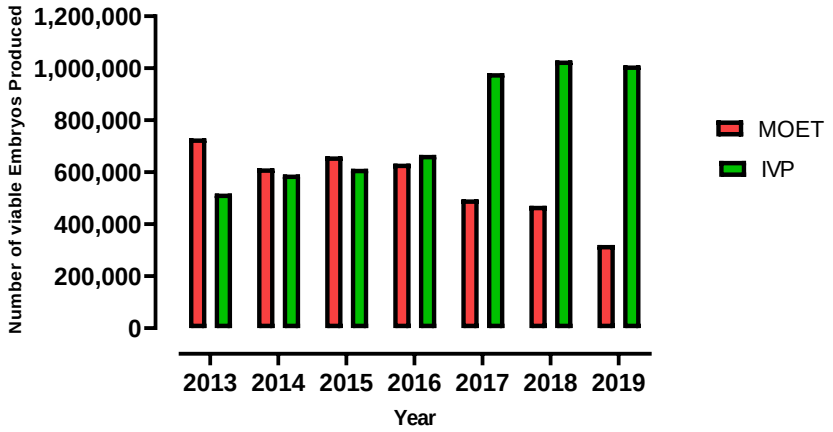

Figure 1.2: The number of embryos produced worldwide annually illustrating the reduction of embryos produced in vivo and increase in IVP embryos produced by OPU according to IETS newsletter reports since 2013.

1.3 *In Vitro* Embryo Production (IVP)

Bovine IVP is now established as a robust system to deliver embryos commercially for farms across the world. Today it is possible to produce embryos capable of delivering healthy calves with superior genetics for all different cattle breeds. Previous IVP systems resulted in embryos that were less tolerant to cryopreservation, using conventional slow-freezing protocols, compared to *in vivo* embryos (Abe et al., 2002), and calves that exhibited abnormal offspring syndrome (Farin et al., 2009, Young et al., 1998) that range from changes in gene expression to abortion or neonatal death to larger calves (Farin et al., 2014) . After several years of research many, but not all, of these problems have been resolved. Contemporary IVP media have largely been remodelled to become serum free, and the incidence of abnormal calves has reduced when serum was replaced for Bovine serum albumin (BSA) (Van Wagtendonk-de Leeuw et al., 2000), increasing the acceptance among dairy and beef producers.

According to the International Embryo Transfer society (IETS Annual newsletter Dec, 2019), the number of bovine embryos produced from OPU-IVP is increasing sharply. North America has recently surpassed South America in embryos produced by this means (**Figure 1.3**) and has become the largest IVP producer in the world. With the technology to accelerate the genetic gain, US have drastically increased the number of cattle genotyped, rising to over 2 million dairy cattle genotyped since 2011, where over 60% of these are heifers (December 2018;

https://www.uscdcb.com). This early selection of higher genetics reduce generation interval increasing the selection for more efficient donors in future IVP programs accelerating dairy and beef cattle efficiency in the country.

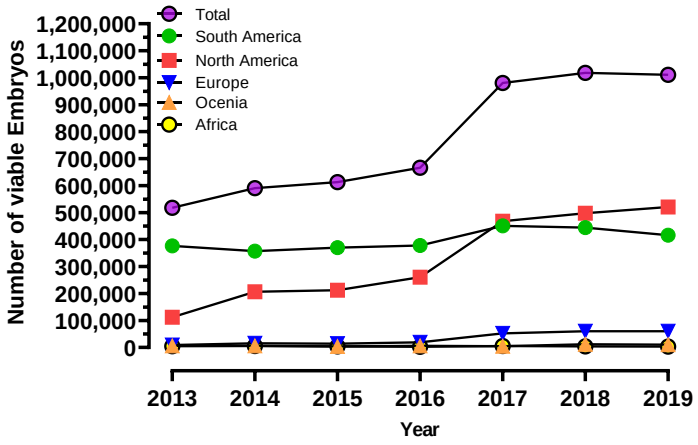

Figure 1.3: Number of IVP embryos produced over the world according to IETS data, focused on top three continents. IETS data from total number of embryos produced in-vitro since 2013 to 2019, and yearly production in South America, North America and Europe. IETS.org

In 2018 approximately 740,000 IVP embryos were transferred worldwide, and over 1,018,168 IVP embryos were produced (IETS newsletter 2019); an increase of 2.6% of transferable embryos produced by IVP from the previous year. There are clearly improvements on the IVP system that enhance the production of viable embryos. It is due to advances in the synchronization of follicular waves to superstimulation of donors which allows a greater number of follicles to emerge at each OPU session. Ireland et al. (2008) identified that antral follicular count (AFC) during follicular waves predict the increase on morphologically healthier oocytes. Also AFC is related to ovarian function and fertility and is a heritable genetic trait (Walsh et al., 2014) that can be used to identify more fertile donors improving early selection. An alternative to predict AFC is the association of Anti-Mullarian Hormone (AMH) that can be measured in the blood before assigning a donor to the program (Baruselli et al., 2018, Cardoso et al., 2018).

It is well known that the dominant follicle (DF) starts to release inhibitors that supress ongoing follicle growth and they undergo to a mechanism of atresia (Celestino et al., 2018) thus reducing the potential number of good quality cumulus oophurus complexes (COC's) collected and the potential number of embryos

produced. Dominant follicle removal (DFR) potentially increases the ability of follicles to continue development thus avoiding atresia. The ablation of the DF increases the homogeneity and quality of COCs increasing the number of viable embryos obtained (Merton et al., 2003). Besides the preparation on stimulation, the superstimulation of donors can greatly impact on the efficiency of IVP embryos. The combination of selecting a donor with higher AFC associated to the synchronisation and homogeneity of follicles has the ability to increase the number of blastocyst following OPU (Taneja et al., 2000, Silva-santos et al., 2014).

Synchronisation of follicular waves helps to support hormonal circulation providing and allowing all nutrients needed to create a more adequate environment for a healthier oocyte to develop. Levels of P4, FSH and estradiol (E2) play an important role in oocyte development and when synchronized properly helps oocyte quality (**Figure 1.4**). FSH also induces expression of NADPH cytochrome P450 reductase, which transfer electrons to aromatase; HSD3B2 which converts DHEA to androstenedione, and type 1 17β-hydroxysteroid dehydrogenase (HSD17B1), the "estrogenic" 17β-HSD that reduces estrone to oestradiol (McNATTY et al., 1979). Studies of isolated granulosa cells from preovulatory follicles have shown that FSH, but not LH, stimulates oestrogen production. Expression of growth factor receptors in the follicle are supported under a pathway of events in order to allow follicles to support high quality oocytes.

Recent studies have shown the utility of granulosa-cell based molecular markers as a predictor of oocyte fertilisation, embryo development and competence to establish pregnancy (Bettegowda et al., 2008, Assou et al., 2010). Thousands of genes are found to be differentially expressed between cumulus/granulosa cells of competent and non-competent oocytes but only few of them (*HAS2, GREM1* and *PTGS2*) are significantly correlated with the developmental competence of oocytes and/or embryos (Assidi et al., 2008, McKenzie et al., 2004, Anderson et al., 2009). UDP-glucose pyrophosphorylase-2 (*UGP2*) and pleckstrin homology-like domain, family A, member 1 (*PHLDA1*) expressed in cumulus cells, have been reported as a reliable predictor of pregnancy outcome (Hamel et al., 2010). In addition, expression of GA-binding protein transcription factor b1 (GABPβ1) has also been found significantly higher in the cumulus of the oocytes belongs to non-pregnancy group compared to the pregnancy group. Furthermore, Phosphoglycerate kinase 1 (PGK1), regulator of G-protein signalling 2 (RGS2), regulator of G-protein signalling 3 (RGS3) and cell division cycle 42 (CDC42) have also been found to be

differentially expressed between the FCs of the follicles leading to the pregnancy and those which failed to produce blastocyst (Hamel et al., 2010).

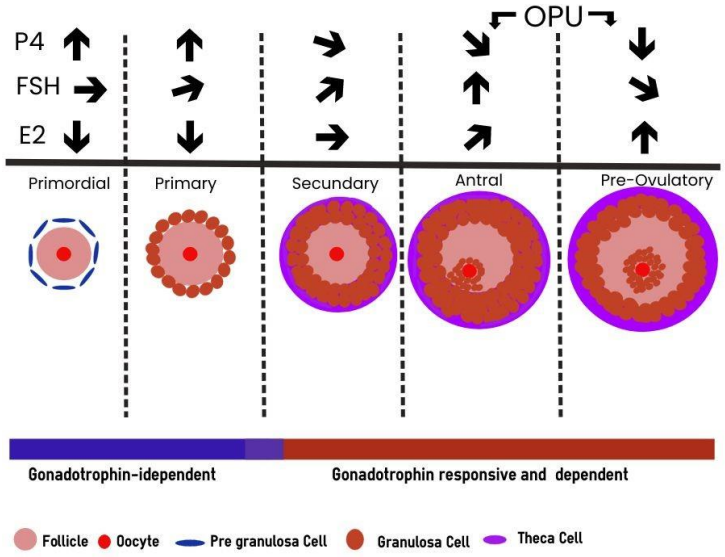

Figure 1.4: Pre-granulosa cells originated from ovarian epithelium, block high molecular-weight substances, such as low-density lipoproteins (LDL) vital to oocyte quality. Granulosa cells are the key for hormonal production The synthesis of these hormones requires a collaborative relationship between theca cells surrounding the follicle, which produce androgens (i.e., dehydroepiandrosterone (DHEA), androstenediol, andostrenedione, testosterone) in response to LH, which then diffuse into granulosa cells and are converted to estrogens (i.e.,estrone, estradiol), by cytochrome P450 aromatase (CYP19A1) in granulosa cells in response to FSH.

Quality control follows where the technician and embryologist in the lab ensures that IVP has received the best attention during development and cryopreservation. It is important to note, however, that each step in the process is interdependent (Lonergan and Fair. 2008) and highly important to succeed. Pregnancy success in cattle after ET using IVP embryos remains suboptimal. Reviews on this topic from around 20 years ago indicate that ~50% of pregnancies will be maintained to term in IVP embryo recipients (Farin et al., 2001; Rizos et al., 2002). The situation has not changed much since.

The possibility to control every step of embryo development increases the ability to predict embryo quality based on its morphology. According to the IETS Manual (2010), embryos go through 9 stages of development where in each stage a quality grade is applied (**Table 1.1 and 1.2**).

Table 1.1 Embryo stage of development according to its structure and visible morphology finds under a microscope

Stage	Cells Development	Findings after fertilization
1	1 cell	Unfertilized ova (UFO) or degenerating and dying ova
2	Cleavage 4-16 cells	After cleavage, embryos from two to 16 cells
3	Cleavage 32 cells /Early morula	Early morulas, embryos have a higher number of cells that start to become difficult to count, usually, this is the stage where embryos can easy be mistaken with UFO.
4	Morula	Full compaction of cells to the centre of the embryo, the cells looks like a berry. Cells present in the embryo are visibly compacting into the centre of the ZP and a format of a berry can be seen when looking in the edge of these cells that are very round. Trophoblast cells are surrounding embryoblast cells.
5	Early blastocyst	Cells start to distinguish in trophectoderm cells (TE) and Inner-cell mass (ICM). A small bag of fluid start to form between both layers. Embryos become to create small sack of blastocoele and ICM and TE start to be easier identified.
6	Blastocyst	A full blastocoele cavity is formed and filled up with Blastocoele fluids (BF's) allowing a clear identification of IMC and TC. Trophoblast cells are now compressed against the ZP and ICM is well compacted.
7	Expanded Blastocyst	Embryo expanded in size and the Zona Pellucida (ZP) start to get thinner prior gastrulation.
8	Hatching Blastocyst	A rupture is seen in the ZP and embryo start to hatching out of ZP. Embryo is bigger than previous stages and ZP start to become thinner and broken in some points to allow the embryo to hatch out.
9	Hatched Blastocyst	Embryo is outside the ZP in a full size and capacity to attachment. Blastulated, hepiblast and hipoblast cells start to grow.

Stage of embryo development according to IETS tutorial 2010.

Table 1.2 Embryo grading of quality and its likelihood to undergo freezing cryopreservation process.

Grade	Overall Grade	Findings	Characteristics	Freezing
1	Good/Excellent	Symmetric blastomeres without cells extrusion, 0% fragmentation or degeneration	Dense, compact and spherical	Very Good
2	Fair	Couple Blastomeres extrusion or degeneration	IMC compacted in advanced stages	Good
3	Poor	Few number of defected cells and shape deformation	25% of IMC compaction with visible degeneration	Poor
4	Dead or Degenerating	Completely degenerated at any stage (UFO's to Blast)	No development	Fail

Accessing embryos at different stages of development helps to predict the quality of these embryos, those cleaving earliest after IVF being more likely to reach the blastocyst stage than their later-cleaving counterparts (Basile et al., 2015, Cruz et al., 2012, Dinnyés et al., 1999; Lonergan et al., 1999). Many factors can affect the developmental competence of IVP embryos, compared to conventional embryos (Merton et al., 2003. Camargo et al., 2018). These include stress during pipetting, which can cause several rapid changes in proteins and mRNA (Xie et al., 2007). Beebe et al. (2002) and Quinn (2004) found that one-step media, without culture change and manipulation, gives best results. Several culture changes during IVP

Commented [j2]: Modified as recommended and corrected typos.

Incorporated legend.

increase the exposure to the environmental oxygen (O_2) and temperatures when transferring into different medium dishes. The mammalian uterus has oxygen levels of between 3 to 8% (Fisher and Bivester. 1993). Higher exposure to a 20% O_2, in air is associated with abnormal embryo development (Iwata et al., 1998, Dickey et al., 2010) reducing the number of viable embryos. High oxygen environment can lead to a reduction in cell division at the 4-cell stage (Lequarré et al,. 2003), reducing the quality of these embryos and increasing stress induced sarcosine oxidase (*SOX*), mitochondrial Mn superoxide dismutase (*MnSOD*), a regulator of box-α (*BAX) stress* genes (Gutiérrez-ad et al., 2004). This in turn can lead to epigenetic alterations in gene expression that affect subsequent developmental stages, including post-transfer development and pregnancy establishment and length (Fang et al., 2019, Siqueira et al., 2020). Contemporary molecular biology techniques now allow us to analyse DNA from embryo biopsies to assess genetic, epigenetic and cytogenic variances that might exist (Niemann et al., 2010; Bermejo-Alvarez et al., 2008).

1.3.1 *In Vitro* Maturation (IVM), Fertilisation (IVF) and Culture (IVC)

Typically, for a session of ultrasound guided follicular aspiration or OPU, we might expect that around 90% of oocytes retrieved will go into IVM but only 35% of these will produce viable grade embryos. Furthermore, only around 50% of transferred embryos will give rise to a live calf (Hasler et al., 1995, Hansen. 2020). Media for IVM, IVF and IVC have been modified over the years with the aim of increasing blastocyst yields and pregnancy outcomes. Back into 1950's, Whitten (1957) confirmed the development of an eight-cell embryo during IVC by using bicarbonate as the physiologic saline supplemented with glucose, penicillin G, streptomycin and egg white. Many of these components are still used today but the egg white was later replaced by bovine serum albumin (BSA). Another important adjustment over the years was the Potassium (K) to sodium (NaCl) ratio in Simplex optimized IVC medium (KSOM) (Lawitts and Biggers, 1993), rendering it one of the most used media in commercial laboratories. KSOM greatly improved when it was supplemented with over 19 amino acids (AAs) (Ho et al., 1995) but it retained BSA. This composition in Bovine IVM can lead to some undesired effects in embryos (Gardner. 1994; Camargo et al., 2018) such large offspring syndrome (Young et al., 1998; Sinclair et al., 2016), making serum-free media highly desirable. Serum free

media can lead to a greater number of blastocysts when compared to serum supplemented media. Brunner et al. (2010) state that embryos cultured in serum-free media have fewer lipid droplets (LD) in their cytoplasm. Also, histochemical examinations have shown that there are more LD in embryos between the morula and blastocyst stages when embryos are cultured in the presence of serum (Abe. 2002), leading to improvements in IVP. According to Rajput et al. (2020), follistatin added to IVM improves embryo development, due to its ability to increase the phosphoralisation of b-catenin enhancing signalling during oocyte maturation. This molecule helps to ensure normal meiotic progression during IVM and can enhance embryo development.

With regard to IVF media, Summers et al. (2000) showed that the addition of AAs to KSOM provided a suitable medium for IVF which increased the percentage of blastocysts that hatched, the number of cells in the ICM, and supported a more organised extracellular matrix based on the pattern of staining of Collagen IV. Further improvements in IVF were also made when L-glutamine was replaced with glycyl-L-glutamine (Summers et al., 2005). When L-glutamine was replaced with glycyl-L-glutamine, ICM cells developed more rapidly than TE cells, and fragmented nuclei in the blastocysts that developed in vitro were reduced.

Sequential media formulations (whereby media is replaced on 2-3 occasions during IVC) can further improve embryo development. These positive effects arise by reducing toxic metabolites such as ammonia and free oxygen radicals accumulated in culture media during IVC (Hashimoto et al., 2008; Takahashi et al., 2000). Several paracrine factors, such as epidermal growth factor (EGF), platelet-activating factors, insulin like growth factors, as well as messenger RNAs (mRNAs) and microRNAs (miRNAs) can all be added at different stages during IVC to aid embryo development (Stokes et al., 2005; Lopera-Vasquez et al., 2016). Several studies have shown that these paracrine factors are packaged into CD9 positive membranous micro-vesicles called exosomes, which are secreted or absorbed by the preimplantation embryo through exocytosis or endocytosis, respectively (Bobrie et al., 2011, Saadelin et al., 2015).

1.3.2 Abattoir Derived Oocytes

The use of abattoir derived ovaries as a source of oocytes for IVP presents an opportunity for research, and has become an attractive field for scientists around

the world. IVP from slaughterhouses ovaries has largely improved the progress and understanding of molecular embryology, explaining many physiological processes. It was through slaughterhouse ovaries that it became possible to distinguish oocyte maturational process from follicular maturational process, making it easier to be reproduced *in vitro* (Thibault, 1977). Media composition and quality can be evaluated and tested with larger numbers of oocytes. Once it was established that meiosis could be induced by placing aspirated oocytes in culture media (Pincus and Enzeman, 1935, Edwards, 1965) it was observed that the rate of metaphase II oocytes was over 85% within 24 h (Sirard et al., 1989).

1.3.3 Ovum Pick-Up (OPU)

Ovary aspiration was first performed by a Canadian researcher by endoscopy via the right paralombar fossa; he had a higher recovery when using a pump instead of a syringe (Lambert et al., 1983). Later a Danish researcher made use of an ultrasound to aspirate bovine follicles laterally (Callesen et al., 1987); but only in 1988 was intravaginal ultrasound-guided follicle aspiration established with a really low recovery rate (RR) of 2.3 oocytes per OPU (Pieterse et al.,1988). The number of oocytes collected by OPU significantly improved by changing the frequency of collection from once to twice weekly (Qi et al., 2013) either stimulated with PMSG or non-stimulated. Through the years many protocols to superstimulate were used to improve RR. Looney et al. (1994) stimulated with 4-5 mg pFSH for 3 days prior to aspiration, increasing oocyte retrieval (to 6.3 COC's per session) and the number of Grade 1–2 embryos per session compared to non-treated animals. A later study also show that was possible to increase recovery by twisting the needle during OPU compared to simple aspiration, (*63.7% vs 33.1%*; SASAMOTO et al., 2003). Vacuum pressure was also optimised in that study.

The success of OPU is determined by the quality of COC's and it is closely associated with follicle development (Baruselli et al., 2012). Oocytes from follicles >3 mm are competent to undergo maturation and culture having a potential to become transferable blastocyst by Day 7 after fertilization. Lonergan et al. (1994) found a higher blastocyst yield from follicles >6 mm, despite the use of FSH. One advantage seen from OPU is that by DFR it is possible to increase the homogeneity of the follicle population, and this has been shown to improve in vitro developmental competence (Hagemann et al., 1999, Lussier et al., 1995).

1.3.4 Cryopreservation

Improvements in cryopreservation, allowing embryos to be frozen using both slow freezing or vitrification processes, facilitates the ability to deliver genetics to other continents in a more biosecure way. Vitrification is an ultrarapid cooling technique that requires a high concentration of cryoprotectant. Slow freezing is a method that uses low concentrations of cryoprotectant and slow cooling rates to avoid ice crystallization. Therefore, vitrification does not require specialized expensive equipment, and it takes only a few seconds to cool embryos. Embryos are firstly placed in the cryoprotectant, secondly, placed in liquid nitrogen, and then immediately solidified in vitrification. Instead of ice crystallizing, the embryos are visibly solidified by extreme elevation in viscosity during cooling and in direct contact with liquid nitrogen, despite rapid procedure it requires longer process to thaw before transfer where slow freezing embryos can simply be thawed and directly transferred into a recipient. According to IETS (2019), 740,002 IVP embryos were transferred worldwide in 2018. Although cryopreservation through slow freezing or vitrification has been demonstrated to be successful (Martínez et al., 2002, Catt et al., 2018) approximately 541,615 embryos were transferred fresh.

Embryos can suffer a number of cryoinjuries during the freezing process, including damage to membrane functions, observed as disruption of the plasma membrane, changes in mitochondrial cristae, matrix swelling of the rough endoplasmic reticulum, and poorly developed desmosomes (Mogas. 2019). Another concern in cryopreserving embryos relates to lipid droplets that are formed during oocyte growth and maturation. Tolerance to cryopreservation of bovine embryos developed from IVM/IVF oocytes cultured in a serum-supplemented medium can be increased after removal of cytoplasmic LD by centrifugation, suggesting that cytoplasmic LD may directly affect the sensitivity of embryos to chilling and freezing (Ushijima et al., 1999).

Several approaches can be taken to reduce lipid levels at the point of embryo freezing (Ushijima et al., 1999; Braga et al 2019., Abe et al., 2002). Bovine embryos cultured in serum-free systems reduces the accumulation of cytoplasmic LD. Similarly, removal of excessive intracellular lipids from early embryos significantly improves their resistance to cryopreservation. Recently, del Collado et al. (2017) reported a reduction in LD accumulation when using cytochalisin B during IVM.

Cytochalasin B acts by blocking tranzonal projections (TZP) between cumulus cells and the oolemma, reducing the entrance of fatty acid binding protein 3 (FABP3) into the oocyte in the first 9 h of maturation. This reduces the risk of poor cryopreservation or loss of embryo viability during the process.

1.3.4.1 Cryopreservation and post-thaw viability

Cryopreservation of embryos led to MOET and IVP technologies to becoming popular. It enabled embryo shipped across the world with higher biosecurity than shipping calves, and didn't require immediately suitable recipients (Mapletoft . 2018). The slow freezing process is widely used today in MOET and can be done on farm where all that's necessary is a freezer machine. Embryos can easily be thawed and transferred directly negating the need for an embryologist to access embryos before transfer. On the other hand, vitrified embryos allow an over look and re-expansion access prior to transfer. New vitrification procedures where embryos are put directly into nitrogen is widely used in North America for IVP. Both techniques have pros and cons and these are discussed elsewhere (Mandawalla et al., 2016). For the slow freezing process embryos are placed in a hypertonic solution with low molecular weight permeable cryoprotectants to facilitate partial embryo dehydration and therefore avoid intracellular ice crystal formation during cryopreservation (Saragusty et al., 2011); while vitrification involves the use of highly concentrated aqueous solutions of cryoprotectants to prevent ice crystal formation transforming in an amorphous glassy during exceedingly fast cooling (Arav and Natan. 2019).

Zona Pellucida Hardening (ZPH) can be a result of cryopreservation, although the trigger of hardening naturally happens after fertilisation. Media presence of calcium in the vitrification solution can also cause ZPH in IVP system (Wiesak et al., 2017). Assisted hatching (AH), an easy procedure to open the ZP discussed further in this thesis, may help embryos to be released from their ZP and to implant in the uterine wall. It is known that AH can increase pregnancy rates in humans (Zeng et al., 2018). It has been shown that collapsing before vitrification has a positive influence on post-thaw blastocyst viability, which makes it possible to increase the chance of pregnancy onset after IVP-ET (Chang et al., 2011, Zhu et al., 2011). In summary cryopreserved embryos still have a lower conception rate after implantation, AH and optimal methods of freezing can help to improve pregnancies.

1.4 Pregnancy Outcomes Following Transfer

As mentioned previously, pregnancy outcomes with IVP embryos are the greatest challenge at this time. In vitro culture reduces the number of embryos capable of establishing pregnancies (Pontes et al., 2009; Ferraz et al., 2016). Compared to previous years, pregnancy rates following ET have not changed by much (Hansen. 2020[b]) (**Figure 1.5).** In 1992, non-surgical unilateral or bilateral transfers in heifers led to a 50% PR at 21 days and a pregnancy loss of 14% between days 35 to 90 (Reichenback et al., 1992). Block and Hansen. (2007) observed a PR of 32% when evaluating P4 levels in plasma at day 21, and reported a 49% pregnancy loss at day 40 of pregnancy. Stewart et al. (2011) successfully achieved 45.5% PR at 30 days but again normal drop of 13% was seen after 40 days. Not too long ago, Denicol et al., (2013), reported a loss of 19% during summer in the US, dropping from 36.4% at 30 days to 29% at 45 days of pregnancy. Pregnancy losses seem to be decreasing over the years, however.

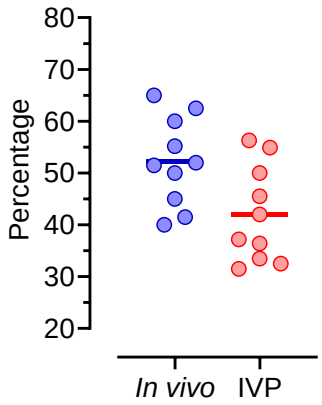

Figure 1.5. Data from several studies undertaken with *Bos taurus and Bos Indicus* cattle summarising pregnancy outcomes (percentage pregnant) at first pregnancy check after 30 of gestation following embryo transfer of either *in vivo* derived (MOET) or *in vitro* produced (IVP) embryos. *In vivo* derived: Lohuis et al (1990); Nogueira et al (2002)*; Nogueira et al (2002)[C]; Nogueira et al (2002)*; Nogueira et al (2002)*; Scenna et al (2005); Scenna et al (2005)[C]; Pontes et al. (2009); Faizah et al (2018); Vettical et al (2019). *In vitro* produced (IVP): Reichenbach et al. (1992); McMillan.(1998) Block et al (2005); Block and Hansen (2007); Loureiro et al. (2009); Pontes et al. (2009); Block et al. (2010); Stewart et al. (2011); Bonilla et al. (2014); Denicol et al. (2014). * = Different treatments in a same study, [C] = Group control. A simple t-test revealed that mean percentages differed (SED = 3.99; P=0.019) between embryo groups

1.4.1. Possible Reasons for Pregnancy Failure

Pregnancy failure can arise due to inherent deficiencies in the embryo or some deficiency in the recipient which undergoes ET. A number of factors are linked to recipient management including nutritional status, health, synchrony (with the embryo being transferred) and stress (Bo and Mapletoft. 2014, 2018[2]). Stressed cows have lower pregnancy rates.

Many changes in molecular and cellular biology occur in the embryo during the IVP, and these can affect pregnancy outcomes following ET (Hansen. 2020). Embryos with no visual morphological defects may harbour chromosomal errors (aneuploidy), associated with spontaneous abortions (Schmutz et al., 1996). Chromosomal abnormalities may also originate by penetration of ovum by more than one sperm cell (polyspermia). Mixoploidy, polyploidy and haploidy are all aberrations that are encountered frequently in IVP embryos (Viuff et al., 1999). Embryo inability to hatch outside the ZP or to implant are significant causes for pregnancy failure. Blastocysts formed in vitro have many characteristics that differentiate them from those generated in vivo by superovulation, such as oxygen consumption, accumulation of intracellular lipids, and changes in gene expression. Additionally, IVP embryos are more likely to contain abnormal chromosomes (Viuff et al., 1999; Tšuiko et al., 2017). The prevalence of mixoploidy in IVP blastocysts was reported to be 72% (Viuff et al., 1999). After the transfer of embryos, there has been evidence of reduced trophoblast growth, loss of the embryonic disk, altered placental function, and dysregulation of the fetal developmental process (Ealy et al., 2019). Evidently, there is not yet an efficient method for producing transferrable embryos in vitro in a high-throughput manner that overcomes all these challenges. On the other hand, tools to identify developmentally competent embryos are available today and these can improve pregnancy outcomes following transfer (**Figure 1.6).**

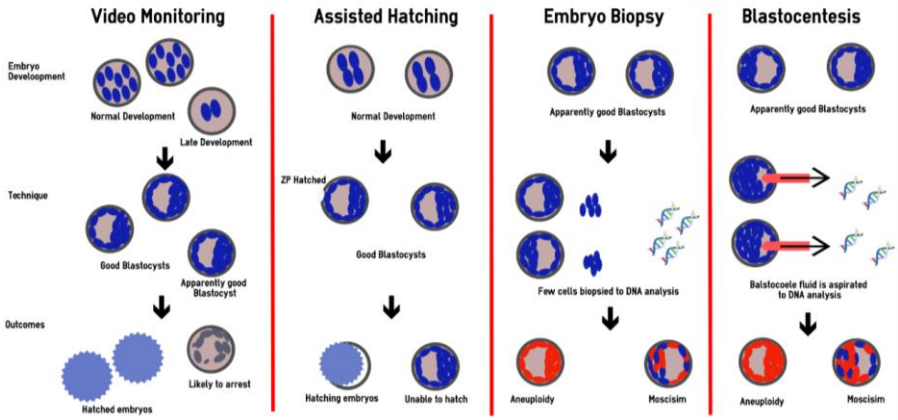

Figure 1.6. Different techniques used to ensure embryo quality prior to transfer. Video monitoring (time-lapse) can identify late development associated with aneuploid embryos. Assisted hatching can help embryos to hatch from the zona pellucida increasing the chances of attachment to the uterine wall. Embryo biopsy can be used to identify embryos that contain chromosomal errors or low Genetic estimated breeding values (gEBV), increasing not only the chances of a recipient to carry a healthy pregnancy to term but to produce high-genetic merit calves. Blastocentesis is considered less invasive than biopsy.

