THESIS

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The failure of IVF in equine reproduction and the subsequent development of OPU and ICSI techniques for the commercial breeding of Sport Horses

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Acronyms and abbreviations

AI – Artificial Insemination

AFC – Antral Follicle Count

ANP A – Atrial Natriuretic Peptide A

ART – Assisted Reproduction Technique

BSA – Bovine Serum Albumin

COC – Cumulus Oocyte Complex

Cp – Compact

EGF – Epidermal Growth Factor

ET – Embryo Transfer

Ex – Expanded

FBS – Foetal Bovine Serum

FSH – Follicular Stimulating Hormone

GnRH – Gonadotropin Releasing Hormone

hCG – human Chorionic Gonadotropin

ICSI – Intracytoplasmic Sperm Injection

IGF – Insulin-like Growth Factor

IP_3 – Inositol 1,4,5-trisphosphate

IVC – *In Vitro* Culture

IVF – *In Vitro* Fertilisation

IVM – *In Vitro* Maturation

IVP – *In Vitro* Production

IVEP – *In Vitro* Embryo Production

LH – Luteinising Hormone

LOS – Large Offspring Syndrome

MPF – Metaphase Promoting Factor

OPU – Ovum Pick-Up

OT – Oocyte Transfer

PLCζ – Phospholipase C zeta

PVP – Polyvinylpyrrolidone

PZD – Partial Zonal Drilling

RFM – Retained Foetal Membranes

ROS – Reactive Oxygen Species

SOF – Synthetic Oviductal Fluid

US – Ultrasound

ZP – Zona Pellucida

1. INTRODUCTION

Assisted reproductive technologies (ARTs) have been applied to domestic animal obstetrics for over a hundred years, with the development of ARTs in the horse dating back as far as the late nineteenth century when the first foals were born through artificial insemination. The techniques have continued to advance since then, and commercial equine breeding now consists of an arsenal of procedures designed to maximise the reproduction of sub-fertile mares and stallions. These practices include oocyte transfer (OT); embryo transfer (ET); intracytoplasmic sperm injection (ICSI); *in vitro* fertilisation (IVF); and nuclear transfer or cloning (Alvarenga *et al.*, 2008). With the ever-increasing commercial availability of these reproductive technologies, there has been a noticeable departure from traditional breeding management in sport horse breeds in the last number of years.

Assisted reproduction has become a necessity in modern day equine breeding for a number of reasons. As mares can naturally produce only one foal per year, these techniques allow for numerous embryos or offspring to be produced from one mare in a single year period. Furthermore, many breeders feel it is now unnecessary to put valuable mares at risk of injury or death from pregnancy-related complications, and the flushing and transfer of embryos into recipient mares presents much lower risks to the donor mare. Another notable benefit is that, with ET and ICSI, embryos can be produced from mares while actively competing, an important factor for elite mares whose performance peak overlaps with their prime fertile years. Ovum pick-up (OPU) and ICSI have the further advantage over conventional ET in that it does not require any exogenous hormone stimulation and it is done as an outpatient procedure, significant elements that have a minimum impact on mares' competition or training schedules (Bols and Stout, 2018). Additionally, with ICSI, breeders can produce embryos outside of the natural breeding season thanks to developments in OPU, *in vitro* embryo production (IVEP) and embryo cryopreservation.

However, similar to human medicine, perhaps the most important driver of equine ART development is fertility issues. Unlike in other domestic animals, reproductive abilities have never been a significant factor in the selection criteria for the breeding of horses; rather, they are selected primarily for conformation and physical performance abilities. As a result, many fertility problems have been unintentionally preserved in mares and stallions, including chronic uterine diseases, physical injuries, or malformations in the female reproductive tract, and sub-fertile sperm or oligozoospermia in males (Salamone *et al.*, 2017). For mares with

these reproductive issues, it can prove impossible to produce embryos or offspring under standard reproductive management, and ART is the only option for successfully breeding these individuals. Additionally, for stallions with poor fertility, or that have died with limited sperm reserves remaining, ICSI is the best option to maximise the production of foals using these sires. The ability to thaw frozen semen straws, dilute and re-freeze them into "ICSI doses", also makes ICSI the most efficient method of fertilisation when semen straws are in finite supply (Hinrichs, 2018).

These fertility challenges are what has prompted the need for IVEP techniques to be developed in equine breeding in the last few decades (Salamone *et al.*, 2017). Furthermore, IVEP has proven to be an important tool in reproductive research to better understand the events of normal *in vivo* fertilisation in all species. It therefore serves to provide an important stepping-stone to other ARTs, and a way to examine the cause of failure in these advanced procedures to continuously improve the efficiency of such methods (Lonergan, 2013). With regards to *in vitro* maturation (IVM) and *in vitro* culturing (IVC) of embryos, the horse has proven to be particularly complex compared to other domestic animals; with breakthroughs in IVM/IVC appearing much later in equine studies, and still no perfect system has been developed to date. The delay in establishing a standardised optimal culture media has kept the IVEP in equine breeding far behind other species in ART developments. By the time the first successful IVM of equine oocytes was achieved, bovine IVEP was already at its peak; a major reason for this delay was the extremely limited availability of abattoir-sourced oocytes in horses compared to bovine, which has severely impeded research in equine ART as a result (Alvarenga *et al.*, 2008).

This thesis will explore the process and evolution of three main assisted reproduction technologies in the horse: *in vitro* fertilisation, transvaginal ovum pick-up, and intracytoplasmic sperm injection; with emphasis on the failures of IVF and the consequent development of OPU-ICSI programmes, that rapidly gained significance as an alternative ART in this species (Salamone *et al.*, 2017). Retrospective analysis reveals a pattern of adaptation of many technologies from cattle to horses; with the exception of ICSI, the various ARTs have historically been primarily developed and implemented successfully in bovine reproduction, before being adapted, sometimes much later, to use in equine breeding programmes. The complexity of equine reproduction, compared to other domestic animals, has also resulted in the need for the development of specific technologies to overcome conventional *in vitro* short falls in this species. This review will look at the hypothesised

reasons for failure of conventional IVF reported in the horse to date, and the subsequent emergence of OPU and ICSI procedures to fill this vacuum in the market.

Initially a last resort for sub-fertile mares and stallions, the efficiency and ease of use has resulted in the now widespread adoption of oocyte recovery and ICSI as a convenient option for the breeding management of normally fertile horses (Hinrichs, 2018). Furthermore, the possibility of cryopreservation of embryos or oocytes also allows for the genetics and progeny of valuable horses to be preserved for indefinite amounts of time; which has sparked the emergence of a new niche market for the sale of these frozen entities as a result. Ultimately, the success of commercial ICSI programmes can be measured in the number and value of the foals produced. In one of the most prestigious foal auctions in September 2020, the Zanghersheide Quality Auction, twenty-eight of the seventy-five foals presented (37%) were conceived through ICSI at the Avantea laboratory in Italy. This was an increase of 19% from the previous year's figures, with these foals reaching a significantly higher average purchase price than those produced by other methods (https://www.avantea.it/en/). These statistics highlight the continuous expansion in popularity of ICSI technology and increasing commercial value of the offspring produced, securing its position as a valuable ART in the equine breeding world.

2. LITERARY REVIEW

2.1 Ovum Pick-Up

2.1.1: The history and development of OPU

2.1.1.1: The origins of OPU in horse breeding

The period of the late 1980s, and into the 1990s, saw researchers take an active interest in developing assisted reproduction techniques in equine breeding. The mid 1990s notably featured many experiments in adapting well-established cattle techniques to the successful application in horses – transvaginal OPU, in particular. In her chapter on equine assisted reproductive biotechnologies, Sansinena (2020) claims that oocyte recovery was first developed as a laparoscopic technique in human medicine, and that the traumatic and invasive nature of this approach prompted the development of a "new, less invasive, ultrasound-guided approach to oocyte collection"; the first of which was reported in Scandinavia in 1981. Thus, this "novel technique" is considered to have revolutionised reproductive medicine and is now the assumed foundation for human IVF technology to date.

Sansinena (2020) credits Pieterse *et al.* (1988) as the first mention of OPU use in cattle breeding, and Brück (1992) as the first report of a successful OPU procedure in the mare; delineating the nature of reproductive technology to be established in cattle and later adapted to the horse. A review on the history of OPU, by Bols and Stout (2018), determined that despite transvaginal follicular aspiration being explored in horses soon after its establishment in cattle reproduction, the technique failed to gain widespread interest for a number of, primarily practical, reasons. These included the poor initial recovery rates of immature follicle oocytes, coupled with the lack of commercially available gonadotrophin hormones needed to stimulate dominant follicles for the successful retrieval of *in vivo* matured oocytes; the conclusion being that a commercial IVEP from live donors was just not viable. This limitation, combined with the fact that conventional IVF is not successful in the horse, impeded the development of any commercial interest in advancing transvaginal OPU techniques in horses. The introduction of oocyte transfer (OT) in the mid-1990s rekindled the interest in OPU that was later boosted by reports of the success of ICSI for embryo production by Cochran *et al*. (1998) and McKinnon *et al.* (1998), as cited by Bols and Stout (2018). However, progress still remained slow, as IVEP was still failing to achieve blastocyst

production rates on a par with those achieved when the ICSI oocytes were transferred to the oviducts of recipient mares or sheep pre-treated with progesterone (Bols and Stout, 2018).

Meintjes *et al.* (1995) conducted a study on transvaginal ultrasound-guided oocyte aspiration in a group of horses and ponies with the aim of assessing this technique as a safe and repeatable option for future breeding programmes, as well as determining its success in early gestational mares. Their paper summarises the various attempts made so far to recover oocytes in equine patients as: (1) paralumbar laparotomy; (2) ovarian manipulation via colpotomy incision or *per rectum* and subsequent follicle puncture with paralumbar needle; and (3) transvaginal ultrasound-guided oocyte aspiration. The first two methods are considered as "invasive, often cumbersome, and of limited repeatability due to adhesion and scar tissue formation." Furthermore, they have been reported with high variability in recovery rates, with paralumbar aspiration methods ranging from 10%, in a study by Vogeslang *et al*. (1998), to 73%, reported by Hinrichs *et al*. (1990) (cited by Meintjes *et al.*, 1995).

2.1.1.2: Risks involved

Despite being described by many as safe and reliable, Stout (2020) draws attention to the risks involved in transvaginal OPU; he lists complications such as rectal tears, peritonitis, haemorrhaging and ovarian abscess formation. In over 2,200 OPU procedures, Stout's team have recorded just one euthanasia, the result of presumably peritonitis-provoked laminitis, and three serious, but non-fatal, abdominal haemorrhages post-OPU. Bleeding due to rectal mucosal damage was a common finding (~16% of procedures), but this may be attributed to operator inexperience in holding the ovary correctly, claiming no serious rectal tears have occurred.

Ortis and Foss (2013) list internal haemorrhaging, adhesion formation and ovarian abscessation as the top risk factors. Even though they have recorded only one case of ovarian abscessation in over a thousand OPU procedures, they emphasise that OPU must not be taken lightly, and attention must be paid in every procedure to correct aseptic technique and appropriate restraint. As every rectal palpation carries a risk of rectal tearing, care must be taken, especially as the palpation can be for long periods with ovarian manipulation as an added factor. Furthermore, the fact that many mares present for OPU due to uterine abnormalities, such as chronic endometritis and pyometra, increases the risk of abdominal contamination from the vagina during the needle puncture. Therefore, a prophylaxis

antibiotic treatment protocol is recommended in every procedure, regardless of the relative infrequency of infections associated with the OPU process (Ortis and Foss, 2013).

Meintjes *et al.* (1995) went a step further by investigating the effect transvaginal OPU could have on mares who were pregnant at the time of the procedure. Their preliminary study concluded that the pregnant mares "were able to maintain adequate luteal function to support their pregnancies, and all gave birth to normal-appearing, healthy foals at term". The study recorded a zero abortion/foetal loss rate despite the transvaginal aspiration being repeated as many as 11 times in the mares, confirming that this method of oocyte recovery had no negative effect on luteal function during early gestation. In fact, according to an early study by Hinrichs *et al*. (1991), as cited by Meintjes *et al.* (1995), the removal of the follicular fluid via aspiration "actually enhances luteinization and progesterone production by the subsequent luteal tissue in the mare". The final results of the experiment by Meintjes *et al.* (1995) concluded that this procedure is "a safe, repeatable, and reliable means of obtaining intact cumulus-oocyte complexes from cyclic as well as pregnant mares". Furthermore, no differences between ponies and horses were established during the procedure, with similar results being achieved overall. At the conclusion of their study on factors affecting OPU, Kanitz *et al.* (1995) inseminated five of their donor mares in order to demonstrate that repeated transvaginal follicular aspiration has no negative effect on future fertility; all five mares became pregnant.

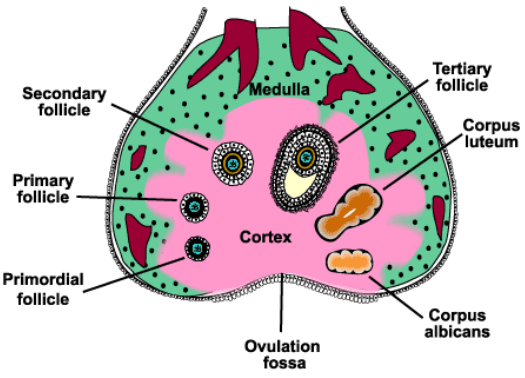

Figure 2.1: Histologically inverse structure of the equine ovary (www.ansci.wisc.edu/jjp1/equine/mare_anat/ovary2.html)

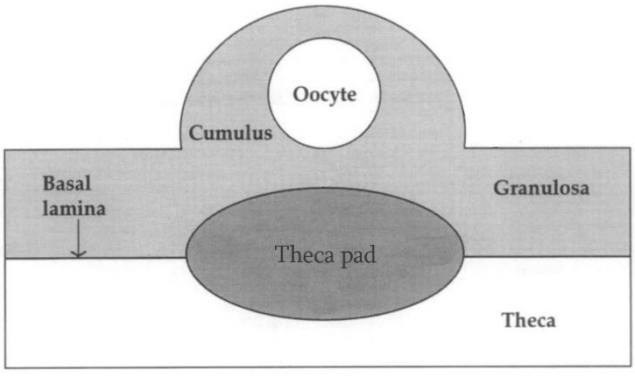

Figure 2.2: A schematic drawing of the structure of an oocyte-cumulus hillock illustrating the position of the thecal pad (Hawley *et al.*, 1995)

2.1.2: The application of OPU

2.1.2.1: The equine cumulus oocyte complex

Sansinena (2020) classifies the equine ovary as histologically unique (Fig. 2.1), drawing attention in particular to the attachment of the cumulus-oocyte complex (COC) to the theca wall of the follicle, which Stout (2020) refers to as a "broad cumulus cell hillock" strongly anchoring the COC to the wall. Stout (2020) further suggests its significance as an impediment to successful recovery of immature oocytes, requiring repeated flushing and vigorous scraping of the follicle wall to achieve clinically acceptable recovery rates (>50%). This differs greatly from humans and other domestic animal species where the connection is much looser, thereby requiring only the negative vacuum pressure to remove the COC effectively. This resulted in the need for adaptations to the original procedure, described in human and cattle reproduction, through a combination strategy of a collapsing and perfusing cycle of aspiration, coupled with gentle rectal massaging of the follicle and a scraping of the theca wall with the needle (Sansinena, 2020).

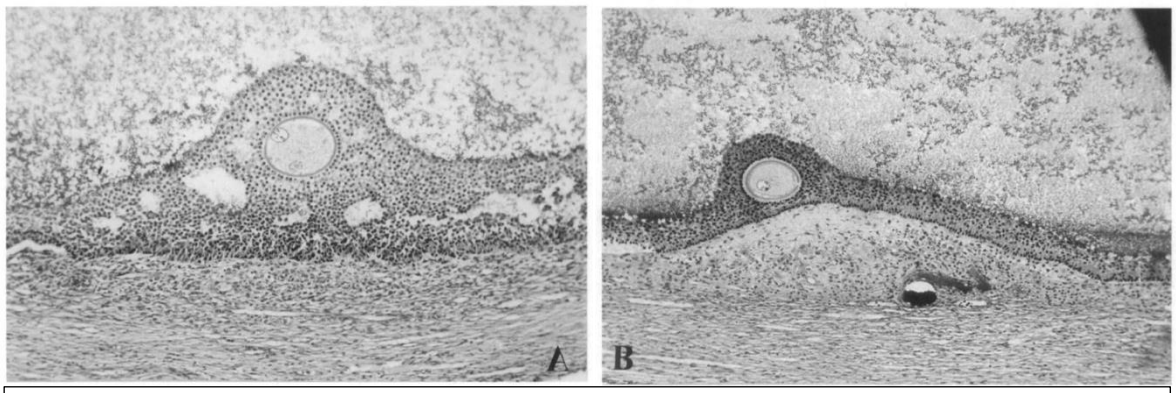

Figure 2.3: photomicrographs illustrating the histology of (A) the larger cumulus mass in bovine follicles; and (B) the distinct thecal pad underlying the cumulus hillock in equine follicles (Hawley *et al.*, 1995)

A histological comparison between the bovine and equine follicular structure elucidates the singular nature of the equine model. Despite having a smaller follicle size when compared to the mare, bovine follicles contain a thicker cumulus layer between the basal lamina and the oocytes. This aspect, coupled with the fact that the COC protrudes further from the follicular wall, results in an easier release of the COC during OPU through aspiration pressure alone. Furthermore, bovine have a higher number of fenestrations within the COC that weaken its attachment to the follicle wall, as well as a narrower base to the hillock that likely facilitates the easier detachment of the oocyte upon aspiration. Conversely, the

structure of equine follicles appears histologically designed to maintain a firm attachment between follicle wall and COC. They have a unique thecal pad, a biconvex lenticular cellular layer beneath the granulosa hillock, that creates a convex shaped area under the zone of cumulus attachment towards the antrum (Fig. 2.2 and 2.3). A significant element of this thecal pad is the granulosa cell processes, that can be observed extending into the structure, acting like an anchor for the attachment of the COC. Studies have shown that in atretic follicles, these thecal pads are smaller or absent, and may be related to the release of the oocyte from the thecal wall in these follicles (Hawley *et al.*, 1995).