1.5. Zona Pellucida Hardening and Assisted Hatching

It is well-known that after fertilization, the ZP surrounding the oocyte undergoes a "hardening" process in order to prevent subsequent sperm from penetrating. After fertilization glycoprotein changes happens on the ZP, there is an increase in the level of calcium that cause ZPH (Sun et al., 2003), the conversion of ZP ZP2 to ZPf seems to contribute to ZPH (Schroeder. 1990).

Recently, failure of the embryonic ZP to rupture following blastocyst expansion has been proposed as a possible contributing factor in implantation failure and this has been attributed ZPH (Das et al., 2012). LAH can help to reduce the thickness of ZP and potentially improve pregnancy outcomes. Assisted hatching (AH) is a procedure of which a small hole is made in the ZP it helps the embryo to hatch outside the ZP. The most common techniques AH involve the use of a laser beam, Tyrode's solution, microblade or microneedles to ZP drill and aim to weaken the ZP for the embryo to hatch.

1.5.1. Zona Pellucida Hardening

ZPH is a natural process that is associated with fertilization (Nichols et al., 1989) serving as a block to polyspermy (Austin et al., 1961). The slow block of polyspermy

consists of a gradual barrier to sperm entry, as opposed to a fast block, which blocks additional sperm from fertilizing the egg initially but temporarily. These vesicles form a barrier by fusing cortical granules with the plasma membrane of the oocyte cortex (the area directly below the plasma membrane). A sperm-impermeable extracellular matrix is formed when the cortical granules are released, modifying the existing cellular extracellular matrix (Haley et al., 2004). ZPH per se does not impair the ability of the embryo to hatch. The imbalance of calcium within media and lack of enzymes involved in proteolytic digestion are more common mechanisms of ZPH associated with failure to hatch (Coy et al., 2008). Before fertilization a protein called ovastacin is stored in cortical granules, it cleaves ZP protein 2 (ZP2) upon fertilization and thereby destroys the ZP sperm ligand and triggers ZPH (Quesada et al., 2004; Burkart et al., 2012). Several factors are associated to ZPH in conventional IVP related to media used. Some constituents in oviductal fluid are known in several species to influence ZPH (Coy et al., 2010). A component of fetal bovine serum (FBS), called Fetuin, inhibits ZPH during oocyte maturation. It has been proposed that fetuin acts by preventing the proteolytic conversion of ZP2 to $ZP2_f$ by precociously released cortical granules (Schroeder et al., 1990). Fetuin has been shown to be effective in reducing ZPH (George et al., 1993). Heparin-like glycosaminoglycans (S-GAGs) can regulate ZP hardening allowing a return to the ‘‘non-resistant’’ state (Coy et al., 2010). They can also improve fertilization rate in IVP whilst apparently not increasing polyspermy (Dietzel et al., 2016).

The thickness and structure of the ZP has been widely investigated to predict the developmental potential of fertilized oocytes, as well as ART outcomes. It is reported that the thickness of ZP varies from 10 to 31 μm in human oocytes and influences sperm penetration during fertilization. Fertilisation success is highest for oocytes with a ZP <18.6 μm in thickness, and deteriorates above 22 μm (Bertrand et al., 1995). Duan et al., 2017, investigated if abnormal ZP thickness would have better results in ICSI rather than in vitro fertilization. They found that from 11,799 cycles of ICSI, fertilisation rate was higher for ICSI than fertilisation in-vitro (85% vs 64.3%) respectively. However, ICSI is not effective in cattle due to the failure of chromatin decondensation and inability to properly activate the egg limiting the application of ICSI in a commercial bovine laboratory. Another reason for ZP hardening is attributed to freeze-thaw process with oocytes, which can reduce fertilisation (Carrol et al., 1990). Bypassing fertilisation the ZPH will lead to implantation failure maintaining the embryo trapped into the ZP, cryopreservation of

embryos might also induce the endurance of the ZP and its structure changes (Moreira da SIlva et al., 2005).

1.5.2 Assisted Hatching

A substantial number of blastocysts after transfer fail to implant. Prolonged embryo culture may impair the embryo's ability to hatch and ultimately implant (Malter et al., 1989). AH is a technique of which a small opening in the ZP is performed, making holes, slots or thinning of different sizes; drilling, cutting, digesting, or melting the zona mechanically, chemically or with a laser beam to induce higher hatching and subsequent implantation rates following transfer (Miyata et al., 2010). It is poorly understood whether or not AH outcomes vary depending on the morphologic characteristics of the blastocyst or it's ZP. Inferring from the evidence that cleavage stage embryos with reduced ZP thickness have higher implantation potential, previous studies attempted to determine if the effectiveness of AH depends on ZP thickness (Nakasuji et al., 2014, Debrock et al., 2011). Only cleavage stage embryos with ZP thickness ≥ 15 µm demonstrated an improvement in implantation rate. Moreover, in cattle, the use of AH in lower quality embryos has the potential to increase pregnancy rate, and has demonstrated to be an easier approach to be performed on farm in ET embryos or when IVP embryos are transferred fresh (Taniyama et al., 2011).

Previous AHs were performed with partial zona dissection (PZD) pipettes, similar to a bevel needle of glass used to slice a small piece of the embryo. In this technique the embryo is held firmly in position by the holding pipette and an opening is made by introducing an injection/dissecting pipette through the ZP, followed by rubbing the embryo gently against the holding pipette until the embryo is released (Kim et al., 2012). ZP dissection can also be achieved by chemical 'drilling' (Handyside. 1991). In fact, the hole created by acidified Tyrode's depends on variables such as the amount of solution deposited, the time of exposure and operator skills. Jones et al. (2006) compared laser and acidified Tyrode's blastocyst hatching techniques and found similar results in terms of embryo quality. Tyrode's assisted biopsy, however, can take around 29 min while laser biopsy can be performed in 7 min (Geber et al., 2011). Tyrode's acid drilling reduces the number of embryos able to reach blastocyst stage due to cytoplasm acidification caused by Tyrode's (Fiorentino et al., 2008). Again, it will depend on the technician and the

quantity of Tyrode's injected during the zona drilling; also, blastomeres are more likely to be intact in laser assisted biopsy compared with Tyrode's.

1.6 Embryo Biopsy

It has been 30 years since the first embryo biopsy used to obtain a genetic sample for identifying embryo sex in cattle [by polymerase chain reaction (PCR)] (Thibier and Nibart. 1995; Bredbacka. 1998; Hasler et al., 2002). Nowadays using molecular-based genotypic selection techniques, there is a great chance to select for multiple production traits (Ponsart et al., 2014; Sirard. 2018). Genomics selection makes it possible to estimate the animal genomic breeding value (gEBV) and reduce the cost and generation interval compared to traditional testing (Bouquet and Juga. 2013). It also has the potential to identify poor quality embryos that are aneuploid leading to an enhancement in pregnancy rates. Chromosomal abnormalities constitute a major cause of embryonic loss in mammals (Jacobs et al., 1990; KINKG. 1990). In humans, chromosomal abnormalities lead to implantation failure, early pregnancy loss or severe chromosomal diseases such as Down's and Patau syndromes (Viaggi et al., 2013). In a more recent study, aneuploidy bovine embryos were related to a slower and regressed development during a time lapse monitoring by micro camera, most of the abnormal blastocyst presented mixploidy by diploid/triploid combination (Magata et al., 2019).

1.6.1 Preimplantation Genetic Testing (PGT), Biopsy Analysis

PGT previously known as preimplantation genetic screen (PGS) is today a well-known and used technique for searching genetic abnormalities on genes and chromosomal errors. Aneuploidy is the most common genetic abnormality in humans, and its high incidence in embryos is a major cause of failed implantation, pregnancy loss, and congenital defects in infants and children (Nagaoka et al., 2012). In Cattle, aneuploidy embryos also fail to implant and the identification of them has the potential to increase pregnancy rate as suggested by Turner et al. (2019) with 100 % concordance between biopsy and offspring single nucleotide polymorphism (SNP) analysis. PGT in bovine embryos favour the identification of gnomically desired traits that appeal to cattle breeders intensifying bovine earlier

selection reducing the costs of an unproductive cow or non-wanted traits in a specific breed.

1.6.2 Biopsy Technique

Currently, embryo biopsy can be performed with several instruments through a variety of techniques. In the most direct approach, the ZP opening is made by a mechanical, chemical or phototermolyses technique and a small number of blastomeres are aspirated using a micropipette (Herrera et al., 2016). The use of micro blade, aspiration pipette and needle are some common techniques of biopsy used for bovine embryos although laser biopsy has become established as a safe method recently and will be discussed further in this review. With microblade biopsy, 5-10 cells are aimed to be recovered, but normally a larger number of cells are collected (Peippo et al., 2007). It is placed on the edge of the embryo and moved down to retrieve cells. During the early development stage, blastomeres can be recovered from any portion of the embryo. Bredbacka et al. (1995) developed a technique to eliminate the need for a holding pipette, making some scratches at the bottom of the dishes in order to hold embryos prior biopsy. Lopes et al. (2001) successively introduced PBS containing 0.8% BSA to the dish, in order to prevent cell attachment to the cutting instruments or dish bottom, thereby improving microblade biopsy efficiency. Microblade biopsy is considered to be a more straightforward and quicker technique compared to needle and aspiration (Cenariu et al., 2012). When an embryo is biopsied at the blastocyst stage, it is important to avoid the inner mass cells, and TE cells should be removed preferentially.

1.6.3 Polar Body Biopsy (PBB)

Polar body biopsy (PBB) was used primarily for aneuploidy screening in humans (Verlisnky et al., 1996). The major application of PBB is the detection of maternally derived chromosomal aneuploidies and translocations in oocytes (Montag et al., 2009). In humans it can be used as an alternative when regulations prohibit embryo biopsy in a particular region or country (Küpker et al., 2001). Potentially less cost effective and limited to errors of maternal origin only, PBB is associated with lower implantation rates compared to blastocyst biopsy (Fragouli et

al., 2010). In livestock species little progress in PBB is found. Technically simpler to do PBB when compared with blastomere or TE cells recovery, a higher number of procedures is necessary and consequently a large number of aneuploidy is found. Fluorescence *in situ* hybridization (FISH), a molecular cytogenetic technique used to identify chromosomal regions through hybridization of flurescetly-labeled DNA and for chromosomal analysis in PGT for chromosomal imbalance, was the most common test used for PBB and blastomeres biopsy (Delhanty et al., 1997). The use of FISH on blastomeres increases drastically the number of false/positives and false/negatives in blastomeres (Harton et al., 2011); the mistaken results can be related to mosaicism, that is natural in early embryonic development where the chromosome set detected in the biopsied single blastomere may not represent the genotype of the embryo (Kuliev et al., 2005). Overlapping signal, split signal, chromosomal self-correction and fragmented DNA are all innate processes during embryo development (DeUgarte et al., 2008) increasing the chances to discard a healthy embryo. PBB may lead to a huge amount of work and a mistaken number of embryos potentially likely to implant. A recent study undertook repeated biopsies for pre-implantation genetic testing. In case of failed genetic diagnosis in the PBB, embryos were re-biopsied during second cleavage or at the blastocyst stage. Analyses at each stage yielded different results and predictions of implantation success (Priner et al., 2019).

1.6.4 Blastomere Biopsies (Cleavage Zygotes)

Cleavage stage biopsies are usually done at the 4-8 cell stage using a mechanical, chemical or laser tool to open the ZP. Usually, one or two cells are removed in a cleavage stage biopsy; a higher number of blastomeres retrieved risks the formation of the ICM and embryo death (Hardy et al., 1990; Tarin et al., 1992). However, biopsy at this stage has been shown to reduce embryonic potential (Scott et al., 2013[1]). Cleavage stage embryos are not associated morphologically with the extent of euploidy, while blastocyst stage is (Majumdar et al., 2017). Although cleavage stage biopsy still accounts for 90% of all reported PGT cycles in humans according to ESHRE PGD (Thornhill. 2019) a few reports of early stage biopsy are mentioned in animal practices nowadays, despite to be consider an easier procedure to undertake. A higher percentage of intact blastomeres can be collected from laser-drilled biopsy and acid Tyrodes (De Vos et al., 2001) specially when free

media with Ca++ and Mg++ are used to reduce the cells adhesion and facilitate the blastomere removal (Abdelmassih et al., 2001). Another procedure undertaken at this stage is called fragment removal; the fragments are commonly seen on embryos cleaving at early stage of live and changes in cells physiology were suggested to cause this fragmentation (Antczak et al., 1999). Removing this fragmentation can improve ART and it's suggested to be used only in embryos with approximately 15-35% fragmentation (Halvaei et al., 2018). At this stage embryos are not genetically diagnosed if there is no fragmentation to be anlaysed, however if embryos are fragmented they should be genetically tested (Hardy et al., 1990; Tarin et al., 1992) because the fragments will appear from apoptosis caused by cellular errors. It is recommended to use IVP where the embryos are genetically diagnosed before transfer (Antczak et al., 1999). In fact this approach is rarely undertaken in bovine embryos, firstly due to the unknown number of embryos that would reach the blastocyst stage, increasing the time of an embryologist to perform biopsies in unknown embryos and secondly due to the imprecision of DNA analysis for traits from one or two cells.

1.6.5 Trophectoderm Biopsy

Harvesting TE cells in a blastocyst has not been shown to have any detrimental effect on subsequent post-transfer development in humans and animal models such as mice (Carson et al., 1993), primates (Summers et al., 1988) and cattle (Agca et al., 1998). TE cell biopsy is widely adopted for biopsy because it analyses multiple cells of the trophoblast lineage, without reducing the ICM. Blastocyst biopsy is becoming more popular due to the higher number of cells collected compared to early biopsies and to represent a small proportion of the total number of cells in the blastocyst. Blastocyst-stage biopsy can be performed by making a hole in the ZP on Day 3-4 in humans (Rubino et al., 2020), however there is no data available in cattle. A procedure on Day 6 in cattle would possibly allow the developing TE cells to protrude after blastulation, facilitating biopsy. When TE cells are collected a higher number of cells and DNA can be harvested. Recently, Oliveira et al. (2017) compared biopsy at different stages of blastocyst development and found biopsy at the expanded blastocyst stage to give the best results (implantation rate of 61% vs 38% for earlier stages).

De Boher et al. (2004) reported one of the first studies to successfully undertake TE biopsy in human embryos, which subsequently led to successful pregnancy outcomes following transfer (McArthur et al., 2005). Embryo biopsy at blastocyst stage is now becoming more popular (Scott et al., 2013[2]). For blastocyst biopsy, McArthur et al. (2005) created a "hatch" on Day 3-4 of embryo development to encourage the herniation of TE cells outside the ZP. He applied a 1.48µm diode laser or a 500µs pulse to create a hole in the ZP and embryos were biopsied on Day 5 in the morning. Embryos were held with a holding pipette and extruded cells were collected at the 3 o'clock position; cells were collected within 3 to 5 laser pulses ranging between 2-9 cells (McArthur et al., 2005). However, this can increase the chances of the ICM extruding. Conventional biopsy in cattle is performed around Day 7 when TE cells are biopsied following expansion when the ZP becomes thinner and TE cells are expressed out. A micropipette is then used to retrieve a certain number of cells (Capalbo et al., 2014), thus avoiding embryo stress in previous development stages.

A recent technique uses a quick movement called "flicking", where the TE cells in the aspiration pipette are pressed against the holding pipette (Costa Borges et al., 2018). This rapid approach has been gaining in popularity among embryologists. Also there appears to be little damage caused to cells after biopsy (Benavent et al., 2019). It can also reduce the number of laser pulses during biopsy. Thus according to Scarica et al. (2018) it reduces the incidence of mosaicism due to laser pulses. The number of cells to be collected is one of the most critical factors for TE biopsy (Neal et al., 2017). Some researchers believe it must be >5 (Zhang et al. 2016, Schoolcraft et al., 2010). However, it is hard to count the exact number of cells when retrieving from the TE layer, there has been existing results from preimplantation genetic testing PGT of trophectoderm cells, (Adler et al., 2014).

1.7 Summary of Pregnancy Outcomes Following Embryo Biopsy

Blade biopsy is still mostly used in cattle with a higher number of procedures done in trophectoderm stage. Pregnancy rate ranges from 30% to 54% (El-Sayed et al., 2006, Souza et al., 2017) (**Table 1.3 and 1.4**); similar to those embryos biopsied by laser. In humans, Laser biopsy is first choice representing 75% of all biopsies (Moutou et al., 2014). Pregnancy rates range from 25% to 67% (**Table 1.5**).

Table 1.3: Studies employing embryo biopsy by blade on trophectoderm cells from bovine embryos produced in vivo and in vitro.

Biopsy	Stage	Re expansion after Biopsy	Re expansion after Cryopreservation	Pregnancy Rate	Author
Blade	Trophectoderm	82%±2.6% (24hs after biopsy)	-	-	(Ramos-Ibeas et al; 2014)A
		Biopsied without supplementation 27.5%±2.5% Biopsied with t10,c12CLA 64.6%±4.4	vitrified Biopsied without supplementation 62.5%±7.9% Biopsied with t10,c12CLA 95%±7	-	(Pereira et al;2008)B
		-	-	In vivo Biopsied at 30d 57.35% 60d 54.21% In vivo Control at 30d 57.64% 60d 55.89% IVP Biopsied at 60d 26.37% IVP control 60d 19.82%	(Souza et al; 2017)C
		94.6%	-	Single embryo transfer 44.2% Embryo Transferred in pair 14.3%	(Peippo et all 2007)D
		72.6%±13.8	-	Biopsied 47.1%±7.9 Control 42%±8.5	(Oliveira et al;2017)E
		-	40% biopsied 61% Control	-	(Gonzalez et al; 2008)F
		85%	-	30%	(El-Sayed et al;2006)G
		-	-	57% on Day 45, 43% on Day 65 (fresh embryos: 45% and 38% respectively)	(Fisher et al; 2012)H

A- 190 d8 hatched, Grade 1 and 2 embryos were biopsied. Monolayer culture system + mouse embryonic fibroblast support the growth of bovine Trophoblast cells. **B-** 47 early to expanded blastocysts were biopsied, 23 with linoleic acid t10, c12 and 24 without it. The aim was improve micromanipulation by supplementing media with trans-10, cis-12 conjugated linoleic acid. **C**- 471 embryos were biopsied and had pregnancy rate (PR) compared to 456 biopsied control embryos. **D-** 74 IVP embryos were biopsied to evaluate re-expansion while 134 were biopsied to evaluate the accuracy of genotypes. The PR data are from 57 in vivo biopsied sex-selected embryos transferred singly and in pairs. **E-** 222 embryos were biopsied to evaluate their implantation compared to non-biopsied embryos. **F-** 110 Grade 1 Blastocyst were used in this experiment, 55 were biopsied and 55 were control. **G-** A total of 138 IVP embryos were biopsied. After re-expansion they were transferred and pregnancy diagnosis checked on days 25, 32, 40 and 50. **H-** 42 in vitro blastocysts (cf. 42 fresh embryos), Vitrification - 2 h incubation and evaluation before transfer.

Table1.4: Studies using either a laser or aspiration pipette in embryo and evaluation of outcomes.

Biopsy	Stage	Re expansion after Biopsy	Re expansion after Cryopreservation	Pregnancy Rate	Author
Laser	Trophectoderm	-	-	41.4%	(Turner et al; 2019)A
		-	Biopsied 84.6% Control 69.4%	Fresh 54.9% Frozen Biopsied 42.1%	(Campos-Chillon et al; 2015)B
Aspiration Pipette	Blastomere	4-8 cells 66.7% 8-16 cells 73.3%	-	-	(Shirazi et al; 2010)C
		-	Biopsied and vitrified 85%	Biopsied and vitrified 44% Frozen 23% Fresh 50%	(Agca et al; 1998)D
		Hatched D2 4-8cell 70% D3 4-8cell 58% D4 8-16cell 53.8%	-	-	(Shirazi et al; 2010)E
		Blastocyst of Biopsied 53.3% and 44.9% hatched. Blastocyst of control were 66% and 42.6% hatched.	-	-	(Polisseni et al; 2010)F
		-	-	47.3%	(Lacaze et al; 2009)G
	Trophectoderm	-	-	62.5%	(Lacaze et al; 2009)H

A- From 111 embryos biopsied, 93 were amplified WGA, 89 SNP typed and 61 fulfil all requirement for Karyomapping. **B-** Two group of embryos were biopsied, one with laser assisted drilling (LAD) and one with Piezo assisted drilling (PAD). The PAD group was cryop reserved within three different methods but they did not differ. **C-** Comparison of bovine embryos at days 2, 3 and 4 of development using aspiration pipette to drill the zona pellucida. **D-** Embryos biopsied and vitrified in comparison with embryos frozen, followed by fresh transfer. **E-** This study evaluated the quality of bovine blastocyst derived from embryos biopsied at day 2, 3 and 4 by assessment of cryosurvivability of the resulting blastocysts. **F-** Evaluation of 8-16 cell-biopsies by assessing blastocyst development. A total of 92 embryos were submitted to biopsy and 103 were used as a control **G**-**H**- 167 embryos were biopsied followed by direct or frozen transfer.

Table 1.5: Evaluation of human blastocysts and cleavage stage embryos biopsied by laser and Tyrode's acid.

Biopsy	Stage	Re expansion after Biopsy	Re expansion after Cryopreservation	Pregnancy rate	Author
Laser	Trophectoderm	100%	-	47.6%	(Kokkali et al; 2007)A
		-	-	43.4%	(McArthur; 2008)B
		-	-	67%	(Ramos et al 2019)C
	Blastomere	50.4%	-	26.7%	(Kokkali et al; 2007)D
		-	-	25.6%	(McArthur; 2008)E
		-	-	Day 3 biopsied 30.8% Day 4 biopsied 43.8%	(Kim et al; 2015)F
		Good Quality (GI)32.5%(GII)32.6% (GIII)32.4% Poor Quality (GI)67.5%(GII)67.4% (GIII)67.6%	-	-	(Taylor et al; 2010)G
Tyrode	Blastomere	BF 53% CF 58%	-	BF 28% CF 28.5%	(Magli et al; 2006)H
		48.9%	-	-	(Geber et al; 1995) I

A- 53 Blastocysts biopsied, with 26 unaffected for β-thalassaemia syndrome, from those 21 were implanted. **B-E** Embryos biopsied at day 3 and transferred at days 5-6. **C-** 55 embryos biopsied where 32 were genetically normal. Only euploid embryos were transferred. **D-** 101 embryos early stage (6-8 cells) embryos biopsied. 76 embryos gave a reliable diagnosis where 47 were unaffected for β-thalassaemia syndrome. 30 blastocysts were implanted. **F-** Laser biopsy was performed on 126 day 3 and 150 embryos on day 4. **G-** 240 embryos were biopsied and divided in 3 groups of a different pulse length. (GI) 0.604mS, (GII) 0.708mS and (GIII) 1.010mS. Embryo re expansion was assessed**. H-** 47 embryos were biopsied and frozen (BF) and 150 were only frozen (CF). **I-** 47 embryos were biopsied at the 8 cell stage and followed daily until day 6.

1.8 Blastocentesis

Blastocentesis is a relatively new technique used to collect DNA from a blastocyst-stage embryo by using a fine ICSI pipette which is inserted between two TE cells, to minimize trauma and contamination, and blastocoel fluid is aspirated (Gianaroli et al., 2014). The technique consists of a rapid procedure to evaluate Blastocyst fluid (BF) and replaces current biopsies offering a non-invasive technique to identify mosaicism and aneuploidy (Zhigalina et al., 2016). Tsuiko et al. (2019) found a high concordance between BF, TE and ICM to karyotype. Despite the relatively small amount of BF that is recovered, there was success using the DNA from BF for whole-genome amplification and polymerase chain reactions. Chromosomal analysis can also be achieved using array comparative genomic hybridization (Palini et al., 2013, Zhigalina et al., 2016). Furthermore, next-generation sequencing of BF-DNA has been successfully completed. Furthermore, the sequencing profiles generated from whole-genome amplified products were similar to those obtained by the corresponding blastomeres both at the chromosome and gene level (Zhang et al., 2016).

However, there are issues with regard to the yield of DNA and possible contamination from the surrounding media that could contribute to diagnostic errors (Hammond et al., 2017). Perloe et al. (2013) reported that embryonic DNA can be detected in the BF of some embryos, although only three of nine BF samples (33.3%) were concordant with the corresponding TE biopsy. In an additional study, the discordance in karyotypes reached up to 50% between BF and TE biopsies (Tobler et al., 2015). The most likely source of cell free DNA is from embryonic cells undergoing apoptosis (Hardy. 1999). Palini. (2013) noted that concentrations of DNA less than 5 pg were insufficient for analysis and that the most successful samples contained around averaged 10 pg DNA.

1.8.1 Additional Considerations of Biopsy vs Blastocentesis

Additionally blastocentesis is technically easier to perform compared to the laser application during a biopsy procedure represents a rapid and replicable procedure for DNA analysis. On the other hand, with laser assisted biopsy a number of issues concerning the timing, precision and effectiveness of the procedure need

to be taken into account; also pregnancy establishment and the risk of congenital disorders (Montag et al., 2009[b]). The laser beam can also induce DNA damage and compromise subsequent development (Wang et al., 2018). The temperature profile in the beam and vicinity is predicted as a function of laser pulse duration and power are important considerations (Douglas-Hemilton et al., 2001). Nd:YAG (Neodymium, Yttrium Aluminum Garnet, or Nd:YAG) lasers were initially used for ART to trap spermatozoa with a wavelength of 1064 (Tadir et al., 1989); however, there are risks associated with these lasers, including UV radiation damage. Later, an erbium:YAG laser with a wavelength of 2900 nm was introduced (Feichtinger et al., 1992). However, it required direct contact, which raises concerns about damage and contamination. Moreover, holmium, yttrium-scandian-gallium-garnet (Ho:YSGG) laser using at 2100 nm wavelength exhibited different absorption behavior in water than earlier lasers; moreover, it required quartz slides. Assisted reproductive technology now includes six types of lasers that use infrared emitting diodes currently available in the market for non-contact micro-drilling using a 1480-nm diode laser (Davidson et al., 2019). Zone breaching and TE biopsy can be shortened by using this approach and complexity is reduced. This strategy, however, has yet to be evaluated in terms of its potential disadvantages. In addition, the toxic compounds released from heat diffusion haven't been determined, which must stay below 100°C. However, in a recent experiment using laser biopsy at 400 mW for 0.25 ms over 41% pregnancy rate was described in cattle following ET (Turner et al., 2019), confirming that good results can be achieved.

1.9 Working Hypothesis, Aims and Objectives.

In the series of experiments reported in this thesis, we set out to identify the most straightforward and reliable method of embryo biopsy to be adopted in a commercial laboratory, considering the practicality of the procedure to be performed for a technician inexperienced in micromanipulation. We hypothesise that the use of laser to perform embryo biopsy, following herniation of the TE would reduce the time taken for each biopsy and improve precision; thereby providing enough cells to analyse while preserving embryo integrity. Our second objective was to assess the benefits of LAH for IVP embryos in order to ensure the ability of cryopreserved embryos to hatch. We hypothesise that LAH would increase the proportion of embryos able to hatch. The aim was to develop LAH as a fast and easy procedure

to incorporate into the commercial laboratory, with the longer-term aim of improving pregnancy rate

2. CHAPTER 2: A COMPARISON OF HIGH-THROUGHPUT APPROACHES FOR BLASTOCYST BIOPSY INVOLVING EITHER A MICROBLADE OR LASER

2.1 Introduction

Embryo biopsy is becoming increasingly used in human assisted reproduction due to its ability to identify monogenic disorders by PGD (Sciorio et al., 2020, Spits et al., 2009), and to identify inherited genetic diseases, Robertsonian translocations and chromosomal aneuploidy through preimplantation genetic testing (PGT-A) (Sciorio et al., 2020). Aneuploidy is the most frequent type of chromosomal abnormality and can increase pregnancy loss during early development (Vitez et al., 2019). Many aneuploid embryos can reach the blastocyst stage and often don't arrest until after transfer (Fragouli et al., 2013).

Several biopsy approaches have been performed since the introduction of PGD and these are still debated today to establish the best embryo development stage for biopsy reducing the risk of damage to the embryo (Thornhill. 2019, Montag. 2019, Wang et al., 2018, Magli et al., 2016). Although biopsy at Day 3 post fertilization was found to be an easier technique to collect cells for PGT-A among different techniques (Tarin and Handdyside. 1993) and was when the majority of biopsies was performed (Capalbo et al., 2017), TE cell biopsy has become more attractive in recent years and has been replacing cleavage stage biopsies (Zacchini et al., 2017) . Scott et al. (2013), in a clinical study, found that biopsied blastocysts gave a higher number of live-born infants compared to biopsied cleavage-stage embryos. Early cleavage-stage biopsies have several disadvantages such as 1) low cell number, 2) choice of blastomeres, 3) possible selection of cells allocated to the inner-cell mass, and 4) a higher proportion of embryonic cells removed (Thornhill. 2019). Recent literature reviews reflect that early-stage biopsies can reduce implantation rates by up to 40% while the impact of later stage trophectoderm biopsies is less than 2% (Leigh. 2019). Another alternative method used to increase the efficiency in human PGT-A is the use of a laser beam pulse given a day prior biopsy, thereby allowing the cells to herniate outside the zona pellucida (ZP). As describe by McArthur et al. (2005), this approach at the early blastocyst stage can facilitate the collection of cells during micromanipulation reducing any procedure harm.

In cattle, the combination of PGT and reproductive biotechnologies in young heifers and genetically superior cows permits a greater rate of genetic progress for selected traits (Ponsart et al., 2014). Genomic selection based on single nucleotide polymorphism (SNP) has increased the dissemination of more productive cattle through early genetic selection, and has led to the ability to derive genetic estimate breeding values (gEBVs) at the embryonic stage. Bovine embryo biopsy to facilitate this has been found to be a safe (Oliveira et al., 2017), can easily determine embryo sex (Sachan et al., 2020), provide a robust estimate of gEBV, (Saadi et al., 2014, Mullaart and Wells. 2018), and have no negative effect on pregnancy rates using either in vivo derived or IVP embryos (de Souza et al., 2017). However aneuploidy is known to be common in IVP embryos. Karyomapping can explore both PGT-A and PGD (Turner et al., 2019, Tutt et al., 2021) and lead to higher pregnancies.

Current embryo biopsy procedures use either a blade, or laser and aspiration pipettes (Cenariu et al., 2012). TE cell biopsy provides a good genetic and cytogenetic record of the whole embryo (Tutt et al., 2021). However, these procedures can be technically challenging, slow, potentially damaging to the embryo and expensive. Given the number of cattle embryos that in future are predicted to require gEBV and PGT, there is a need to develop a high-throughput system for cattle-embryo biopsy. In this study we evaluated the use of blade and laser assisted biopsy in herniated and non-herniated embryos at the blastocyst stage to identify the easiest, quickest and most precise procedure for future gEBV and PGT-A analyses.

2.2 Materials and Methods

2.2.1 Oocyte Source

Ovaries from regional slaughterhouses in the UK were collected and immediately put into a thermal flask (~36°C). No fluid was added to the flask and ovaries were transported to the laboratory. At the laboratory, the temperature of the flask was measured and maintained at 35°C±2°C in a water bath (Grant, Cambridge, UK). Ovaries were then rinsed with aspiration (IVF Bioscience®, Copenhagen, Denmark) media and dried with blue roll individually prior aspiration. An 18G aspiration needle, attached to a 4mm Brazilian tubbing (Partnar®, Holland, USA) in a Rocket Pump R29700 (Watford, England, UK), was used to aspirate the

contents of all follicles between 4-10mm of diameter into a 50ml corner tubing. The follicular aspirant was rinsed using a filter cup, held in a warmed filter stand (~35°C), using IVF Bioscience® media. This aspirant was then searched using a 100mm petri dish under a 40X magnification microscope. All cumulus-oocyte complexes (COCs) were graded from 1-4 according to IETS manual where COCs with expanded or absent cumulus, or with fragmented, pale or irregular cytoplasm were classed Grade 4 and rejected.

2.2.2 *In Vitro* Maturation (IVM)

Selected COCs were then placed in 1 ml maturation vials from Boviteq Canada Laboratories at ~4 h following collection from the slaughterhouse. Groups of around 30 COCs were matured for 20-24 h in HEPES buffered TCM199 media supplemented with 10% (v/v) FBS, 4 mg/ml fatty acid free BSA, 0.2 mM pyruvate, 50 µg/ml gentamicin, 5 µg/ml FSH, 0.5 µg/ml LH, 1 µg/mE 2). During this period, COCs were incubated in a Cryologic transportable incubator (Blackbourn, Australia) at 38°C. Fertilization dishes were prepared with fertilization media (tyrodes lactate media supplemented with 0.6% (w/v) fatty acid free BSA, 1.5 µg/ml heparin, 0.2 mM sodium pyruvate, 0.08 mM penicillamine, 0.04 mM hypotaurine, 10 mM epinephrine, 50 µg/ml gentamicin) and incubated in a Binder® incubator at 38°C, 6.7 CO_2 and 5% O_2 to equilibrate for the next day.

2.2.3 *In Vitro* Fertilization (IVF)

At the end of IVM, COCs were washed 3 times in IVF Bioscience wash media® and 5 COCs were placed in each 40 µL drop of fertilisation media in a 30 mm petri dish. Prior to insemination, semen were prepared using a gradient of percoll 45% and 90% (Bovipure™, Nidacon, Mölndal, Sweden). Semen from a sire with proven fertility (Emphaisis Boldi) was used for this experiment at 0.5 $X10^6$/ml concentration. Frozen straw of semen was thawed in a 37°C water flask for 45 s and placed in a small PCR tube with 150 µL of 45% gradient and 150 µL of 90% gradient on the bottom. The tube was spun in the centrifuge at the speed of 600rpm for 5 min, the supernatant sperm on the top of the tube was removed and 30 µL of the sperm on the bottom were transferred into a 400 µL of gradient media in a second PCR tube. The tub was spun at 1300 rpm for 3 min. A 30 µL volume containing the sperm pellet

was taken from the bottom of the tube and transferred to an empty PCR tube; 2 µL of sperm from this tube was inserted into 40 µL of water in another tube for sperm counting. Then, 10 µL of the sperm mixed with water was placed in a Neubauer chamber (NC) and spermatozoa were counted individually. The total number of spermatozoa present in the NC were entered into this formula: n° (number of sperm) X 0.042 (expected concentration of sperm) X 28 (total volume of media, remained from the 30 µL) = X (total value of the formula) - 28 (total volume of media). The result number from the formula represents the amount in µL of gradient medium to be added to the sperm tube. A 5 µL aliquot of 1% heparin was added to the fertilization drop containing 2 µL of mixture of penicillamine, hypotaurine and epinephrine (PHE), and then 2µL of spermatozoa was added to this drop containing COCs. Fertilization dishes were placed into a Binder® incubator (Tuttlingen, Germany) at 38°C humidified atmosphere with 6.7% CO_2 and 5% O_2.

2.2.4 *In Vitro* Culture (IVC)

After 20-22 h, presumptive zygotes (Day 1) were stripped and transferred to a 14 µl drop of culture media '1' consisting modified SOF based sequential culture media (mSOF), as previously described (Nivet et al., 2012), in a humidified environment at 6.7% CO_2 and 5% O_2, at 38°C within a Miri® incubator (Kringelled, Denmark). Cleavage was evaluated after 72 h and embryos (Day 4) moved into 14 µl drops of culture media '2'. In the morning of Day 6, embryos were transferred into culture media '3', and split into 4 different treatment groups across 4 different 30 mm petri dishes.

2.2.5 Embryo Biopsy-Treatment Groups

The study involved four biopsy treatments set out in a 2 x 2 factorial arrangement (Herniation vs Non-Herniation; and Blade vs Laser). A total of 139 Day 6 embryos were split into four biopsy-treatment groups. Group I (Non-herniated, Blade biopsy): 39 embryos, from the early blastocyst to expanded blastocyst stages, were biopsied using a microblade (Bioniche Quebec, Canada) on Day 7 post-fertilization. Group II (Non-herniated, Laser biopsy): 39 embryos were submitted to laser-assisted biopsy on Day 7. Group III (Herniated + Blade biopsy): 30 embryos that had their ZP punctured by laser on Day 6 – 6.5 allowing cells to herniate from

the ZP, were biopsied on Day 7 using a microblade adapted in the micromanipulator. Group IV (Herniated + Laser biopsy): 31 embryos that had their ZP punctured on Day 6 – 6.5 (described above) were biopsied on Day 7 with the aid of a laser following herniation.

2.2.6 Embryo Biopsy

Prior to biopsy, which used a RI integra 3^{TM} micromanipulator (Knardrupvej, Denamrk), embryos were transferred to 50µl drops of culture '3' media equilibrated within a biopsy dish (falcon® 50mm X 9mm, BD, USA). There were four drops in each dish covered by mineral oil. Each drop contained one embryo for biopsy. In Group I, embryos were placed with Inner cell mass (ICM) at the 6 to 9 o'clock position held by a holding pipette (Orgio®, MPH-MED-35, USA) at a 35°angle (120-150µm of diameter outer diameter and 15-20 µm inner diameter). The blade was placed horizontally on the edge of the embryo at the 12' o'clock position. A scratch was made on the bottom of the biopsy dish to prevent the embryo rolling during the procedure as described elsewhere (Bredbacka et al., 1995), and trophectoderm cells were biopsied aiming for no more than 20% of the whole embryo in those not herniated. Embryos were released and holding free blade biopsy was performed, moving to the next drop until the dish was completed. In Group 2, embryos were biopsied on Day 7 post-fertilization using the same type of biopsy dish. Embryos were held with a holding pipette with ICM at the 9 o'clock position, and trophectoderm cells located at 3 o'clock (**Figure 2.1**). Using an aspiration pipette 30°angle (Orgio®,USA) (23-27 µm inner diameter) a few soft pushes were made on the ZP to release any attached trophectoderm cells before laser beam shots. Between 1 and 3 laser pulses were administered at 164 µw in order to open the ZP. The aspiration pipette was then inserted and a target number of ~10 cells were aspirated into the pipette. When these cells were located in the pipette another few pulses of 164 µw were made in order to detach the sample cells. In Groups III and IV, the culture petri dish was kept with lid on and the ZP opening was performed without the need of holding pipette. Between Day 6 and 7, at early blastocyst stage (stage 5), the ZP was punctured using a laser beam. Embryos that could not have the ZP opened on the opposite side of the ICM were then rolled with a handmade glass pipette. In order to create a hole in the ZP 1 to 3 laser pulses at 164 µw were applied. Embryos were placed back into the culture incubator to be biopsied the

following day when cells were extruding from the ZP. The extruded cells were collected by blade biopsy in Group 3 and aspiration pipette and laser biopsy in Group 4 (**Figure 2.1**). At the end of the biopsy procedure, blastocysts were transferred to culture '3' dish in order to re-expand. Re expansion was analysed after 3 h following biopsy or accessed on the morning of the following day.

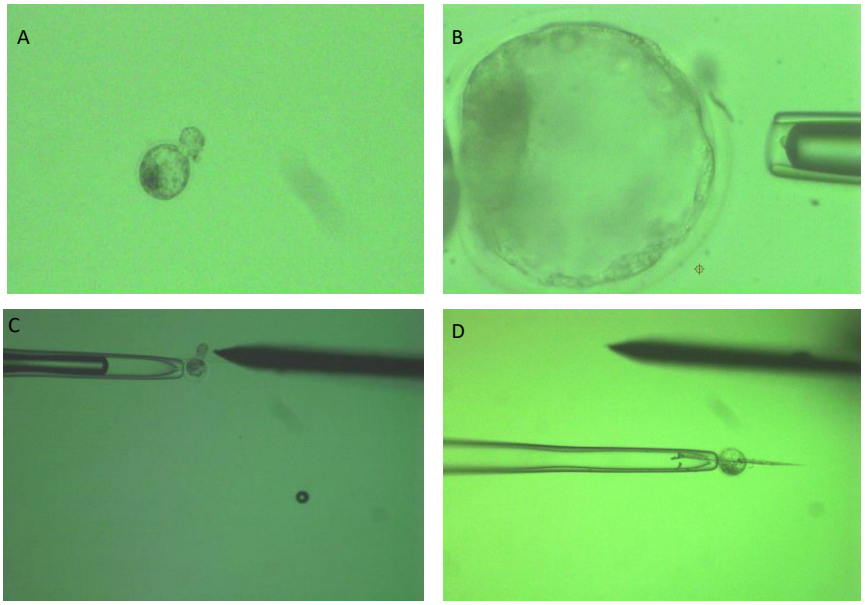

Figure 2.1. Embryo biopsies. **A.** Herniarted blastocyst with IMC at the opposite side of extrusion ready to undergo laser biopsy one day after the ZP was punctured at the early stage of blastocyst development (IETS Stage 5). **B.** Non-hernitated blastocyst (IETS Stage 6) on Day 7 ready for laser biopsy with aspirating pipette .**C.** Herniated blastocyst using the blade biopsy procedure; extruded cells are aligned with the scratch on the bottom of biopsy dish to avoid embryo rolling. **D.** Blastocyst ready for biopsy by blade held in the screatch to avoid it rolling during procedure. Blastocyst in these images are around 110 µM

2.2.7 Embryo Freezing preparation Following Biopsy

Approximately 3 hours after biopsy embryos were prepared to immunostaining. Embryos that were re-expanded partially or had not re-expanded were left to be accessed the next day. After re-expansion all embryos were transferred into an Eppendorf ® tube (Hamburg, Germany) of 1% Polyvinyl alcohol (PFA) (150 µl Phosphate buffer saline (PBS) and Polyvinylpyrrolidone (PVP) diluted in 50µl of 4% PFA), labelled and it was left to rest during 20min before put into a -10°C freezer.

2.2.8 Biopsy Cell Counts

Cells were collected with a striping pipette orgio® EZ-grip 0.09mm (Knardrupvej, Denamark) in minimal media and placed on a slide under a NIKON SMZ18 microscope. The media was allowed to evaporate (around 10-20 min), and 1 µl of 70% EtOH was gently placed on top of the sample and also allowed to evaporate. Then 0.5 µl of Heamatoxylin was added to pigment the nuclei of the cells. Another 5 µl of 5% acetic acid in 70% EtOH was then added on top of the cells and allowed to dry. The number of cells collected during biopsy was counted under a microscope using a 20X objective lenses (**Figure 2.2**).

2.3 Statistical Analyses

Analyses were performed using the GenStat statistical package (19th Edition, VSN International, 2018; https://www.vsni.co.uk/). All proportion data, associated with embryo biopsy and re-expansion, were analysed using generalized linear models that assumed binomial errors and used logit-link functions. Terms fitted to these models were 'Replicate', 'Herniation (No v Yes)', 'Instrument (Blade v Laser)' and interactions between these terms. Cell and laser-pulse count data were analysed using the same models but assumed Possion errors. Data are presented as adjusted means ± SEM.

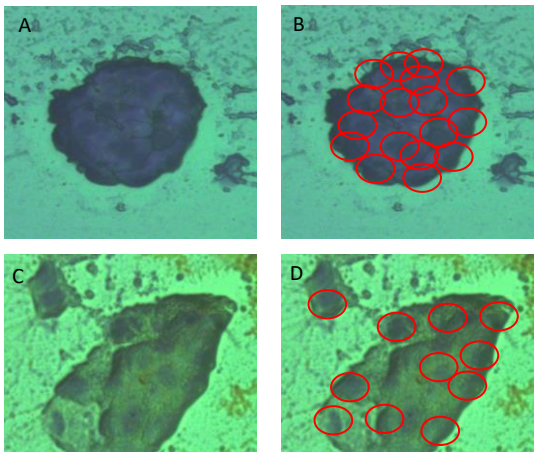

Figure 2.2. Biopsy cell counts. **A.** An 18-cell sample collected by blade biopsy from a herniated blastocyst. It is possible to see a high number of cells agglomerated together. **B.** Red cycles demonstrate were nuclei from cells

are in the figure a. **C.** An 11-cell sample collected by laser biopsy from a herniated blastocyst. Cells can be laid flat making it easier to count lower numbers. **D.** Red cycles are showing nuclei cells from laser biopsy more distant compared to blade sample.

2.4 Results

A total of 4,832 follicles were aspirated and 2,331 (recovery rate 48%) COC's collected, of which 1,072 (78 IETS Grade 1, 281 Grade 2 and 756 Grade 3) were placed into maturation. The proportion cleaved of matured averaged 74% (796/1072) and the proportion cleaved that reached the blastocyst stage averaged 24% (186/796). Of these blastocysts, 139 were used in this study, while the other 47 served for training purposes.

Embryos that had their ZP breached on Day 6 to 6.5, thus allowing herniation of cells, were generally easier (P=0.001) to biopsy compared to those that were not induced to herniated; this was independent of the instrument used (i.e., blade vs laser) which did not differ statistically (**Figure 2.3 A**). The criteria for difficulty concerned the number of cells recovered (with respect to the target number [5-10 cells]) and embryo survival after the procedure. Related to the ease of biopsy was the fact that induction of herniation reduced (P<0.001) the time taken to undertake biopsy on Day 7 (**Figure 2.3 B**). On this occasion, however, it was evident that biopsy time was less (P<0.001) for the microblade compared to the laser.

A total of 122/139 (88%) embryos re-expanded following biopsy, and this value did not differ between biopsy procedures (**Figure 2.3 C**). However, there was a difference (P=0.031) between herniation and instrument which indicated that the proportion blastocysts that re-expanded following biopsy was greater (90%) when laser biopsy was undertaken on herniated blastocysts compared to the other biopsy combinations (66%) (**Figure 2.3 D**).

The number of blastomeres retrieved was associated (P=0.014) on whether or not the blastocyst had herniated prior to biopsy; it also differed (P<0.001) between biopsy instruments (**Figure 2.4**). Herniation prior to biopsy led to a small increase in the number of cells recovered (13.7 vs 11.5 blastomeres), whereas the use of the laser compared to the microblade for biopsy led to a substantial decrease in the number of recovered cells (8.9 vs 16.1 blastomeres). Herniation prior to biopsy also increased the proportion of biopsies that contained blastomeres from within the ICM (**Figure 2.5**).

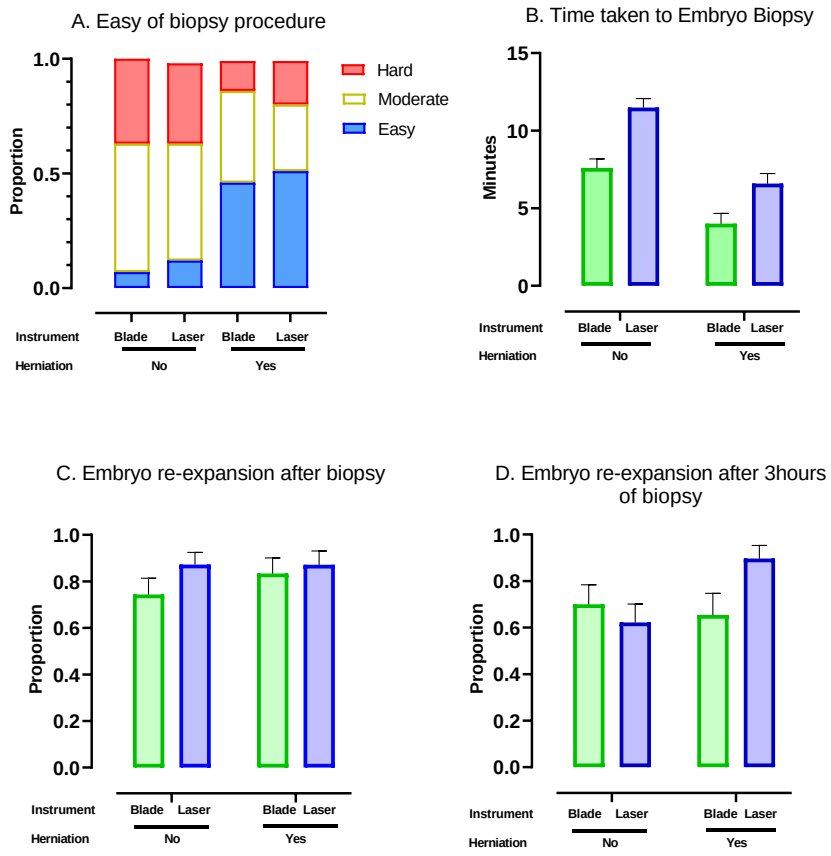

Figure 2.3. Operational parameters associated with embryo biopsy. **(A)** Proportion of biopsy occasions classified as being easy, moderately difficult or difficult. **(B)** Time required for biopsy. **(C)** Proportion blastocysts that had re-expanded within 24 h of biopsy. **(D)** Proportion blastocysts that had re-expanded with 3 h of biopsy. Herniation prior to biopsy was considered easiest (P=0.001) irrespective of which instrument (blade vs laser) was used (**A**). Time required for biopsy was less (P<0.001) for herniated than non-herniated blastocysts and when the microblade rather than the laser was employed for biopsy (**B**). Proportion re-expanded blastocysts within 24 h did not differ between biopsy treatment combinations (**C**). Herniation followed by laser biopsy led to a greater proportion re-expanded blastocysts within 3 h (**D**). * Significant different between herniation and non-herniation. ** Instrument significant different.

Finally, the cumulative number of laser pulses required for the entire biopsy procedure was greatest (P<0.001) when laser-assisted biopsy followed herniation (**Figure 2.6**). In fact, the application of laser pulses to perform embryo herniation, added to the number of laser pulses for biopsy, it is expected to be greater than for those embryos where the biopsy was performed by the use of the blade.

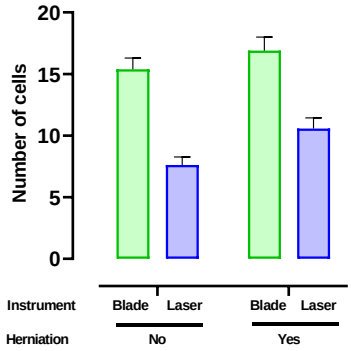

Figure 2.4. Number of blastomeres present in blastocyst biopsies. Herniation prior to biopsy increased (P=0.014), whereas laser biopsy decreased (P<0.001), the number of cells recovered on each occasion. *Laser biopsy have significant smaller number of cells in both groups.

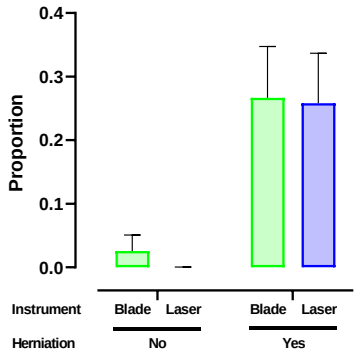

Figure 2.5. The proportion of biopsies that contained blastomeres from the ICM. Herniation prior to biopsy increased (P<0.001) the chance of containing cells from the ICM. *Significant proportion of ICM cells.

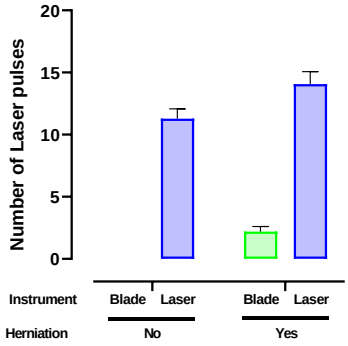

Figure 2.6. Cumulative number of laser pulses required for each method blastocyst biopsy. The number of laser pulses applied was greatest (P<0.001) when laser-assisted biopsy followed herniation. Means with a different superscript differed at P<0.05.* Significant number of laser pulses in both groups.

2.5 Discussion

In accordance with the more recent literature, we focussed on the blastocyst stage where TE cells can provide a sufficient number of cells to be analysed which have high concordance to the ICM (Tufekci et al., 2019, Victor et al., 2019, Tutt et al., 2021). Also, determined whether the approach influences the speed and precision of biopsy. Three main findings emerged from this study. Firstly, our hypothesis that herniated TE cells facilitate biopsy for a non-experienced technician, and does not lead to any obvious technical harm to the embryo, such as complete destruction of the blastocyst or visible embryo disjunction, is supported. Secondly, we identified that laser biopsy is more precise in collecting the desired number of cells when compared to blade biopsy, making it easier to predict the number of cells collected. However, herniation followed by laser biopsy was easier for the non-experienced technician. Finally, the incidence of ICM inclusion in the section of the embryo herniated is increased, and this could have implications for subsequent embryo development and survival, although this has yet to be determined.

2.5.1 Herniation vs Non-Herniation

It is clear that herniation of the pre-hatching embryo at the early blastocyst stage facilitated the biopsy procedure by reducing the time taken compared to those embryos that were not herniated (**Figure 2.3 A**). This was mainly due to the reduced effort of pulling TE cells away from the ZP at the blastocyst stage while holding them with a pipette. Aggregated time of embryo exposure to manipulation over four hours is sufficient for the occurrence of molecular and morphology changes (Gordon. 1994); for example, proteins belonging to the family of the heat shock proteins (Hsp) of 70 kilodaltons (kDa) are expressed as a reflection of cell stress (Pedersen et al., 2005). These proteins are one of the first to be produced during embryonic development and is of great importance for cell function (Basu et al., 2002). Reducing the combined time of blastocyst exposure was one of the key factors to avoid molecular (possibly epigenetic) changes. When opening the ZP a day before biopsy, the speed of procedure was prioritized to reduce embryo exposure to the environment. For this reason we kept the petri dish lid on and ZP opening was performed without the holding pipette. Our method of not using a holding pipette for ZP drilling accelerated the process of micromanipulation, reducing the time to open

the ZP to less than 20 seconds per embryo. It facilitates introduction to a commercial laboratory setting during regular media change protocols, without significantly increasing the time that embryos are exposed to atmospheric conditions. Capalbo et al. (2014) described a non-herniation technique for biopsy of the TE without ZP opening prior the procedure. This study showed some benefit for the laboratory routine. Because the timing of biopsy for an embryo at hatching stage is critical, excess herniation of TE may induce arrest. However, technicians should still avoid excessive TE herniation. Continuous observation using time-lapse monitoring technology and direct TE aspiration with non-assisted hatching may be helpful for detecting and preventing early arrest (Campbell et al., 2013). Non-assisted hatching has disadvantages. For example, if blastocyst collapse occurs during TE cell aspiration, the biopsy procedure may be difficult and be associated with a higher risk of losing the ICM. Furthermore, blastocyst collapse may extend the biopsy time.

Laser assisted aspiration of TE cells was quicker in herniated embryos overall. However, compared to the first three replicates, the average time taken to perform laser biopsy in non-herniated embryos reduced by 33% in Replicate 4, confirming that experience improves technique speed. Blade biopsies were quick in both herniated and non-herniated groups. As described by Bredbacka et al. (1995), we made a scratch at the bottom of the biopsy dish in order to avoid embryos rolling during the procedure and to increase precision on cell numbers recovered. It was efficient in avoiding embryo rolling, but it did not help to accurately predict the number of cells collected.

2.5.2 Sample Accuracy

To collect a small number of cells from an embryo may be less invasive but increase the risk of amplification failure when analysing DNA (Treff et al., 2011). On the other hand, a higher number of cells may lead to a more reliable amplification but may be harmful for the blastocyst. In this experiment we found a higher proportion of re-expansion at 3 h in those embryos biopsied using the laser technique that consequently had a smaller number of cells collected (**Figures 2.3.** and **2.4**). In our study we aimed to collect between 5-10 cells from each embryo, following the consensus among researchers (Schoolcraft et al., 2010, Zhanhg et al., 2016). Also, Fisher et al. (2012) found that single-cell or three-cell sample failed to yield enough DNA when genotyping, however the test chosen to amplify DNA has

an influence (Munne and Wells., 2017); the number of cells collected should be in accordance to the blastocyst biomass (Cimadomo et al., 2018) increasing the chances of a more accurate chromosomal analysis.

Although chromosomal abnormality, a biologic event occurring at any stage of embryo development, can have an impact on the accuracy of diagnosis based on the analysis of only 5–10 cells from a blastocyst with >100 cells (Capalbo[b] et al., 2016), biopsy of TE cells is likely to provide a higher yield of DNA and present a higher accuracy compared to cleavage stage biopsy (Vera-Rodriguez and Rubio. 2017; Treff et al., 2017). Although, retrieving a smaller number of embryonic cells has a less harmful effect on the embryo development (Treff et al., 2011), Cimadomo et al. (2018) showed that eight TE cells were necessary for conclusive DNA amplification after biopsy of ~9,000 embryos. Thus, the most suitable cell number for biopsy may be 5-10 cells rather than 1-5 cells, or more than 10 cells which would represent a larger portion of the embryo and could cause a reduction in euploid-embryo implantation according to Neal et al. (2017).

Blade biopsy was easy to perform, however it was more difficult to accurately predict the number of cells biopsied. A higher number of cells were collected with blade in both herniated and non-herniated embryos compared to the laser plus aspiration pipette, as seen in the **Figure 2.4**. In accordance with de Souza et al. (2017), biopsies obtained from the trophoblast of a blastocyst with a microsurgical knife had a call rate following whole genome amplification and genotyping similar to that obtained with a 30- to 40-cell morula and a 50-cell bisected blastocyst, but this larger number of sample cells can reduce the ability of the embryo to continue to develop. Laser biopsy provides a more accurate number of desired cells. It was possible to observe the cells getting into the aspiration pipette and apply the laser beam in the junction of cells to be left behind with lower damaged caused to the cells. Biopsy with herniated embryos increases the speed of biopsy and causes no severe damage to the embryos.

2.5.3 Inner-Cell Mass Herniation

The ICM can herniate outside the ZP following micromanipulation prior to biopsy (Capalbo et al., 2016). However, only a small number of embryos in this study had their ICM herniated. We believe that the developmental stage chosen to puncture the ZP (i.e., late morula (IETS Stage 4) or early blastocyst (IETS Stage 5)

was important in reducing the incidence of ICM extrusion. Generally, ZP opening at early blastocyst stage can reduce the incidence of ICM herniation due to the fact that, at this stage, the ICM is visible allowing the technician to make the branch on the opposite side, once that naturally ZP hatching tend to be near to the embryonic pole (Negrón-Pérez et al., 2017). Even so, around 36% of embryos hatch near the ICM (Niimura et al., 2010). Physical damage to the ICM during the biopsy can be irreversible and may arrest embryo development. Also, a double ZP opening to avoid ICM during the biopsy, can increase the time of each biopsy and hinder sample collection. In our experiment approximately 24% of embryos that were in group III and IV had the ICM extruded. In order to avoid extra ZP opening and ICM damage, we collected cells during herniation, collecting TE cells from the single ZP gap performed a day before, thereby avoiding an increase in the time of the procedure. With existing techniques, when the ICM is herniated a second ZP opening is made to biopsy trophectoderm cells and to avoid the ICM (Rubino et al., 2014). We simply push the TE under the ICM, thereby collapsing the blastocoele cavity providing more space to manage the TE through the ZP opening without harming the ICM.

2.5.4 Supplementary Findings

Re-expansion of all embryos didn't differ after biopsy. In fact there was a suggestion that laser biopsy could increase re-expansion, either in those herniated or not, and it was higher when herniated embryos were biopsied by laser, reaching almost 90% re-expansion after approximately 3 hours (**Figure 2.3 D**). Embryo biopsy followed by cryopreservation and thawing, could also lead to identification of safe procedures that would help embryos to continue to development and improve hatching after implantation. It is necessary to further investigate this technique to ensure a high survival rate after standard cryopreservation in ethylene glycol, most commonly used for direct transfer practices.

There is a sense that embryos that receive several laser pulses are not adversely affected, even when going from 10-20 laser pulses at 290 µw/s to 40 laser pulses at 400 µw/s using an infra-red indiumgallium-arsenic-phosphorous (InGaAsP) semiconductor diode laser (Johnson et al., 2019). In our protocol we applied a small InGaAsp diode laser pulse length at 164µw/s with as few pulses as possible in each technique **(Figure 2.5).** Its unique absorption characteristics in

water and culture media allowed for non-contact application in standard culture dishes and avoided mutagenicity and contamination (Davidson et al., 2018). The reason for a small pulse is because the laser pulse can increase the heat emitted in the media (Taylor et al., 2010). Thus creating a smaller gap in the ZP, allowing a slower and smaller portion of TE cells to be expelled, reduces the need of rigor in estimating cells.