Kanitz *et al.* (1995) studied closely the effect that the OPU technique had on the cumulus oocyte complexes in the aspirated follicles. They found that there was no significant difference in recovery rates of oocytes (28.8% *vs.* 26.4%) when the vacuum pressures were altered from 0.2bar to 0.4bar. This is in direct contrast to results found previously in cattle studies, where an increased pressure used to aspirate small and medium follicles resulted in a loss of cumulus cells, and therefore a decreased quality in the aspirated oocyte. The reason for this, is due to the aforementioned physiologically stronger connection between the oocyte and the follicular wall in horses. Pre-ovulation, this connection becomes looser in order for ovulation to occur, and as a result, the recovery rate of oocytes increases in the preovulatory period. Kanitz *et al.* (1995) cite the various works by Palmer *et al.* (1986), Vogelsang *et al.* (1988) and Cook *et al.* (1993), and their experimental proof of this phenomenon; that preovulatory follicles, more specifically those under gonadotrophin influence – both endogenous or exogenously applied – have increased oocyte recovery rates. In fact, Hinrichs (2018) notes potentially higher recovery rates were achieved from dominant follicles in older mares when both hCG and GnRH are administered for stimulation of the follicles.

In addition, Kanitz *et al.* (1995) further concluded that the recovery rate of oocytes in horses had the tendency to decrease as the diameter of the follicle increased, independent of the vacuum pressure applied, as well as the stage of oestrus cycle the mares were currently in. In their study, they aspirated the follicles of mares in three different cycle stage: Acyclic, Day 5 and Day 12 of their oestrus cycle. By directly comparing the recovery results of each group, a higher recovery rate was achieved overall in follicles that were ≤20mm; this resulted in higher oocyte recovery rates in the Acyclic and Day 5 groups, recording that up to 90% of all Day 5 follicles were <20mm, compared to only 79% in Day 12 mares. The study cites similar findings by Becker *et al*. (1994), supporting their hypothesis. Kanitz *et al*. (1995) postulates the nature of the COC as a reason, where smaller follicles, i.e. those ≤20mm, had more compact cumulus layers than the larger follicles observed; the large follicles containing

either fewer cumulus cell layers surrounding the oocyte, or denuded oocytes. There have been many further studies over the years into other factors that affect the success rates of OPU in mares.

2.1.2.2: Biological factors affecting OPU outcome

There are many additional biological factors that can be described as influencing the success of OPU in horses. In particular, the age of the mare is of chief concern in many studies. Through his comparison with other farm animals, Gordon (2004) characterises horses as having a considerably longer reproductive lifespan than that of domestic ruminants and swine, making their age a significant consideration in determining the quality of oocytes to be aspirated. Recent studies have shown evidence of morphological anomalies in oocytes from mares exceeding 19 years of age, that were otherwise not evident in oocytes from younger mares under 10 years of age, suggesting a notable decline in mare reproductive efficiency with advancing age. Gordon's continued comparison between species further outlines the remarkable difference in primordial follicle numbers in the ovary; estimating a mere 36,000 primordial follicles present in the equine ovary, *versus* 120,000 and 160,000 in the cow and ewe, respectively.

Using the data from the previous two years of their commercial OPU programme at Utrecht University, Stout (2020) established a similar link between a reduction in mean oocyte recovery and mare age. Mares over 18 years of age showed a decreased mean follicle number, as well as a decline in recovery rate, possibly influenced by cystic structures on the ovaries being misidentified as follicles. Additionally, follicle size and breed influenced overall recovery rates; a higher recovery rate was recorded in mares in which follicles <10mm made up the larger percentage of aspirated follicles. Studies have also noted a difference in average number of follicles in different breeds – but Stout postulates that this may be affected by the selection process of mares with adequate follicle number and affected by the interval between OPU procedures, rather than a clear breed distinction.

Claes *et al.* (2016) conducted a study to retrospectively identify different biological factors that affected recovery rates of oocytes and the overall success of IVEP in a group of Warmblood horses. A total of 159 mares were subjected to the OPU-ICSI procedure between 1-10 times, with data recorded on the (a) season/time of year; (b) age of the mare; (c) previous reproductive and performance history; and (d) antral follicle count (AFC), which was regarded as the total number of follicles exceeding 4mm in diameter. Applying linear and logistic regression, the team found that oocyte recovery during OPU decreased with

advancing mare age and differed between seasons; follicle production decreased in mares with increasing age, and transition periods exhibited the highest follicle number – with Spring yielding higher results than Autumn seasons. However, mare age, season and athletic performance had no impact on actual viable embryo production after OPU recovery of the oocytes.

Furthermore, the AFC did influence embryo production, as mares with a higher AFC were observed as having a better chance of producing viable embryos though IVP (Claes *et al.*, 2016). In fact, the Utrecht University's clinical protocol is to ideally delay OPU procedure until a mare produces a minimum of 15 follicles exceeding 10mm, if possible (Bols and Stout, 2018). For reproductive history, Claes *et al.* (2016) divided the mares into fertile, sub-fertile and infertile categories; while no significant difference was recorded between fertile and sub-fertile groups, the infertile mares were 4-6 times less likely to produce a viable IVP embryo in comparison. If a severe uterine abnormality was registered, notably chronic intractable uterine fluid or uterine infections, these odds dropped to 13 times less likely than the first two groups. Thus, Claes *et al.* (2016) concluded that mare age, seasonal period, AFC and reproductive history are all important markers for predicting successful IVEP in mares, with chronic infertility remaining a significant limiting factor for this assisted reproduction technique.

2.1.2.3: Technical factors affecting OPU outcome

Ultimately, the success of OPU and IVP of equine oocytes can be determined on the basis of two interrelated components: (1) the recovery rate of oocytes, and (2) the blastocyst production rate, which is subsequently measured against the eventual pregnancy and foaling rates after transfer of these embryos. Regarding recovery rates, technical factors such as the needle size used during OPU must be considered. From a review of previous studies, the recovery rate of oocytes increased as the needle increased in diameter; with one study recording a 38% rate with 15-gauge *versus* a 48% rate with 12-gauge needle. This phenomenon is attributed to the larger needle's ability to create more turbulence and quicker flow during perfusion-aspiration cycles, as well as more effectively scraping the COC from the follicle wall. However, there still remains inadequate data published regarding pregnancy and foal rates of OPU derived oocytes to make a firm conclusion on what factors ultimately affect the success of OPU-IVP (Bols and Stout, 2018).

Various reports have indicated a significantly higher rate of early pregnancy loss compared with other methods of breeding like AI or ET (>20% compared to 5-10%). Moreover, the

interval between OPU procedures has an impact on follicle production. Studies performed on a set 14-day interval resulted in a decrease in follicle number in subsequent aspirations, compared to a system of ultrasonographic monitoring of the mare's ovaries and delaying the next procedure until adequate follicles were produced (Bols and Stout, 2018). The protocol followed in an Avantea-based study collected oocytes ideally when there were at least 5-7 medium sized (10-20mm) antral follicles per ovary, monitoring the mare until this was achieved before collection. It was preferable to have no dominant follicle present, a picture best seen during the transition periods. This same protocol is used commercially for their OPU programme currently offered to breeders (Galli *et al.*, 2013).

In addition, due to the incredibly sensitive nature of oocytes, the entire OPU procedure – from ambient temperature, time taken for oocyte removal, materials and solutions used for follicle aspiration, and even drugs administered to the mare – must be closely controlled and very precise to ensure optimal developmental competence of the oocyte. By simply altering the flushing medium, from a commercial embryo flushing medium, with clinical grade heparin, to a medium designed especially for oocyte recovery, containing laboratory-grade heparin, one study recorded a significant improvement in blastocyst production (from 0.88 to 1.24 blastocysts per OPU, and a jump from 49% to 63% of OPUs yielding 1 embryo) (Stout, 2020).

2.1.2.4: Aspiration of Dominant *versus* Immature follicles

Another factor greatly influencing OPU is the difference between the recovery of oocytes from dominant follicles and immature follicles, a factor that has been studied extensively over the years. In her review of ART in horses, Hinrichs (2018) compares the two different approaches to OPU based on follicle stage (dominant *vs.* immature). She describes the dominant follicle aspiration as an easier procedure that is particularly beneficial to practitioners inexperienced with OPU, as these follicles are more visible (i.e. appear larger on the US screen), and have a higher recovery rate owing to the loosening of the COC complex from the follicular wall. Hinrichs reviews various studies that show dominant follicles as having a higher developmental competence compared to those matured *in vitro*, as high as 70% *vs.* ~30% in a 2013 study by Foss, Ortis and Hinrichs. The disadvantages associated with dominant follicle aspiration are the necessity for close and frequent monitoring of the follicle progression, as well as accurate timing of hCG/GnRH administration. Furthermore, the application of exogenous gonadotrophins resumes meiotic division in the oocyte, and therefore requires accurate ICSI timing (36-42 hours after), and

thus careful coordination with transport to a central ICSI laboratory. Additionally, the limitations of superovulation in the mare means only one, or occasionally two, follicles can be aspirated in one OPU procedure (Hinrichs, 2018).

In comparison, aspirating immature follicles is less exact and allows for OPU to be done on a set schedule, or at a time selected through ultrasound examination. It can also be performed year-round, whereas dominant follicles are present only in the breeding season. The immature oocytes are still in arrested meiosis, a "resting phase", and can be held in a simple holding medium at room temperature for an extended period of time, or placed immediately in maturation medium, with ICSI scheduled at a convenient time; thus there is less of a time constraint on them *versus* the oocytes recovered from dominant follicles (Hinrichs, 2018).

However, the aspiration of the smaller follicles is more difficult, and the COC is still firmly attached to the follicle wall, therefore requiring more vigorous flushing and physical scraping with the needle to remove the oocyte sufficiently (Galli *et al.*, 2013). In addition, the oocyte needs further IVM to metaphase II stage of meiosis before ICSI can be performed. Hinrichs (2018) outlines that a portion of the follicles aspirated are juvenile or atretic, and some oocytes (~ 40%) will not successfully mature enough to fertilise; moreover, the success rate of embryo production with ICSI is lower in immature follicle oocytes than those from dominant follicles. However, Jacobson *et al.* (2010), as cited by Hinrichs *et al*. (2018), highlights how a higher number of oocytes is recovered per OPU procedure in immature follicles, resulting in a far higher rate of blastocyst development overall (~1 blastocyst per immature follicle aspiration *versus* 0.33 blastocysts per dominant follicle aspiration). Additionally, immature follicles can be aspirated at the same time as dominant follicles. Unpublished observations suggest that exogenous gonadotropin administration for the dominant follicle does not appear to affect maturation or blastocyst rates of the immature oocytes, but a different ICSI schedule would be needed between the harvested oocytes to allow for the maturation time of the immature oocytes (Hinrichs, 2018).

2.1.3: OPU procedure

2.1.3.1: Equipment required

The OPU system equipment is made up of three main parts: the ultrasound scanner, an aspiration pump, and the needle guidance system that is connected to a special collecting tube for the oocytes (Fig 2.4 and 2.5). The ultrasound requires a specific transducer unit that has the needle guide built in; this allows the needle to be visualized on the ultrasound monitor

and enables a more accurate manipulation of the needle by the operator. Furthermore, it is helpful to use needles with an echogenic tip, a roughened area behind the tip that traps a small amount of air, to make it more visible in the sonographic field as it is advanced into the follicles. It is also recommended to have a biopsy guide on the ultrasound screen to aid the correct angle of advancement of the needle. A vacuum pump is connected to the needle with silicon/Teflon tubing, with a collection device in between to catch the oocytes and follicular fluid; usually an embryo filter or a simple sealed Falcon tube (Bols and Stout, 2018).

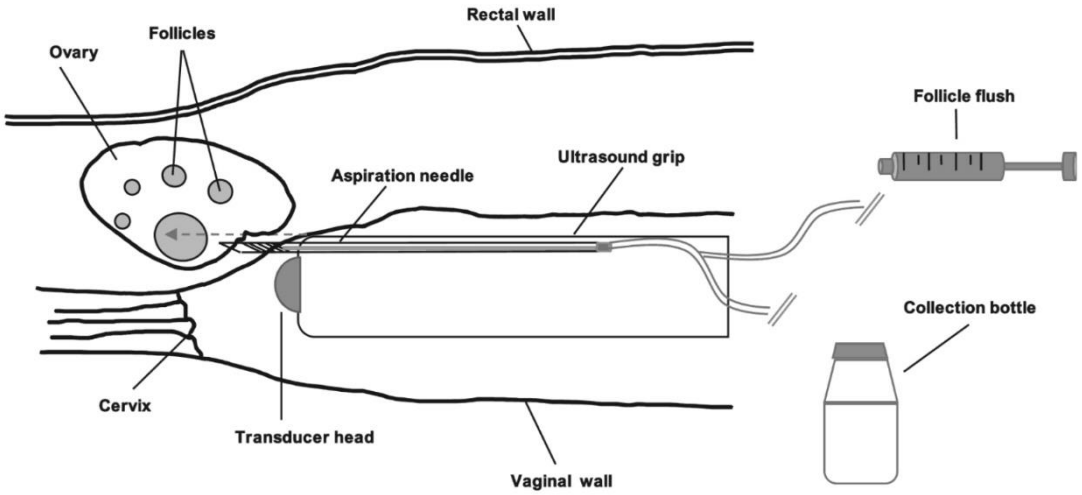

Figure 2.4: A labelled schematic of the OPU setup and equipment (Sansinena, 2020)

This is the basic system used in cattle OPU; however, mares require some modifications due to their behavioural and anatomical differences. The more firm attachment of the COC to the follicular wall means the oocytes cannot be simply aspirated in one step, like they are in cattle; repeated flushing and aspiration, along with careful scraping of the follicle wall with the needle tip, is required to successfully dislodge the COC, as explained in a previous section. This process requires a double lumen, usually 12-gauge and 60cm long; the inner stylet is connected to the vacuum pump with the collecting vessel in between, and the outer needle is used to introduce the flushing medium once the initial follicular fluid is removed. The double lumen is associated with a notably reduced chance of an oocyte remaining in the needle's dead space and consequently being flushed repeatedly into the follicle. The vacuum pressure is calculated to achieve approximately 20-25ml per minute to avoid damaging the COC, and the medium used is generally commercial embryo flushing medium with heparin (5-20 IU/ml) added to prevent clotting of any blood or follicular fluid (Bols and Stout, 2018).

2.1.3.2: Procedure protocol

The donor mare is secured in stocks and sedated with an alpha-2 agonist potentiated with an opioid analgesic, usually a detomidine hydrochloride and butorphanol combination; hyoscine-N-butylbromide is also advised to induce rectal relaxation, and to minimise any peristaltic waves that may result in rectal wall damage. In addition, a 2% lidocaine epidural anaesthesia can be administered to prevent the mare straining in response to the vaginal probe and the rectal manipulation of the ovaries. This step is particularly recommended in the more time consuming immature follicular aspiration procedures. It is further advised to use an NSAID (e.g. flunixin meglumine) to alleviate pain associated with the OPU, along with antibiotics administered peri-operatively to combat any contaminants introduced into the abdominal cavity by the procedure (Bols and Stout, 2018).