2.5.5. Conclusions

Herniation of TE cells help to increase the speed of embryo biopsy when laser assisted, but it is unpredictable to collect a desired number of cells when using a blade for biopsy. Also, for a non-experienced technician the performance of herniated biopsy helps in the reduction of time per biopsy although after a few sessions it is basically similar to the non-herniated laser biopsy procedure. We also conclude that the use of laser biopsy during the blastocyst stage represents the best method for incorporation in a commercial laboratory, accelerating the precision of biopsy for DNA analysis. More studies evaluating the speed of biopsy and viability after cryopreservation will support the laser biopsy for commercial applications.

3. CHAPTER 3. LASER BIOPSY OF HERNIATED AND NON-HERNIATED TE CELLS ARE EQUALLY SAFE FOR BOVINE EMBRYOS AND HAS POTENTIAL USE IN THE COMMERCIAL LABORATORY

3.1 Introduction

Biopsy for preimplantation cytogenetic testing (PGT-A) and genetic analyses (PGD) has been developed as a clinical tool for increasing implantation rates and reducing the risk of miscarriage while helping to identify desired genetic traits in cattle (Mullaart and Wells. 2018). However, its effectiveness is still in dispute. There are different motivations to use embryo biopsy, such as selection of female embryo (Park et al., 2001), high reproductive traits selected by Marker assisted selection (MAS) (Ponsart et al., 2014, Humblot et al., 2010), better feed efficiency and other traits (Mullaart and Wells. 2018). In addition, there are some other concerns regarding the potential harm caused by biopsy for embryo development and implantation rate (Makhijani et al., 2021, Zacchini et al., 2017). Several studies have shown that the adverse effects of biopsy for PGT/PGD on embryo development and pregnancy rate might depend on the stage of embryo development (Zacchini et al., 2017, Scott et al., 2013, Treff et al., 2011), and some other factors such as cell type, the number of cells to be biopsied, instrumentation used and embryo quality, (Cenariu et al., 2012, Cimadomo et al., 2018, Cimadomo et al., 2016). Studies have also shown that even a single TE biopsy at the blastocyst stage may cause a decrease in implantation rate and fetal growth (Rubino et al., 2020), while more recent studies have demonstrated that TE cell biopsy can be more accurate than early embryo stages causing lower harm to embryo development (Leigh. 2019). In our previous experiment (Chapter 2) we showed that the use of laser biopsy has a more accurate number of cells biopsied and can lead to a rapid and safe system of blastocyst biopsy in a commercial laboratory with embryos recovering after the procedure.

Commercial application of in vitro embryo biopsy relies on the ability to perform it accurately within a reduced amount of time in order to avoid higher risks of atmospheric exposure which is toxic for embryos (Karagenc et al., 2004, Catt et al., 2000, Harvey et al., 2007). Also, in reducing the risk of cryopreservation of embryos

with an opening in their ZP, IVF embryos are less resistant to cryopreservation compared to those produced in vivo due to the higher lipid accumulation (Sanches et al., 2018), where lowering the temperature can cause irreversible damage such chromosome and DNA abnormalities (Fuijihira et al., 2004, Arav et al., 2008). Also, it is debated whether the amount of laser contact with the medium and embryo can impact DNA. Herrero et al. (2019) stated that TE biopsy may increase the risk of mosaicism while Kelk et al. (2017) concluded that TE biopsy cause no harm to DNA. There are many studies claiming that lasers do not have negative effect on DNA using different sources of laser from diode to solid-state lasers and some non-laser systems have been used in different animal models as well as with human embryos (Taylor et al., 2010).

In our previous chapter we found the optimum time, instrument and number of cells to be followed in a commercial laboratory system without cryopreservation of the embryos after biopsy. In this experiment we hypothesize that opening the ZP a day before the biopsy, could accelerate the procedure on the day of biopsy and increase survivability of these embryos that would be shortened exposed to the environment and consequently cryopreserved. In the present study we aimed to identify the easiest approach for TE biopsy in herniated or non-herniated cells from blastocysts, and to investigate if TE biopsy in both groups would affect embryo re-expansion and survivability after cryopreservation in comparison with non-biopsied embryos.

3.2 Material and Methods

3.2.1 Ovaries Preparation and Follicles Aspiration

Fresh slaughterhouse ovaries were collected, when Covid19 regulations allowed us to, from a local abattoir in Carlisle, UK, placed in a flask container at 36°C and transported to the Paragon Veterinary Group laboratory in Penrith, UK about 1h away. Prior to follicle aspiration, ovaries were washed with Phosphate Buffer Saline (PBS) and IVF Bioscience® (Copenhagen, Denmark) washing media warmed to 35°C in a water bath (Grant, Cambridge, UK). A 20G 1/5 inch needle attached to a 20ml syringe was used to aspirate follicles between 4-15 mm in diameter. 15ml conical tubes were placed in a warmer rack and all aspirants placed

into the tube. An average of 177 oocytes were collected from each of the 7 replicates where 120 were put into maturation per cycle.

3.2.2 Oocyte Retrieval and Grading

Aspirants were then filtered in 120 ml Emcon filters (Agthech UK) warmed at 36°C with IVF Bioscience® OPU media also maintained in a warmer water bath at 36°C. The filter was rinsed with OPU media and poured into a 90 mm petri dish for searching under a 40X magnification microscope. All oocytes were collected from the dish with a 20 µm pipette and placed into a small 30 mm petri dish containing IVF Bioscience® wash media before grading and placed into maturation vials. Grade I oocytes were defined in those with compact cumulus investment of more than three cell layers surrounding the oocyte. Cytoplasm consistency and colour were also evaluated to determine gamete grade. Those classified as Grade II had a small number of cumulus layers around the oocyte, typically up to 3 layers of cumulus cells. Grade III COCs with fair and consistent cytoplasm, and with very low degradation, were also put into maturation despite having only one cumulus cells layer. Those oocytes that have no cumulus cells or high degradation on their cytoplasm, or those atretic with no cumulus compaction, were discarded.

3.2.3 *In Vitro* Maturation

Grade I, II and III oocytes were placed in 1 ml maturation vials from Boviteq Canada Laboratories at ~4 h following the process described in (Chapter 2) using the same equipment. 4 vials containing 30 oocytes each were maturated in a Cryologic transportable incubator (Blackbourn, Australia) at 38°C while fertilisation medium was incubated in a Binder® incubator at 38°C, 6.7 CO_2 and 5% O_2 to equilibrate medium for the fertilization on the next day, as described in Chapter 2.

3.2.4 *In Vitro* Fertilisation

COCs were washed 3 times in IVF Bioscience wash media® where 5-6 COCs were placed in each 40 µL drop of fertilisation media in a 30 mm petri dish. The fertilisation procedure was the same as that used in Chapter 2. In this experiment a semen check evaluation was performed 20 hours after fertilisation under the

microscope (Olympus SZ 61TR, Tokyo, Japan) to ensure semen quality. Where all samples were the same quality with sperms spinning and swimming forwards after the period.

3.2.5 *In Vitro* Culture

After 20 hrs, presumptive zygotes (Day 1) had the cumulus cells detached from the ZP with a 3 µl stripping pipette (RI, EZ-GripVITROMED, Wildenbruchstrasse, Germany), and transferred to a 14 µl drop of culture media consisting of modified SOF based sequential culture media (mSOF), as previously described (Nivet et al., 2012). They were cultured in a humidified environment at 6.7% CO_2 and 5% O_2, at 38°C within a Miri® incubator (Kringelled, Denmark). Cleavage was evaluated after 72 h and embryos (Day 4) moved to 14 µl drops of culture media '2' (Boviteq, Canada). Day 6, embryos were transferred into culture media '3', and split into three different treatment groups across four different 30 mm petri dishes, before initiating the treatment. Each culture drop followed the minimal volume/embryo suggested (6-14 µl) by the Boviteq laboratory, in order to provide sufficient nutrients for development. All embryos were graded according to IETS manual (2010).

3.3 Treatment Groups

On Day 6 post fertilization, embryos that had developed at least into the morula stage were identified and divided in three groups: Group Control (Con), Group II Laser (L), Group III Herniation + Laser (H+L). In each group, embryos were transferred into a 14µl single drop that allowed individually each embryo to be tracked. A table of identification was made in each dish for each group, allowing to observe the development of embryos at every assessment individually.

Group Con 55 embryos were assigned to this group where no biopsy was undertaken. Group L, 55 embryos were biopsied on Day 7 post fertilization using our laser biopsy system (Chapter 2). Approximately 55% of these embryos were biopsied at the mid-blastocyst stage, and 12% and 32% were at early blastocyst and expanded blastocyst stages respectively. In Group H+L 51 embryos were assigned at the mid-blastocyst, early blastocyst and late morula stages, when the beginning of ICM compaction was evident. These embryos had the ZP punctured using 1-3 diode laser pulses (at 164µw/s) 16-20 h before embryo biopsy. All groups

had their diameter measured across the embryo, with crossing lines at the centre using the calliper settings adjusted in microns by the a RI software in the integra 3™ micromanipulator (Knardrupvej, Denmark) on Day 6 post fertilisation, Day 7 biopsy and before freezing at Day 8.

3.4 Biopsy

Group Con had no biopsies performed, they were assessed at the same stage as the other treatment groups on Day 6, 7 and 8 post fertilisation. Group Con blastocysts were cryopreserved on Day 8 together with re-expanded embryos from Group L and H+L. All embryos were punctured 1-3 times with a 240 µw/s laser beam to ablate the blastocoele cavity prior to freezing. In Group H+L, the ZP opening was perforated after transferring these embryos into the single culture drop using diode laser from a RI integra 3™ micromanipulator (Knardrupvej, Denmark). There was no necessity of holding or aspirating pipettes to perform ZP opening, the lid was also kept on the dish to reduce environmental exposure and increase speed of biopsies per embryo. Day 7 embryos were assessed and graded according to their morphology. The embryo and the extrusion of trophectoderm cells outside the ZP opening (**Figure 3.1 C and D**) were measured in the micromanipulator individually. Four embryos were placed in individual 50ul drops of culture media in a biopsy dish (falcon® 50mm X 9mm, BD, USA) in order to be biopsied. The biopsy was performed under the micromanipulator using a holding pipette Orgio® (MPH-MED-35, USA) at 35°angle (120-150µm of diameter outer diameter and 15-20 µm inner diameter), and an aspiration pipette at 30°angle Orgio® (USA) (23-27 µm inner diameter). The aspiration pipette was washed in PVP to prevent cells getting attached in the internal pipette wall. Embryos were held with the Inner-Cell Mass cells at the 9 o'clock position when possible or on the opposite side of the extrusion to be biopsied. The aspiration pipette was positioned as close as possible to the corner of the extruded cells and a small number of cells were collected. To detach TE cells from the embryo, a small number of diode laser pulses were used in a small length of 164µw/s. Biopsied embryos were then placed in the culture dish again to allow re-expansion until Day 8 and then cryopreserved of biopsied embryos. In the morning of Day 8, embryos were cryopreserved in a slow freezing protocol in an ethylene glycol freezer.

In Group L embryos were biopsied at Day 7 post fertilization using the micromanipulator at 37°C. Embryos in this group had no extruded cells (**Figure 3.1 A and B**) so ZP opening was performed on the day of biopsy. Embryos were held using the holding pipette at the embryonic pole at the 9 o'clock position and 1 to 3 diode laser beam (164µw/s) was applied to the ZP at the 3 o'clock position in order to create an access point for the aspiration pipette. The aspiration pipette was positioned against the TE cells and a small number of cells aspirated from the embryo. Embryos that had their cells aspirated were placed back into the culture dish to re-expand overnight and be cryopreserved on Day 8.

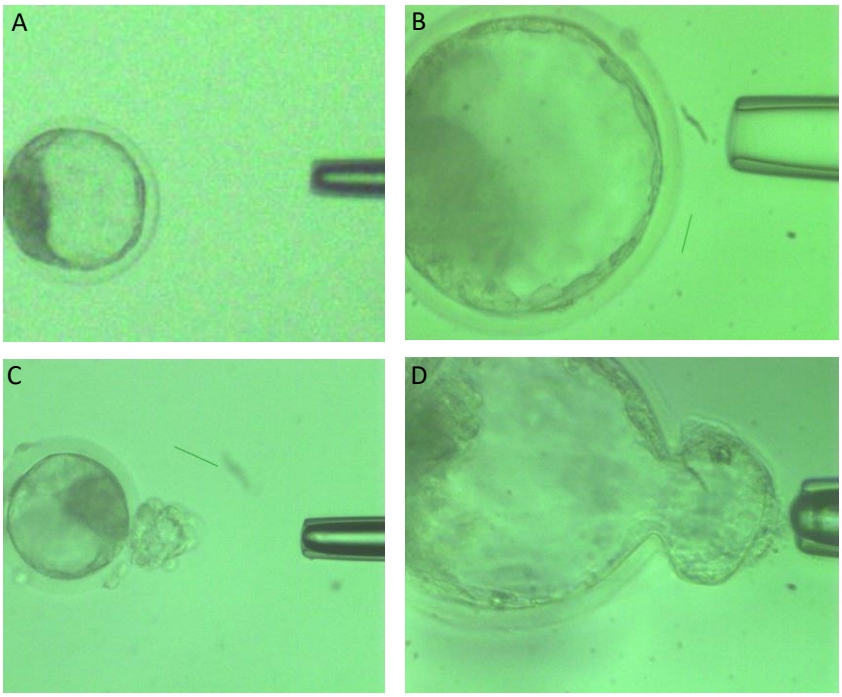

Figure 3.1, A. Intact ZP in non-herniated embryo before biopsy with TE cells attached to the internal wall. **B**. TE cells of non-herniated embryos detaching the ZP internal wall. **C**. Herniated TE cells with small ICM going out the ZP. **D**. Herniated TE cells outside the ZP with a blastocoele fluid enlarging the gap in the ZP. Blastocyst diameter in these images are around 104µM

3.5 Cryopreservation

On Day 8 post fertilization, approximately 12-18 hours after biopsy, embryos that had re-expanded were cryopreserved using a slow freezing process in an Ethylene glycol freezer Biocool® FTS system (Pensilvania,USA) at -0.5°C/min rate.

Embryos were measured and blastocoele cavity was collapsed with laser beam before embryos were washed in holding and freezing media. No more than 18 embryos were cryopreserved at each time to respect the time of dehydration on the protocol. Each well of a six well dish had a tag to identify each embryo place before cryopreservation. Embryos were then transferred to a dish with freezing media and kept there for 15 to 25 minutes before being placed in the freezer (Biocool controlled FTS System). They remained in the freezer for 10 minutes after seeding and temperature reduced at a rate of 0.5°C /min until they reached -34°C. Embryos were then placed into a liquid nitrogen tank.

3.5.1 Embryo Thawing and Re-expansion

To assess biopsied embryos after cryopreservation, we rehydrated them with holding (Boviteq®) media. Embryos were thawed in a 30°C semen-thaw cup (Dairymac, Hempshire-UK), for 30 seconds. Embryo straws were then dried with blue roll and the plug containing the information of them was pulled out. The back of the embryo straw was cut and embryos was pushed into a 90mm search dish for identification and consequently transferred to a 6 well dish. Using a 200ul pipette, each embryo was placed in a six-well dish and 50 µl of warm holding media was mixed together. After 1 minute, another 100 µl of holding media was added. One minute later, 200 µl of holding media was added to the well and each embryo was washed one minute later in two 100 µl drops of holding media. After the final wash embryos were placed in a 14µl culture drop to allow re-expansion and were observed at intervals of 2h until 12h, and then the next day after 24hs. After 2hours those embryos that expanded were kept in the culture drop of expansion until 6 hours then cryopreserved with other embryos that were expanded at 4 or 6 hours after placed at the re-expansion culture drop.

3.5.2 Re-Thawing and Immunostaining

After re-expansion, embryos were cryopreserved for a second time to be immunostained with fluorescent stain 4'6-diamidino-2-phenylindole (DAPI) at the University of Nottingham, Biosciences laboratory. A total number of 135 embryos from L, H+L and Con groups were re-thawed in Boviteq freezing medium in three 20 µl drops before being washed and placed to incubation for 19h. Embryos that re-

expanded were submitted to a 1%PFA PCR tube with 16 µl PBS/PVP, 1 µl DAPI and 0.1% Triton x100. The staining solution contained 0.1% PVP/PBS + 2 µl of 1mg/ml of DAPI and 20 µl of 10% Triton. Afterwards, immuno-stained embryos were captured under the microscope and cell counting performed using Image J software. The Image J settings were adjusted to better identify overlapping cells with process of binary and filter image with 12.0 unsharp mask, revealing higher accuracy of cells counting. (**Figure 3.2**)

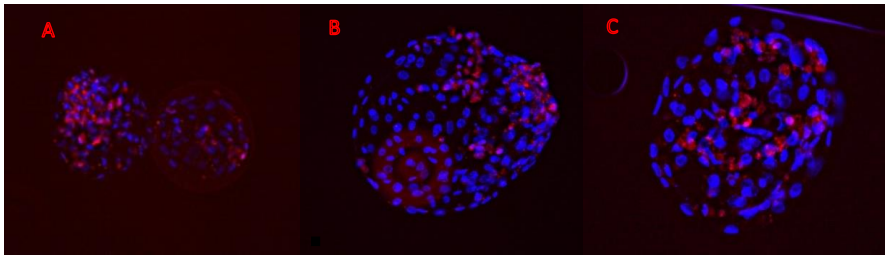

Figure 3.2. Immunostained blastocysts with two different colour,Dapi=Blue and Red=SOX2 of cells that were identified by 8 and 16-bit, to increase the accuracy of counting . **A**. Immunostained embryo from H+L group with extruded cells outside the ZP with more blacoele fluid between both structures compared to L and Con groups. **B.** Embryo from Group L. **C**. Group Con embryo. Blastocyst diamater in these images were around 98µM.

3.6 Statistical Analysis

Analyses were performed using the GenStat statistical package (19th Edition, VSN International, 2018; https://www.vsni.co.uk/). All proportion data, associated with embryo biopsy and re-expansion, were analysed using generalized linear models that assumed binomial errors and used logit-link functions. Terms fitted to these models were 'Replicate', and 'Treatment'. Cell and laser-pulse count data were analysed using the same models but assumed Possion errors. Data are presented as adjusted means ± SEM.

3.7 Results

Over 7 replicates a consistent number of 120 oocytes were put into maturation followed by fertilization 24 hours later. Proportion cleaved averaged 53% (445/840) and 36% (163/445) developed to produce Grade I and II blastocysts. A total 163 embryos were assigned to this experiment, 55 Con, 55 in the L and 51 in the H+L. All embryos were approximately 150 microns in diameter at Day 6. By Day 7, Embryos from Group H+L were smaller in size compared to those in Group Con and L ($P<0.01$) but were larger by Day 8, when the herniation extruded (**Figure 3.3**).

Following biopsy on Day 7, only 6% of embryos in Group H+L were completely hatched before freezing compared to 16% from Group Con and L.

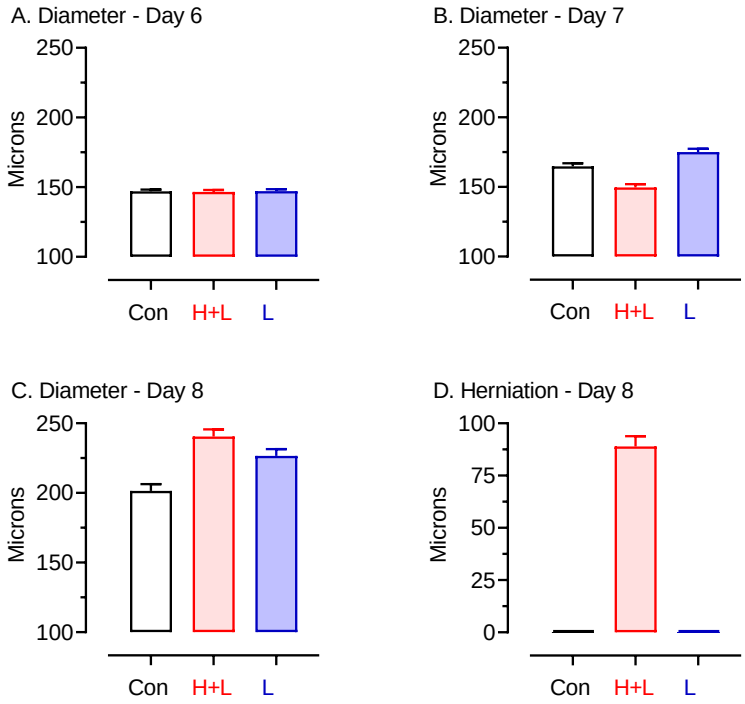

Figure 3.3: Embryo diameter. **A.** Day 6 embryos from three treatment groups at the early blastocyst stage. H+L embryos were submitted to ZP opening with a 164µw/s. **B.** Day 7 Con and L groups expanded more (P<0.01) than H+L.* H+L Significant smaller 24hs after ZP opening **C.** Diameter of Day 8 embryos from H+L increased rapidly in size compared to Con and L groups; mainly due to herniation that averaged 88 microns reaching 230 microns in embryo size after biopsy. **D.** Illustration of the herniation size in microns after ZP opened at Day 6.

Biopsy procedure was faster in Group H+L(P<0.05) compared to Group L (2 min38 s vs 3 min 37s) However, during last three replicates, Group L and H+L were similar in time taken (2min:51s and 2min50s respectively), indicating that the herniation technique might not increase speed when more experience is acquired to perform laser biopsy. Time for biopsies at different stages of development did not differ (Group H+L [BL and EXBL] vs Group L [BL and EXB] of 2min37s, 2min30s vs 3min31s, 3min37s) respectively.

The number of cells collected during biopsy did not differ between treatments. The Group H+L mean 9.1 ± 0.79 cells per biopsy compared to 8.2 ± 0.91 from Group L. When biopsying Group H+L, EXBL embryos averaged 9.3 ± 1.33 cells compared to 7.2 ± 2.09 EXBL embryos from Group L. The number of cells collected during

biopsy of BL embryos in H+L and L group were similar at 8.4 ± 0.62 and 9.0 ± 0.57 respectively.

The survivability of embryos following biopsy did not differ from the Group Con, demonstrating that biopsy is secure and safe to perform in bovine embryos not causing any obvious harm to embryo expansion after thawing (**Figure 3.4)**. The proportion re-expanded was not significant different. Although the Group I reached it maximum re-expansion by 6 h following thawing with 0.8208 ± 0.04825 re-expanded, Group L and H+L increased between 6 to 12 hours after thawing (Group L, from 0.8167 ± 0.04925 to 0.8532±0.04498 and Group H+L from 0.8027 ± 0.05220 to 0.8218 ± 0.05033). Embryos from Group Con, L and H+L had 0.8205±0.04824, 0.8542 ± 0.04510 and 0.8410± 0.04876 re-expansion consequently after 24 h.

Immunostained embryos did not show any difference in cell number between the three treatment groups (**Figure 3.5**). The average number of cells was 100.2 ± 3.533, 105.4 ± 3.517 and 100.5 ± 3.472 in Group Con, L and H+L respectively. All groups had at least 48h of culture after cryopreservation and were thawed twice before immunostaining.

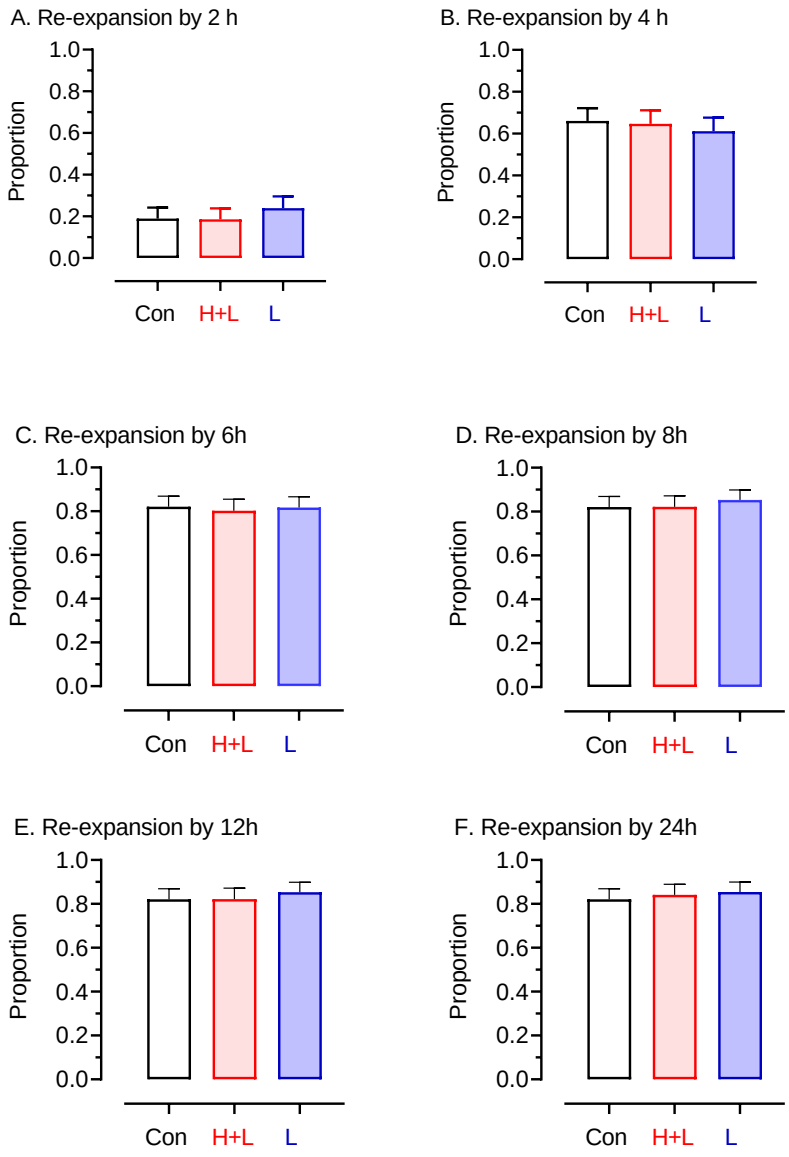

Figure 3.4: Post thaw re-expansion. **A**. Two hours after thawing, re-expansion was slightly higher but not significantly so in Group L compared to Group Con and H+L. **B.** A slightly higher proportion of Group Con embryos have achieved full re-expansion after thawing compared Group L and H+L. All groups continue to increase re-expansion at 4h after thawed. **C**. Group Con reach the full re-expansion. **D** All groups achieve full re-expansion at this point. **E**. Full re-expanded groups with non-significant difference, the same can be seen in figure **F** where Group L have a small proportion of the embryos higher expanded followed by Group H+L and Con (non-significant)

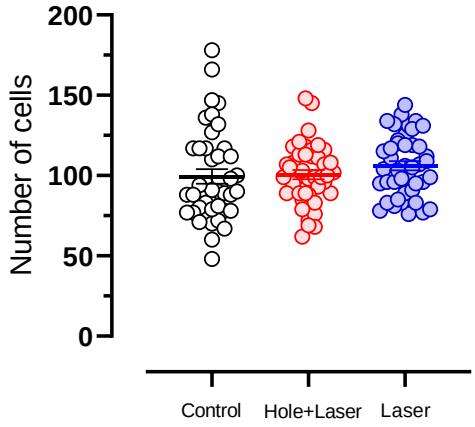

Figure 3.5. Number of cells present in the embryo after second cycle of re-expansion and thawing, with a more constant number of cells present in those embryos biopsied compared to control.

3.8 Discussion

The aim of this study was to ensure that laser assisted biopsy does not reduce embryo survivability and that it is safe to perform in the commercial laboratory using our system. Two main findings emerge from this study. Firstly, that embryo biopsies involving herniation or straight laser-assistance are equally quick in the hands of an experienced technician in a commercial laboratory. Secondly, that laser biopsy in our system inflicts no apparent harm on embryo development.

3.8.1 Biopsy Speed

Generally, single TE cells biopsy needs to be performed within 3 minutes (Capalbo et al., 2016) so it can avoid longer period of embryo exposure to the environment outside the incubator. This has been demonstrated to be detrimental to mammalian embryos (Zhang et al., 2010). In our system, the time spent to perform biopsy for the Group H+L was approximately, 2min 38s, considering the time of exposure to open the ZP on Day 6, keeping the technique below the maximum 3 minutes. In Group L, a longer period was taken to perform the biopsy, which exceeds the target to remain under 3 minutes per procedure. However, after the second replicate, the time spent per biopsy reduced and it was similar for Group L and to Group H+L; maintaining both techniques under the desired time. It is

evident that some procedures from Group H+L had the perfect herniation with no resistance when taking TE cells accelerating the biopsy to less than 1min30s, where Group L had a biopsy that took 11min 00s to perform. It also demonstrates that speed of operation with straight laser biopsy increases with experience. Capalbo et al. (2016) found among embryologists that about 20 procedures are needed as a minimum for training to perform TE biopsy when the embryologist was already trained at other biopsies stages. This is similar to what we have found during our two replicates with a total of 24 procedures in TE cells biopsy. Group L was comparable to herniated embryos from Group H+L once the technician was trained, reducing the time for positioning the aspiration pipette on the TE cells accurately, and being more confident to apply aspiration force in the pipette. When biopsying embryos from Group L, making a small hole between 20-25 µm, helped to accelerate the procedure. The aspiration pipette used was between 23-27 µm of diameter carefully enlarging the ZP hole to hold TE cells. The small gap improved the ability to take the sample with lower laser pulses, without breaking apart the ZP, the small hole prevents the embryo from coming out of the ZP when aspirating the TE cell, thus reducing the need of several laser beam shots to take the sample.

Herniated TE cells help to increase the speed of biopsy for the non-experienced technician but it is crucial that, on the ZP opening, only a small gap of between 10-25 µm is performed to avoid a creation of a large extrusion outside the ZP. In our experiment, we identified that it is common even performing small holes, to have a large herniation that can be sometimes as big as the embryo, reducing the ability to collect a more compact sample. However, when extruded cells are not in a large herniation the technique improved time efficiency to perform biopsy during the early stages of the experiment (i.e., first couple of replicates). However, as reported in Chapter 2, the Group H+L, had 17% of extrusions containing ICM.