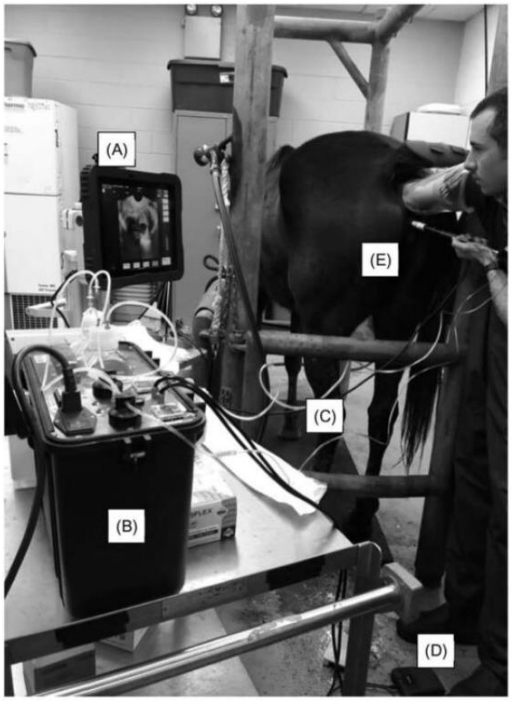

Figure 2.5: OPU components: (A) ultrasound; (B) aspiration pump with collection and flush medium bottles; (C) aspiration/flush lines; (D) foot pedal; and (E) transducer grip and needle guide (Sansinena, 2020)

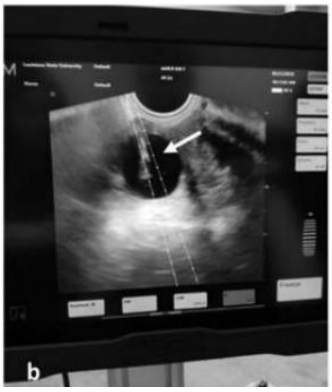

Figure 2.6: (a) Inserting the needle into the guide; and (b) the ultrasound image of the needle puncturing a follicle with the guiding system (Sansinena, 2020)

After rectal evacuation of any faeces, the tail is bandaged and tied to one side out of the way, and a thorough cleaning and disinfection of the perineum and vulva is performed. Obstetrical lubricant is applied to the OPU device (transducer and needle unit), and it is then inserted into the vagina and manipulated into position with one hand from the outside; meanwhile, the other hand is used *per rectum* to manipulate the ovary and position it against the head of the transducer, such that the follicles appear in the sonographic image on the scanner (Bols and Stout, 2018). This is done by grasping the suspensory ligament of the ovary between two fingers and lifting the ovary up over the uterus; this can be painful for mares with less pliable suspensory ligaments, in which case NSAID administration pre-OPU is essential (Ortis and Foss, 2013). The use of the biopsy guide on the scanner screen will identify the correct positioning of the follicle for successful puncture, making the process easier and more efficient (Fig. 2.6). The needle is advanced slowly, piercing the vaginal wall until it becomes visible on the screen, where the ovary can be manipulated to the correct position for follicular puncture (Bols and Stout, 2018). Firm pressure of the probe against the vaginal wall is opposed by firm pressure placed on the ovary against the vaginal wall by the other hand (Ortis and Foss, 2013). Once the needle is positioned in the centre of the follicle, the aspiration and flushing can begin, usually controlled via foot pedal by the operator. Initially, an assistant may be needed to advance the needle while the operator controls the ovarian position, but with practice this can be done by one person efficiently (Ortis and Foss, 2013).

2.1.3.3: Aspiration of different follicle types

The process further differs slightly depending on which type of follicles are being aspirated. For immature follicles, once punctured, the follicle content is completely aspirated then refilled immediately, while the pump keeps running to create as much turbulence as possible within the follicle. The operator can further increase the turbulence by rectally balloting the follicle during the evacuation phase. This flushing is repeated approximately 10 times, while a gentle scraping is simultaneously applied to the follicle wall to release the oocyte. The operator can puncture several small follicles successively in a single puncture, by manipulating the ovary and needle guide through the stroma; the pump remains running in this case, but must be stopped once the needle is withdrawn from the ovary, either to initiate a new puncture or the end of the procedure. Although less sensitive than dominant follicle oocytes, temperature shock must still be avoided to minimise cellular stress and ensure oocyte developmental competence (Ortis and Foss, 2013).

Dominant follicles are aspirated and flushed in the same manner, with similar turbulence used to dislodge the oocyte, but they require less physical manipulation, due to the loosening of the COC by the gonadotrophins; the OPU procedure must be more accurately timed to correspond with the administration of these hormones. These oocytes are far more temperature sensitive, and any decrease in temperature can cause fertilisation abnormalities through depolymerisation of the meiotic spindle. Therefore, all equipment and solutions used must be kept at body temperature (37°C); prewarm flushing medium, keep collection bottles in a water bath, and Petri dishes for identification are kept on warming trays at all times (Ortis and Foss, 2013).

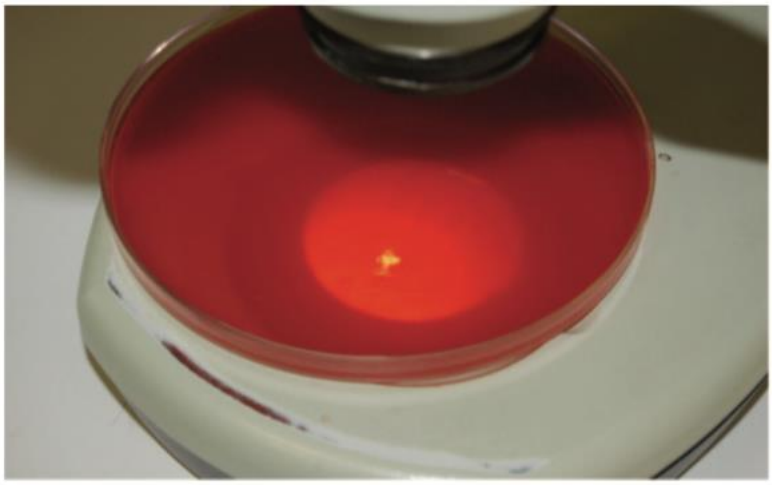

Figure 2.7: A COC collected from a dominant follicle in blood tinged fluid in a Petri dish (Ortis and Foss, 2013)

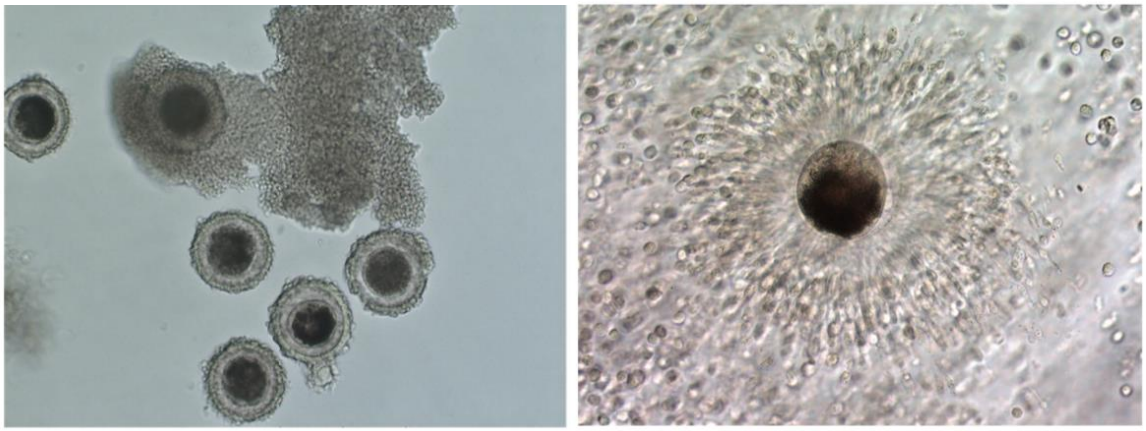

Figure 2.8: (*left*) A compact COC recovered from an immature follicle and (*right*) an expanded COC from a dominant follicle (Ortis and Foss, 2013)

2.1.3.4: COC identification measures

Once the OPU is complete, the contents of the collection bottles are flushed into the Petri dishes and placed under a stereomicroscope to search for the COC. For dominant follicles, the aspirated fluid usually contains blood once the follicle has responded appropriately to the gonadotropin stimulation. It has been suggested that the presence of blood may, in some cases, indicate an increased chance of a mature oocyte recovery, and that clear fluid implies a lack of response to gonadotropins. The COC is generally easy to spot in the bloody fluid, manifesting as a clear, sizeable, "fluffy mass" of cells (Fig 2.7); but all cell masses should be carefully examined at x10 and x25 magnification. The appearance of the dominant follicle oocyte can be seen in Figure 2.8; a generally expanded cumulus mass encloses the darkened oocyte surrounded by its corona radiata. In a healthy oocyte, the cytoplasm contains heterogeneously dark and light areas, indicating meiotic competence. Manipulation of the oocytes can be done using a 0.25ml semen straw or a 1.5mm diameter glass pipette (Ortis and Foss, 2013).

Fluid from immature follicles is only occasionally bloody, and due to their higher recovery numbers, the fluid is filtered through an embryo filter, which is then washed with more fluid before its content is transferred to a Petri dish. The cumulus cloud is generally more dense in immature oocytes (Fig. 2.8) but can appear somewhat expanded also. They may be found in clumps with cellular debris, or individually with only a few cumulus cell layers, and therefore careful examination of the whole dish is required. Their smaller size allows for handling using a simple pipettor with 10µL tips to transfer them into maturation medium (Ortis and Foss,2013).

After their respective maturation periods, these oocytes are ready for either fertilisation or cryopreservation; the standard procedure in commercial equine programmes is ICSI fertilisation, due to the repeated failure of IVF to produce viable offspring in horses to date, as discussed in the following chapter.

2.2: In Vitro Fertilisation

2.2.1: The introduction of IVF to animal breeding

2.2.1.1: What is IVF?

Sansinena (2020) describes IVF as "a complex sequence of events initiated by the penetration of a metaphase-II, mature oocyte by a capacitated sperm cell". In simpler terms, it is the coincubation of a capacitated sperm and a fully matured female oocyte in a medium that adequately supports fertilisation. The embryos are cultured up to blastocyst stage and then either transferred into the uterus or cryopreserved. IVF found its first human success with the birth of Louise Brown in 1978, and has become a routine method of treating infertility in humans since then. Its success prompted the adaptation of IVF to domestic animals, including cattle, pigs, sheep, goats, and buffalos, and is now a key component of commercial *in vitro* embryo production in cattle breeding technology. As was the case with OPU, the technique of IVF was first implemented in cattle reproduction before attempts were made to later adapt the process to equine breeding programmes. Despite many advances achieved in bovine, equine breeding has yet to establish a reliable protocol for IVF, with continuously disappointing success rates since the initial two foals born in 1991 (Leemans *et al.*, 2016). The ready availability of oocytes from slaughterhouse ovaries in cattle and pigs, has made research advances into the technique in these species far more attainable compared to the horse (Sirard, 2018). Moreover, the limitations in conditions for testing, and low number of oocytes used in each case, remain important factors for the difficulty in identifying why IVF is so unsuccessful in the horse (Mugnier *et al.*, 2009).

2.2.1.2: The development of IVF technology in cattle breeding

The use of IVF has become widespread, with a study in 2013 marking 6.8% of all calves born in the previous year were as a result of IVF; a similar focus on IVF programmes has also been adapted to laboratory animals (Leemans *et al.*, 2016). IVF was first introduced into cattle breeding as an experimental procedure, with a similar objective to that in human reproduction – to treat infertility. This quickly morphed into a method of decreasing generation intervals and improving the genetic selection for many breeds. After decades of IVF experimentation in rodents, the birth of Louise Brown in 1978 intensified the interest in adapting the technology for use in large mammals, especially the cow. The first calf produced by IVF was done via surgical oocyte recovery and embryo transfer back into the

oviduct. This later developed into a laparoscopic recovery technique and uterus transfer of the embryo after incubation in a rabbit or sheep oviduct, which was later replaced with co-culture with oviductal cells (Sirard, 2018).

Heldman *et al.* (2010) describes IVF as "one of the most rapidly expanding areas of embryo transfer technologies" in cattle breeding. The first calf born from IVF technology was born in the early 1980s, however, it was over a decade later before IVF would be used worldwide, as it was a complex procedure that required special equipment and trained technicians. The use of IVF, primarily in dairy cattle, has grown exponentially in the last number of years, with the number of embryos produced *in vitro* now exceeding that produced *in vivo* worldwide. IVF allows breeders to maximise on the selective breeding of genetically superior animals, with the ability to use frozen semen and cryopreserved oocytes removing any previous geographical barriers. IVF has also become the most common ART used for sexed semen, increasing the efficiency of the genetic selection. Despite the continuous advancements in IVF technology, the embryo production rate remains a rather low 30-40%, and optimising methods to improve this value remains a priority in laboratories and breeding organisations all over the world (Sanches *et al.*, 2019).

When IVF was adapted for research purposes, it opened the door to abattoir-sourced oocytes. These oocytes allowed for the development of *in vitro* maturation methods, negating the need for oviductal incubation using rabbits and sheep, that was standard practice up until this point. The composition of the culture media is an element that has been heavily researched and is still under constant analysis and modification today to achieve optimal conditions. The first media to be widely used was TCM-199 supplemented with serum, follicular stimulating hormone (FSH), and oestradiol. It remained the same for over thirty years and is still used in most laboratories in some capacity. The embryo culture medium, conversely, was modified frequently over this initial period, and is still revised regularly nowadays. The requirements of a growing embryo are dynamic, and they need different substrates at different cell stages. A synthetic oviductal fluid was developed as early as the 1970s, and later enriched with specific amino acids that allowed the embryos to successfully reach blastocyst stage. An interesting phenomenon observed, was that the bovine embryos had higher blastocyst formation rates when cultured in groups, compared to individual culture of single oocytes. Despite trying an extensive recipe of maturation mediums, success rates of blastocyst formation remained plateaued at 30-40% by 2000, and focus shifted to the source of the oocytes and the COC morphology to improve these rates (Sirard, 2018).

The follicular wave process in cyclic bovine ovaries means that a certain percentage of the oocytes obtained for IVM will be atretic, and the rest will be either in their growth phase or plateau phase, and this is reflected in their COC morphology. Atretic oocytes will have a partially denuded cumulus, linked to a lower developmental competence; oocytes in the growth phase will have a bright, compact cumulus and are not yet fully competent; and plateau phase oocytes will show an expansion to their cumulus, associated with the highest competence. However, a trend emerged where preovulatory follicle oocytes sometimes demonstrated the highest developmental potential, but as dominant follicles do not all ovulate, these oocytes often underwent atresia. Such observations are credited for the development of ovum-pick up treatments and improvement of hormonal pre-treatments (Sirard, 2018).

It was observed that oocytes collected in the growth phase, under continuous FSH influence, had a lower developmental competence than those that were allowed a window of LH-supported growth, by arresting FSH for a few days. A new protocol emerged to treat the donor cows before aspirating oocytes, giving FSH stimulation until a wave of dominant follicles with sufficient LH receptors appeared (~8mm in bovine), and then allowing a natural cycle to continue where the follicles grow and develop without FSH influence, only a basal LH level. This post-FSH period has been linked to an increase in oocyte quality and blastocyst formation rates after IVF. In fact, these experiments revealed that culture conditions are not detrimental to successful blastocyst formation if the oocyte quality is excellent to begin with. In the words of Pat Lonergan, "there is considerable evidence of a significant influence of follicular origin on oocyte developmental potential, and it appears that once the oocyte is removed from the follicle, its developmental capacity is capped" (Sirard, 2018).

2.2.1.3: Summary of the IVF process

In IVF procedures, 25µl of sperm suspension is combined with 25 µl of medium, that contains 10µg/mL heparin to stimulate capacitation. Then four or five COCs, retrieved from a cyclic donor via OPU and allowed to mature *in vitro*, are placed in a droplet of special fertilisation culture medium with the capacitated sperm under a layer of paraffin oil, and incubated for 24 hours at 39°C with 5% CO_2 and 95% air. The basic process can be seen in Figure 3.1. The medium contains serum albumin, usually bovine serum albumin (BSA) or foetal calf serum, and an energy source, usually glucose or pyruvate, that supports oocyte metabolism, sperm motility and acrosomal reaction. Most mediums also contain antibiotics,

such as streptomycin and penicillin. The ideal pH is between 7.2 and 7.6. Common media widely used are Krebs-Ringer's bicarbonate, TCM199 or modified Dulbecco's (Jainudeen *et al.*, 2013).

Beyond the ideal environmental conditions, the key to success is having the optimal ratio of fertilisable sperm that are highly motile, and a good quality oocyte containing a distinct first polar body. After fertilisation, the embryos are cultured to a stage of blastocyst development before being implanted in the uterus of a recipient cow, or cryopreserved. This IVC can be through co-culture with oviductal cells or with simple medium supplemented with a synthetic oviductal fluid (Jainudeen *et al.*, 2013).