There is a hypothesis that ICM splitting could cause monozygotic twining during the process of blastulation (Gu et al., 2018). The chorionicity (the number of chorionic membranes that surround the fetuses in a multiple pregnancy) and aminionicity (the number of amniotic inner mebrane surrounding the foetuses in a multiple pregnancy) of the cells also play a significant role in fetal development after embryo splitting (McNamara et al., 2016). Additionally, the fate of blastocyst cells is already known. Two cell lines, epiblast and primitive endoderm are formed at the early blastocyst stage (Morris et al., 2010). Therefore, full attention should be paid to TE biopsy to avoid ICM.

3.8.2 Re-expansion

In this study we found no difference in re-expansion overall among the three groups, enhancing our confidence with the system applied in the laboratory. Also, after biopsy no embryo died and 100% of the embryos assigned to the project were cryopreserved. All embryos were then collapsed with a laser beam pulse at the blastocoele cavity prior to freezing to reduce the possibility of crystal formation and consequently cell damage (Mandawala et al., 2016).

Group L had a slightly higher proportion of re-expansion after two hours compared to Group Con and H+L (0.2391 vs 0.1894 and 0.1855) (**Figure 3.3 A**), The shrunken blastocyst requires some time to seal and fill blastocoel (Harper et al., 2012, Mc Arthur et al., 2005). Due to the decreased pressure, when collapsing, it can alter integrity of tight junctions, causing morphological changes and cell loss, as tight junctions are essential to the differentiation or polarization of epithelial cells (Cockburn et al., 2010). Nevertheless, in our biopsies, Group L started to re-expand earlier but by 24 hours a similar proportion had re-expanded across all three treatment groups (**Figure 3.3 F**). This could also be associated with the smaller number of cells retrieved from Group L biopsies compared to Group III (Lauri et al., 2013), and due to the fact that biopsy can assist embryos to expand and hatch as in laser-assisted hatching (Hammadeh et al., 2011). Between the three groups we achieved an average re-expansion rate of 83.6%, embryos that fail to re-expanded were considered dead. Therefore, either biopsy technique can be used to collect TE cells for DNA analysis, both appears to be safe for embryo development.

3.8.3 Number of Cells in the Embryo After Immunostaining

The number of cells collected by biopsy is important. The general consensus is that around 10 cells will yield sufficient DNA for amplification using standard methodologies, but also that the number of cells collected in a biopsy needs to be limited according to the biomass of the blastocyst (Cimadomo et al., 2018). In this experiment we aimed to collect around 10 cells from all embryos (estimated at the time of biopsy by observation without actually counting the cells; but then counting them retrospectively when immunostained after cryopreservation).

We counted the number of cells present in the embryos after staining them with DAPI and found no significant difference in the number of cells present in the embryos from all groups, despite embryos been biopsied. Mammalian embryos before implantation average 100µm and have about 50-100 cells (Eckert and Fleming. 2008), where bovine have approximately between 80-200 cells at this stage (Valadão et al., 2018). The proportion of cells in all groups was over 100 cells (100.2, 105.4, and 100.5). We found no difference in the number of cells present in embryos from all groups (**Figure 3.5**). Interestingly biopsied embryos presented equal or similar number of cells from those in Group Con. There is no evidence in the literature that embryo biopsy increases the number of cells in an embryo, raising the possibility to further investigate the potential increase in cell number after TE biopsy. We associate the similar number of cells with the fact that Group II and III were exposure for two thawing cycles totally 48 hours which was enough time to allow compensation in cell number.

3.9 Conclusions

The application of laser biopsy procedure either in herniated or non-herniated embryos take a similar period of time to be performed' also that both techniques are safe and represent an efficient method to perform TE embryo biopsy in a commercial laboratory. Although herniated embryos from Group III are initially a faster approach to perform TE biopsy, after approximately 20 procedures a non-experienced technician has the ability to equally perform biopsy between herniated and non-herniated blastocyst embryos.

4. CHAPTER 4. EFFECTIVENESS OF LASER ASSISTED HATCHING TO INCREASE THE HATCHABILITY OF CRYOPRESERVED EMBRYOS.

4.1. Introduction

Despite improvements to in vitro embryo production (IVP) and transfer, implantation rates in cattle remains low. It has been estimated that between 50-75% of bovine blastocysts fail to implant following transfer (Valadão et al., 2018). The inability of an embryo to hatch is one of the main reasons for implantation failure (Seshagiri et al., 2009). The ability of an embryo to develop and implant depends on gamete quality such chromosomal constitution, and the quality of the cytoplasm (Huisman et al., 2000). Although morphologic grading is widely used in animal reproduction today to select embryos, it does not represent embryo quality with certainty and many of lower grade embryos that are transferred have a lower pregnancy rate (Hasler et al., 2001, Erdem et al., 2020).

Capability to hatch after transfer is a prerequisite for implantation; the embryo needs to attach to the uterine wall and initiate placentation (Valadão et al., 2018). This process occurs following the increase of blastocoelic pressure in the blastocyst (Bergström et al., 1972, Lopata et al., 1989). The capability to hatch is poor if the ZP is triggered into a process called “hardening” (ZPH). This is characterized by an increased resistance of the ZP to proteolytic digestion not by its stiffness. Mechanical forces applied to the ZP lead to ZPH (Papi et al., 2010). These are forces applied when pipetting and in the pressure used during an OPU session. ZPH naturally happens to protect the gamete from polyspermy by changing the properties of ZP after the sperm penetration in the previteline space. ZPH occurs within minutes upon syngamy encapsulating the oocyte, preventing any other sperm from penetrating the egg (Körschgen et al., 2017). Embryonic and endometrium enzymes are essential to the embryo to hatch, (O’Sullivan et al., 2002). One important mediator to help embryos to hatch is Prostaglandin (PG) E2; levels of which are higher in better quality embryos (Boruszewska et al., 2019). Magata et al., (2019) found that embryos with slower development during cleavage had a lower hatchability compared from those with normal cleavage. It is known that timing of cleavage is a parameter for embryo quality (Lechniak et al., 2008). Increasing

evidence indicates that one of the causes of lower implantation with poorer graded embryos might be associated with the inability to hatch and implant.

ZPH can also be triggered during vitrification (Wiesak et al., 2017), mainly due to calcium used during cryopreservation and fertilisation medium (Gualtieri et al., 2011). The absence of calcium in the media leads to improvements in sperm penetration in the ZP (Fujiwara et al., 2010). However, when oocytes are exposed to dimenthylsulphoxide, ethylene glycol and propylene glycol (Cryoprotectants) there is an increase in intramembrane calcium levels resulting in ZPH (Marques et al., 2018, Tian et al., 2007). Ethylene glycol for instance is a common cryoprotectant used in bovine practices to cryopreserve embryos.

Cryopreservation of the embryos in an IVP system allow embryos to be thawed and transferred whenever suitable recipients are available, improving convenience and reducing costs of a synchronisation protocol. The decrease in pregnancy rates can be approximately 10 to 13% between fresh and frozen-thawed bovine embryos of comparable quality grades (Hasler et al., 2001), but poor-quality embryos are known to produce much lower pregnancy rates compared to higher quality grades (Kara et al., 2020); and are frequently discarded resulting in considerable economic loss. Taniyama et al. (2011) reported an increase in pregnancy rate after assisted hatching of poor-quality embryos. Nevertheless, Pryor et al. (2011) concluded that laser-assisted hatching (LAH) by its own is beneficial to embryo development following cryopreservation. Nevertheless, other studies have found LAH to be beneficial for embryo hatching and implantation (Hsieh et al., 2002, Balaban et al., 2006, and Ueno et al., 2016). Conversely, some studies indicate that LAH produces no significant improvement in embryo hatching (Schimmel et al., 2014).

Currently three LAH methods are used clinically according to the degree of invasiveness: Firstly, total ZP opening (a small gap across the inner membrane and external ZP layer is performed); secondly, partial ZP opening (where only a small proportion of the external area of the ZP is thinned); and thirdly, laser assisted thinning of a large portion of the outside part of the zona pellucida to facilitate hatching (a procedure known as quarter laser assisted hatching) (Davidson et al., 2018, Petersen et al., 2005)

Nowadays the use of LAH is not extensive in commercial bovine IVP laboratories. Firstly, due to the cost of reliable equipment for laser application and secondly because of the need of trained embryologists to undertake micromanipulation. In the current study we aim to identify whether LAH improves

hatchability of cryopreserved embryos during the first 48 hours following thawing in a commercial laboratory setting.

4.2 Material and Methods

4.2.1. Ovary Collection and Follicle Aspiration

Due to pandemic restrictions, slaughterhouse ovaries were collected on only three available days from a local abattoir (Carlisle, UK) and the protocol was followed as it is in Chapter 3.

4.2.2. Oocyte Recovery and Grading

Contents in the 50ml tube were filtered in a warmed filter cup, held at 36°C with IVF Bioscience® OPU media. The filter was rinsed three to four times until the fluid in the filter cup remained colourless. Subsequently, the fluid was poured into a 90 mm petri dish on a warm stage platform at 37°C for searching under a 40X magnification microscope (Nikon SMZ10- Japan). All oocytes were collected from the dish and placed into a small 30mm petri dish containing IVF Bioscience® wash media with antibiotics before grading and placed into maturation.

Grade I oocytes were defined as those oocytes with a compact cumulus investment with more than three cell layers of cumulus cells surrounding the oocyte; cytoplasm consistency and colour were evaluated to determine the gametes' grade. Grade I COCs were colour consistent and had no white spots in the nucleus or any minimal degeneration in the surrounding layer. Those classified as Grade II were also carefully evaluated on their morphology but with a small number of cumulus layers around the oocyte, some visible inconsistent cytoplasm or nucleus homogeneity. Grade III COCs with good and solid cytoplasm were put into maturation despite having only one cumulus cells layer and uneven nucleus colour. Those oocytes that had no cumulus cells or degradation of their cytoplasm were discarded.

4.2.3 *In Vitro* Maturation

Grade I, II and III COCs were placed in 1 ml maturation media vials with round bottom from Boviteq Canada Laboratories at ~4 h after collection from the slaughterhouse. Groups of 30 COCs were matured for 23 h in HEPES buffered TCM199 media supplemented with 10% (v/v) FBS, 4 mg/ml fatty acid free BSA, 0.2 mM pyruvate, 50 µg/ml gentamicin, 5 µg/ml FSH, 0.5 µg/ml LH, 1 µg/mE 2). COCs were incubated in a Cryologic transportable incubator (Blackbourn, Australia) at 38°C. Fertilization dishes were prepared with fertilization media (Tyrodes lactate media supplemented with 0.6% (w/v) fatty acid free BSA, 1.5 µg/ml heparin, 0.2 mM sodium pyruvate, 0.08 mM penicillamine, 0.04 mM hypotaurine, 10 mM epinephrine, 50 µg/ml gentamicin) and incubated in a Binder® incubator at 38°C, 6.7 CO_2 and 5% O_2 to equilibrate medium for the fertilization on the next day.

4.2.4 *In Vitro* Fertilization

After 22 hours of IVM, COCs were washed 3 times in IVF Bioscience wash media®. Five COCs were placed in each 40 µL drop of fertilisation media in a 30 mm petri dish. Prior to fertilization, semen was prepared using a gradient of percoll 45% and 90% (Bovipure™, Nidacon, Mölndal, Sweden). Semen from the same bull (Boldi Emphasis) was used for this experiment at 0.5 $X10^6$/ml concentration in a 0.5ml straw. Frozen semen was thawed in a 37°C semen-thaw cup (Dairymac, Hempshire-UK per 45s and placed in a small PCR. The sperm was evaluated prior fertilisation to ensure motility. After certifying as prior described in Chapter 3, the aliquoted sperm was added to the oocytes in the fertilisation drops containing 5 µL aliquot of 1% heparin and 2 µL of PHE. Fertilisation dishes were placed into a Binder® incubator (Tuttlingen, Germany) at 38°C humidified atmosphere with 6.7% CO_2 and 5% O_2.

4.2.5 *In Vitro* Culture

Grade I, II and III COCs were placed in 1 ml maturation vials from Boviteq Canada Laboratories at ~4 h following the process described in Chapter 3.

4.2.6 Treatments

On Day 7 post fertilization, embryos were placed under a 40X objective microscope and assessed morphologically. Only Grade 1 and 2 blastocysts (BL, Stage 6) and expanded blastocysts (EXBL, Stage 7) were assigned to either the treatment or control group.

Treatment group: Embryos were kept in the culture dish with lid on and were placed under the micromanipulator RI (as described in Chapter 1). When required, a round glass pipette was used to turn blastocysts into the desired position. Their zona pellucida was then opened horizontally with 2 or 3 diode laser beams at 120µm/s. ZP opening was performed on the abembryonic pole near the TE cells with approximately ¼ of distance from the ICM in a total ZP opening. TE cells and Blastocoele fluids (BF's) were intact in all 88 embryos.

All embryos were cryopreserved immediately afterwards using the protocol described in Chapter 3. They were graded and labelled, then frozen in a direct transfer system of cryopreservation in ethylene glycol at -0.5°C /min and kept in a nitrogen tank. Embryos were dehydrated with holding medium (Boviteq Canada) before freezing and subsequently washed four times in frozen medium before loading into straws.

4.2.7 Thawing and Expansion/Hatching Evaluation

Frozen embryos were thawed in a semen-thaw cup (Dairymac, Hempshire-UK) at 30°C per 30 s. The straw containing the embryo was dried with blue roll and using a scissors, the back of the embryo straw was cut. Embryos were pushed with a rod into a 90 mm search dish. Using a 200µl pipette, the embryo was placed in a six-well dish and 50µl of warm holding media (Boviteq® Canada) was mixed together, after 1 minute, another 100ul of holding media was added to the embryo. One minute later, 200µl of holding media was added to the well and after 1 minute each embryo was washed in two 100µl drops of holding media. After the final wash, embryos were placed in a 14µl culture drop to allow re-expansion after thawing and were observed for an interval of 6, 24, 30, 40 and 48 h.

4.3 Statistical Analysis

GenStat (19th Edition, VSN International, 2018; http://www.vsni.co.uk/) was used for the analysis. Generalized linear models with binomial errors and logit-link functions were used to analyse all proportion data associated with embryos hatching and hatched. This model was fitted with the terms 'replicate' and 'treatment'. Data are presented as adjusted means ± SEM.

4.4 Results

Overall, three replicates were performed undertaking COC classification and embryo development (**Table 1.6**), before blastocyst stage on day 7 was determined (**Table 1.7**).

Table 1.6 Number of oocytes and COC's quality

Number of Oocytes	GI %	GII %	GIII %	Cleavage %	Embryos%
330	17	44	39	70.2(231/330)	38(88/231)

Table 1.7 Stage of embryo development assigned to each group

Number of Embryos	BL %	EXBL %	BL LAH %	EXBL LAH %	Control BL %	Control EXBL %
88	42 (37/88)	57.9(51/88)	48.6(18/37)	50.9(26/51)	51.3(19/37)	49(25/51)

The proportion of viable embryos following 6 h thawing was 0.941 ± 0.0398 and 0.883 ± 0.0760 for EXBLs from assisted hatching (AH) and Control groups respectively; while the proportion viable BLs from AH and Controls were 0.922 ± 0.0524 and 0.824 ± 0.1091 with no difference between groups or embryo stage (**Figure 4.1A**). By 6 h, the proportion hatched embryos was greater (P=0.011) for EXBL compared to BL and following AH (P = 0.007) compared to controls (**Figure 4.1B**). These treatment-group differences were broadly maintained up to 30 h post thawing **(Figure 4.1C, D)**, but treatment differences were not evident thereafter (**Figure 4.1E, F**). Numerically, by 48 h post thaw, the highest proportion of hatched embryos were for EXBL that had undergone AH (0.892 ± 0.0597).

The pattern for embryos that had completely hatched and out of the ZP (**Figure 4.2**) matched that for hatching and hatched embryos presented in **Figure 4.1**, although effect of stage of embryo development (i.e., BL vs EXBL) was less evident.

A positive effect of AH was evident (P=0.007) up to 40 h post thaw (**Figure 4.2D**). There was an indication (P=0.059) that the portion of embryos that failed to hatch after 48 h was reduced following AH (**Figure 4.2F**).

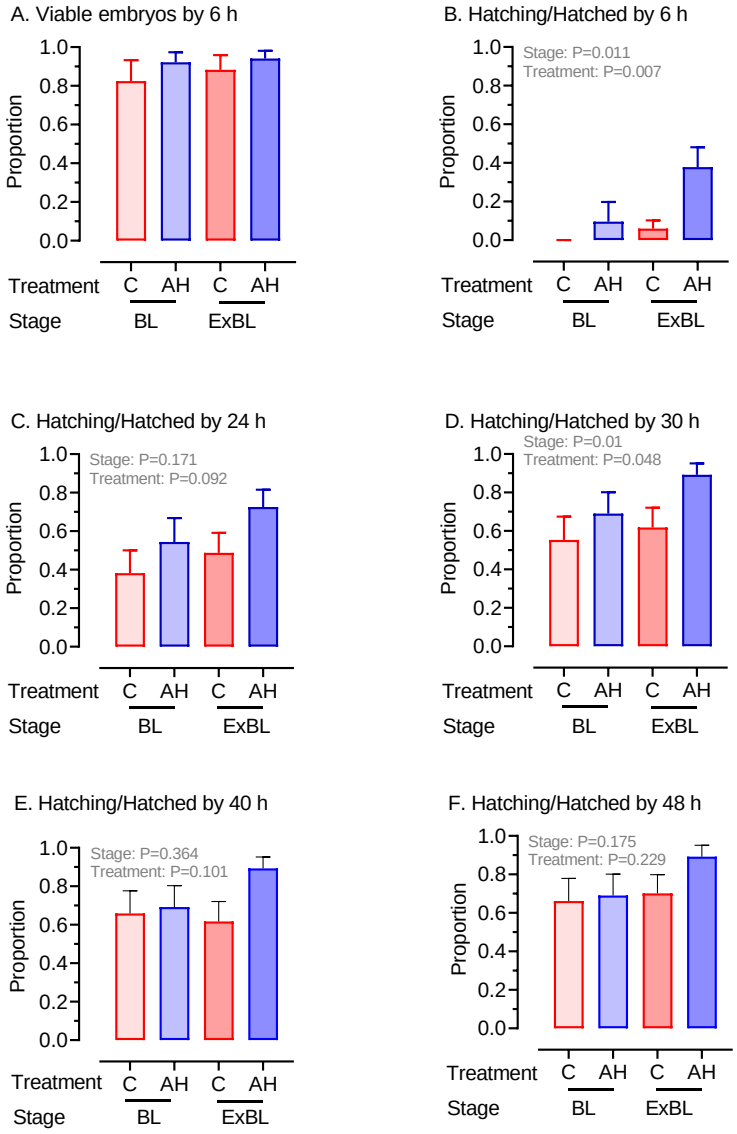

Figure 4.1. Proportion of embryos that were hatching or had hatched at different time points following thawing. Proportion of viable embryos after 6 h **A**. Proportion embryos hatching or hatched after 6 h **B**, 24 h **C,** 30 h **D,** 40 h **E,** and 48 h **F.**

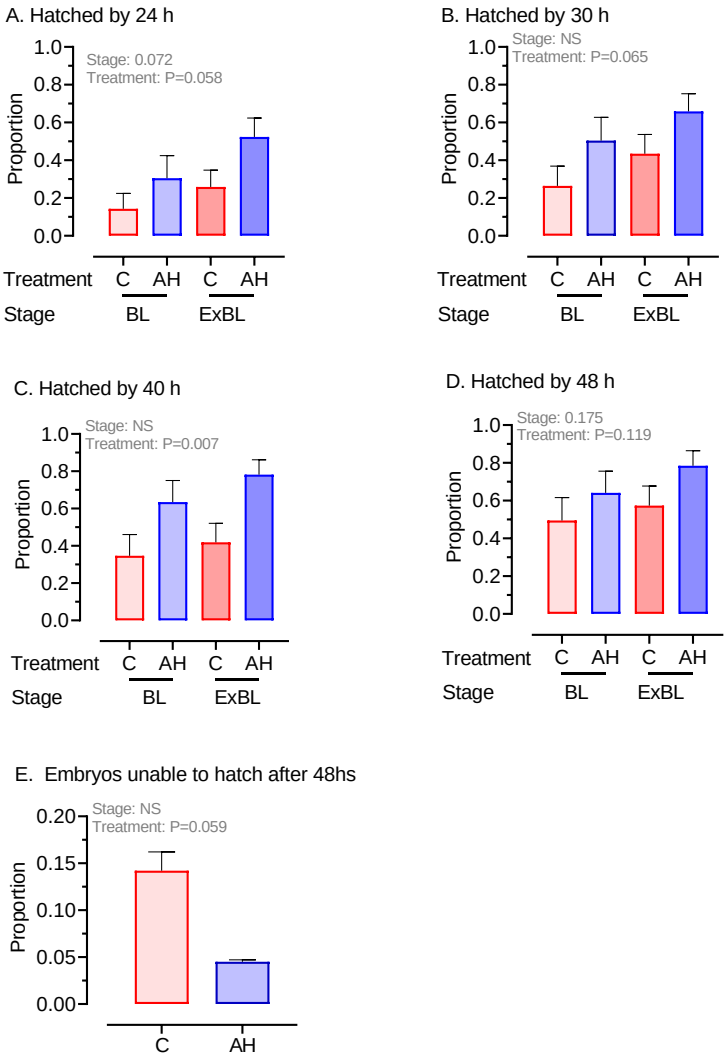

Figure 4.2. **A**. Proportion embryos hatching or hatched after 24 h **B**, 30 h **C,** 40 h **D** and 48 h **E.** Finally, the proportion embryos that failed to hatch after 48 h.

4.5 Discussion

The current study evaluated the ability of LAH to increase the proportion bovine BL and EXBL that hatched at different time points following cryopreservation in ethylene glycol. The main finding to emerge from this study was the tendency of LAH embryos to hatch faster during the first few hours compared to the control

group, confirming its significance at 40 hours after thawing. Although the number of hatched embryos did not differ after 48 hours between both groups, there was also a tendency for embryos to die because of their inability to hatch from the ZP; and LAH helped to reduce this proportion (**Figure 4.2E**).

4.5.1. Faster Hatching in LAH Embryos

LAH promotes a rapid release of the embryo from the ZP derived by bovine IVP. The presence of a complete artificial gap in the ZP facilitates hatching. The proportion of embryos that start hatching during the first 6 hours was significant higher in those at the expanded blastocyst stage from the LAH group (**Figure 4.1 B**). This can be accredited to the development of good quality embryos that develop faster compared to lower quality embryos associated with aneuploidy; and also to poorer quality embryos that when assisted improve hatchability (Campbell et al., 2013, Chawla et al., 2015, Magata et al., 2019).

In our experiment we prioritized blastocyst and expanded blastocyst stage embryos in order to increase the accuracy of the position for LAH. According to Negrón-Pérez & Hansen (2017) a higher number of embryos start hatching at the abembryonic pole (i.e., the opposite side of ICM position). This was the chosen area to undertake LAH. It not only to avoid direct laser beam contact with the ICM, but it also reduces heat generated in the procedure. A larger number of pulses in a larger laser length may increase medium temperature up to 366ºC (Douglas-Hamilton and Conia. 2001). We aimed at a lower time in the TE cell using a 120mW with 2ms duration diode laser pulse to undertake LAH. Pulse durations over 5ms has been shown to increase temperature by over 160°C when 100mW laser power was applied. To avoid damage to the embryos it is preferred to secure the diode laser lower than λ 1,480nm. (Hartshorn et al., 2005, Hsieh et al., 2002); that is about 148mW, but most important is ensuring the time is less than 5ms.

LAH was cautiously applied, thereby avoiding any TE cells. This technique has been demonstrated to allow complete hatching compared to partial or ZP thinning. It has been reported, using time-lapse imaging in a mouse model, that zona thinning, unlike partial hatching, does not facilitate hatching but results in an increased incidence of multiple hatching sites and incomplete hatching (Schimmel et al., 2014). It is important to remember that the inner pressure caused by the embryo can help the ZP to break but most important is that ZP hatching is mainly

characterised by the enzymes that work to facilitate hatching. After 30 hours the proportion of control group embryos hatching was smaller than EXBL embryos LAH (**Figure 4.1 D**), Morishita et al. (1993) suggested that protein synthesis in the hatching embryos is higher than in the embryos that failed to hatch. Furthermore, the TE secretes trypsin-like substances, plasmin, glutamine, insulin and epidermal growth factor, which are products of amino acids. Protein synthesis in embryos is considered a critical factor in blastocyst hatching.

Embryos were produced in vitro using conventional medium (Biviteq® Canada). Medium composition is undeclared but fetal calf serum (FCS) is added during maturation and is likely to contain calcium. Calcium is one of the major components that leads to ZPH. Cryopreservation in ethylene glycol could trigger ZPH as well (Dobrinsky. 1996). Both elements that could trigger ZPH were present in the experiment and in our commercial laboratory system. We could increase the proportion of embryos to fully hatch compared to those in the control group (**Figure 4.2 C**). The LAH before cryopreserving BL and EXBL embryos accelerates hatching.

4.5.2 Re-expanded Embryos Unable to Hatch

An interesting finding in this experiment arose when a proportion of the embryos in both groups achieved full re-expansion after thawing but were unable to hatch. These embryos formed a blastocoele at timepoints when they began to degenerate and they were effectively dead by 48 hours. We also identified a small proportion of embryos that were trapped in the ZP following LAH compared to the group control (**Figure 4.2 E**).

4.5.3 Summary

In Summary LAH is a safe and rapid procedure to perform, however there was no difference in the proportion of embryos that hatched after 48 hours. The approach can accelerate hatching. Good and fair quality EXBL and BL embryos can benefit from LAH to accelerate hatching in a commercial bovine laboratory setting. This could lead to improved pregnancy rates following embryo transfer, but this awaits to be determined.

5. CHAPTER 5 GENERAL DISCUSSION

5.1 Project Outcomes

In accordance with our hypothesis, laser biopsy of herniated TE cells increased the speed of the procedure in a commercial bovine IVP laboratory setting. Herniated TEs are sometimes used for biopsy in humans (McArthur et al., 2005) but not used at a commercial scale in cattle. We found that the technique requires less sophisticated skill with regard to laser biopsy compared to non-herniated embryos irrespective of whether a blade or laser was used. Working with herniated TEs is relatively straightforward. We found that it's easier and quicker to place the embryo in the best position and recover a consistent number of cells. Laser biopsy is a safe approach for commercial embryos and did not reduce the proportion of embryos that survived and re-expanded. Non-herniated TE biopsy requires more refined work. The biopsy requires to be detached from the ZP and can lead to the collapse the blastocoele cavity making it harder to identify the TE from the ICM; thereby increasing the time required (Aoyama et al., 2020). It is also possible to detach the TE by the application of medium with a fine pipette in the periviteline space creating a gap as reported by Capalbo et al. (2014) and Aoyama et al. (2020); but this requires greater technical skill.

To allow herniation on Day 7 it was necessary to assess the embryos one day prior to biopsy. However, on Day 6 there was a scheduled medium change when all embryos were assessed. Herniated embryos received a higher number of laser pulses compared to non-herniated embryos and have the risk of medium temperature to rising which could induce epigenetic changes (Honguntikar et al., 2017), despite the fact that embryo development might not be affected (Taylor et al., 2010). Also, the size and position of the ZP opening was crucial to allow a precise number of cells to be extruded.

5.1.1 What was Different about this Project?

The project was performed in the commercial laboratory using standardised protocols so that it could be incorporated in practice. In the Industry embryologists have to integrate learning and new techniques/skills with ongoing protocols. Normally there is a group of people with specific roles and the process is undertaken

quickly to avoid adverse embryo exposures. When undertaking biopsies, a few things were considered such as new dish preparation during routine work and equipment calibration prior biopsy (where the laser needs to be calibrated before each cycle (day)). These procedures increase the cumulative hours of work.

5.1.2 Economic Aspects in a Commercial Laboratory

Despite the benefits of the laser biopsy the laser and associated equipment represents an expensive investment for a small laboratory. A micromanipulator requires servicing annually to ensure its integrity and operation. The diode laser has an operational lifespan of between 25,000 to 50,000 hours. It is therefore important to identify the return on investment when applying such procedures in a commercial laboratory. Further larger-scale laser applications are therefore required to balance this investment. Selection of high gEBV embryos associated with low levels of chromosomal abnormalities represents one such key application (Mullaart and Wells. 2018, Mclean et al., 2020).

On the other hand, the microblade has the potential to perform the procedure but potentially at lower cost. It is also a fast approach but it is not as precise as laser biopsy and requires more experience in order to develop a high-throughput system that avoids collecting excess cells and compromising embryo development. However, biopsy at any scale prevents embryos from being exported. Current regulations do not accept embryos with incomplete ZP due to possible disease transmission (Mapletoft. 2018), thereby reducing the market for export of high quality and genomically evaluated embryo.

5.1.3 Embryo Survival Following Biopsy

We found no difference in embryo survival following biopsy by herniation or non-herniation and cryopreservation. We conclude that laser biopsy of either herniated or non-herniated TE is the easier method to rapidly learn without compromising embryo integrity; an observation consistent with others (Oliveira et al., 2017, Turner et al., 2019). Normally farmers express concern in discarding low quality embryos if they have any potential recipients, and they are likely to transfer poor quality embryos. We found no difference in embryo survivability after laser biopsy in comparison to blade biopsy and non-biopsied embryos. It can be attributed

to the minimally invasive nature of this procedure and the precise thermal control of the micromanipulator (Wheat et al., 2014).

5.1.4 Future Considerations for Embryo Biopsy

Further applications of TE laser biopsy would benefit from a formal return on investment considering the cost of preparing recipients for embryos prone to fail and technician transfer fees. Early embryo selection (based on gEBVs and/or aneuploidy screening) may of greater interest to UK cattle breeders once new incentives for livestock production materialise (Gov UK, 2020, AHDB, 2020). There are 6.9 million female cattle in the UK (Defra farming statistics. 2021). Selection within 1.9 million dairy cows with a replacement rate of 30% could increase returns by £20/heifer/annum or £11.3 million pa. The genotyping of TE cells could accelerate cattle production efficiency across the UK, moving towards achieving the plan of net zero CO_2 by 2050 (UNFCC, 2015).

The commercial application of laser biopsy can also benefit the earlier selection of bulls. The use of bull semen is today the most used reproductive technology where the field is expected to reach USD 5.86 billion by 2027 (Size, 2021). A few years ago the selection of a bull was entirely depended on the proven history of a substantial number of daughters, what could take up to 7 years to reach 80% reliability. Today a newly born calf can start life with 70% reliability based on DNA analysis. DNA genotyping test can be performed from embryo biopsies, further advancing the onset of decision making and avoiding the birth of unwanted calves.

5.2 Laser Assisted Hatching (LAH)

LAH is a relatively straightforward technique and easily applied in the commercial laboratory. We had no difficulty in implementing the technical aspects of LAH. However, there are several incidents recurrent from a small ZP opening such as abnormal expansion of the embryo, trapping phenomena, with only few TE projections outside the ZP, and incomplete zona hatching (Alteri et al., 2018). However, we encountered no such incidents. On the other hand, we found a higher proportion of embryos following LAH hatch and hatching earlier. Earlier hatching could overcome the challenge of a slightly asynchronous uterus. From our data we predict a potential increase of 6% pregnancy rate (following transfer). Also, the

possible LAH could benefit low grade embryos, reducing number trapped in the ZP, leading to higher PR in the field.

5.2.1 Application of LAH in the Field

We currently have agreed a contract with interested partners to progress our investigation of LAH embryos to consider ET under field conditions. We will provide a LAH service for OPU- IVP embryos and collect field data from pregnancy diagnosis.

5.3 Final Conclusion

We conclude that the application of laser biopsy following TE herniation is a safe and precise option to be introduced to a commercial laboratory and that this can be followed by laser biopsy in non-herniated embryos once some experience is acquired. We also conclude that application of LAH has the potential to speed up the hatching process of embryos and may help embryos to implant. LAH is a safe and quick procedure to adopt in order to potentially improve pregnancy rates following ET.

6. BIBLIOGRAPHY

Abdelmassih, S.T., Munne, S., Abdelmassih, V.G., Abdelmassih, R. and Nagy, Z.P., 2001. The effect of the biopsy procedure and the removal of a single blastomere from day 3 human embryos on their implantation potential. *Fertility and Sterility*,*76*(3), pp.S136-S137.

Abe, H., Yamashita, S., Satoh, T. and Hoshi, H., 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Molecular reproduction and development*, *61*(1), pp.57-66.

Adams, G.P., 1994. Control of ovarian follicular wave dynamics in cattle: implications for synchronization & superstimulation. *Theriogenology*, *41*(1), pp.19-24.

Adler, A., Lee, H.L., McCulloh, D.H., Ampeloquio, E., Clarke-Williams, M., Wertz, B.H. and Grifo, J., 2014. Blastocyst culture selects for euploid embryos: comparison of blastomere and trophectoderm biopsies. *Reproductive biomedicine online 28*(4), pp.485-491.

Agca, Y., Monson, R.L., Northey, D.L., Peschel, D.E., Schaefer, D.M. and Rutledge, J.J., 1998. Normal calves from transfer of biopsied, sexed and vitrified IVP bovine embryos. *Theriogenology*, *50*(1), pp.129-145.

Ahdb.org.uk. Dairy genetics evaluations

Alteri, A., Viganò, P., Maizar, A.A., Jovine, L., Giacomini, E. and Rubino, P., 2018. Revisiting embryo assisted hatching approaches: a systematic review of the current protocols. *Journal of assisted reproduction and genetics*, *35*(3), pp.367-391.

Ambrose, J.D., Drost, M., Monson, R.L., Rutledge, J.J., Leibfried-Rutledge, M.L., Thatcher, M.J., Kassa, T., Binelli, M., Hansen, P.J., Chenoweth, P.J. and Thatcher, W.W., 1999. Efficacy of timed embryo transfer with fresh and frozen in vitro produced embryos to increase pregnancy rates in heat-stressed dairy cattle. *Journal of Dairy Science*, *82*(11), pp.2369-2376.

Anderson, R.A., Sciorio, R., Kinnell, H., Bayne, R.A.L., Thong, K.J., De Sousa, P.A. and Pickering, S., 2009. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction*, *138*(4), pp.629-637.

Antczak, M. and Van Blerkom, J., 1999. Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Human Reproduction*, *14*(2), pp.429-447.

Aoyama, N. and Kato, K., 2020. Trophectoderm biopsy for preimplantation genetic test and technical tips: A review. *Reproductive Medicine and Biology*, *19*(3), pp.222-231.

Arav, A. and Natan, Y., 2019. The near future of vitrification of oocytes and embryos: looking into past experience and planning into the future. *Transfusion Medicine and Hemotherapy*, *46*(3), pp.182-187.

Arav, A. and Zvi, R., 2008. Do chilling injury and heat stress share the same mechanism of injury in oocytes?. *Molecular and cellular endocrinology*, *282*(1-2), pp.150-152.

Assidi, M., Dufort, I., Ali, A., Hamel, M., Algriany, O., Dielemann, S. and Sirard, M.A., 2008. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. *Biology of reproduction*, *79*(2), pp.209-222.

Assou, S., Haouzi, D., De Vos, J. and Hamamah, S., 2010. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *MHR: Basic science of reproductive medicine*, *16*(8), pp.531-538.

Austin CR (1961) “The mammalian egg” Oxford Blackwell ch2, pp 92-96.

Ax, R. L., Armbrust, S., Tappan, R., Gilbert, G., Oyarzo, J. N., Bellin, M. E. … McCauley, T. C. (2005). Superovulation and embryo recovery from peripubertal Holstein heifers. Animal Reproduction Science, 85(1-2), 71–80. doi:10.1016/j.anireprosci.2004.04.034 2004

Balaban, B., Urman, B., Yakin, K. and Isiklar, A., 2006. Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: a prospective randomized study. *Human Reproduction*, *21*(8), pp.2136-2140.

Baruselli, P.S., Batista, E.O.S., Vieira, L.M. and Souza, A.H., 2018. Relationship between follicle population, AMH concentration and fertility in cattle. *Animal Reproduction (AR)*, *12*(3), pp.487-497.

Baruselli, P.S., de Sá Filho, M.F., Martins, C.M., Nasser, L.F., Nogueira, M.F., Barros, C.M. and Bó, G.A., 2006. Superovulation and embryo transfer in Bos indicus cattle. *Theriogenology*, *65*(1), pp.77-88.

Baruselli, P.S., Ferreira, R.M., Sales, J.N.S., Gimenes, L.U., Sá Filho, M.F., Martins, C.M., Rodrigues, C.A. and Bó, G.A., 2011. Timed embryo transfer programs for management of donor and recipient cattle. *Theriogenology*, *76*(9), pp.1583-1593.

Baruselli, P.S., Sá Filho, M.F., Ferreira, R.M., Sales, J.N.S., Gimenes, L.U., Vieira, L.M., Mendanha, M.F. and Bó, G.A., 2012. Manipulation of *Reproduction in Domestic Animals*, *47*, pp.134-141.

Basile, N., Caiazzo, M. and Meseguer, M., 2015. What does morphokinetics add to embryo selection and in-vitro fertilization outcomes?. *Current Opinion in Obstetrics and Gynecology*, *27*(3), pp.193-200.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M. and Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. *Gene*, *295*(2), pp.173-183.

Beebe, D., Wheeler, M., Zeringue, H., Walters, E. and Raty, S., 2002. Microfluidic technology for assisted reproduction. *Theriogenology*, *57*(1), pp.125-135.

Benavent, M., Escriba, M., Miret, C., Vanrell, I., Costa-Borges, N., Calderón, G., Crespo, J. and Teruel, J., 2019. Evaluation of the impact of the pulling and flicking trophectoderm biopsy procedures on the integrity of the biopsied cells and their correlation to PGT-A results. *Fertility and Sterility*, *112*(3), p.e242.

Bergström, S., 1972. Shedding of the zona pellucida of the mouse blastocyst in normal pregnancy. *Reproduction*, *31*(2), pp.275-277.

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P. and Gutierrez-Adan, A., 2008. Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiological Genomics*, *32*(2), pp.264-27

Bertrand, E., Van den Bergh, M. and Englert, Y., 1995. Fertilization and early embryology: does zona pellucida thickness influence the fertilization rate?. *Human reproduction*, *10*(5), pp.1189-1193.

Bettegowda, A., Patel, O.V., Lee, K.B., Park, K.E., Salem, M., Yao, J., Ireland, J.J. and Smith, G.W., 2008. Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications. *Biology of reproduction*, *79*(2), pp.301-309.

Block, J., Bonilla, L. and Hansen, P.J., 2010. Efficacy of in vitro embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium. *Journal of dairy science*, *93*(11), pp.5234-5242.

Block, J., Rivera, R.M., Jousan, F.D., Silvestre, F.T., Paula-Lopes, F.F., Ocon, O.M., Rosson, H., Bilby, T.R., Hansen, P.J., Drost, M. and Monson, R.L., 2005. Effects of bovine somatotropin and timed embryo transfer on pregnancy rates in non-lactating cattle. *The Veterinary Record*, *156*(6), p.175.

Block, J., and P. J. Hansen. 2007. Interaction between season and culture with insulin-like growth factor-1 on survival of in vitro produced embryos following transfer to lactating dairy cows. Theriogenology 67:1518–1529. doi:S0093-691X(07)00114-8

Blondin, P., 2018. Status of embryo production in the world. *Animal Reproduction (AR)*, *12*(3), pp.356-358.

Bó, G.A. and Mapletoft, R.J., 2014. Historical perspectives and recent research on superovulation in cattle. *Theriogenology*, *81*(1), pp.38-48.

Bó, G.A. and Mapletoft,[2] R.J., 2018. Embryo Transfer Technology in Cattle. In *Animal Biotechnology 1* (pp. 107-133). Springer, Cham.

Bo, G.A., Adams, G.P., Pierson, R.A. and Mapletoft, R.J., 1995. Exogenous control of follicular wave emergence in cattle. *Theriogenology*, *43*(1), pp.31-40.

Bó, G.A., Baruselli, P.S., Moreno, D., Cutaia, L., Caccia, M., Tríbulo, R., Tríbulo, H. and Mapletoft, R.J., 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology*, *57*(1), pp.53-72.

Bobrie, A., Colombo, M., Raposo, G. and Théry, C., 2011. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic*, *12*(12), pp.1659-1668.

Boni, R., 2018. Ovum pick-up in cattle: a 25 yr retrospective analysis. *Animal Reproduction (AR)*, *9*(3), pp.362-369.

Bonilla, L., Block, J., Denicol, A.C. and Hansen, P.J., 2014. Consequences of transfer of an in vitro-produced embryo for the dam and resultant calf. *Journal of dairy science*, *97*(1), pp.229-239.

Boruszewska, D., Grycmacher, K., Kowalczyk-Zieba, I., Sinderewicz, E., Staszkiewicz-Chodor, J. and Woclawek-Potocka, I., 2019. Expression of enzymes involved in the synthesis of prostaglandin E2 in early-and late-cleaved bovine embryos at different stages of preimplantation development. *Theriogenology 133*, pp.45-55.

Bouquet, A. and Juga, J., 2013. Integrating genomic selection into dairy cattle breeding programmes: a review. *Animal*, *7*(5), pp.705-713.

Braga, J.V., Komninou, E.R., Vieira, A.D. and Mondadori, R.G., 2019. Approaches to reduce lipids: a review of its impacts on in vitro embryo production. *Rev Bras Reprod Anim*, *43*(1), pp.3-7.

Bredbacka, P., 1998. Recent developments in embryo sexing and its field application. *Reproduction Nutrition Development* , *38* (6), pp. 605-613.

Bredbacka, P., Kankaanpää, A. and Peippo, J., 1995. PCR-sexing of bovine embryos: a simplified protocol. *Theriogenology*, *44*(2), pp.167-176.

Brunner, D., Frank, J., Appl, H., Schöffl, H., Pfaller, W. and Gstraunthaler, G., 2010. The serum-free media interactive online database. *ALTEX-Alternatives to animal experimentation*, *27*(1), pp.53-62.

Burkart, A.D., Xiong, B., Baibakov, B., Jiménez-Movilla, M. and Dean, J., 2012. Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermyOvastacin cleaves ZP2 and prevents polyspermy. *The Journal of cell biology*, *197*(1), pp.37-44.

Callesen, H., Bak, A. and Greve, T., 1992. Use of PMSG antiserum in superovulated cattle?. *Theriogenology*, *38*(5), pp.959-968.

Callesen, H., Greve, T. and Christensen, F., 1987. Ultrasonically guided aspiration of bovine follicular oocytes. *Theriogenology*, *27*(1), p.217.

Camargo, L.S.D.A., Viana, J.H.M., Sá, W.F.D., Ferreira, A.D.M., Ramos, A.D.A. and Vale Filho, V.R., 2018. Factors influencing in vitro embryo production. *Animal Reproduction (AR)*, *3*(1), pp.19-28.

Campbell, A., Fishel, S., Bowman, N., Duffy, S., Sedler, M. and Thornton, S., 2013. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reproductive BioMedicine Online*, *27*(2), pp.140-146.

Campos-Chillon, F., Mancino, J., Barcelo-Fimbres, M., Altermatt, J.L. and AniCell Biotech, L.L.C., 2015. Progress in genotyping in vitro-produced embryos: are we close. *Proceedings, Applied Reproductive Strategies in Beef Cattle*, *17*, pp.258-265.

Capalbo, A., Rienzi, L., Cimadomo, D. and Ubaldi, F.M., 2017. Polar Body, Cleavage Stage and Trophectoderm Biopsy. *Handbook of In Vitro Fertilization*, pp.245-258.

Capalbo, A., Rienzi, L., Cimadomo, D., Maggiulli, R., Elliott, T., Wright, G., Nagy, Z.P. and Ubaldi, F.M., 2014. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. *Human reproduction*, *29*(6), pp.1173-1181.

Capalbo, A., Romanelli, V., Cimadomo, D., Girardi, L., Stoppa, M., Dovere, L., Dell'Edera, D., Ubaldi, F.M. and Rienzi, L., 2016. Implementing PGD/PGD-A in IVF clinics: considerations for the best laboratory approach and management. *Journal of assisted reproduction and genetics*, *33*(10), pp.1279-1286.

Capalbo[b], A., Ubaldi, F.M., Cimadomo, D., Maggiulli, R., Patassini, C., Dusi, L., Sanges, F., Buffo, L., Venturella, R. and Rienzi, L., 2016. Consistent and reproducible outcomes of blastocyst biopsy and aneuploidy screening across different biopsy practitioners: a multicentre study involving 2586 embryo biopsies. *Human reproduction*, *31*(1), pp.199-208.

Cardoso, C.J.T., de Oliveira, J.S., Junior, H.K., da Silva, W.A.L., da Silva Arruda, E.D., Souza-Cáceres, M.B., de Oliveira, F.A.M., Nogueira, É., de Paula Nogueira, G. and de Andrade Melo-Sterza, F., 2018. Anti-Müllerian hormone (AMH) as a predictor of antral follicle population in heifers. *Animal Reproduction*, *15*(1), p.12.

Carroll, J., Depypere, H. and Matthews, C.D., 1990. Freeze–thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen–thawed mouse oocytes. *Reproduction*, *90*(2), pp.547-553.

Carson, S.A., Gentry, W.L., Smith, A.L. and Buster, J.E., 1993. Trophectoderm microbiopsy in murine blastocysts: comparison of four methods. *Journal of assisted reproduction and genetics*, *10*(6), pp.427-433.

Catt, J.W. and Henman, M., 2000. Toxic effects of oxygen on human embryo development. *Human Reproduction*, *15*(suppl_2), pp.199-206.

Catt, S., Amaya, G., Batsiokis, M., Walton, S. and Taylor-Robinson, A.W., 2018. Comparison of pregnancy in cattle when non-vitrified and vitrified in vitro-derived embryos are transferred into recipients. *Theriogenology*, *120*, pp.105-110.

Celestino, J.J.H., Chaves, R.N., Matos, M.H.T., Silva, M.S.J., Bruno, J.B., Maia-Júnior, J.E. and Figueiredo, J.R., 2018. Mechanisms of atresia in ovarian follicles. *Animal Reproduction (AR)*, *6*(4), pp.495-508.

Cenariu, M., Pall, E., Cernea, C. and Groza, I., 2012. Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability.*BioMed Research International*, *2012*.

Chang, E.M., Han, J.E., Kim, Y.S., Lyu, S.W., Lee, W.S. and Yoon, T.K., 2011. Use of the natural cycle and vitrification thawed blastocyst transfer results in better in-vitro fertilization outcomes. *Journal of assisted reproduction and genetics*, *28*(4), pp.369-374.

Chawla, M., Fakih, M., Shunnar, A., Bayram, A., Hellani, A., Perumal, V., Divakaran, J. and Budak, E., 2015. Morphokinetic analysis of cleavage stage embryos and its relationship to aneuploidy in a retrospective time-lapse imaging study. *Journal of assisted reproduction and genetics*, *32*(1), pp.69-75.

Cimadomo, D., Capalbo, A., Ubaldi, F.M., Scarica, C., Palagiano, A., Canipari, R. and Rienzi, L., 2016. The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. *BioMed research international*, *2016*.

Cimadomo, D., Rienzi, L., Romanelli, V., Alviggi, E., Levi-Setti, P.E., Albani, E., Dusi, L., Papini, L., Livi, C., Benini, F. and Smeraldi, A., 2018. Inconclusive chromosomal assessment after blastocyst biopsy: prevalence, causative factors and outcomes after re-biopsy and re-vitrification. A multicenter experience. *Human Reproduction*, *33*(10), pp.1839-1846.

Cockburn, K. and Rossant, J., 2010. Making the blastocyst: lessons from the mouse. *The Journal of clinical investigation*, *120*(4), pp.995-1003.

Costa-Borges., N, Mestres., E, Garcia., M, Vanrell., I, Rink., K, Levtonov., M, Calderon., G., 2018.eppendorf No 351. www.eppendorf.com/uploads/media/Appl_351_GB_2018_final_01.pdf

Coy, P. and Avilés, M., 2010. What controls polyspermy in mammals, the oviduct or the oocyte?. *Biological Reviews*, *85*(3), pp.593-605.

Coy, P., Grullon, L., Canovas, S., Romar, R., Matas, C. and Aviles, M., 2008. Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction*, *135*(1), p.19.

Cruz, M., Garrido, N., Herrero, J., Pérez-Cano, I., Muñoz, M. and Meseguer, M., 2012. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reproductive biomedicine online*, *25*(4), pp.371-381.

Das, M. and Holzer, H.E., 2012. Recurrent implantation failure: gamete and embryo factors. *Fertility and sterility*, *97*(5), pp.1021-1027.

Davidson LM, Liu Y, Griffiths T, Jones C, Coward K. Laser Technology in the assisted reproductive technology laboratory: a narrative review. Reprod Biomed Online. 2018;38:725–739. doi: 10.1016/j.rbmo.2018.12.011.

Davidson, L.M., Liu, Y., Griffiths, T., Jones, C. and Coward, K., 2019. Laser technology in the ART laboratory: a narrative review. *Reproductive biomedicine online*, *38*(5), pp.725-739.

De Boer, K.A., Catt, J.W., Jansen, R.P., Leigh, D. and McArthur, S., 2004. Moving to blastocyst biopsy for preimplantation genetic diagnosis and single embryo transfer at Sydney IVF. *Fertility and Sterility*, *82*(2), pp.295-298.

de Sousa, R.V., da Silva Cardoso, C.R., Butzke, G., Dode, M.A.N., Rumpf, R. and Franco, M.M., 2017. Biopsy of bovine embryos produced in vivo and in vitro does not affect pregnancy rates. *Theriogenology*, *90*, pp.25-31.

De Vos, A. and Van Steirteghem, A., 2001. Aspects of biopsy procedures prior to preimplantation genetic diagnosis. *Prenatal diagnosis*, *21*(9), pp.767-780.

Debrock, S., Peeraer, K., Spiessens, C., Willemen, D., De Loecker, P. and D'Hooghe, T.M., 2011. The effect of modified quarter laser-assisted zona thinning on the implantation rate per embryo in frozen/vitrified-thawed/warmed embryo transfer cycles: a prospective randomized controlled trial. *Human reproduction*, *26*(8), pp.1997-2007.

Defra farming statistics: March 2021 assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/973322/structure-dec20-ukseries-25mar21i.pdf

del Collado, M., da Silveira, J.C., Sangalli, J.R., Andrade, G.M., da Silva Sousa, L.R., Silva, L.A., Meirelles, F.V. and Perecin, F., 2017. Fatty acid binding protein 3 and transzonal projections are involved in lipid accumulation during in vitro maturation of bovine oocytes. *Scientific reports*, *7*(1), pp.1-13.

Delhanty, J.D., Harper, J.C., Ao, A., Handyside, A.H. and Winston, R.M., 1997. Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. *Human genetics*, *99*(6), pp.755-760.

Denicol,A.C., K.B.Dobbs, K.M.McLean, S.F.Carambula, B. Loureiro, and P. J. Hansen. 2013. Canonical WNT signaling regulates development of bovine embryos to the blastocyst stage. Sci. Rep. 3:1266. doi:10.1038/srep01266

DeUgarte, C.M., Li, M., Surrey, M., Danzer, H., Hill, D. and DeCherney, A.H., 2008. Accuracy of FISH analysis in predicting chromosomal status in patients undergoing preimplantation genetic diagnosis. *Fertility and sterility*, *90*(4), pp.1049-1054.

Dickey, R.P., Wortham, J.W.E., Potts, A. and Welch, A., 2010. Effect of IVF laboratory air quality on pregnancy success. *Fertility and Sterility*, *94*(4), p.S151.

Dietzel, E., Floehr, J., Van de Leur, E., Weiskirchen, R. and Jahnen-Dechent, W., 2016. Recombinant fetuin-B protein maintains high fertilization rate in cumulus cell-free mouse oocytes. *Mhr: Basic science of reproductive medicine*, pp.1-9.

Dinnyés, A., Lonergan, P., Fair, T., Boland, M.P. and Yang, X., 1999. Timing of the first cleavage post-insemination affects cryosurvival of in vitro–produced bovine blastocysts. *Molecular Reproduction and Development: Incorporating Gamete Research*, *53*(3), pp.318-324.

Dobrinsky, J.R., 1996. Cellular approach to cryopreservation of embryos. *Theriogenology*, *45*(1), pp.17-26.

Douglas-Hamilton, D.H. and Conia, J.D., 2001. Thermal effects in laser-assisted pre-embryo zona drilling. *Journal of biomedical optics*, *6*(2), pp.205-214.

Duan, L., Bao, S., Li, K., Teng, X., Hong, L. and Zhao, X., 2017. Comparing the long-acting and short-acting forms of gonadotropin-releasing hormone agonists in the long protocol of IVF/ICSI Cycles: A retrospective study. *Journal of Obstetrics and Gynaecology Research*, *43*(6), pp.1037-1042.

Ealy, A.D., Wooldridge, L.K. and McCoski, S.R., 2019. BOARD INVITED REVIEW: Post-transfer consequences of in vitro-produced embryos in cattle. *Journal of animal science*, *97*(6), pp.2555-2568.

Eckert, J.J. and Fleming, T.P., 2008. Tight junction biogenesis during early development. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1778*(3), pp.717-728.

Edwards, R.G., 1965. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*, *208*(5008), pp.349-351.

Egger-Danner, C., Cole, J.B., Pryce, J.E., Gengler, N., Heringstad, B., Bradley, A. and Stock, K.F., 2015. Invited review: overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. *Animal*, *9*(2), pp.191-207.

El-Sayed, A., Hoelker, M., Rings, F., Salilew, D., Jennen, D., Tholen, E., Sirard, M.A., Schellander, K. and Tesfaye, D., 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological genomics*, *28*(1), pp.84-96.

Elsden, R.P., Hasler, J.F. and Seidel Jr, G.E., 1976. Non-surgical recovery of bovine eggs. *Theriogenology*, *6*(5), pp.523-532.

Erdem, H., Karasahin, T., Alkan, H., Dursun, S., Satilmis, F. and Guler, M., 2020. Effect of embryo quality and developmental stages on pregnancy rate during fresh embryo transfer in beef heifers. *Tropical animal health and production*, *52*, pp.2541-2547.

Faizah, H.M.S., Richard, F., Meena, P., Stanley, K.L., Amriana, H., Alhassany, A., Yadav, S.B., Marie, L., Crouch, B. and Saipul, B.A.R., 2018. Multiple ovulation embryo transfer (MOET) in dairy cattle in Gatton. *Malaysian journal of veterinary research*, *9*(2), pp.109-116.

Fang, L., Jiang, J., Li, B., Zhou, Y., Freebern, E., Vanraden, P.M., Cole, J.B., Liu, G.E. and Ma, L., 2019. Genetic and epigenetic architecture of paternal origin contribute to gestation length in cattle. *Communications biology*, *2*(1), pp.1-11.

FAO 2009 http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf

FAO World Agriculture (2012) https://ourworldindata.org/meat-and-seafood-production-consumption

Farin, C.E., Barnwell, C.V. and Farmer, W.T., 2014. Abnormal offspring syndrome. *Bovine Reproduction*, pp.620-638.

Farin, C.E., Farmer, W.T. and Farin, P.W., 2009. Pregnancy recognition and abnormal offspring syndrome in cattle. *Reproduction, Fertility and Development*, *22*(1), pp.75-87.

Farin, P.W., Crosier, A.E. and Farin, C.E., 2001. Influence of in vitro systems on embryo survival and fetal development in cattle. *Theriogenology*, *55*(1), pp.151-170.

Feichtinger, W., Strohmer, H. and Radner, K., 1992. Erbium YAG laser for micromanipulation of oocytes and spermatozoa. *The Lancet*, *340*(8811), pp.115-116.

Ferraz, P.A., Burnley, C., Karanja, J., Viera-Neto, A., Santos, J.E.P., Chebel, R.C. and Galvão, K.N., 2016. Factors affecting the success of a large embryo transfer program in Holstein cattle in a commercial herd in the southeast region of the United States. *Theriogenology*, *86*(7), pp.1834-1841. *Fertil Steril. 2013 Sep; 100(3):608-14.*

Fiorentino, F., Biricik, A., Nuccitelli, A., De Palma, R., Kahraman, S., Sertyel, S., Karadayi, H., Cottone, G., Baldi, M., Caserta, D. and Moscarini, M., 2008. Rapid protocol for pre-conception genetic diagnosis of single gene mutations by first polar body analysis: a possible solution for the Italian patients. *Prenat Diagn*, *28*(1), pp.62-4.

Fischer, B. and Bavister, B.D., 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *Reproduction*, *99*(2), pp.673-679.

Fisher, P.J., Hyndman, D.L., Bixley, M.J., Oback, F.C., Popovic, L., McGowan, L.T., Berg, M.C. and Wells, D.N., 2012. Brief communication: potential for genomic selection of bovine embryos. In *Proc. NZ Soc. Anim. Prod* (Vol. 72, pp. 156-158).

Fisher, P.J., Hyndman, D.L., Bixley, M.J., Oback, F.C., Popovic, L., McGowan, L.T., Berg, M.C. and Wells, D.N., 2012. Brief communication: potential for genomic selection of bovine embryos. In *Proc. NZ Soc. Anim. Prod* (Vol. 72, pp. 156-158).

Foote, R.H., 2010. The history of artificial insemination: Selected notes and notables. *J. Anim. Sci*, *80*, pp.1-10.

Fragouli, E., Alfarawati, S., Spath, K., Jaroudi, S., Sarasa, J., Enciso, M. and Wells, D., 2013. The origin and impact of embryonic aneuploidy. *Human genetics*, *132*(9), pp.1001-1013.

Fragouli, E., Katz-Jaffe, M., Alfarawati, S., Stevens, J., Colls, P., Goodall, N.N., Tormasi, S., Gutierrez-Mateo, C., Prates, R., Schoolcraft, W.B. and Munne, S., 2010. Comprehensive chromosome screening of polar bodies and blastocysts from couples experiencing repeated implantation failure. *Fertility and sterility*, *94*(3), pp.875-887.

Fujihira, T., Kinoshita, M., Sasaki, M., Ohnishi, M., Ishikawa, H., Ohsumi, S. and Fukui, Y., 2004. Comparative studies on lipid analysis and ultrastructure in porcine and southern minke whale (Balaenoptera bonaerensis) oocytes. *Journal of Reproduction and Development*, *50*(5), pp.525-532.

Fujiwara K, Sano D, Seita Y, Inomata T, Ito J, Kashiwazaki N (2010) Ethylene glycol-supplemented calcium-free media improve zona penetration of vitrified rat oocytes by sperm cells. J Reprod Dev 56:169–175

Gardner, D.K., 1994. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biology International*, *18*(12), pp.1163-1180.

Geber, S., Bossi, R., Lisboa, C.B., Valle, M. and Sampaio, M., 2011. Laser confers less embryo exposure than acid tyrode for embryo biopsy in preimplantation genetic diagnosis cycles: a randomized study. *Reproductive Biology and Endocrinolog 9*(1), p.58.

Geber, S., Winston, R.M. and Handyside, A.H., 1995. Proliferation of blastomeres from biopsied cleavage stage human embryos in vitro: an alternative to blastocyst biopsy for preimplantation diagnosis. *Human Reproduction*, *10*(6), pp.1492-1496.

George, M.A. and Johnson, M.H., 1993. Use of fetal bovine serum substitutes for the protection of the mouse zona pellucida against hardening during cryoprotectant addition. *Human reproduction (Oxford, England)*, *8*(11), pp.1898-1900.

Gianaroli, L., Magli, M.C., Pomante, A., Crivello, A.M., Cafueri, G., Valerio, M. and Ferraretti, A.P., 2014. Blastocentesis: a source of DNA for preimplantation genetic testing. Results from a pilot study. *Fertility and sterility*, *102*(6), pp.1692-1699.

Gonzalez, C., Le Bourhis, D., Joly, C.G., Moulin, B., Heyman, Y. and Humblot, P., 2008. Pregnancy rates after single direct transfer of biopsed frozen-thawed bovine embryos according to quality. *Proceedings of the 24th AETE*.