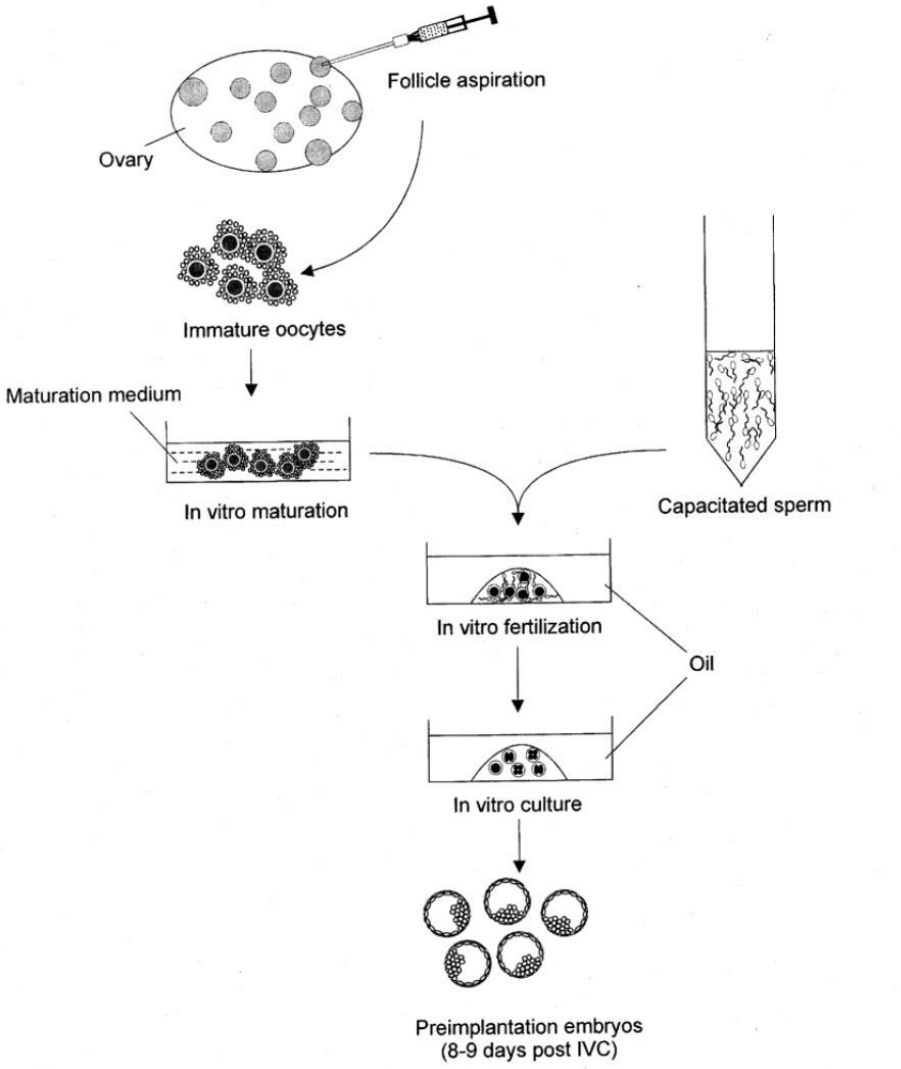

Figure 3.1: The basic steps of IVF procedure currently used in bovine reproduction (Jainudeen *et al.*, 2013)

2.2.2: IVF in Equine breeding

2.2.2.1: Current statistics

"For more than 25 years, the co-incubation of mature oocytes with capacitated spermatozoa has been the standard method for producing *in vitro* embryos in several species, including man, cattle, pigs and many laboratory animals. However, conventional IVF is still not reliably successful using equine gametes" (Leemans *et al.*, 2016). Despite the decades of research and work in equine IVF technology, only two foals have been born to date (both in 1991), with overall fertilisation rates reported as ranging from 0-33%, and are highly inconsistent, even within the same laboratory (McPartlin *et al.*, 2009). Furthermore, of these two foals born, both were from oocytes that were matured *in vivo* (Hinrichs *et al.*, 2002). As cited by Li *et al.* (1995), the success reported by Palmer *et al.* (1991) of one of these live foals, required eight adequately fertilised embryos transferred to the oviducts of eight recipient mares to result in that single pregnancy.

An early report by Li *et al.* (1995) characterises the limited progress of IVF in equine by that point as surprising, considering that both *in vitro* maturation of oocytes and capacitation of stallion sperm cells have been individually successful; with the sperm cells having the capability, after capacitation and acrosomal reaction, to penetrate zona-free oocytes of hamsters *in vitro*. Furthermore, oocytes that were matured *in vitro* and then implanted back into the oviduct of a recipient mare, were fully capable of *in vivo* fertilisation and establishing pregnancy. Attempts up until then at fertilising *in vitro* repeatedly failed to impress, with even the most advanced embryos proving incapable of developing beyond the 4- to 6-cell stages during *in vitro* culturing.

2.2.2.2: Zona pellucida as an influential factor

The initial suspicion was on the nature of the zona pellucida (ZP) of equine oocytes; thicker than that of other animals species, it was assumed that the ZP was abnormally hardened by the *in vitro* maturation process, acting as a barrier to sperm cells that were perhaps less adequately prepared by the *in vitro* capacitation process (Li *et al.*, 1995). In their study, Li *et al.* (1995) explored the effect on *in vitro* fertilisation rates of equine oocytes, after zonal drilling was performed in an effort to facilitate a motile spermatozoon to overcome the ZP barrier. Previous studies in lab animals, and later human IVF programmes, proved zona drilling to be a successful procedure in boosting IVF rates, making it an important technique applied in human cases of male infertility. Li *et al.* (1995) found that mechanical cutting and

renting caused unacceptable damage to the oocyte, and instead, opted for using an acidic Tyrode's solution (Fig. 3.2) to dissolve a small hole in the equine ZP, recording >98% oocyte survival rate. After maturation and selection of good quality oocytes, zona drilling was performed, and the oocytes were co-cultured with motile sperm previous activated using a calcium ionophore solution. The fertilised oocytes were then cultured on a bovine oviduct cell monolayer and evaluated for development over an 8-day period. From the various control groups, it was observed that neither zona-drilled oocytes, cultured with native sperm cells (i.e. not treated with calcium ionophore solution), nor intact ZP oocytes, cultured with artificially activated sperm cells, yielded fertilisation success. From the group of drilled oocytes and capacitated sperm, 79% cleavage rate was achieved, with 45.5% of those cleaved developing to morula and blastocyst stage; highlighting the need for both measures in order to achieve successful fertilisation. Despite these encouraging results, actual pregnancy rates and live foal births remain remarkably low from IVF (Li *et al.*, 1995). Although a milestone achievement, it highlighted the fact that some step was still missing, an unknown entity preventing commercially repeatable results from this procedure.

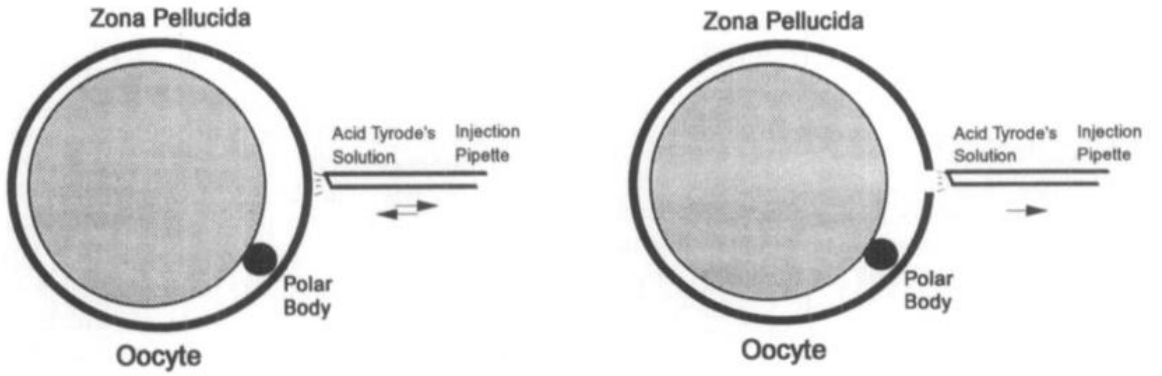

Figure 3.2: Zonal drilling of an oocyte using acidic Tyrode's solution to facilitate oocyte penetration of the ZP by the spermatozoa in IVF (Li *et al*., 1995)

A later study by Hinrichs *et al.* (2002) also hypothesised that the key barrier to successful IVF in the horse is the penetration of the ZP, demonstrated by increased fertilisation rates after techniques such as zona drilling and partial or total zona removal. However, frequency of polyspermy in these cases means they are not viable options of circumventing the zona issue in IVF. It has been suggested that maturation of the oocytes *in vitro* is responsible for their zonal hardening and resistance to penetration. The inappropriate culture conditions may trigger the cortical granules to release prematurely, a phenomenon that also happens in aged oocytes. However, studies found that when fetuin, a protein prove to stop the ZP hardening

in mouse oocytes, was added to the IVM culture medium, the hardening of the equine oocytes' ZP did decrease, and yet fertilisation rates did not increase as a result (Hinrichs *et al.*, 2002).

Studies on zona drilling, like the previously mentioned experiment by Li *et al.* (1995), and partial zonal dissection (PZD), have been shown to improve initial sperm penetration rates. However, Leemans *et al.* (2016) also believe these are not viable options in practice, due to the high rates of polyspermy and subsequent developmental incompetence. They attribute the failure of equine IVF as mostly likely due to the inadequate capacitation and subsequent penetration of the ZP by spermatozoa, dismissing theories of the ZP hardening in response to suboptimal *in vitro* maturation conditions, or missing oviduct-dependent changes in the ZP penetrability, due to lack of sufficient evidence.

2.2.2.3: Sperm capacitation as an influential factor

IVF has been unsuccessful with both *in vivo*- and *in vitro*-matured oocytes, but, if oocyte transfer (OT) is performed, where *in vivo* matured oocytes are placed in the oviduct of an inseminated mare, then excellent pregnancy results can be achieved (75% to 82%). Unfortunately, a similar process using *in vitro* matured oocytes failed to reproduce the same success, with a low blastocyst recovery rate of 17%, and low pregnancy rates of 10% at day 16 (Hinrichs *et al.*, 2002). This suggests that the missing link is most likely to be with sperm capacitation and hyperactivation, presumed independent processes that must be induced *in vitro* for successful IVF in the horse (McPartlin *et al.*, 2009).

"Capacitation is the process by which the glycoprotein coat and the seminal proteins are removed from the surface of the sperm's acrosome by substances secreted by the uterus or fallopian tubes of the female genital tract, thereby permitting the acrosome reaction to occur" (https://medical-dictionary.thefreedictionary.com/capacitation). Thus, capacitation is a necessary change that spermatozoa undergo to enable them to fertilise the oocyte. The discovery in bovine reproduction that bull semen could be capacitated *in vitro* by glycosaminoglycan heparin, significantly advanced the IVF technology in domestic farm animals (Jainudeen *et al.*, 2013). Conversely, previous attempts to successfully capacitate equine sperm *in vitro* have failed to result in sufficient IVF pregnancies, with reduced ZP penetration rates, acrosomal exocytosis and protein tyrosine phosphorylation. A study by McPartlin *et al.*, (2009) demonstrated that successful IVF required hyperactivation of stallion sperm, achieved through exposure to procaine *in vitro*. Through a combination of procaine-induced hyperactivation and capacitation conditions, they achieved significant and

reproducible percentages of successfully fertilised oocytes in their IVF experiments. Understanding the mechanism of capacitation and hyperactivation that occurs *in vivo* may clarify elements of equine oocyte fertilisation that could be useful in improving other assisted reproduction techniques, as well as IVF.

When co-cultured with untreated spermatozoa, the oocyte fertilisation rate is practically 0%. The use of calcium ionophore treatment in several studies has yielded good results of penetration and pronucleus formation after IVF (Hinrichs *et al.*, 2002). In their study, Hinrichs *et al.* (2002) attempted to evaluate several steps of the IVF process: the effects of duration of oocyte maturation, the components of maturation medium, and sperm preparation, on IVF rates. The *in vivo* fertilisation rates of IVM oocytes that were transferred to oviducts of inseminated mares are used for comparison, with an analysis of embryo development 40-44 hours post-fertilisation recorded. It concluded that normal fertilisation rates remained low in the *in vitro* fertilised oocytes (16%) *versus* the *in vivo* fertilised OT oocytes (77%), throughout the various oocyte and sperm treatments; indicating that IVM oocytes are capable of sperm activation and ZP penetration, and that irreversible zona hardening is not the reason for failure of IVF. It may be that changes occur in the permeability of the ZP *in vivo*, or a more effective sperm capacitation occurs in the oviduct that allows for successful fertilisation. By using a variety of culture mediums and calcium ionophore concentrations, based on reports of other research studies, Hinrichs *et al.* (2002) concluded that no basic method emerged capable of obtaining reproducible results in equine IVF. The duration of maturation and sperm treatments ultimately did not emerge as important factors, whereas the medium composition was notably influential. More detailed work on the effects of culture medium and sperm capacitation has been done in other species and this level of research needs to be applied to the horse.

Later attempts to induce hyperactivated motility of equine spermatozoa using procaine, by McPartlin *et al.* (2009) and Ambruosi *et al.* (2013), as cited by Leemans *et al.* (2016), brought into question what definition of fertilisation was being applied to the experiments; whether true fertilisation could be differentiated from parthenogenic activation of unfertilised oocytes in the results amassed. The suggestion is made for the closer evaluation of fertilisation, through visualisation of sperm mid-piece or tail to register successful ZP penetration, and evidence of second polar body extrusion; not to rely on the formation of a pronucleus as the primary indicator for fertilisation (Leemans *et al.*, 2016). Hinrichs *et al.* (2002) also express difficulty in directly comparing published reports on fertilisation rates

due to differences in the way fertilisation is assessed; however, they observed that any high rates achieved were not repeatable even within the same laboratory.

2.2.2.4: The role of the oviduct

2.2.2.4.1: The oviduct cell model

The high fertilisation rates achieved with *in vivo* fertilisation in the horse, lends itself to the theory that the oviductal environment itself is a key factor in sperm capacitation and the maturation and interaction of both gametes. The recreation of accurate *in vivo* oviduct conditions in an *in vitro* oviduct model, could bring a successful IVF programme within reach, but a more advanced knowledge is needed on the activation of stallion spermatozoa by the oviductal microenvironment in order to identify the capacitation triggers missing from medium used in current IVF protocols (Leemans *et al.*, 2016).

The limited data available on sperm-oviduct interactions in the horse can be linked to the lack of equine reproductive tissue samples made available for study, and the invasive nature of *in vivo* oviductal studies. Many attempts at oviductal cell models have been made over the years, from oviduct cell monolayers to explants and apical plasma membrane preparations. Studies in bovine and humans reported that spermatozoa binding showed preference to explants over monolayers, with a particular affiliation to the cilia or deeper regions of the epithelial cells. A 2014 study observed the oviduct explants mimicking certain functions of *in vivo* conditions, where the stallion spermatozoa bound to the explants underwent capacitation-related events, like tail-associated protein tyrosine phosphorylation. They concluded that the ability of explants to maintain oviduct secretory activity within *in vivo* ranges, make it the current superior choice for *in vitro* fertilisation attempts (Leemans *et al.*, 2016).

Previous studies have shown in other species – such as humans, cattle, and pigs– that co-culture with oviduct epithelial cells promotes the production of embryos *in vitro*, while oviductal proteins also improve the efficiency of IVF in these species. In equine, co-culture with oviduct epithelium has proven to improve sperm capacitation and selection of spermatozoa for superior motility and morphology, based on their binding to the epithelium (Mugnier *et al.*, 2009). Mugnier *et al.* (2009) conducted a study to establish if oviductal cells and oviductal fluid have a direct beneficial effect on IVF rates. They observed that coincubation with both equine and porcine oviductal cells yielded similar improvements in IVF rates. As is seen with previous practices of the culture of ICSI equine embryos in sheep

oviducts, heterologous oviduct cells can have a positive effect on oocyte maturation, fertilisation, and later embryo development. The oviduct cells used were harvested during the *in vivo* fertilisation period, to ensure an optimum environment for fertilisation. Various studies have shown a notable increase in IVF rates in other species after oviductal cell co-culturing, with clear modification of the ZP seen in pig oocytes, indicating an improved competence for fertilisation in this species. In this study, however, oviductal cell co-incubation of both *in vitro*- and *in vivo* matured equine oocytes still yielded low IVF rates. In addition, unlike bovine and porcine species, the presence or absence of cumulus cells does not appear to impact the fertilisation rates of equine oocytes and are therefore deemed non-essential components. Furthermore, hormonal stimulation of the oviductal cells resulted in no beneficial effect on the equine oocytes, despite reports of notably increased oviductal protein secretion and blastocyst formation rates in similar hormone-stimulated bovine and porcine oviduct cell co-cultures (Mugnier *et al.*, 2009).

2.2.2.4.2: The oviduct sperm reservoir

The high rates of fertilisation achieved through *in vivo*- compared to *in vitro* fertilisation, gives merit to theories on the oviductal sperm reservoir, which postulate that the final stages of capacitation are triggered by factors within the oviduct itself. One study ruled out the presence of a hormone-based signal, as none of the recipient mares used were treated with progesterone before recovery of the embryo; rather, all were under constant oestrogen stimulation and high fertilisation rates were achieved in the absence of preovulatory follicles, or exposure of the oocytes to follicular fluid. The lack of ovulation supports the theory that, in the mare, capacitated sperm are released from the caudal isthmus and move through the oviduct continuously, not in response to ovulation-specific signals (Hinrichs *et al.*, 2002).
It is worth considering that an *in vitro* replication of the sperm reservoir created by the isthmus *in vivo*, may assist in creating a subpopulation of competent viable sperm capable of prolonged presence until fertilisation occurs, at which point it remains viable and able to activate successfully. Studies have shown that, if the stallion spermatozoa comes into direct membrane contact with oviduct cells *in vitro*, the sperm maintain better motility that those without this contact, and further exhibit lower calcium concentrations intracellularly, allowing them to delay capacitation and prolong their viability for fertilisation (Leemans *et al.*, 2016).

2.2.2.4.3: The oviduct-spermatozoa binding

Oviduct-spermatozoa binding serves a number of aims: (1) provide a supportive environment for sperm storage and prolonged survival until ovulation; (2) select morphologically correct and motile sperm population; (3) ready the sperm for fertilisation through capacitation; and (4) inhibit polyspermic fertilisation occurring. It remains questionable whether equine sperm-oviduct binding *in vivo* is nonspecific to a certain degree. Because the nature of oviduct cell receptors are still unknown, studies hypothesise that only a proportion of pre-ovulatory sperm-oviduct binding reaction is suspected as specific lectin interactions *in vitro*; supported by evidence of sperm having a similar binding capacity to other ciliary epithelium types. Many theories as to the complex mechanism of binding of equine sperm and oviduct cells are currently in circulation, the full nature of which is yet to be clearly understood (Leemans *et al.*, 2016).