Gordon, I., 1994. Laboratory production of cattle embryos. CAB International. *Biotechnology in Agriculture*, (11), p.640.

Gov UK 2020 (Executive summary): https://www.gov.uk/government/publications/farm-animal-genetic-resources-fangr-technology-review/review-of-current-and-new-technologies-relevant-to-fangr-october-2018

Granleese, T., Clark, S.A., Swan, A.A. and van der Werf, J.H., 2015. Increased genetic gains in sheep, beef and dairy breeding programs from using female reproductive technologies combined with optimal contribution selection and genomic breeding values. *Genetics Selection Evolution*, *47*(1), p.70.

Greve, T., 1976. Egg transfer in the bovine: Effect of injecting PMSG on different days. *Theriogenology*, *5*(1), pp.15-19.

Greve, T., Lehn-Jensen, H. and Rasbech, N.O., 1977. Non-surgical recovery of bovine embryos. *Theriogenology*, *7*(4), pp.239-250.

Gu, Y.F., Zhou, Q.W., Zhang, S.P., Lu, C.F., Gong, F., Tan, Y.Q., Lu, G.X. and Lin, G., 2018. Inner cell mass incarceration in 8-shaped blastocysts does not increase monozygotic twinning in preimplantation genetic diagnosis and screening patients. *Plos one*, *13*(1), p.e0190776.

Gualtieri, R., Mollo, V., Barbato, V., Fiorentino, I., Iaccarino, M. and Talevi, R., 2011. Ultrastructure and intracellular calcium response during activation in vitrified and slow-frozen human oocytes. *Human Reproduction*, *26*(9), pp.2452-2460.

Gutiérrez-ad´ n, A., Rizos, D., Fair, T., Moreira, P.N., Pintado, B., Fuente, J.D.L., Boland, M.P. and Lonergan, P., 2004. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Molecular Reproduction and Development: Incorporating Gamete Research*, *68*(4), pp.441-448.

Hagemann, L.J., Beaumont, S.E., Berg, M., Donnison, M.J., Ledgard, A., Peterson, A.J., Schurmann, A. and Tervit, H.R., 1999. Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. *Molecular Reproduction and Development: Incorporating Gamete Research*, *53*(4), pp.451-458.

Haley, S.A. and Wessel, G.M., 2004. Regulated proteolysis by cortical granule serine protease 1 at fertilization. *Molecular biology of the cell*, *15*(5), pp.2084-2092.

Halvaei, I., Ghazali, S., Nottola, S.A. and Khalili, M.A., 2018. Cleavage-stage embryo micromanipulation in the clinical setting. *Systems biology in reproductive medicine*, *64*(3), pp.157-168.

Hamel, M., Dufort, I., Robert, C., Léveillé, M.C., Leader, A. and Sirard, M.A., 2010. Identification of follicular marker genes as pregnancy predictors for human IVF: new evidence for the involvement of luteinization process. *MHR: Basic science of reproductive medicine*, *16*(8), pp.548-556.

Hammadeh, M.E., Fischer-Hammadeh, C. and Ali, K.R., 2011. Assisted hatching in assisted reproduction: a state of the art. *Journal of assisted reproduction and genetics*, *28*(2), pp.119-128.

Hammond, E.R., McGillivray, B.C., Wicker, S.M., Peek, J.C., Shelling, A.N., Stone, P., Chamley, L.W. and Cree, L.M., 2017. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. *Fertility and sterility*, *107*(1), pp.220-228.

Handyside, A.H., 1991. Biopsy of human cleavage stage embryos and sexing by DNA amplification. In *Preimplantation genetics* (pp. 75-83). Springer, Boston, MA.

Hansen, P.J., 2020. Implications of assisted reproductive technologies for pregnancy outcomes in mammals. *Annual review of animal biosciences*, *8*, pp.395-413.

Hansen, P.J., 2020[b]. The incompletely fulfilled promise of embryo transfer in cattle—why aren't pregnancy rates greater and what can we do about it?. *Journal of Animal Science*, *98*(11), p.skaa288.

Hardy, K., 1999. Apoptosis in the human embryo. *Reviews of Reproduction*, *4*(3), pp.125-134.

Hardy, K., Martin, K.L., Leese, H.J., Winston, R.M. and Handyside, A.H., 1990. Human preimplantation development in vitro is not adversely affected by biopsy at the 8-cell stage. *Human Reproduction*, *5*(6), pp.708-714.

Harper, J.C. and SenGupta, S.B., 2012. Preimplantation genetic diagnosis: state of the art 2011.*Human genetics*, *131*(2), pp.175-186.

Harton, G.L., Magli, M.C., Lundin, K., Montag, M., Lemmen, J. and Harper, J.C., 2011. ESHRE PGD Consortium/Embryology Special Interest Group—best practice guidelines for polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS). *Human reproduction*, *26*(1), pp.41-46.

Hartshorn, C., Anshelevich, A. and Wangh, L.J., 2005. Laser zona drilling does not induce hsp70i transcription in blastomeres of eight-cell mouse embryos. *Fertility and sterility*, *84*(5), pp.1547-1550.

Harvey, A.J., 2007. The role of oxygen in ruminant preimplantation embryo development and metabolism. *Animal reproduction science*, *98*(1-2), pp.113-128.

Hashimoto, S., Nishihara, T., Murata, Y., Oku, H., Nakaoka, Y., Fukuda, A. and Morimoto, Y., 2008. Medium without ammonium accumulation supports the developmental competence of human embryos. *Journal of Reproduction and Development*, pp.0806230072-0806230072.

Hasler, J.F., 2001. Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle. *Theriogenology*, *56*(9), pp.1401-1415.

Hasler, J.F., Cardey, E., Stokes, J.E. and Bredbacka, P., 2002. Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment. *Theriogenology*, *58*(8), pp.1457-1469.

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E. and Trimmer, S.A., 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*, *43*(1), pp.141-152.

Herrera, C., 2016. Clinical applications of preimplantation genetic testing in equine, bovine, and human embryos. *Journal of Equine Veterinary Science*, *41*, pp.29-34.

Herrero, L., Cascales, L., Ortiz, J.A., Aparicio, M., Ten, J. and Bernabeu, R., 2019. Does the trophectoderm biopsy technique, pulling or flicking, affects the diagnostic rate, mosaic rate or

transferability rate displayed by the genetic analysis in PGT-A cycles?. *Oral presentation at European Society of Human Reproduction and Embryology.(Vienna, Austria)*.

Heyman, Y., Tamassia, M., Richard, C., Renard, J.P. and Chastant-Maillard, S., 2003. Preliminary results on variability in oocyte recovery and developmental competence in cattle derived from embryonic cloning: work in progress. *Theriogenology*, *60*(5), pp.891-900.

Ho, Y., Wigglesworth, K., Eppig, J.J. and Schultz, R.M., 1995. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Molecular reproduction and development*, *41*(2), pp.232-238.

Honguntikar, S.D., Salian, S.R., D'souza, F., Uppangala, S., Kalthur, G. and Adiga, S.K., 2017. Epigenetic changes in preimplantation embryos subjected to laser manipulation. *Lasers in medical science*, *32*(9), pp.2081-2087.

Hsieh, Y.Y., Huang, C.C., Cheng, T.C., Chang, C.C., Tsai, H.D. and Lee, M.S., 2002. Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. *Fertility and sterility*, *78*(1), pp.179-182.

Huisman, G.J., Fauser, B.C., Eijkemans, M.J. and Pieters, M.H., 2000. Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture. *Fertility and Sterility*, *73*(1), pp.117-122.

Humblot, P., Le Bourhis, D., Fritz, S., Colleau, J.J., Gonzalez, C., Guyader Joly, C., Malafosse, A., Heyman, Y., Amigues, Y., Tissier, M. and Ponsart, C., 2010. Reproductive technologies and genomic selection in cattle. *Veterinary Medicine International*, *2010*.

IETS manual 2010: https://www.iets.org/Career-Development/Bovine-Ova-Tutorials

IETS newsletter: http://www.iets.org/Newsletter Dec 2020 Embryo Technology newsletter.

IETS newsletter :https://www.iets.org/pub_newsletter.asp

Ireland, J.L.H., Scheetz, D., Jimenez-Krassel, F., Themmen, A.P.N., Ward, F., Lonergan, P., Smith, G.W., Perez, G.I., Evans, A.C.O. and Ireland, J.J., 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biology of reproduction*, *79*(6), pp.1219-1225.

Iwata, H., Akamatsu, S., Minami, N. and Yamada, M., 1998. Effects of antioxidants on the development of bovine IVM/IVF embryos in various concentrations of glucose. *Theriogenology*, *50*(3), pp.365-375.

Jacobs, P.A., 1990. The role of chromosome abnormalities in reproductive failure. *Reproduction Nutrition Development*, *30*(Suppl. 1), pp.63s-74s.

Johnson, D., Haimowitz, Z., Arifova, M., Akopians, A., Welch, C. and Barritt, J., 2019. Repeated high intensity laser biopsy pulses do not alter genetic testing results nor increases mosaicism following human embryo blastocyst biopsy of trophectoderm cells. *Reproductive BioMedicine Online*, *39*, pp.e4-e5.

Jones, A.E., Wright, G., Kort, H.I., Straub, R.J. and Nagy, Z.P., 2006. Comparison of laser-assisted hatching and acidified Tyrode's hatching by evaluation of blastocyst development rates in sibling embryos: a prospective randomized trial *Fertility and sterility*, *85*(2), pp.487-491.

Kara, U., Çoban, S., Say, E., Hizli, H., Mutlu, H., Nak, Y., SAĞIRKAYA, H., Ayaşan, T., Çoşkun, M.A., Asarkaya, A. and Erten, H.E., 2020. Effect of embryo quality on pregnancy outcome in recipient cows and heifers. *Journal of the Hellenic Veterinary Medical Society*, *71*(2), pp.2235-2242.

Karagenc, L., Sertkaya, Z., Ciray, N., Ulug, U. and Bahceci, M., 2004. Impact of oxygen concentration on embryonic development of mouse zygotes. *Reproductive biomedicine online*, *9*(4), pp.409-417.

Kelk, D.A., Sawarkar, S.S., Liu, Y., Dufton, M., Ribustello, L. and Munne, S., 2017. Does laser assisted biopsy introduce mosaic or chaotic changes to biopsied cells?. *Fertility and Sterility*, *108*(3), p.e88.

Kim, E.K., Kim, E.A., Kim, E.H., Kim, N.H. and Choi, D.H., 2015. Day 4 Biopsy Improves Pregnancy Outcome Comparing to Day 3 Biopsy in Preimplantation Genetic Screening. *Gynecol Obstet (Sunnyvale)*, *5*(330), pp.2161-0932.

Kim, H.J., Kim, C.H., Lee, S.M., Choe, S.A., Lee, J.Y., Jee, B.C., Hwang, D. and Kim, K.C., 2012. Outcomes of preimplantation genetic diagnosis using either zona drilling with acidified Tyrode's solution or partial zona dissection. *Clinical and experimental reproductive medicine*, *39*(3), p.118.

KING, W.A., 1990. Chromosome abnormalities and pregnancy failure in domestic animals. In *Advances in veterinary science and comparative medicine* (Vol. 34, pp. 229-250). Academic Press.

Kokkali, G., Traeger-Synodinos, J., Vrettou, C., Stavrou, D., Jones, G.M., Cram, D.S., Makrakis, E., Trounson, A.O., Kanavakis, E. and Pantos, K., 2007. Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of β-thalassaemia: a pilot study. *Human Reproduction*, *22*(5), pp.1443-1449

Korhonen, K., Julkunen, H., Kananen, K., Bredbacka, P., Tiirikka, T., Räty, M., Vartia, K., Kaimio, I., Kontinen, A., Halmekytö, M. and Vilkki, J., 2012. The effect of ascorbic acid during biopsy and cryopreservation on viability of bovine embryos produced in vivo. *Theriogenology*, *77*(1), pp.201-205.

Körschgen, H., Kuske, M., Karmilin, K., Yiallouros, I., Balbach, M., Floehr, J., Wachten, D., Jahnen-Dechent, W. and Stöcker, W., 2017. Intracellular activation of ovastacin mediates pre-fertilization hardening of the zona pellucida. *MHR: Basic science of reproductive medicine*, *23*(9), pp.607-616.

Kuliev, A. and Verlinsky, Y., 2005. Preimplantation genetic diagnosis in assisted reproduction. *Expert review of molecular diagnostics*, *5*(4), pp.499-505.

Küpker, W., Huber, G., Ludwig, M. and Diedrich, K., 2001. Preimplantation genetic diagnosis in Germany: ethical responsibility and law. *Reproductive biomedicine online*, *2*(2), pp.84-87.

Lacaze, S., Ponsart, C. and Humblot, P., 2009, September. Influence of embryo stage on pregnancy rates following transfer of bovine biopsied embryos under on-farm conditions. In *25 ème COLLOQUE SCIENTIFIQUE _ 25 th SCIENTIFIC MEETING*.

Lambert, R.D., Bernard, C., Rioux, J.E., Beland, R., D'amours, D. and Montreuil, A., 1983. Endoscopy in cattle by the paralumbar route: technique for ovarian examination and follicular aspiration. *Theriogenology*, *20*(2), pp.149-161.

Lauri, A., Lazzari, G., Galli, C., Lagutina, I., Genzini, E., Braga, F., Mariani, P. and Williams, J.L., 2013. Assessment of MDA efficiency for genotyping using cloned embryo biopsies. *Genomics*, *101*(1), pp.24-29.

Lawitts, J.A. and Biggers, J.D., 1993. [9] Culture of preimplantation embryos. *Methods in enzymology*, *225*, pp.153-164.

Lechniak, D., Pers-Kamczyc, E. and Pawlak, P., 2008. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reproductive biology*, *8*(1), pp.23-42.

Leigh D. Improvement and standardization of biopsy procedures. Reprod BioMed Online. 2019. 38:e6-e7.

Lequarré, A.S., Marchandise, J., Moreau, B., Massip, A. and Donnay, I., 2003. Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. *Biology of reproduction*, *69*(5), pp.1707-1713.

Lohuis, M., Smith, C. and Burnside, E.B., 1990. Embryo transfer results in a dispersed nucleus MOET/AI testing scheme. *Proc. 4th World Congr. Genet. Livestock Prod*, *14*, pp.221-4.

Lohuis, M.M., Smith, C. and Dekkers, J.C.M., 1993. MOET results from a dispersed hybrid nucleus programme in dairy cattle. *Animal Science*, *57*(3), pp.369-378.

Lonergan, P. and Fair, T., 2008. In vitro-produced bovine embryos—dealing with the warts. *Theriogenology*, *69*(1), pp.17-22.

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P. and Boland, M.P., 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *Reproduction*, *117*(1), pp.159-167.

Lonergan, P., Monaghan, P., Rizos, D., Boland, M.P. and Gordon, I., 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Molecular reproduction and development*, *37*(1), pp.48-53.

Looney, C.R., Bondioli, K.R., Hill, K.G. and Massey, J.M., 1988. Superovulation of donor cows with bovine follicle-stimulating hormone (bFSH) produced by recombinant DNA technology. *Theriogenology*, *29*(1), p.271.

Looney, C.R., Lindsey, B.R., Gonseth, C.L. and Johnson, D.L., 1994. Commercial aspects of oocyte retrieval and in vitro fertilization (IVF) for embryo production in problem cows. *Theriogenology*, *41*(1), pp.67-72.

Lopata, A. and Hay, D.L., 1989. The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture. *Human reproduction*, *4*(suppl_1), pp.87-94.

Lopera-Vasquez, R., Hamdi, M., Fernandez-Fuertes, B., Maillo, V., Beltran-Brena, P., Calle, A., Redruello, A., Lopez-Martin, S., Gutierrez-Adan, A., Yañez-Mó, M. and Ramirez, M.Á., 2016. Extracellular vesicles from BOEC in in vitro embryo development and quality. *PloS one*, *11*(2).

Lopes, A.S., Martinussen, T., Greve, T. and Callesen, H., 2006. Effect of days post-partum, breed and ovum pick-up scheme on bovine oocyte recovery and embryo development. *Reproduction in domestic animals*, *41*(3), pp.196-203.

Lopes, R.F.F., Forell, F., Oliveira, A.T.D. and Rodrigues, J.L., 2001. Splitting and biopsy for bovine embryo sexing under field conditions. *Theriogenology*, *56*(9), pp.1383-1392.

Loureiro, B., Bonilla, L., Block, J., Fear, J.M., Bonilla, A.Q. and Hansen, P.J., 2009. Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced in vitro. *Endocrinology*, *150*(11), pp.5046-5054.

Lussier, J.G., Lamothe, P. and Pacholek, X., 1995. Effects of follicular dominance and different gonadotropin preparations on the superovulatory response in cows. *Theriogenology*, *1*(43), p.270.

Macmillan, K.L. and Peterson, A.J., 1993. A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. *Animal Reproduction Science*, *33*(1-4), pp.1-25.

Macmillan, K.L., Taufa, V.K., Barnes, D.R. and Day, A.M., 1991. Plasma progesterone concentrations in heifers and cows treated with a new intravaginal device. *Animal Reproduction Science*, *26*(1-2), pp.25-40.

Magata, F., Ideta, A., Okubo, H., Matsuda, F., Urakawa, M. and Oono, Y., 2019. Growth potential of bovine embryos presenting abnormal cleavage observed through time lapse cinematography. *Theriogenology*, *133*, pp.119-124.

Magli, M.C., Gianaroli, L., Grieco, N., Cefalu, E., Ruvolo, G. and Ferraretti, A.P., 2006. Cryopreservation of biopsied embryos at the blastocyst stage. *Human Reproduction*, *21*(10), pp.2656-2660.

Magli, M.C., Pomante, A., Cafueri, G., Valerio, M., Crippa, A., Ferraretti, A.P. and Gianaroli, L., 2016. Preimplantation genetic testing: polar bodies, blastomeres, trophectoderm cells, or blastocoelic fluid?. *Fertility and sterility*, *105*(3), pp.676-683.

Majumdar, G., Majumdar, A., Verma, I.C. and Upadhyaya, K.C., 2017. Relationship between morphology, euploidy and implantation potential of cleavage and blastocyst stage embryos. *Journal of human reproductive sciences*, *10*(1), p.49.
Makhijani, R., Bartels, C.B., Godiwala, P., Bartolucci, A., DiLuigi, A., Nulsen, J., Grow, D., Benadiva, C. and Engmann, L., 2021. Impact of trophectoderm biopsy on obstetric and perinatal outcomes following frozen–thawed embryo transfer cycles. *Human Reproduction*,*36*(2), pp.340-348.

Malter, H.E. and Cohen, J., 1989. Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. *Gamete research*, *24*(1), pp.67-80.

Mandawala, A.A., Harvey, S.C., Roy, T.K. and Fowler, K.E., 2016. Cryopreservation of animal oocytes and embryos: Current progress and future prospects. *Theriogenology*,*86*(7), pp.1637-1644.

Mapletoft, R.J., 2018. History and perspectives on bovine embryo transfer.*Animal Reproduction (AR)*,*10*(3), pp.168-173.

Marques CC, Santos-Silva C, Rodrigues C, Matos JE, Moura T, Baptista MC, Horta AEM, Bessa RJB, Alves SP, Soveral G, Pereira RMLN (2018) Bovine oocyte membrane permeability and cryosurvival: effects of different cryoprotectants and calcium in the vitrification media. Cryobiology 81:4–11

Martínez, A.G., Brogliatti, G.M., Valcarcel, A. and De Las Heras, M.A., 2002. Pregnancy rates after transfer of frozen bovine embryos: a field trial. *Theriogenology*, *58*(5), pp.963-972.

McArthur, S.J., Leigh, D., Marshall, J.T., de Boer, K.A. and Jansen, R.P., 2005. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertility and sterility*, *84*(6), pp.1628-1636.

McArthur, S.J., Leigh, D., Marshall, J.T., Gee, A.J., De Boer, K.A. and Jansen, R.P.S., 2008. Blastocyst trophectoderm biopsy and preimplantation genetic diagnosis for familial monogenic disorders and chromosomal translocations. *Prenatal Diagnosis: Published in Affiliation With the International Society for Prenatal Diagnosis*, *28*(5), pp.434-442.

McKenzie, L.J., Pangas, S.A., Carson, S.A., Kovanci, E., Cisneros, P., Buster, J.E., Amato, P. and Matzuk, M.M., 2004. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Human reproduction*, *19*(12), pp.2869-2874.

MCLEAN, Z., OBACK, B. and LAIBLE, G., 2020. Embryo-mediated genome editing for accelerated genetic improvement of livestock. *Frontiers of Agricultural Science and Engineering*, *7*(2), pp.148-160.

McMillan, W.H., 1998. Statistical models predicting embryo survival to term in cattle after embryo transfer. *Theriogenology*, *50*(7), pp.1053-1070.

McNamara, H.C., Kane, S.C., Craig, J.M., Short, R.V. and Umstad, M.P., 2016. A review of the mechanisms and evidence for typical and atypical twinning. *American journal of obstetrics and gynecology*, *214*(2), pp.172-191.

McNATTY, K.P., MAKRIS, A., DEGRAZIA, C., RAPIN, O. and RYAN, K.J., 1979. The production of progesterone, androgens, and estrogens by granulosa cells, thecal tissue, and stromal tissue from human ovaries in vitro. *The Journal of Clinical Endocrinology & Metabolism*, *49*(5), pp.687-699.

Merton, J.S., De Roos, A.P.W., Mullaart, E., De Ruigh, L., Kaal, L., Vos, P.L.A.M. and Dieleman, S.J., 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology*, *59*(2), pp.651-674.

Miyata, H., Matsubayashi, H., Fukutomi, N., Matsuba, J., Koizumi, A. and Tomiyama, T., 2010. Relevance of the site of assisted hatching in thawed human blastocysts: a preliminary report. *Fertility and sterility*, *94*(6), pp.2444-2447.

Mogas, T., 2019. Update on the vitrification of bovine oocytes and in vitro-produced embryos. *Reproduction, Fertility and Development*, *31*(1), pp.105-117.

Montag, M., 2019. Polar body biopsy. In *In Vitro Fertilization* (pp. 583-590). Springer, Cham.

Montag, M., Van der Ven, K., Rösing, B. and Van der Ven, H., 2009. Polar body biopsy: a viable alternative to preimplantation genetic diagnosis and screening. *Reproductive biomedicine online*, *18*, pp.6-11.

Montag[b], M.H., Klose, R., Köster, M., Rösing, B., van der Ven, K., Rink, K. and van der Ven, H., 2009. Application of non-contact laser technology in assisted reproduction. *Medical Laser Application*, *24*(1), pp.57-64.

Moreira da Silva, F. and Metelo, R., 2005. Relation between Physical Properties of the Zona Pellucida and Viability of Bovine Embryos after Slow-freezing and Vitrification. *Reproduction in domestic animals*, *40*(3), pp.205-209.

Morishita, T., Yokoyama, M., Nozaki, M., Sano, M. and Nakano, H., 1993. Improvement in blastocyst hatching of mouse embryos cocultured with human placental cells. *Journal of assisted reproduction and genetics*, *10*(7), pp.463-467.02.

Morrell, J.M. and Mayer, I., 2017. Reproduction biotechnologies in germplasm banking of livestock species: a review. *Zygote*, *25*(5), pp.545-557.

Morris, S.A., Teo, R.T., Li, H., Robson, P., Glover, D.M. and Zernicka-Goetz, M., 2010. Origin and formation of the first two distinct cell types of the inner cell mass in the mouse embryo. *Proceedings of the National Academy of Sciences*, *107*(14), pp.6364-6369.

Moutou, C., Goossens, V., Coonen, E., De Rycke, M., Kokkali, G., Renwick, P., SenGupta, S.B., Vesela, K. and Traeger-Synodinos, J., 2014. ESHRE PGD Consortium data collection XII: cycles from January to December 2009 with pregnancy follow-up to October 2010. *Human Reproduction*, *29*(5), pp.880-903.

Mullaart, E. and Wells, D., 2018. Embryo biopsies for genomic selection. *Animal Biotechnology 2*, pp.81-94.

Munné, S. and Wells, D., 2017. Detection of mosaicism at blastocyst stage with the use of high-resolution next-generation sequencing. *Fertility and sterility*, *107*(5), pp.1085-1091

Nagaoka, S.I., Hassold, T.J. and Hunt, P.A., 2012. Human aneuploidy: mechanisms and new insights into an age-old problem. *Nature Reviews Genetics*, *13*(7), pp.493-504.

Nakasuji, T., Saito, H., Araki, R., Nakaza, A., Kuwahara, A., Ishihara, O., Irahara, M., Kubota, T., Yoshimura, Y. and Sakumoto, T., 2014. Validity for assisted hatching on pregnancy rate in assisted reproductive technology: Analysis based on results of J apan A ssisted R eproductive T echnology Registry System 2010. *Journal of Obstetrics and Gynaecology Research*, *40*(6), pp.1653-1660.

Neal, S.A., Franasiak, J.M., Forman, E.J., Werner, M.D., Morin, S.J., Tao, X., Treff, N.R. and Scott Jr, R.T., 2017. High relative deoxyribonucleic acid content of trophectoderm biopsy adversely affects pregnancy outcomes. *Fertility and sterility*, *107*(3), pp.731-736.

Negrón-Pérez, V.M. and Hansen, P.J., 2017. The bovine embryo hatches from the zona pellucida through either the embryonic or abembryonic pole. *Journal of assisted reproduction and genetics*, *34*(6), pp.725-731.

Neto, A.C., Sanches, B.V., Binelli, M., Seneda, M.M., Perri, S.H. and Garcia, J.F., 2005. Improvement in embryo recovery using double uterine flushing. *Theriogenology*, *63*(5), pp.1249-1255.

Nicholas, F.W. and Smith, C., 1983. Increased rates of genetic change in dairy cattle by embryo transfer and splitting. *Animal Science*, *36*(3), pp.341-353.

Nichols, J. and Gardner, R.L., 1989. Effect of damage to the zona pellucida on development of preimplantation embryos in the mouse. *Human Reproduction*, *4*(2), pp.180-187.

Niemann, H., Carnwath, J.W., Herrmann, D., Wieczorek, G., Lemme, E., Lucas-Hahn, A. and Olek, S., 2010. DNA methylation patterns reflect epigenetic reprogramming in bovine embryos. *Cellular Reprogramming (Formerly "Cloning and Stem Cells")*, *12*(1), pp.33-42.

Niimura, S., Ogata, T., Okimura, A., Sato, T., Uchiyama, Y., Seta, T., Nakagawa, H., Nakagawa, K. and Tamura, Y., 2010. Time-lapse videomicrographic observations of blastocyst hatching in cattle. *Journal of Reproduction and Development*, pp.1008250299-1008250299.

Nivet, A.L., Bunel, A., Labrecque, R., Belanger, J., Vigneault, C., Blondin, P. and Sirard, M.A., 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction*, *143*(2), p.165.

Nogueira, M.F.G., Barros, B.J., Teixeira, A.B., Trinca, L.A., Michael, J.D. and Barros, C.M., 2002. Embryo recovery and pregnancy rates after the delay of ovulation and fixed time insemination in superstimulated beef cows. *Theriogenology*, *57*(6), pp.1625-1634.

Oliveira, C.S., Quintão, C.C.R., de Freitas, C., dos Reis Camargo, A.J., Serapião, R.V. and de Almeida Camargo, L.S., 2017. Post implantation development reveals that biopsy procedure can segregate "healthy" from "unhealthy" bovine embryos and prevent miscarriages. *Animal reproduction science*, *184*, pp.51-58.

O'Sullivan, C.M., Liu, S.Y., Karpinka, J.B. and Rancourt, D.E., 2002. Embryonic hatching enzyme strypsin/ISP1 is expressed with ISP2 in endometrial glands during implantation. *Molecular Reproduction and Development: Incorporating Gamete Research*, *62*(3), pp.328-334.

Palini, S., Galluzzi, L., De Stefani, S., Bianchi, M., Wells, D., Magnani, M. and Bulletti, C., 2013. Genomic DNA in human blastocoele fluid. *Reproductive biomedicine online*, *26*(6), pp.603-610.

Papi, M., Brunelli, R., Sylla, L., Parasassi, T., Monaci, M., Maulucci, G., Missori, M., Arcovito, G., Ursini, F. and De Spirito, M., 2010. Mechanical properties of zona pellucida hardening. *European Biophysics Journal*, *39*(6), pp.987-992.

Park, J.H., Lee, J.H., Choi, K.M., Joung, S.Y., Kim, J.Y., Chung, G.M., Jin, D.I. and Im, K.S., 2001. Rapid sexing of preimplantation bovine embryo using consecutive and multiplex polymerase chain reaction (PCR) with biopsied single blastomere. *Theriogenology*, *55*(9), pp.1843-1853.

Pedersen, K.S., Kristensen, T.N. and Loeschcke, V., 2005. Effects of inbreeding and rate of inbreeding in Drosophila melanogaster–Hsp70 expression and fitness. *Journal of evolutionary biology*, *18*(4), pp.756-762.

Peippo, J., Viitala, S., Virta, J., Räty, M., Tammiranta, N., Lamminen, T., Aro, J., Myllymäki, H. and Vilkki, J., 2007. Birth of correctly genotyped calves after multiplex marker detection from bovine embryo microblade biopsies. *Molecular Reproduction and Development: Incorporating Gamete Research*, *74*(11), pp.1373-1378.

Penna, V.M., Madalena, F.E. and Alvim, M.T.T., 1998. Open MOET selection nucleus in Guzerá. In *Proc. Sixth World Congr. Genet. Appl. Livest Prod.* (Vol. 23, pp. 439-442).

Pereira, R.M., Carvalhais, I., Pimenta, J., Baptista, M.C., Vasques, M.I., Horta, A.E.M., Santos, I.C., Marques, M.R., Reis, A., Pereira, M.S. and Marques, C.C., 2008. Biopsied and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture. *Animal reproduction science*, *106*(3-4), pp.322-332.

Perloe, M., Welch, C., Morton, P., Venier, W., Wells, D. and Palini, S., 2013. Validation of blastocoele fluid aspiration for preimplantation genetic screening using array comparative genomic hybridization (aCGH). *Fertility and Sterility*, *100*(3), p.S208.