Similarly, the data on the releasing mechanism of the sperm from the oviduct cells is limited. It is assumed that the sperm must first undergo capacitation before release, however, Leemans *et al.* (2016) found that commercial capacitating media did not trigger the release of sperm from oviductal explants. A further study on physiological triggers found that specifically processed follicular fluid was able to generate hyperactive motility and release the sperm from the explants, but only in small numbers. This led to the question of how many sperm are generally released from the isthmus reservoir to fertilise the egg at ovulation; to prevent polyspermy *in vivo*, a low ratio of sperm:oocyte is vital, therefore, it follows that the oviduct plays a role in regulating this low number of released sperm. This phenomenon may explain the limited release of sperm in *in vitro* conditions, despite artificial induction of capacitation or hyperactivity. Leemans *et al.* (2016) further postulate, however, that the isthmus sperm binding is not obligatory for sperm capacitation and activation for fertilisation *in vivo*. McCue *et al.* (2000) demonstrated that sperm surgically introduced the other direction via the infundibulum yielded similar pregnancy rates to those achieved after AI into the uterine body, highlighting that neither the uterine body nor the ampulla are specifically necessary for sperm to acquire the capacity to fertilise.

2.2.2.4.4: Oviductal fluid

However, converse to other species, where oviduct epithelia is not required *in vitro* for capacitation, it is likely that the interaction with cells or fluids in the oviduct is necessary for stallion sperm to become capable of fertilising an oocyte. The general composition of

oviductal fluid has been known for decades, yet its exact effect on sperm capacitation and fertilisation remains poorly understood. It is a mixture of follicular, peritoneal, and oviduct cell secretory fluids, that contain hormones, proteins and various factors that likely modulate the preparation of gametes for fertilisation. Its electrolyte, lipid and carbohydrate composition is designed to create the optimal environment for fertilisation, and its volume and activity is cycle- and region-dependent. Proteins that influence maturation of the oocyte, or have an affinity to plasma membranes of the sperm, were observed as early as 1995, but their function within the oviduct of horses is still ambiguous (Leemans *et al.*, 2016).

Studies suggest that specific proteins secreted by the oviduct epithelium have a positive effect on fertilisation rates of equine oocytes. In human, bovine, and porcine species, some of these proteins have been identified – such as osteopontin, oviductin and Atrial Natriuretic Peptide A (ANP A). These have all been shown to be actively secreted in oviductal fluid and improve IVF rates, as well as embryonic development in these species. In horses, oviductin is a suspected pseudogene, i.e. one that has become inactive from repeated mutations over time, due to its stop codon within the genome; suggesting it is unlikely to be involved in the fertilisation process. On the other hand, osteopontin has been demonstrated to be expressed by equine oviductal cells. However, when the IVF medium was supplemented with a wide concentration range of bovine osteopontin, only an insignificant increase was noted in equine IVF rates; but it may be possible that the bovine derived protein was not able to interact with equine gametes on an efficient level. An equivalent osteopontin supplementation in bovine and porcine IVF, yielded convincing improvements in fertilisation and cleavage rates. A similarly disappointing trend resulted from ANP A supplementation in equine, but further studies using these proteins from different species and in different concentrations are needed to entirely rule out their significance. More research must also be conducted into identifying the equivalent influential proteins present in equine oviductal fluid and their role in fertilisation, in order to improve IVF rates in this species (Mugnier *et al.*, 2009).

The pH of the luminal fluids of mare genital tracts is slightly alkaline, with a higher pH than that found in seminal fluid, as demonstrated by Gonzalez-Fernandez *et al.* (2012), cited by Leemans *et al.* (2016); sperm that was introduced into the uterus of a mare, and then retrieved a short time later, increased in pH from 7.45 to 7.85. After the demonstration of alkaline vesicles in secretory oviduct cells by Leemans *et al.* (2014), they speculated that an alkaline gradient is formed on explants *in vitro*, or an alkaline microenvironment *in vivo*, that is capable of inducing sperm protein tyrosine phosphorylation, without the sperm having to bind to the oviduct. A 2012 study similarly concluded that a local increase in the bicarbonate

concentration in oviductal fluid may be an essential step in successful sperm capacitation in the horse (Leemans *et al.*, 2016).

The presence of a small volume of follicular fluid as a component of the oviductal fluid has been proven. It may play a significant role in influencing the oviductal microenvironment of mares, despite its negligible volume (Leemans *et al.*, 2016). When 20% follicular fluid from preovulatory follicles was added to the culture medium of equine oocytes, it resulted in an increase in pronucleus formation after ICSI in compact cumuli oocytes. However, no such improvement could be reproduced using similar supplementation in IVF. Culturing the oocytes in 100% follicular fluid did increase the rate of IVF *versus* using standard maturation medium, but it had an opposite effect on *in vivo* fertilised oocytes, by significantly lowering their cleavage rates (Hinrichs *et al.*, 2002). Furthermore, follicular fluid has been demonstrated in humans, cattle, and laboratory animal species, to contain certain factors capable of activation/capacitation of spermatozoa. While these exact factors have yet to be identified, progesterone – already defined as the key factor in humans – seems a likely candidate (Leemans *et al.*, 2016).

It has also been hypothesised that, apart from the follicular fluid released at ovulation, both cumulus cells, still anchored to the oocyte, and the oviduct epithelium itself, may secrete some of these unidentified follicular fluid factors. However, follicular fluid is not alkaline, and therefore poses the question of whether it is a necessary factor within oviductal fluid for successful capacitation. The fact that Leemans *et al.* (2016) demonstrated only tyrosine phosphorylation occurring in sperm bound to oviduct explants, suggests that it may be missing pro-capacitating factors from follicular fluid that hindered events such as plasma membrane fluidity and hyperactivated motility. Similarly, unpublished data has observed that no capacitation-related parameters were triggered when stallion spermatozoa was co-incubated with COCs (Leemans *et al.*, 2016).

Overall, it appears that oviductal secretions may be more instrumental in inducing capacitation than the actual binding of the sperm to the oviduct epithelium, suggesting a facultative rather than obligatory role of binding and creation of a sperm reservoir. In this case, timing of insemination must be taken into consideration. If sperm arrives in the ampulla in the early pre-ovulatory period, then a substantial population will bind the epithelium and form a reservoir, lying dormant until the release of 'pro-capacitating factors' occurs around ovulation. However, if sperm enter the ampulla in the periovulatory period, they will meet the capacitation triggers directly in the oviductal fluid, and will not bind the oviduct epithelium, but rather lose their binding affinity altogether. Fertilisation happens within a

short space of time in this case, and therefore negates the step of sperm storage. Nevertheless, new technologies, such as 3D oviduct printing and microfluidic automation currently under development, may further elucidate the exact role of oviductal fluid and its many components on equine IVF. Ultimately, if the capacitation triggers can be correctly identified, a sufficient capacitating medium may be established to enable IVF to become a successful commercial operation in equine breeding (Leemans *et al.*, 2016).

Yet, the question remains whether a failure of fertilisation itself, or the failure of the embryo to develop properly after fertilisation, is the cause of such poor statistics achieved in equine IVF studies to date. Furthermore, recovery of the *in vivo* fertilised IVM oocytes from the recipient mares in one study, highlighted a delay in development to 4-cell stage compared to naturally ovulated and fertilised oocytes; suggesting the IVM oocytes may fail to develop to the blastocyst stage *in vivo*, or there is a failure in signalling their transport through the oviduct to the uterus by day 5. This apparent developmental delay may account for poor embryonic development observed in previous studies of *in vivo* fertilised IVM oocytes. Further research into adequate IVM of equine oocytes is necessary to overcome its shortfalls thus far (Hinrichs *et al.*, 2002).

2.2.2.5 The significance of developing IVF in the horse

Why is it so important to crack the code for equine IVF? In other species, a comparison between IVF and ICSI techniques observed differences in the epigenetic imprinting of genes during embryonic development of the offspring, suggesting that IVF has physiological relevance over ICSI (McPartlin *et al.*, 2009). Epigenetics is defined as "the study of changes in gene function that are mitotically and/or meiotically heritable, and that do not entail a change in DNA sequence" (Ventura-Juncá *et al.*, 2015). The ICSI technique can be said to bypass certain physiological events, namely natural selection of the sperm that fertilises the oocyte, and subsequent capacitation, acrosomal reaction and membrane fusion. Studies in mice, cattle, and non-human primates, have observed "an asynchronous remodelling of chromatin decondensation of the male pronucleus". Mice, in particular, have shown transcriptome disturbances and abnormal development, health and behaviour associated with DNA fragmentation (Ventura-Juncá *et al.*, 2015). These epigenetic alterations are a significant factor that has prevented ICSI becoming a viable option in species other than equine, where IVF is already regarded as an efficient technology. Furthermore, it is reason enough to continue research into perfecting equine IVF as a commercially viable technique.

However, it is evident that all *in vitro* embryo techniques have their drawbacks. From early on in bovine IVF research, a difference in the appearance and behaviour of *in vivo* produced embryos, compared to *in vitro* matured embryos, was observed. Many studies have shown a lower developmental competence after implantation and a higher sensitivity to cryopreservation in *in vitro* produced embryos. Genomic analysis technology now allows us to compare and observe the impact of culture conditions on the transcriptome, and the presence of different molecules that result in alterations of gene expression. Perhaps unsurprisingly, a higher embryonic and foetal loss is recorded with IVF pregnancies than other ART, like AI or ET, from *in vivo* origins. In studies on all species involved, differences can be found between IVF and naturally conceived offspring, and more research is needed into the potential consequences on the future health of the individuals born through such assisted reproduction techniques (Sirard, 2018).

2.3: Intracytoplasmic Sperm Injection

2.3.1: The history and development of ICSI

2.3.1.1: The origins of ICSI in horse breeding

Intracytoplasmic sperm injection (ICSI) is an assisted reproduction technique that utilises micromanipulation to inject a single spermatozoon into the cytoplasm of a mature oocyte. The first record of successful formation of a pronuclei with this method, was reported as early as 1976 in hamsters. By 1992, the first human baby was born via ICSI, and it has since become an important technique applied to modern human assisted reproduction. The use of ICSI has been documented in other animals, such as cattle, small ruminants, pigs, and even domestic cats and wild felids. The first reports of its application in the horse are from 1996, and it has since developed into a popular commercial enterprise, with the combination of transvaginal OPU, ICSI and ET being the current routine protocol for *in vitro* embryo production (Salamone *et al.*, 2017). The first ICSI foal was born in the USA in 1996, using *in vitro* matured oocytes that were injected with sperm and transferred directly to the oviducts of recipient mares. A period of highly variable results followed, and it was many years before this success could be repeated, mostly due to failures in achieving blastocyst formation (Alm *et al.*, 2010). However, later advancements in culture medium, and the development of the Piezo drill method, have greatly improved cleavage rates and repeatability of this technology in recent years (Salamone *et al.*, 2017).

Initially, the embryos produced through ICSI were cultured *in vivo* using sheep oviducts, before being recovered and transferred to recipient mares; this technique saw successful cleavage rates of up to 36%, but for ethical and practical reasons, new *in vitro* culture methods needed to be developed in which the embryo is transferred only once directly to the recipient mare. (Alm *et al.*, 2010). With the development of a transvaginal OPU technique in mares, as well as improvements in efficiency of IVM and culture media, ICSI has seen increasing popularity amongst breeders in the last few years. Current statistics in equine breeding programmes show that ~60% of OPU-ICSI sessions yield at least one blastocyst, and an average of 1.8 blastocysts are produced per successful OPU-ICSI procedure (Claes *et al.*, 2018). However, there are still highly variable blastocyst formation rates reported between ICSI laboratories, and the technique is subject to many influential factors, such as stallion fertility, mare age, and oocyte quality. Nevertheless, of the blastocysts formed, there is generally acceptable pregnancy rates achieved after transfer to recipient mares, with highs

of 50-80% reported (Salamone *et al.*, 2017). These impressive statistics dictate that the combination of OPU and ICSI can be considered as "a competitive alternative to conventional embryo flushing" (Galli *et al.*, 2016). In addition, production of embryos through ICSI technology has also been further applied to the development of more efficient embryo *in vitro* culture systems in the horse (Alm *et al.*, 2010).

2.3.1.2: ICSI in cattle breeding

Unlike in the previously described sections on OPU and IVF, ICSI represents a break in the pattern where the technology is first developed in cattle and later adapted to the horse. Despite the extensive efforts made, ICSI fertilisation rates have remained critically low in cattle, and the number of offspring born through this technique is disappointing (Salamone *et al.*, 2017). In general, the low embryo production rates achieved in domestic animals other than the horse, have resulted in ICSI not being the ART of choice to overcome male infertility; unless the male is very valuable or a limited sperm reservoir exists, as is often the case in equine. Furthermore, the failure of IVF in the equine species translates to ICSI being the only viable option in many cases involving sub-fertile individuals (Oseguera-López *et al.*, 2019).

The application of ICSI in bovine reproduction has failed to gather much interest and has been used mainly for research purposes to date. The reason for this is two-fold: efficient IVF programmes are already established that achieve acceptable production rates; and the failure of ICSI to reproduce similar success rates to IVF in this species. One of the main setbacks observed by bovine ICSI studies, is the poor oocyte activation after sperm injection and asynchronous pronucleus formation (Wrenzycki, 2018). Studies have reported as high as 90% of bovine oocytes fail to achieve calcium oscillations necessary for oocyte activation after the sperm is injected, showing variable pronuclei formation and inconsistent nuclear decondensation of sperm heads (Salamone *et al.*, 2017). These obstacles have proven difficult to overcome by exogenous manipulation protocols, limiting the use of this ART commercially in cattle so far. Additionally, bovine sperm are larger in size than equine, and therefore, require larger diameter injection pipettes that may cause damage to the cytoskeleton of the oocytes, resulting in lower developmental competence and embryo production (Wrenzycki, 2018).

Ultimately, it remains unclear whether the fault lies with the sperm or the oocyte in these species, but it is generally accepted that ICSI bovine embryos require some form of artificial activation to successfully reach the blastocyst stage of development; as such, no reliable

standardised protocol has been developed so far that allows for acceptable enough embryo production rates in bovine to justify the use of ICSI in this species (Salamone *et al.*, 2017).

2.3.1.3: Advantages and disadvantages of ICSI in equine breeding

An important advantage of ICSI in the horse is the ability to perform this technology outside of the breeding season and at any stage in the mare's reproductive cycle. Thanks to improvements made in transvaginal OPU in horses, oocytes can be collected through the winter and transitional periods and fertilised with ICSI. The embryos can then be cryopreserved, with good pregnancy results being reached after later transfer to recipients (Salamone *et al.*, 2017). For the most part, the cryopreservation of ICSI embryos is now the standard procedure, not direct implantation into recipient mares. There are a number of reasons for this, namely that a lot of OPU-ICSI sessions are carried out in the non-breeding season/winter months, and therefore recipients are not readily cycling for the transfer to occur. Furthermore, these ICSI embryos are mostly from very valuable and genetically elite animals, and as such, a specialised international market has now been established for the sale of the embryos in their cryopreserved state. The ability to cryopreserve the IVP embryos is also an important method of banking these valuable genetics (Galli *et al.*, 2016).

In addition, the value in this technique is that ICSI requires only a single spermatozoon to fertilise the oocyte, and is considered the most effective method available for fertilisation of oocytes with immotile sperm or immature spermatids (Salamone *et al.*, 2017; Salgado *et al.*, 2018). The possibility of using sperm with very low fertility rates, or in limited reserve, is one of the main advantages ICSI has over other ARTs, like IVF and OT. The procedure can be performed with fresh, cooled, or frozen-thawed semen (Alvarenga *et al.*, 2008). The cryopreservation of stallion semen has become an invaluable technology in modern equine breeding, but unfortunately it has its drawbacks, such as requiring special liquid nitrogen storage containers that need constant maintenance and can make the transport of straws cumbersome and dangerous. Furthermore, there is always the potential for damage of the sperm and viral contamination between straws kept in the same tank. Before ICSI was developed, these straws could only be used as one AI dose in a single mare, which was problematic in stallions of high value, or stallions no longer alive/breeding with a limited number of straws remaining. ICSI is the most efficient alternative in this case, as one straw can be used to fertilise many more than just one oocyte. A study in 2006 showed that frozen straws can be thawed, diluted and refrozen, without any effect on blastocyst formation after ICSI; meaning multiple smaller ICSI doses or "cuts" can be made from a single straw of

semen and refrozen. It also further offers an alternative method to cloning these stallions in order to preserve key bloodlines (Choi *et al*., 2011; Salamone *et al.*, 2017).

Furthermore, ICSI provides an important last resort option for breeding mares that die or are euthanised unexpectedly; by recovering the oocytes from the ovaries post-mortem, they can be fertilised with ICSI and transferred to a recipient, or the valuable embryos/oocytes can be cryopreserved for future use. These advantages, coupled with the failures of IVF technology, has kept the equine species at the forefront of the ICSI technique to date, with the popularity of this method continuing to grow exponentially (Salamone *et al.*, 2017).

The main disadvantage of ICSI is that, alongside IVM/IVC and cryopreservation, it requires a specialised laboratory and equipment, quality control systems, and highly trained operators. Therefore, the procedure is limited to a small number of facilities offering commercial ICSI programmes around the world. As a result, it is a very expensive process, far more costly than conventional embryo flushing, and thus, is only used in the most valuable of horses. While general equine practitioners are capable of the OPU and ET steps in the process, the recovered oocytes must be shipped as quickly as possible to these central specialised ART laboratories. The need to transport the oocytes is a further cost, and creates an additional risk factor, but one necessary to allow a wider accessibility of ICSI programmes to breeders (Galli *et al.*, 2016). Furthermore, there is a major limiting factor for using ICSI in sub-fertile mares – oocyte quality; if the fertility problem stems from poor quality oocytes, then ICSI will not be helpful in the breeding of such mares (Hinrichs, 2018).

2.3.2: Step of the ICSI procedure

2.3.2.1: OPU and IVM of oocytes

Transvaginal OPU is used to obtain cumulus oocyte complexes (COCs) from the donor mare, which are then evaluated and characterised as expanded (Ex) or compact (Cp) cumulus oocytes. The oocytes are washed in synthetic oviductal fluid (SOF) medium and transferred to an appropriate culture medium; Ex are cultured *in vitro* for 24 hours, and Cp for 30-32 hours, at 38.5°C in 5% CO_2-in-air atmosphere (Alm *et al.*, 2010; Claes *et al.*, 2018). After IVM is complete, the cumulus cells are then removed from the COCs; first enzymatically, by incubation for 5 minutes in a SOF medium containing hyaluronidase, then manually with gentle pipetting. Vortexing is preferably avoided in horses to minimise damage and loss of

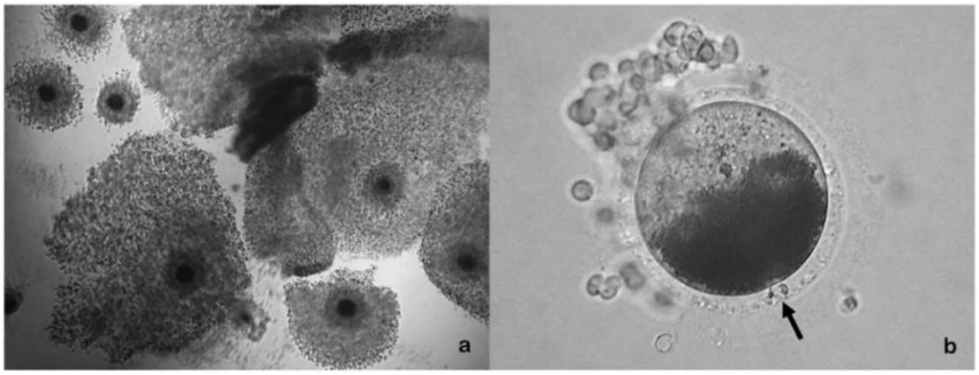

Figure 4.1: Equine oocytes in IVC: (a) full COCs; and (b) a mature oocyte after cumulus cell stripping, with arrow indicating the presence of a visible first polar body (Sansinena, 2020)

oocytes, given the high value nature of the horses used (Claes *et al.*, 2018; Salamone *et al.*, 2017). Oocytes that contained a clearly visible first polar body are then selected for the ICSI procedure (Fig. 4.1) (Claes *et al.*, 2018).

2.3.2.2: Sperm preparation

2.3.2.2.1: Thawing of semen straw

ICSI can be performed with fresh, cooled, or frozen semen; however, in the vast majority of cases, frozen semen straws are used, as it is usually lower quality sperm, or sperm of limited supply, that is applied in ICSI. The straws are thawed in a 39°C water bath for 30 seconds, and then subjected to a selection method to isolate the motile sperm (Alm *et al.*, 2010).

2.3.2.2.2: Sperm selection methods

As ART expanded to include ICSI, methods of sperm isolation have developed in parallel. The development of ICSI techniques initially dampened the need for more sophisticated sperm selection methods, due to the nature of needing a single sperm that is manually injected in, resulting in quality issues in the beginning (De Jonge, 2017). Nowadays, the sperm preparation is considered a critical step in the ICSI process to select only viable spermatozoa (Salamone *et al.*, 2017). Sperm selection techniques should be simple and economic to perform, but also effectively remove any low quality, immotile or dead spermatozoa, as well as eliminate bacteria, leukocytes, and toxic substances, such as reactive oxygen species (ROS). As they satisfy all these requirements, swim-up and density gradient centrifugation methods are the most often used in animals (Fig. 4.2). Swim-up methods can involve centrifugation, straight migration, migration-sedimentation or recovery of

spermatozoa from non-resuspended pellets. Conversely, density gradient methods are based on motile sperm being able to move through density gradients, formed by colloidal particles, during centrifugation (Oseguera-López *et al.*, 2019). Perhaps the simplest methods of selection are those based on sperm migration. These work by mimicking the natural separation of spermatozoa from seminal plasma *in vivo* via self-propelled, directional migration, i.e. forward progressive motility. However, these methods do not work as well for semen with poor quality spermatozoa without sufficient motility, and therefore, alternative methods are better suited for ICSI sperm selection (De Jonge, 2017).

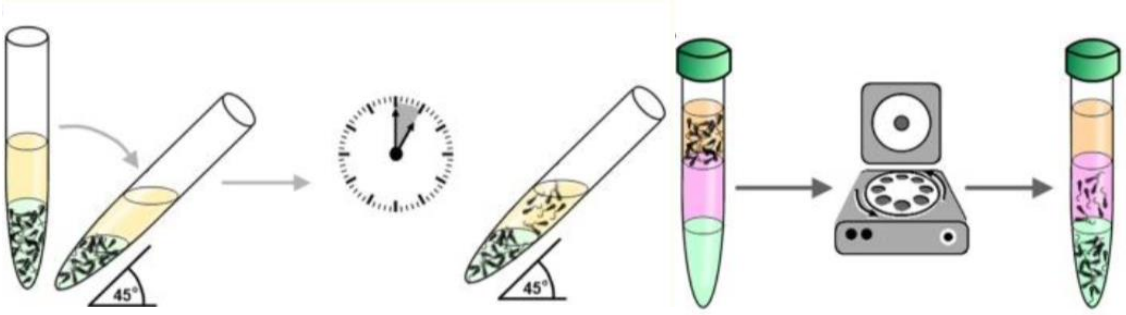

Figure 4.2: Schematic of the basic method of (*left*) swim-up and (*right*) density gradient centrifugation sperm selection techniques (Oseguera-López *et al.*, 2019)

The main two swim-up methods used are (a) swim-up from washed pellet, and (b) direct swim-up from semen. The swim-up from washed pellet method involves the separation of spermatozoa from seminal fluids through repeated centrifugation and dilution until a loosely compacted pellet is formed at the bottom of the tube, which can be carefully resuspended in a small volume of medium, or left intact with culture medium carefully layered over the pellet. The tube is incubated at body temperature for one hour at a 45° angle, allowing progressively motile sperm to swim up from the pellet through the medium; alternatively, a four-well plate can be used to increase the interface between the pellet and medium. An increase in medium turbidity is suggestive of motile sperm "swimming up" from the sediment. The upper layer of the medium is then aspirated and transferred to a clean tube, where the spermatozoa quality can be assessed. This is an effective method of gathering healthy motile sperm and has been linked with increased rates of acrosomal reaction and nuclear maturity. However, not all motile sperm can escape the pellet and the overall number recovered can be low, therefore, the nature of the pellet is an important factor. The centrifugation process can also cause damage to the sperm cells, associated with sudden production of ROS from some cells, that in turn damage the healthy spermatozoa in the suspension (De Jonge, 2017).

In the direct swim-up from semen technique, instead of centrifugation, the semen is layered under medium in test tubes angled at 45°, or four-well plates, and incubated for one hour. After the sperm swim up, indicated by medium turbidity, the upper lay is aspirated and transferred to a centrifuge tube. After gentle centrifugation, the supernatant is removed and then the sediment pellet is resuspended in culture medium. This method has a higher rate of recovery of motile spermatozoa than the last method, and is notably absent of any additional cellular debris. Studies comparing different methods have found this one to yield the best sperm motility. In addition, conducting the centrifugation step after the initial separation of sperm from other cellular elements, reduces the ROS production by damaged sperm cells and leucocytes (De Jonge, 2017).

The migration-sedimentation method combines the techniques of sedimentation and swim-up using special tubes that contain an inner cone. After the spermatozoa swim up to the surface, they then settle down into the inner cone due to gravity. After incubation for an hour, the inner cone contents are aspirated, centrifuged, and the pellet resuspended. This method has similar advantages and disadvantages to the previous two. Studies have shown it is particularly effective in sub-fertile males, showing increased motility and morphology with this method, and should therefore be the method of choice in males with oligozoospermia (De Jonge, 2017).

In density gradient centrifugation, semen is layered on top of a colloid gradient in a tube and centrifuged, then motile sperm are collected from the bottom of the tube where the highest gradient density is situated. The gradients can be continuous or discontinuous, and different types of colloid substances can be used, such as Percoll PVP-coated silica particles or current commercial variants (De Jonge, 2017). Percoll is considered to be toxic in human studies, but still remains the colloid of choice in animal ART (Oseguera-López *et al.*, 2019). Although more expensive, this technique is considered ideal for stallions with issues such as oligozoospermia or asthenozoospermia, as the whole volume of ejaculate is used in this method and therefore yields a high number of total motile spermatozoa. Furthermore, research has suggested that this method selects sperm that have a higher DNA quality. The use of silica particles can also reduce bacterial contamination, as well as effectively "clean up" samples infected with venerally transmitted viruses (De Jonge, 2017).

Studies on both these categories of selection methods have demonstrated that recovered sperm samples have normal morphology and optimum motility; in humans, they have further observed that these techniques specifically select sperm cells with the longest telomeres, an aspect now considered as an indicator of correct spermatogenesis (Oseguera-López *et al.*,

2019). Nowadays, sperm quality parameters extend beyond just motility, morphology, and sperm count; the epigenome, transcriptome and proteome have come under focus. However, with detailed testing comes the inevitable risk of detrimental iatrogenic influences on fertilisation or embryonic development, so this must be weighed in as a factor when considering more advanced sperm selection methods (De Jonge, 2017).

2.3.2.2.3: Sperm Immobilisation

After the selection step is completed, right before ICSI is performed, the sperm is suspended in Tyrode's medium with 10% Polyvinylpyrrolidone (PVP) to slow down the flagellar movement of the sperm, and allow it to be captured by the injection pipette, i.e. immobilise it (Alvarenga *et al.*, 2008). The operator aspirates the sperm cell into the pipette, rolling the tip of the pipette over the sperm tail in the process in order to damage its membrane. This action is believed to facilitate the nuclear decondensation of the sperm head and subsequent oocyte activation; therefore, it is a step that should not be bypassed, even when dead spermatozoa are used (Salamone *et al.*, 2017).

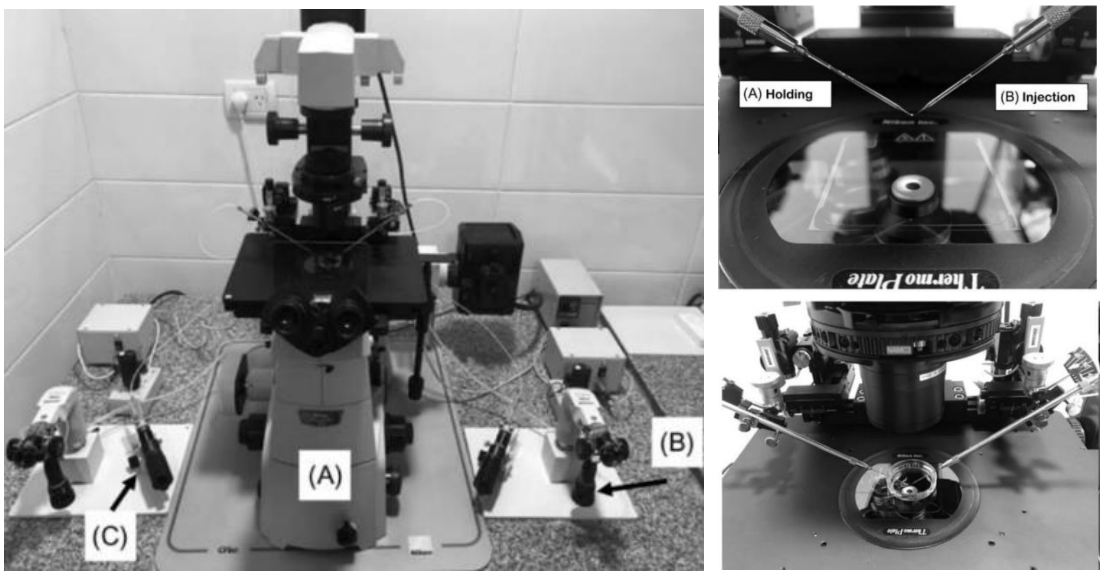

Figure 4.3: (*left*) An ICSI micromanipulation station with (A) inverted microscope; (B) micromanipulation joysticks and (C) the microinjectors; and (*right*) a close-up image of the microtools (Sansinena, 2020)

2.3.2.3: ICSI equipment and methods

2.3.2.3.1: Basic equipment

The ICSI procedure itself requires specialised equipment, including an inverted microscope attached to a micromanipulation system that allows precise handling of the microscopic

gametes (Salamone *et al.*, 2017). The entire ICSI procedure is done under oil in an inverted microscope using a micromanipulator (Alvarenga *et al.*, 2008). The micromanipulator has two arms, one connected to a 120µm holding pipette that secures the oocyte, and the other arm connected to the 5-7µm injection pipette containing the sperm, shown in Figure 4.3.
The injection pipette passes through the ZP of a metaphase II oocyte and deposits a single spermatozoon into the cytoplasm. The key element in this process in the position of the first polar body, where the genetic material of the oocyte is presumed to be located near (Fig 4.4). Therefore, it is vital that this is not damaged by the injection pipette, which is inserted with the polar body clearly visible at the 12- or 6 o'clock position to prevent any chromosomal or spindle damage. The pipette design is also a specific factor, with sperm size dictating the diameter of the injection pipette, and therefore, different pipette sizes are used in different species. In the horse, pipettes commercialised for use in humans, with a 7µm inner diameter, can be used. Furthermore, the shape of the injection pipette differs based on the system used (Salamone *et al.*, 2017).

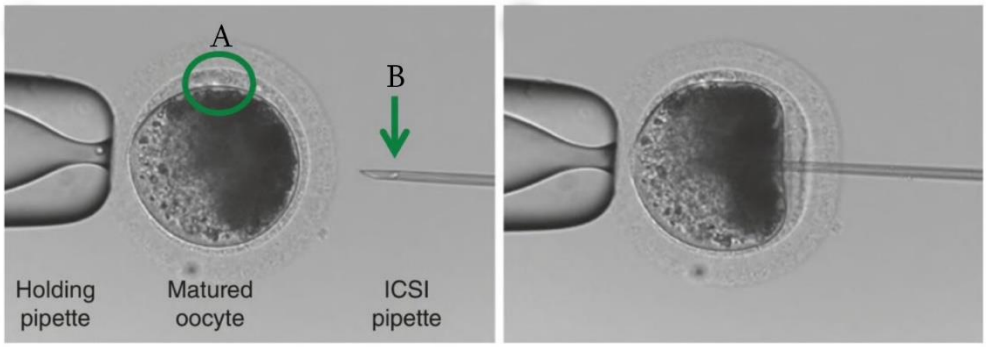

Figure 4.4: ICSI procedure in an oocyte with (A) location of the oocyte polar body at 12 o'clock; and (B) a sperm loaded into the tip of injection pipette (Wrenzycki, 2018)

2.3.2.3.2: ICSI methods available

Two main methods of ICSI procedures exist today: the conventional ICSI and the Piezo-driven ICSI. In the conventional method, a sharply bevelled pipette is used to manually crush the tail of the sperm, by rolling it against the bottom of the Petri dish; the sperm is immobilised and its plasma membrane ruptured, and then aspirated into the pipette tip, tail first. With the oocyte held with the polar body at 12- or 6 o' clock position, the injection pipette is pushed through the ZP and advanced through approximately 90% of the oocyte diameter to the opposite side. Once suction pressure breaks through the oolemma and

cytoplasm appears in the pipette, the sperm is ejected out of the pipette with the minimum amount of medium possible (Salgado *et al.*, 2018).

For the Piezo method, the pipette used is more blunt and micro-vibrations are applied to immobilise the sperm, disrupt the tail, and break the plasma membrane, as well as infiltrate the ZP and oolemma. The injection pipette is introduced into the sperm droplet and a sperm cell is aspirated tail first before being slowly ejected until the midpiece exits the tip, at which point the pipette is moved into direct contact with the tail and pulses applied. This process is repeated until the sperm is fully immobilised and then it is reloaded into the pipette. The oocyte is fixed in position, with the polar body visible at 12- or 6 o' clock, and pulses are applied by the Piezo drill to the ZP until a hole is formed. The pipette is fed through this hole into the oocyte and another pulse is applied to break the oolemma membrane. No cytoplasm is aspirated into the pipette in this method, and the pipette is advanced through 90% of the cytoplasm before the sperm ejected (Salgado *et al.*, 2018).