Petersen, C., Mauri, A., Baruffi, R., Oliveira, J., Massaro, F., Elder, K., & Franco, J. (2005). *Implantation failures: success of assisted hatching with quarter-laser zona thinning. Reproductive BioMedicine Online, 10(2), 224–229* doi:10.1016/s1472-6483(10)60944-3.

Petyim, S., Båge, R., Hallap, T., Bergqvist, A.S., Rodríguez-Martínez, H. and Larsson, B., 2003. Two different schemes of twice-weekly ovum pick-up in dairy heifers: effect on oocyte recovery and ovarian function. *Theriogenology*, *60*(1), pp.175-188.

Pieterse, M.C., Kappen, K.A., Kruip, T.A. and Taverne, M.A.M., 1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. *Theriogenology*, *30*(4), pp.751-762.

Pincus, G. and Enzmann, E.V., 1935. The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs. *The Journal of experimental medicine*, *62*(5), pp.665-675.

Polisseni, J., de Sá, W.F., de Oliveira Guerra, M., Machado, M.A., Serapião, R.V., de Carvalho, B.C., de Almeida Camargo, L.S. and Peters, V.M., 2010. Post-biopsy bovine embryo viability and whole genome amplification in preimplantation genetic diagnosis. *Fertility and sterility*, *93*(3), pp.783-788.

Ponsart, C.A.H.D., Le Bourhis, D., Knijn, H., Fritz, S., Guyader-Joly, C., Otter, T., Lacaze, S., Charreaux, F., Schibler, L., Dupassieux, D. and Mullaart, E., 2014. Reproductive technologies and genomic selection in dairy cattle. *Reproduction, Fertility and Development*,*26*(1), pp.12-21.

Pontes JH, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, et al. 2009. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. Theriogenology 71:690–97. https://doi.org/10.1016/j.theriogenology.2008.09.031 83.

Pontes, J.H.F., Sterza, F.M., Basso, A.C., Ferreira, C.R., Sanches, B.V., Rubin, K.C.P. and Seneda, M.M., 2011. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. *Theriogenology*, *75*(9), pp.1640-1646.

Priner, S., Altarescu, G., Schonberger, O., Holzer, H., Rubinstein, E., Dekel, N., Peretz, A. and Eldar-Geva, T., 2019. The effect of repeated biopsy on pre-implantation genetic testing for monogenic diseases (PGT-M) treatment outcome. *Journal of assisted reproduction and genetics*, *36*(1), pp.159-164.

Pryor, J.H., Looney, C.R., Romo, S., Kraemer, D.C. and Long, C.R., 2011. Cryopreservation of in vitro produced bovine embryos: effects of lipid segregation and post-thaw laser assisted hatching. *Theriogenology*, *75*(1), pp.24-33.

Qi, M., Yao, Y., Ma, H., Wang, J., Zhao, X., Liu, L., Tang, X., Zhang, L., Zhang, S. and Sun, F., 2013. Transvaginal ultrasound-guided ovum pick-up (OPU) in cattle. *J. Biomim. Biomater. Tissue Eng.*, *18*, p.118.

Quesada, V., Sánchez, L.M., Álvarez, J. and López-Otín, C., 2004. Identification and Characterization of Human and Mouse Ovastacin A NOVEL METALLOPROTEINASE SIMILAR TO HATCHING ENZYMES FROM ARTHROPODS, BIRDS, AMPHIBIANS, AND FISH. *Journal of Biological Chemistry*, *279*(25), pp.26627-26634.

Quinn, P., 2004. The development and impact of culture media for assisted reproductive technologies. *Fertility and sterility*, *81*(1), pp.27-29.

Rajput, S.K., Yang, C., Ashry, M., Folger, J.K., Knott, J.G. and Smith, G.W., 2020. Role of BMP signaling in bovine early embryonic development and stage specific embryotropic actions of follistatin. *Biology of Reproduction*.

Ramos, N., Johnson, D., Eisman, L., Haimowitz, Z., Surrey, M., Danzer, H. and Barritt, J., 2019. The survival, biopsy, ploidy and pregnancy rates of previously vitrified blastocysts subjected to warming/biopsy/revitrification for PGT-A analysis. *Fertility and Sterility*, *111*(4), pp.e36-e37.

Ramos-Ibeas, P., Calle, A., Pericuesta, E., Laguna-Barraza, R., Moros-Mora, R., Lopera-Vásquez, R., Maillo, V., Yáñez-Mó, M., Gutiérrez-Adán, A., Rizos, D. and Ramírez, M.Á., 2014. An eficient system to establish biopsy-derived trophoblastic cell lines from bovine embryos. *Biology of reproduction*, *91*(1), pp.15-1.

Reichenbach, H. D., J. Liebrich, U. Berg, and G. Brem. 1992. Pregnancy rates and births after unilateral or bilateral transfer of bovine embryos produced in vitro. J. Reprod. Fertil. 95:363–370. doi:10.1530/jrf.0.0950363

Reis, A., Staines, M.E., Watt, R.G., Dolman, D.F. and McEvoy, T.G., 2002. Embryo production using defined oocyte maturation and zygote culture media following repeated ovum pick-up (OPU) from FSH-stimulated Simmental heifers. *Animal reproduction science*, *72*(3-4), pp.137-151.

Rizos, D., Ward, F., Duffy, P.A.T., Boland, M.P. and Lonergan, P., 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular reproduction and development*, *61*(2), pp.234-248.

Roche, J.F., Ireland, J. and Mawhinney, S., 1981. Control and induction of ovulation in cattle. *J Reprod Fertil*, *30*(Supp l), pp.211-222.

Rubino, P., Cotarelo, R.P., Alteri, A., Rega, E., Viganò, P. and Giannini, P., 2014. Trophectoderm biopsy performed by a double laser zona drilling: first safety evidence. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, *181*, pp.344-345.

Rubino, P., Tapia, L., de Assin Alonso, R.R., Mazmanian, K., Guan, L., Dearden, L., Thiel, A., Moon, C., Kolb, B., Norian, J.M. and Nelson, J., 2020. Trophectoderm biopsy protocols can affect clinical outcomes: time to focus on the blastocyst biopsy technique. *Fertility and sterility*, *113*(5), pp.981-989.

Ruiz, S., Romero-Aguirregomezcorta, J., Astiz, S., Peinado, B., Almela, L. and Poto, A., 2013. Application of Reproductive Biotechnology for the Recovery of Endangered Breeds: Birth of the First Calf of M urciana–L evantina Bovine Breed Derived by OPU, In vitro Production and Embryo Vitrification. *Reproduction in Domestic Animals*, *48*(6), pp.e81-e84.

Saadeldin, I.M., Oh, H.J. and Lee, B.C., 2015. Embryonic–maternal cross-talk via exosomes: potential implications. *Stem cells and cloning: advances and applications*, *8*, p.103.

Saadi, H.A.S., Vigneault, C., Sargolzaei, M., Gagné, D., Fournier, É., de Montera, B., Chesnais, J., Blondin, P. and Robert, C., 2014. Impact of whole-genome amplification on the reliability of pre-transfer cattle embryo breeding value estimates. *BMC genomics*, *15*(1), p.889.

Sachan, V., Kumar, B., Kumar Agrawal, J., Kumar, A. and Saxena, A., 2020. Methods of Embryo Sexing in Cattle Breeding: A Review. *Iranian Journal of Applied Animal Science*, *10*(1), pp.1-8.

Sanches, B.V., Zangirolamo, A.F., da Silva, N.C., Morotti, F. and Seneda, M.M., 2018. Cryopreservation of in vitro-produced embryos: challenges for commercial implementation. *Animal Reproduction (AR)*, *14*(3), pp.521-527.

Saragusty, J. and Arav, A., 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*, *141*(1), p.1.

Sartori, R., Suarez-Fernandez, C.A., Monson, R.L., Guenther, J.N., Rosa, G.J.M. and Wiltbank, M.C., 2003. Improvement in recovery of embryos/ova using a shallow uterine horn flushing technique in superovulated Holstein heifers. *Theriogenology*, *60*(7), pp.1319-1330.

SASAMOTO, Y., SAKAGUCHI, M., KATAGIRI, S., YAMADA, Y. and TAKAHASHI, Y., 2003. The effects of twisting and type of aspiration needle on the efficiency of transvaginal ultrasound-guided ovum pick-up in cattle. *Journal of Veterinary Medical Science*, *65*(10), pp.1083-1086.

Scarica, C., Montalvo, V., Novo, S., Castelló, C. and López-Teijón, M., 2018. Mosaicism in human blastocysts and technical factors of laser trophectoderm biopsy. *Culture*, *6*, pp.23-2.

Scenna, F.N., Hockett, M.E., Towns, T.M., Saxton, A.M., Rohrbach, N.R., Wehrman, M.E. and Schrick, F.N., 2005. Influence of a prostaglandin synthesis inhibitor administered at embryo transfer on pregnancy rates of recipient cows. *Prostaglandins & other lipid mediators*, *78*(1-4), pp.38-45.

Schimmel, T., Cohen, J., Saunders, H. and Alikani, M., 2014. Laser-assisted zona pellucida thinning does not facilitate hatching and may disrupt the in vitro hatching process: a morphokinetic study in the mouse. *Human Reproduction*, *29*(12), pp.2670-2679.

Schmutz, S.M., Moker, J.S., Clark, E.G. and Orr, J.P., 1996. Chromosomal aneuploidy associated with spontaneous abortions and neonatal losses in cattle. *Journal of Veterinary Diagnostic Investigation*, *8*(1), pp.91-95.

Schoolcraft, W.B., Fragouli, E., Stevens, J., Munne, S., Katz-Jaffe, M.G. and Wells, D., 2010. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertility and sterility*, *94*(5), pp.1700-1706.

Schroeder, A.C., Schultz, R.M., Kopf, G.S., Taylor, F.R., Becker, R.B. and Eppig, J.J., 1990. Fetuin inhibits zona pellucida hardening and conversion of ZP2 to ZPZ during spontaneous mouse oocyte maturation in vitro in the absence of serum. *Biology of reproduction*, *43*(5), pp.891-897.

Sciorio, R., Tramontano, L. and Catt, J., 2020. Preimplantation genetic diagnosis (PGD) and genetic testing for aneuploidy (PGT-A): status and future challenges. *Gynecological Endocrinology*, *36*(1), pp.6-11.

Scott Jr, R.T., Upham, K.M., Forman, E.J., Hong, K.H., Scott, K.L., Taylor, D., Tao, X. and Treff, N.R., 2013. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertility and sterility*, *100*(3), pp.697-703.

Scott Jr, R.T., Upham, K.M., Forman, E.J., Zhao, T. and Treff, N.R., 2013[1]. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertility and sterility*, *100*(3), pp.624-630.

Scott, K.L., Hong, K.H. and Scott Jr, R.T., 2013[2]. Selecting the optimal time to perform biopsy for preimplantation genetic testing. *Fertility and sterility*, *100*(3), pp.608-614.

Seshagiri, P.B., Roy, S.S., Sireesha, G. and Rao, R.P., 2009. Cellular and molecular regulation of mammalian blastocyst hatching. *Journal of reproductive immunology*, *83*(1-2), pp.79-84.

Shirazi, A., Borjian, S., Nazari, H., Ahmadi, E., Heidari, B. and Bahiraee, A., 2010. Effects of timing on cell biopsy from pre-compacted morula stage bovine embryos on subsequent embryonic development. *Journal of reproduction & infertility*, *11*(1), p.25.

Silva-Santos, K.C., Santos, G.M.G., Koetz Júnior, C., Morotti, F., Siloto, L.S., Marcantonio, T.N., Urbano, M.R., Oliveira, R.L., Lima, D.C.M. and Seneda, M.M., 2014. Antral Follicle Populations and Embryo Production–In Vitro and In Vivo–of B os indicus–taurus Donors from Weaning to Yearling Ages. *Reproduction in Domestic Animals*, *49*(2), pp.228-232.

Sinclair, K.D., Rutherford, K.M.D., Wallace, J.M., Brameld, J.M., Stöger, R., Alberio, R., Sweetman, D., Gardner, D.S., Perry, V.E.A., Adam, C.L. and Ashworth, C.J., 2016. Epigenetics and developmental programming of welfare and production traits in farm animals. *Reproduction, Fertility and Development*, *28*(10), pp.1443-1478.

Siqueira, L.G., Silva, M.V., Panetto, J.C. and Viana, J.H., 2020. Consequences of assisted reproductive technologies for offspring function in cattle. *Reproduction, Fertility and Development*, *32*(2), pp.82-97.

Sirard, M.A., 2018. 40 years of bovine IVF in the new genomic selection context. *Reproduction*, *156*(1), pp.R1-R7.

Sirard, M.A., Florman, H.M., Leibfried-Rutledge, M.L., Barnes, F.L., Sims, M.L. and First, N.L., 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovne oocytes. *Biology of reproduction*, *40*(6), pp.1257-1263.

Size, V.A.I.M., 2021. Share & Trends Analysis Report By Animal Type (Cattle, Swine, Sheep, Canine, Equine), By Product (Normal Semen, Sexed Semen), by End Use, and Segment Forecasts, 2021–2028. *Report ID: GVR-3-68038-570-0*.

Sousa, R.V., da Silva Cardoso, C.R., Butzke, G., Dode, M.A.N., Rumpf, R. and Franco, M.M., 2017. Biopsy of bovine embryos produced in vivo and in vitro does not affect pregnancy rates. *Theriogenology*, *90*, pp.25-31.

Spits, C. and Sermon, K., 2009. PGD for monogenic disorders: aspects of molecular biology. *Prenatal Diagnosis: Published in Affiliation With the International Society for Prenatal Diagnosis*, *29*(1), pp.50-56.

Sreenan, J.M., 1978. Non-surgical embryo transfer in the cow. *Theriogenology*, *9*(1), pp.69-83.

Stewart, B. M., J. Block, P. Morelli, A. E. Navarette, M. Amstalden, L. Bonilla, P. J. Hansen, and T. R. Bilby. 2011. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced in vitro with sex-sorted semen

Stokes, P.J., Abeydeera, L.R. and Leese, H.J., 2005. Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk'in vitro. *Developmental biology*, *284*(1), pp.62-71.

Summers, P.M., Campbell, J.M. and Miller, M.W., 1988. Normal in-vivo development of marmoset monkey embryos after trophectoderm biopsy. *Human Reproduction*, *3*(3), pp.389-393.

Summers, M.C., McGinnis, L.K., Lawitts, J.A. and Biggers, J.D., 2005. Mouse embryo development following IVF in media containing either L-glutamine or glycyl-L-glutamine. *Human Reproduction*, *20*(5), pp.1364-1371.

Summers, M.C., McGinnis, L.K., Lawitts, J.A., Raffin, M. and Biggers, J.D., 2000. IVF of mouse ova in a simplex optimized medium supplemented with amino acids. *Human reproduction*, *15*(8), pp.1791-1801.

Sun, Y., Wan, K.T., Roberts, K.P., Bischof, J.C. and Nelson, B.J., 2003. Mechanical property characterization of mouse zona pellucida. *IEEE transactions on nanobioscience*, *2*(4), pp.279-286.

Tadir, Y., Wright, W.H., Vafa, O., Ord, T., Asch, R.H. and Berns, M.W., 1989. Micromanipulation of sperm by a laser generated optical trap. *Fertility and sterility*, *52*(5), pp.870-873.

Takahashi, M., Keicho, K., Takahashi, H., Ogawa, H., Schulte, R.M. and Okano, A., 2000. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology*, *54*(1), pp.137-145.

Taneja M, Bols PE, Van De Velde A, Ju JC, Schreiber D, Tripp MW, Levine H, Echelard Y, Riesen J, Yang X. 2000. Developmental competence of juvenile calf oocytes in vitro and in vivo: influence of donor animal variation and repeated gonadotrophin stimulation. Biol Reprod, 62:206-213.

Taniyama, A., Watanabe, Y., Nishino, Y., Inoue, T., Taura, Y., Takagi, M., Kubota, C. and Otoi, T., 2011. Assisted hatching of poor-quality bovine embryos increases pregnancy success rate after embryo transfer. *Journal of Reproduction and Development*, pp.1105180372-1105180372.

Tarín, J.J. and Handyside, A.H., 1993. Embryo biopsy strategies for preimplantation diagnosis. *Fertility and sterility*, *59*(5), pp.943-952.

Tarín, J.J., Conaghan, J., Winston, R.M. and Handyside, A.H., 1992. Human embryo biopsy on the 2nd day after insemination for preimplantation diagnosis: removal of a quarter of embryo retards cleavage. *Fertility and sterility*, *58*(5), pp.970-976.

Taylor, T.H., Gilchrist, J.W., Hallowell, S.V., Hanshew, K.K., Orris, J.J., Glassner, M.J. and Wininger, J.D., 2010. The effects of different laser pulse lengths on the embryo biopsy procedure and embryo development to the blastocyst stage *Journal of assisted reproduction and genetics*, *27*(11), pp.663-667.

Tervit, H.R., Cooper, M.W., Goold, P.G. and Haszard, G.M., 1980. Non-surgical embryo transfer in cattle. *Theriogenology*, *13*(1), pp.63-71.

Thibault, C., 1977. Hammond memorial lecture. *Reproduction*, *51*(1), pp.1-15.

Thibier, M. and Nibart, M., 1995. The sexing of bovine embryos in the field. *Theriogenology*, *43*(1), pp.71-80.

Thibier, M. and Wagner, H.G., 2002. World statistics for artificial insemination in cattle. *Livestock Production Science*, *74*(2), pp.203-212.

Thornhill, A.R., 2019. Cleavage-Stage Embryo Biopsy. In *In Vitro Fertilization* (pp. 591-601). Springer, Cham.

Tian, S.J., Yan, C.L., Yang, H.X., Zhou, G.B., Yang, Z.Q. and Zhu, S.E., 2007. Vitrification solution containing DMSO and EG can induce parthenogenetic activation of in vitro matured ovine oocytes and decrease sperm penetration. *Animal reproduction science*, *101*(3-4), pp.365-371.

Tobler, K.J., Zhao, Y., Ross, R., Benner, A.T., Xu, X., Du, L., Broman, K., Thrift, K., Brezina, P.R. and Kearns, W.G., 2015. Blastocoel fluid from differentiated blastocysts harbors embryonic genomic material capable of a whole-genome deoxyribonucleic acid amplification and comprehensive chromosome microarray analysis. *Fertility and sterility*, *104*(2), pp.418-425.

Treff, N.R. and Franasiak, J.M., 2017. Detection of segmental aneuploidy and mosaicism in the human preimplantation embryo: technical considerations and limitations. *Fertility and sterility*, *107*(1), pp.27-31.

Treff, N.R., Ferry, K.M., Zhao, T., Su, J., Forman, E.J. and Scott, R.T., 2011. Cleavage stage embryo biopsy significantly impairs embryonic reproductive potential while blastocyst biopsy does not: a novel paired analysis of cotransferred biopsied and non-biopsied sibling embryos. *Fertility and Sterility*, *96*(3), p.S2.

Tšuiko, O., Catteeuw, M., Zamani Esteki, M., Destouni, A., Bogado Pascottini, O., Besenfelder, U., Havlicek, V., Smits, K., Kurg, A., Salumets, A. and D'Hooghe, T., 2017. Genome stability of bovine in vivo-conceived cleavage-stage embryos is higher compared to in vitro-produced embryos. *Human reproduction*, *32*(11), pp.2348-2357.

Tufekci, M.A., Cetinkaya, M., Yapan, C.C., Colakoglu, Y.K., Yelke, H., Cetinkaya, C.P. and Kahraman, S., 2019. 9. Assessment of aneuploidy and mosaicism concordance between different trophectoderm biopsy sites and the inner cell mass evaluated with next-generation sequencing. *Reproductive BioMedicine Online*, *39*, pp.e19-e20.

Turner, K.J., Silvestri, G., Black, D.H., Dobson, G., Smith, C., Handyside, A.H., Sinclair, K.D. and Griffin, D.K., 2019. Karyomapping for simultaneous genomic evaluation and aneuploidy screening of preimplantation bovine embryos: The first live-born calves. *Theriogenology*, *125*, pp.249-258.

Tutt, D.A.R., Silvestri, G., Serrano-Albal, M., Simmons, R.J., Kwong, W.Y., Guven-Ates, G., Canedo-Ribeiro, C., Labrecque, R., Blondin, P., Handyside, A.H. and Griffin, D.K., 2021. Analysis of bovine blastocysts indicates ovarian stimulation does not induce chromosome errors, nor discordance between inner-cell mass and trophectoderm lineages. *Theriogenology*, *161*, pp.108-119.

Ueno, S., Ezoe, K., Yabuuchi, A., Uchiyama, K., Okimura, T., Okuno, T., Kato, K. (2016). Complete zona pellucida removal from vitrified-warmed human blastocysts facilitates earlier in-vitro attachment and outgrowth. Reproductive BioMedicine Online, 33(2), 140–148. doi:10.1016/j.rbmo.2016.05.007.

UN 2017 https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html

UNFCC 2015: https://unfccc.int/process-and-meetings/the-paris-agreement/the-paris-agreement

Ushijima, H., Yamakawa, H. and Nagashima, H., 1999. Cryopreservation of bovine pre-morula-stage in vitro matured/in vitro fertilized embryos after delipidation and before use in nucleus transfer. *Biology of reproduction*, *60*(2), pp.534-539.

Valadão, L., da Silva, H.M. and da Silva, F.M., 2018. Bovine Embryonic Development to Implantation. In *Embryology-Theory and Practice*. IntechOpen.

Van Wagtendonk-de Leeuw, A.M., Mullaart, E., De Roos, A.P.W., Merton, J.S., Den Daas, J.H.G., Kemp, B. and De Ruigh, L., 2000. Effects of different reproduction techniques: AI, MOET or IVP, on health and welfare of bovine offspring. *Theriogenology*, *53*(2), pp.575-597.

Vera-Rodriguez, M. and Rubio, C., 2017. Assessing the true incidence of mosaicism in preimplantation embryos. *Fertility and sterility*, *107*(5), pp.1107-1112.

Verlinsky, Y., Cieslak, J., Freidine, M., Ivakhnenko, V., Wolf, G., Kovalinskaya, L., White, M., Lifchez, A., Kaplan, B., Moise, J. and Valle, J., 1996. Polar body diagnosis of common aneuploidies by FISH. *Journal of assisted reproduction and genetics*, *13*(2), pp.157-162.

Vettical, B.S., Hong, S.B., Umer, M.A. and Wani, N.A., 2019. Comparison of pregnancy rates with transfer of in vivo produced embryos derived using multiple ovulation and embryo transfer (MOET) with in vitro produced embryos by somatic cell nuclear transfer (SCNT) in the dromedary camel (Camelus dromedaries). *Animal reproduction science*, *209*, p.106132.

Viaggi, C.D., Cavani, S., Malacarne, M., Floriddia, F., Zerega, G., Baldo, C., Mogni, M., Castagnetta, M., Piombo, G., Coviello, D.A. and Camandona, F., 2013. First-trimester euploid miscarriages analysed by array-CGH. *Journal of applied genetics*, *54*(3), pp.353-359.

Victor, A.R., Griffin, D.K., Brake, A.J., Tyndall, J.C., Murphy, A.E., Lepkowsky, L.T., Lal, A., Zouves, C.G., Barnes, F.L., McCoy, R.C. and Viotti, M., 2019. Assessment of aneuploidy concordance between clinical trophectoderm biopsy and blastocyst. *Human Reproduction*, *34*(1), pp.181-192.

Villanueva, B., Woolliams, J.A. and Simm, G., 1995. The effect of improved reproductive performance on genetic gain and inbreeding in MOET breeding schemes for beef cattle. *Genetics Selection Evolution*, *27*(4), pp.347-363.

Vitez, S.F., Forman, E.J. and Williams, Z., 2019, March. Preimplantation genetic diagnosis in early pregnancy loss. *In Seminars in perinatology* (Vol. 43, No. 2, pp. 116-120). WB Saunders.

Viuff, D., Rickords, L., Offenberg, H., Hyttel, P., Avery, B., Greve, T., Olsaker, I., Williams, J.L., Callesen, H. and Thomsen, P.D., 1999. A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biology of reproduction*, *60*(6), pp.1273-1278.

Walsh, S.W., Mossa, F., Butler, S.T., Berry, D.P., Scheetz, D., Jimenez-Krassel, F., Tempelman, R.J., Carter, F., Lonergan, P., Evans, A.C. and Ireland, J.J., 2014. Heritability and impact of environmental effects during pregnancy on antral follicle count in cattle. *Journal of dairy science*, *97*(7), pp.4503-4511.

Wang, A.Y., Sullivan, E.A., Li, Z. and Farquhar, C., 2018. Day 5 versus day 3 embryo biopsy for preimplantation genetic testing for monogenic/single gene defects. *The Cochrane Database of Systematic Reviews*, *2018*(12).

Wheat, S., Vaughan, K. and Harbottle, S.J., 2014. Can temperature stability be improved during micromanipulation procedures by introducing a novel air warming system? *Reproductive BioMedicine Online*, *28*, pp.S16-S17.

Whitten, W.K., 1957. Culture of tubal ova. *Nature*, *179*(4569), pp.1081-1082.

Wiesak, T., Wasielak, M., Złotkowska, A. and Milewski, R., 2017. Effect of vitrification on the zona pellucida hardening and follistatin and cathepsin B genes expression and developmental competence of in vitro matured bovine oocytes. *Cryobiology*, *76*, pp.18-23.

Xie, Y., Wang, F., Puscheck, E.E. and Rappolee, D.A., 2007. Pipetting causes shear stress and elevation of phosphorylated stress-activated protein kinase/jun kinase in preimplantation embryos. *Molecular reproduction and development*, *74*(10), pp.1287-1294.

Young, L.E., Sinclair, K.D. and Wilmut, I., 1998. Large offspring syndrome in cattle and sheep. *Reviews of reproduction*,n *3*(3), pp.155-163.

Zacchini, F., Arena, R., Abramik, A. and Ptak, G.E., 2017. Embryo biopsy and development: the known and the unknown. *Reproduction*, *154*(5), pp.R143-R148.

Zeng, M., Su, S. and Li, L., 2018. The effect of laser-assisted hatching on pregnancy outcomes of cryopreserved-thawed embryo transfer: a meta-analysis of randomized controlled trials. *Lasers in medical science*, *33*(3), pp.655-666.

Zhang, J.Q., Li, X.L., Peng, Y., Guo, X., Heng, B.C. and Tong, G.Q., 2010. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. *Reproductive biomedicine online*, *20*(4), pp.510-515.

Zhang, S., Luo, K., Cheng, D., Tan, Y., Lu, C., He, H., Gu, Y., Lu, G., Gong, F. and Lin, G., 2016. Number of biopsied trophectoderm cells is likely to affect the implantation potential of blastocysts with poor trophectoderm quality. *Fertility and sterility*, *105*(5), pp.1222-1227.

Zhigalina, D.I., Skryabin, N.A., Artyukhova, V.G., Svetlakov, A.V. and Lebedev, I.N., 2016. Preimplantation genetic diagnosis by blastocentesis: problems and perspectives. *Russian Journal of Genetics*, *52*(1), pp.1-7.

Zhu, D., Zhang, J., Cao, S., Zhang, J., Heng, B.C., Huang, M., Ling, X., Duan, T. and Tong, G.Q., 2011. Vitrified-warmed blastocyst transfer cycles yield higher pregnancy and implantation rates compared with fresh blastocyst transfer cycles—time for a new embryo transfer strategy?. *Fertility and sterility*, *95*(5), pp.1691-1695.

APPENDIX 1:

Table A1: Demonstrate the number of viable embryos flush in beef and dairy cattle donors along the years

Embryos per Flushing	Breed	Article
6.8	Holstein	(Lohuis et al 1993)
7.4	Beef cattle	(Villanueva et al 1995)[BeefIS]
5.8	Guzera	(Penna et al 1998)
6.3	Holstein Heifers	(Sartori et al 2003)
5.6	Holstein Heifers	(Ax et al 2005)
7.9	Guzera (Bos indicus)	(Neto et al 2005)
11.5	Guzera (Bos Indicus)	(Neto et al 2005)[Beef**]
8.3	Limousion	(Neto et al 2005)
12.7	Limousin	(Neto et al 2005)[Beef**]
9.8	Nelore (*Bos Indicus*)	(Baruselli et al 2006)[Beef!]
8.6	Ayshire and Holstein Frisian	(Korhonen et al 2012)

Beef** shown higher embryo recovery rate by double flushing, after finishing flushing 1L of media was filled in the whole uterus to a second flush. **BeefIS**, a selection of donor to reduce inbreeding was prioritized. **Beef!** Average between 2 protocols that did not differ (CIDR+7 days or 7.5 days, pLH day 8 and AI 12 to 24hs later) were combined in the results.

Table A2: Several studies demonstrating the number of oocytes collected after OPU and blastocyst rate from the number of oocytes put into maturation

OPU	Follicles Aspirated	Oocytes retrieved	Into maturation	Blastocyst Rate	Author
133	-	818	659	29%	(Heyman et al 2003)
171	-	980	844	34.97%	(Heyman et al 2003)[a]
21	564	330	153	-	(Petym et al 2003)[b]
32	792	369	191	-	(Petym et al 2003)
656	-	20,848	15,747	34%	(Pontes et al 2011)[c]
-	3234	1241	152	32.2%	(Reis et al 2002)[d]
25	-	743	743	23%	(Lopes et al 2006)[e]
6	-	66	35	34%	(Ruiz et al 2013)[f]

a OPU performed in cloned and twins cows *bos Taurus* with the use of Crestar to stim program **b**-Two different systems were used, continuously(OPU performed through the period of OPU proposed) and discontinuously(OPU restrict between day 0 and day 12), of OPU to evaluate oocyte recovery and ovary effect in bos taurus non-stimulated. **c** OPU in a huge scale *Bos indicus* non-stimulated, **d**- *Bos Taurus* Simental cows had in vivo maturation of oocytes between OPU session, a 4-week rest period between embryo collection and the first aspiration procedure and, following the second OPU, a 6-week rest period before the next 15-week cycle began Stimulated. **e**- Different stage of lactation cows from 3 farms are analysed together, all oocytes retrieved including grade IV were put into maturation. **f**- 2 bos Taurus dairy cows had OPU followed pregnancy non-stimulated.