With the Piezo method, the piezoelectric actuator works by driving the tip of the pipette through the oocyte membrane in a fast, precise movement. Some researchers believe this makes the procedure easier and more successful by mechanically disrupting the tail and oolemma membrane, allowing for cleaner ZP penetration. If a Piezo drill is not used, it can be difficult to manually break through the oolemma due to its elastic nature. This is done in conventional methods by aspirating until a speed change of the ooplasm entry rate is observed inside the injection pipette, then the sperm and aspirated ooplasm are injected back into the oocyte. This step is an area of frequent mistakes in inexperienced operators. Furthermore, high lipid content in the ooplasm of domestic animals can inhibit proper visualisation of the sperm as it enters the oocyte, but this is generally not an issue in horses (Salamone *et al.*, 2017). As a small hole is formed in the ZP by the Piezo drill, several presumptive maternal cells have been observed in the perivitelline space in these embryos. In other species, the presence of these cells is usually associated with embryonic death; however, in the horse, it appears that the embryo is capable of excluding these cells as it forms the capsule. More studies using agar embedding may be able to determine how much these cells effect embryos fertilised with the Piezo technique (Choi *et al.*, 2004b).

2.3.2.3.3: Post-ICSI embryo culturing

It is important that the injected oocyte is kept at room temperature for at least 20 minutes after the procedure, to allow its punctured plasma membrane to heal. After this point, it can be placed in an appropriate medium for further *in vitro* culturing. Alternatively, immediate

oviductal transfer can be performed in a recipient mare, but this is used less frequently nowadays (Alvarenga *et al.*, 2008). For IVC, the injected oocytes are usually cultured for 5-6 days in modified synthetic oviductal fluid (SOF) medium – often supplemented with additives like bovine serum albumin (BSA) and amino acids – for 5-6 days, at which point they are assessed for blastocyst formation. The viable blastocysts can then be either transferred into recipient mares transcervically, or cryopreserved, usually via slow-freezing, and stored in −196°C liquid nitrogen (Claes *et al.*, 2018).

2.3.2.3.4: ET of frozen ICSI embryos

It has been suggested that ICSI produced embryos are more resistant to damage during cryopreservation, as they are smaller and are missing a capsule at the point of blastocyst formation; thus, acceptable pregnancy rates can still be achieved after ET of these embryos. Before the transfer, the straw containing the frozen embryo is removed from the liquid nitrogen storage tank and held in the air for ~8 seconds before being placed in a water bath for 30 seconds at 22-24°C. The straw is then withdrawn, dried, and can be wiped with an ethanol-soaked cloth to clean it. Afterwards, the sealed end is cut, and the contents are expelled into a Petri dish which is placed under a microscope. The embryo is located and then washed in a decreasing concentration gradient of 8%, 6%, 4%, 2% glycerol in SOF for 5 minutes at a time. It is then transferred into a SOF holding medium where its quality can be assessed, before being loaded into an ET straw and implanted in the recipient mare (Claes *et al.*, 2018).

2.3.2.3.5: ICSI embryonic loss rate after ET

McCue *et al.* (2016) recorded that conventional ET, i.e. *in vivo* developed embryos that are flushed from donor mares and transferred directly to recipients, is 20% more likely to result in pregnancy than freshly transferred ICSI embryos. A study was carried out by Claes *et al.* (2018) to determine pregnancy rates and possible causes of embryonic and foetal losses after the transcervical transfer of cryopreserved ICSI embryos in horses. Following ET, the recipients were scanned for pregnancy between day 7 and day 10, and again on day 23, day 37, and day 43 after transfer. If any abnormality was detected, the mares were scanned more often to closer pinpoint the time of pregnancy loss. Of the implanted recipients, 56% scanned in foal at the first post-ET pregnancy detection (day 7-10), but only 48% were still pregnant at day 37; overall, a 16% embryo loss rate was recorded between day 7 and day 43, far higher than the rates seen in flushed embryos. Furthermore, the percentage of embryonic loss was

highest during early embryonic development (before day 28) of gestation, and blastocysts that took longer to form in IVC (≤7days *vs.* ≥8days) had much higher loss rates. This indicates that embryos slower to develop in IVP could be indicative of reduced quality or viability, but a larger sample size is needed to confirm this suspicion (Claes *et al.*, 2018). There are many factors that can affect the success of ICSI embryo production, and they will be outlined in following sections.

2.3.3: The mechanism of fertilisation in ICSI embryos

2.3.3.1: The major fertilisation steps

2.3.3.1.1: Oocyte activation

Oocyte activation refers to the resumption of meiosis, extrusion of the second polar body, cortical reaction of the oocyte, and formation of the two pronuclei, male and female (Salamone *et al.*, 2017). In all mammalian species studied to date, calcium oscillations are required in the oocyte cytoplasm to trigger this activation sequence and progression of normal embryonic cleavage (Bedford *et al.*, 2004). In the horse, as well as human, mice and cats, the injection of the spermatozoon into the oocyte during ICSI is enough to trigger the activation of the oocyte and induce embryo development. Studies have recorded a 43-74% rate of pronuclei formation after sperm injection alone, and therefore it appears that no other mechanisms are needed to support embryo formation and development (Salamone *et al.*, 2017). It has been further suggested that the frequency and durations of these calcium transients could impact later embryo development before and after implantation stages (Bedford *et al.*, 2004).

The mechanism of calcium oscillation generation in the oocyte is widely attributed to a 'sperm factor', released after fusion of the sperm to the oocyte. This is supported by experiments that demonstrated a fertilisation-like calcium response in oocytes injected with sperm extracts through ICSI. It is widely accepted that a sperm-specific phospholipase C zeta (PLC_ζ) enzyme is responsible for the production of inositol 1,4,5-triphosphate (IP_3) in the oocyte, which in turn binds the endoplasmic reticulum and induces calcium efflux into the cytosol. It is possible that other mechanisms may be involved in different species, but these are yet to be identified (Bedford *et al.*, 2004).

2.3.3.1.2: Nuclear and cytoplasmic changes

It is known that for fertilisation to occur, a series of precisely orchestrated nuclear and cytoplasmic changes must take place within the oocyte. Nuclear changes notably include the formation of male and female pronuclei, and the subsequent migration and apposition of these structures to allow for the mixing of the two genomes; all processes that depend highly on the microtubular and microfilamentar element reorganisation within the oocyte and sperm. In the majority of mammalian species, the centrosome, i.e. the microtubule organising centre, that is responsible for coordinating the union of the pronuclei and formation of the mitotic spindle in the zygote, comes from the sperm and is therefore paternally inherited; the maternal oocyte centrosome is lost during initial gametogenesis. Up until the early 2000s, the pattern of centrosome inheritance was unknown in the horse, and a study was conducted to elucidate this factor, as well as characterise the nuclear and cytoskeletal dynamics in the oocyte after ICSI fertilisation, using confocal laser scanning microscopy (CLSM) analysis. Moreover, it sought to examine the points of deviation in the fertilisation process of zygotes where development was delayed or failed (Tremoleda *et al.*, 2003).

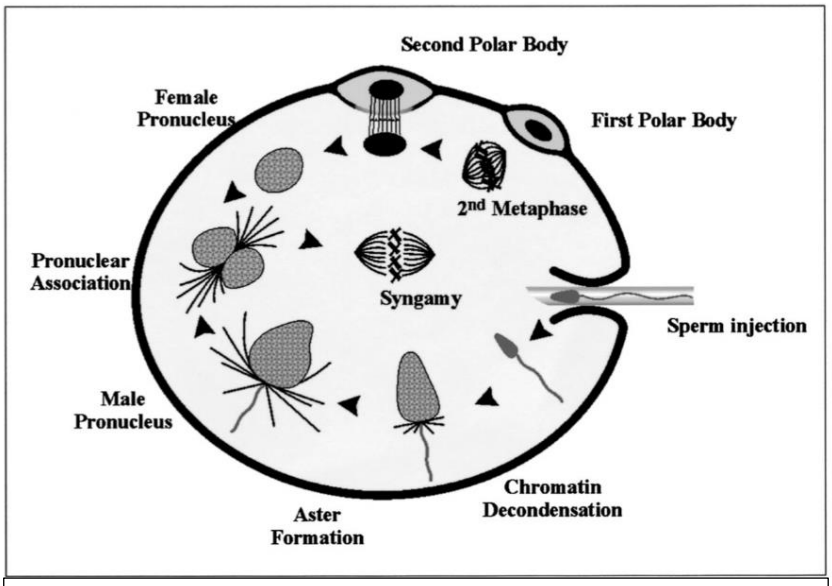

Figure 4.5: A schematic representation of the microtubule and chromatin reorganisation that occurs in equine oocytes after ICSI (Tremoleda *et al.*, 2003)

The oocytes in this study were cultured for 24- and 48 hours, before being labelled with special stains for chromatin, microfilaments and microtubules; the injected sperm were labelled with a mitochondrion-specific dye to track the sperm incorporation and subsequent

transformation of sperm-derived structures into zygotic elements (Fig. 4.5). The injected oocytes were further cultured for 6-, 12- or 18 hours, before being stained and their microtubules and chromatin studied for changes in their distribution and pronucleus formation. By using sham-injected oocytes, they sought to observe the microtubule and chromatin organisation to characterise the specific role of oocyte-derived structures in the formation of the maternal pronucleus and activation of the oocyte.

It appeared that the horse has a primarily paternally inherited centrosome, like that of other mammalian species; the microtubule organisation was initiated by the sperm midpiece, which orchestrated the formation of the sperm aster. However, 76% of the sham-injected oocytes also demonstrated microtubule organisation, with 16% resulting in parthenogenetic development. Previous studies have reported that parthenogenic activation of horse oocytes can result in cell division, indicating that oocytes in this species have sufficient material to form their own functional centrosome. In other species like cattle, pigs, and humans, the sperm aster is usually the only microtubule-containing structure present in the zygote, but it does not follow that there are not maternal and paternal components within the zygotic centrosome. This study's observations of microtubule nucleation is suggestive of a biparental origin to the centrosome formed in the equine zygote (Tremoleda *et al.*, 2003).

2.3.3.2: ICSI fertilisation *vs. in vivo* fertilisation

The variable culture periods of the ICSI oocytes in the study by Tremoleda *et al.* (2003), allowed for the observation of different stages of the fertilisation process and the sequence of remodelling of the cytoskeleton and nuclear elements. It is difficult to evaluate their efficiency without the direct comparison to events that occur in *in vivo* fertilisation, which remain undefined for the most part. It is also unclear how long after ovulation sperm penetration takes place, as sperm transport and capacitation within the reproductive tract takes time; but studies suggests that fertilisation occurs rapidly once this sperm transport is complete.

In the present study, oocyte activation signs were visible as early as 6 hours after sperm injection, however, a large variance was observed in the timing of sperm chromatin decondensation and sperm aster formation, indicative of an issue with synchrony between the gametes after ICSI fertilisation. Pronucleus formation is completed as early as 12 hours post-ovulation *in vivo*, but only 50% of the zygotes reached this point by 12 hours in the current study, with the rest needing 18 hours to reach this landmark. A possible reason for this delay could be that certain structures, usually removed by the oolemma under normal *in*

vivo fertilisation circumstances, were observed in the injected sperm in this experiment, resulting in the need for more extensive sperm remodelling. Moreover, cleavage to 2-cell stage *in vivo* occurs around 20-24 hours after ovulation, but the same stage was only observed in the majority of ICSI zygotes at 48 hours. Similar studies in IVF zygotes revealed a delay in cleavage and early embryonic development, suggesting the IVM of oocytes results in irregularities in oocyte maturation that can also cause delay in the fertilisation process (Tremoleda *et al.*, 2003).

Observation of the oocytes that failed to fertilise in this study, showed the most common reason (65%) for failure was the result of the oocytes not activating; moreover, this was accompanied, for the most part, by the incomplete decondensation of the sperm, suggestive of a communication issue between the two gametes during fertilisation. It is likely that the metaphase-promoting factor (MPF) is responsible for the maintenance of oocyte meiotic arrest in metaphase II, and its subsequent inactivation is a significant event in the resumption of meiotic division and activation of the oocyte. The inactivation of this factor and the activation of the oocyte are initiated by calcium oscillations, triggered by the entry of the sperm into the oocyte cytoplasm. However, ICSI has reported inconsistent occurrence of these oscillations in both *in vivo* and *in vitro* matured oocytes, with subsequent abnormalities of oocyte activation resulting in fertilisation failure (Tremoleda *et al.,* 2003). Bedford *et al.* (2004) previously recorded calcium concentration transients after injection of a horse oocyte with sperm extract; however, they did not observe any calcium fluctuations in oocytes injected with a single motile sperm within the same study; suggesting the oocytes themselves may fail to promote the sperm factor release or fail to respond to its signal.

Bedford *et al.* (2004) conducted a study to investigate the reason for failure of consistent calcium oscillation initiation of ICSI fertilised horse oocytes; the Piezo-driven method was used, and the oocytes were monitored using fluorescence. By using oocytes prepared through *in vivo* and *in vitro* maturation methods, they found no significant difference in the calcium responses post-ICSI; suggesting the source of oocytes is not an influential factor in this case. There was also a high variation in the onset and interval between oscillations, with some oocytes showing a calcium peak just 5 minute after fertilisation, whereas others did not respond for up to 80 minutes after; intervals between peaks ranged from 17 to 79 minutes. Surprisingly, a certain proportion of the oocytes where calcium oscillations were not detected still underwent activation, indicating that an initial rise from the injection process of the sperm itself may have been enough to activate the oocytes (Bedford *et al.*, 2004).

Furthermore, failure of decondensation of the sperm chromatin was observed by Tremoleda *et al.* (2003) in oocytes that failed to activate, suggesting the sperm was unable to transmit the activating signal or the oocyte failed to respond. By injecting mouse oocytes with stallion sperm, Bedford *et al.* (2004) readily observed calcium oscillations, proving that the sperm factor for initiating calcium response in the oocyte is indeed present in stallion spermatozoa. The failure of ICSI to consistently induce the calcium oscillations suggests there may be an issue with the release of the sperm factor. To rule out the inability of spermatozoa to release the sperm factor due to membrane permeability issues, three stallion spermatozoa were injected into horse oocytes, to theoretically increase the availability of the sperm factor; however, the polyspermy resulted in a similar variation in calcium oscillations, with only 40% recording calcium changes, and a longer mean spike interval and duration than the previous single sperm injected oocytes was observed. In other species, the micro-pulses from the Piezo drill immobilisation have proven adequate in facilitating the release of the sperm factor. To this end, in the study by Bedford *et al.* (2004) more aggressive methods of facilitating the release of the factor were applied, using chemical detergents and sonication to forcefully disrupt the sperm plasma membrane; in some cases, a complete head-tail separation resulted. Neither of these methods yielded more successful results than the Piezo drill in horse oocytes, however, the sperm were all still able to activate mouse oocytes.

To test if the factor is in fact released into the ooplasm of the equine oocyte, sperm were injected into the oocyte and then withdrawn after 15-30 minutes and re-injected into a mouse oocyte; only 22% of the mouse oocytes displayed calcium oscillations, indicating that the sperm factor had been rapidly released into the equine ooplasm. To assess whether the sperm factor was inactivated, despite its release into the oocyte, they fused zona-free equine oocytes – fertilised via ICSI but not showing any oscillation pattern – to zona-free unfertilised mouse oocytes. Surprisingly, after fusion occurred, calcium oscillations were observed concomitantly in both oocytes in all cases, appearing first in the mouse oocytes despite the fertilisation of the equine oocytes. This insinuates that the factor was completely released and highly soluble, with the ability to remain active in the ooplasm for long periods of time. The threshold IP_3 concentration in the equine oocyte may be significantly higher than that in the mouse oocyte, explaining its delay in calcium efflux to the cytosol. It has already been demonstrated that bovine oocytes have a less sensitive IP_3 receptor than that of the mouse, but this phenomenon was yet to be tested in equine. Furthermore, the equine oocyte may simply require more time than the mouse oocyte to process the sperm factor (Bedford *et al.*, 2004).

Salgado *et al.* (2018) observed evidence of rapid oocyte activation, such as loss of cortical granule-associated fluorescence and evidence of meiotic resumption, in a small percentage of oocytes immediately after sperm injection; this signifies that ICSI can initiate developmental events in a very short period of time. However, a similar cortical granule loss was seen in both sperm-injected oocytes and sham-injected control oocytes, insinuating that the act of injecting itself may be responsible for the cortical reaction, rather than the rapid release of activating factors from the sperm. It is likely that the injection process triggers calcium oscillations that are responsible for the granule exocytosis. It is also possible that the calcium content of the sperm and oocyte culture media, introduced in the process of ICSI, may play a role in the calcium concentration changes. When the presence of cortical granules was evaluated later in the maturation period of these oocytes, a tendency emerged in which the proportion of prominent granules increased in sham-injected oocytes, but decreased in both groups of sperm-injected oocytes; suggesting that sperm-related factors are responsible for loss of the granules. It is likely that these factors maintain a continued stimulus for activation that cause the granules to translocate to the periphery, whereas sham-injected oocytes showed a pattern of granule recrudescence (Salgado *et al.*, 2018).

Overall, it remains plausible that a step is missing in the activation and/or provision of the sperm factor that allows it to trigger calcium oscillations in the equine oocyte after ICSI. It is possible that some molecular change happens in *in vivo* fertilisation conditions, during capacitation and acrosomal reaction of gamete fusion, that serves this purpose. Further research is needed into methods for improving the ability of the oocytes to initiate a calcium response post-ICSI, with the hope of improving the commercial efficiency of the ICSI technique as a result (Bedford *et al.*, 2004).

2.3.4: Other factors affecting the success of ICSI

2.3.4.1: IVM/IVC of oocytes

2.3.4.1.1: In vivo vs. in vitro

ICSI could not be used commercially in equine breeding until a standardisation of the technique was developed. One of the initial major setbacks was with the IVC of zygotes after ICSI was performed, and in the beginning, immediate oviductal transfer to a recipient mare was the only viable option available. Therefore, in order for ICSI to become a commercial programme, it was necessary for the development of a suitable *in vitro* embryo culture system (Alvarenga *et al.*, 2008). Despite the clinical successes of ICSI by the early 2000s,

there remained significant differences in fertilisation rates reported by different research groups, with cleavage rates ranging from 20-80%, and male pronucleus formation between 21-71%. This indicates that there were notable failures still occurring in zygote development with the ICSI technique (Tremoleda *et al.*, 2003).

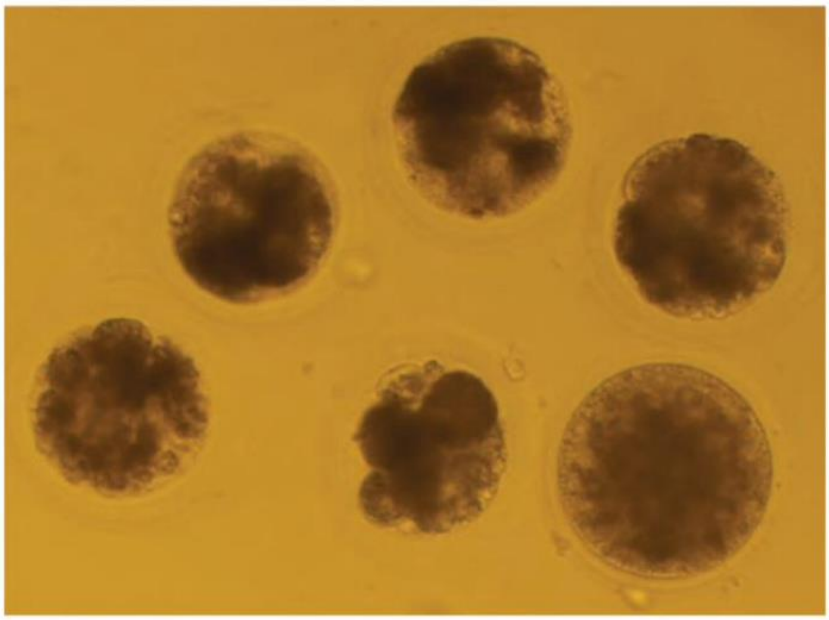

Figure 4.6: ICSI embryos on day 8 of IVC with only one (*bottom right*) forming a viable blastocyst; the rest have cleaved but are now in various stages of degeneration (Bols and Stout, 2018)

In general, the IVC of equine embryos had experienced exceptionally low success rates in comparison to other species, with the delay in its development most likely a consequence of the failure of IVF technology in the horse. With the introduction of ICSI as a viable commercial program, focus shifted to the issues experienced with the *in vitro* culturing system that was yielding low blastocyst formation rates amongst the zygotes that had achieved cleavage (Fig. 4.6); especially when compared to the rates reached through early transfer into the oviduct of recipient mares, which yielded the highest pregnancy rates for ICSI embryos. This highlights the detrimental effect that *in vitro* culturing was having on developmental competence of ICSI zygotes, and the necessity to develop a more reliable system for IVEP in horses (Tremoleda *et al.*, 2003). In a 2004 study, high fertilisation and cleavage rates (69-89%) were observed post-ICSI, but blastocyst formation remained disappointing (9-14% using Piezo drill method; 3-4% using the conventional method) and only three laboratories had reported the birth of live foals from an IVM/ICSI/IVC protocol by this point (Choi *et al.*, 2004b). For this reason, beyond the production of live offspring,

ICSI evolved into a powerful tool to study fertilisation mechanisms and evaluate IVM systems and IVEP protocols to improve the embryo success rates with this ART in equine breeding (Alvarenga *et al.*, 2008).

Limited information is available in the horse as to the sequence of events that happens *in vivo* from fertilisation to early embryo development, as this occurs within the oviduct and therefore, can only be recovered surgically or post-mortem after slaughter of the mare (Tremoleda *et al.*, 2003). It was questioned whether the failure of equine zygotes to develop *in vitro* was the result of the inappropriate embryo culture conditions or related to a general poor developmental competence of equine embryos matured *in vitro*. Various studies were conducted in which ICSI oocytes were transferred to the oviducts of different animals, including mice, sheep, and recipient mares; with the general consensus being that the *in vivo* cultured zygotes showed significantly higher blastocyst formation rates than their IVC counterparts. This suggests that IVM/ICSI embryos are capable of developing if the proper environment is provided after fertilisation, and that the failure of blastocyst formation most likely lies with the inappropriate culture conditions post-ICSI, and not with an inherent developmental defect in oocytes that have been matured *in vitro* (Choi *et al.*, 2004b). In a similar 2010 study, embryo cleavage rates of 40-47% were achieved through ICSI, but blastocyst formation rates remained low, in comparison to the group transferred to ovine or equine oviducts for a "mid-culture" *in vivo* step. This suggests that despite improvements in *in vitro* culture media, it needs further development to achieve rates similar to those observed with *in vivo* culturing methods (Alm *et al.*, 2010).

Furthermore, abnormal expansion of the blastocysts produced *in vitro* has been reported, as the ZP fails to break off and a capsule to form, as normally happens under *in vivo* conditions; in these cases the blastocysts can be seen to hatch through the hole made in the ZP by the Piezo drill around day 7.5. Similar findings can be seen in some embryos collected *ex vivo* from day 5 to day 7 and cultured *in vitro*. As a result, it has been hypothesised that the uterine environment may be necessary for both ZP thinning and capsule formation in the horse. With IVC, the zona remains rigid and prevents the normal expansion of the blastocyst, causing the ZP to rupture or hatching to occur through the Piezo drill hole. However, once they are free of the ZP, the blastocysts will usually expand *in vitro* without apparent long-term effects (Choi *et al.*, 2004b).

2.3.4.1.2: Culture medium composition

The composition of the optimal culture medium for IVM and IVC of equine embryos has been extensively researched. Choi *et al. (*2004b) observed from their experiment of different commercial mediums, that equine embryos thrive in media that are reportedly detrimental to other species, like those containing high glucose and phosphate concentrations. This indicates that the requirements for early embryonic development in equine differs significantly from that of bovine and human embryos. By culturing bovine zygotes in the most equine-suitable media tested, none of the embryos developed past day 4, supporting the above hypothesis.

Various studies conducted on culture medium have failed to observe any effect on the nuclear maturation, cleavage rate, or developmental competence of ICSI produced embryos by medium supplementation with the likes of epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and/or hormones. However, glucose has been described as one of the most influential components of equine embryo culture medium. Choi *et al.* (2004a) cites Azuma *et al.* (1995) as the only previous report published on the effect of glucose concentration on early embryonic development in the horse, which concluded that morula development by day 8 was highest when the glucose concentration was kept low for the first 4 days and then increased. In their study, Choi *et al.* (2004a) observed that embryo development after ICSI was improved by a high concentration of glucose (up to 12mM in compact cumulus oocytes) in the media, exhibiting notably higher cleavage rates and nucleus formation. Conversely, studies in other species observed a higher early embryonic development in low concentrations of glucose only, warranting further exploration into the beneficial glucose effect seen on equine embryonic development in this study.

2.3.4.2: The fertilising sperm

As outlined in a previous section, sperm selection is an important influential factor on the outcome of assisted reproduction technologies. In natural conditions, the female reproductive tract forms a hostile labyrinth environment, that reduces the original millions of spermatozoa to a fractional subpopulation by the point of fertilisation in the oviduct. This is presumably a physiological method of selecting spermatozoa with the highest fertilisation capability. Since sperm selection methods are far less drastic in ARTs, this has been suggested as a major factor for their low embryo production efficiency. This is especially relevant in the case of ICSI and IVF, which circumvent any physiological *in vivo* sperm

selection methods; ICSI in particular bypasses the very last ZP barrier the sperm meets in fertilisation, resulting in an inevitable increased risk of fertilisation with defective spermatozoa, and subsequent failure in embryo production. Therefore, in theory, IVF should be the first choice, but this is not always the case in humans nowadays, and certainly not a viable option for equine ART (Oseguera-López *et al.*, 2019).

Furthermore, while ICSI can be performed with fresh, cooled, or frozen stallion semen, studies have found that fresh semen requires additional chemical activation of the oocyte to achieve acceptable pronuclei formation rates. Conversely, frozen semen does not appear to need exogenous oocyte activation, and it is possible that the freezing process itself may affect the sperm membrane in a way that is later beneficial for the diffusion of the 'sperm factor' into the oocyte cytoplasm that triggers activation. A study by Lazzari *et al.* (2002) used frozen semen with different degrees of fertility and/or motility to assess the impact these factors had on developmental competence of embryos after ICSI. Interestingly, no difference was detected in cleavage rates or embryonic development between oocytes injected with sperm of good, poor or zero fertility status, so long as they all showed some motility capacity. Conversely, non-motile spermatozoa failed to fertilise the oocytes sufficiently, showing markedly low cleavage rates. In the non-motile and low fertility sperm group of this study, no embryos were capable of developing to blastocyst stage. Consequently, it follows that a potential issue with the sperm selection methods used for ICSI in the horse is that they are exclusively based on sperm motility, but not all motile sperm are of efficient quality. The use of centrifugation in the applied methods has also been linked to ROS generation that has a detrimental effect on the sperm quality. Therefore, the development of new sperm selection methods may be required in the future that focus on other sperm characteristics better correlated with the capacity for fertilisation and support of embryonic development (Oseguera-López *et al.*, 2019).

Conversely, Choi *et al*. (2011) argued that spermatozoa do not necessarily need to be motile for fertilisation with ICSI, and as a result, lyophilised (freeze-dried) sperm could potentially be used. To the knowledge of Choi *et al.* (2011), no research had been done previously on the use of lyophilised sperm in equine ICSI programs, and the aim of their study was to assess the fertilisation success and subsequent pregnancy rates achieved using lyophilised spermatozoa, in conjunction with sperm cytosolic extract injection applied to initiate oocyte activation. The sperm cytoplasmic extract used contains the 'sperm factor', i.e. PLC_{ζ}, needed to induce the calcium oscillations in the oocyte for activation The stallion sperm appeared to be resistant to any DNA fragmentation during lyophilisation, and the experiment reported

successful blastocyst formation, normal gestation, and birth of healthy foals; these are believed to be the first offspring of non-laboratory animal species to be born from this method. The sperm extract is used for oocyte activation by this laboratory as standard after nuclear transfer in cloning procedures, and it proved to be an essential element in this experiment; oocytes injected with lyophilised sperm failed to develop into blastocysts without the sperm factor from the extract. Unlike other species, equine oocytes are not widely capable of parthenogenic activation, and therefore ICSI-associated manipulations alone are not enough to trigger spontaneous activation (Choi *et al*., 2011).

This experiment yielded promising results that suggest lyophilisation may become a future alternative to standard cryopreservation of stallion spermatozoa, however, further research is needed to widen the understanding of aspects of this technique. If it proves viable, this technique would negate the need for liquid nitrogen sperm storage, and the sperm could be packaged with only a few sperm cells per dose, allowing a significant number of aliquots to be produced from a single ejaculate. In theory, these lyophilised aliquots could also be prepared from already frozen straws of stallions that are in finite supply, but the effect of freeze drying already frozen sperm is yet undetermined. In addition, storage of lyophilised sperm in this experiment suggested a resistance in equine sperm nuclei to damage, and similar embryo production was observed in freshly lyophilised sperm and those that were stored for 1.5 years (Choi *et al*., 2011).

2.3.4.3: Factors directly affecting developmental competence of oocytes

2.3.4.3.1: Oocyte source

While the majority of ICSI procedures are now performed on oocytes recovered from live mares using transvaginal OPU, a small percentage are still sourced through post-mortem collection from deceased/euthanised mares. As such, it remains an important question on whether the origin of the oocytes has a significant influence on the outcome of the ICSI embryo production. Lewis *et al.* (2016) carried out a study to determine the difference between OPU and abattoir-sourced oocytes on maturation, cleavage, and blastocyst rates following ICSI. Using the same IVM system for both oocyte groups, the nuclear maturation rates of the abattoir oocytes was 39%, compared to 67% for OPU derived oocytes. The maturation rate of the abattoir oocytes was not significantly affected by transport time (from 2.25 hours up to 10 hours) in this study.

Similar findings were reported by Guignot *et al.* (1999) with time ranging 1.5 hours to 8 hours; however, Hinrichs *et al.* (2014) reported that maturation rates were highest when the oocytes were collected within 1 hour of slaughter, suggesting that rapid processing may avoid changes in meiotic competence of these oocytes. It is worth noting that other factors are involved in the developmental competence of oocytes sourced post-mortem, including transport temperature, the health status and age of the mare when she perished, and possible exposure of the oocytes to toxic compounds at the abattoir itself. Various studies have been done on the transport temperature factor, concluding that the ovaries can be kept at 30-37°C if they are processed within 1 hour, but for longer periods, 22-24°C (room temperature) resulted in the highest developmental competence. Considering the many factors involved, it is easy to see why oocytes collected from live donors via OPU have a higher developmental competence than those collected post-mortem (Lewis *et al.*, 2016).

However, it is important to remember that oocytes recovered from live donors through OPU often have to undergo overnight transport to central ICSI facilities before they can be fertilised. A study by Galli *et al.* (2016) compared IVEP rates and pregnancy rates after ET of oocytes collected on-site *versus* oocytes from remote locations shipped overnight to their laboratory. Oocytes recovered on-site were placed in an SOF medium for immediate maturation, whereas oocytes shipped overnight were kept in tubes of SOF at 20-22°C for delayed maturation; these were processed in the same way as the on-site oocytes once they arrived at the facility. After IVM for 26-28 hours, the oocytes were denuded of cumulus cells and those that achieved metaphase II status were selected and Piezo-driven ICSI performed. Afterwards, the injected oocytes were cultured *in vitro* for a further six days before replacing half the medium, and then allowed to culture further.

Embryos that formed blastocysts between day 6 and day 8 of IVC were cryopreserved using 10% glycerol and a slow-freezing protocol. These were later thawed and transferred transcervically into recipient mares on day 5 post-ovulation. The results of this experiment showed a higher percentage of degeneration in the remote oocytes shipped to the laboratory, compared to the group recovered on-site. However, the shipped oocytes that did mature to metaphase II stage showed a high developmental competence. After ICSI, both groups had similar rates of embryo production per OPU and reached pregnancy rates of >60% following thawing and ET. From this, Galli *et al.* (2016) concluded that, although oocyte degeneration occurred in transit, the remote collection and shipping at room temperature before IVM can still be considered a viable commercial option for breeders and equine clinics that are not equipped for such a specialised ART.

2.3.4.3.2: Oocyte morphology: expanded vs compact cumulus

Successful *in vitro* maturation (IVM) is a well-established procedure in domestic species like ruminants and swine, with over 90% of oocytes progressing to metaphase II after IVM. However, the rate of metaphase II achieved through IVM of equine oocytes is still only 40-70%, and highly variable within and between laboratories. Much of this variation can be explained through the oocyte selection process for *in vitro* culturing. When oocytes <20mm are aspirated, the resulting oocytes show a lower meiotic competence than those aspirated from follicles in early atresia. Follicles that have begun the atretic process appear to allow meiotic competence to develop in the enclosed oocyte, while oocytes of viable follicles, even of a similar size, remain in meiotic arrest; once they are released from the follicle and placed in *in vitro* culture medium, meiosis will resume spontaneously in the majority of these oocytes (Dell'Aquila *et al.*, 2003).

Research in other species have observed apoptosis in granulosa cells of the follicle, a process that has been highlighted as a potential molecular mechanism of follicular atresia in mammals. Few studies have been done on follicular atresia in horses, or on the connection between granulosa cell apoptosis and oocyte maturation and developmental competence. One study sought to define this connection, using DNA laddering analysis with agarose gel electrophoresis to evaluate the degree of apoptosis in follicles in different atretic stages. The study found that significantly higher numbers of expanded cumulus (Ex) than compact cumulus (Cp) oocytes originated from follicles in advanced stages of atresia; and that the oocytes from follicles in advanced apoptosis had a much higher incidence of metaphase II being reached after IVC, than those from non-apoptotic follicles. It follows, then, that granulosa cell apoptosis can be closely correlated with cumulus expansion, since Ex oocytes are associated with atretic follicles, and therefore, granulosa cell apoptosis can be connected to meiotic competence as a result (Dell'Aquila *et al.*, 2003).

The COC is the final part of the follicle to be affected by atretic processes. Expanded cumulus morphology is widely accepted as associated with atretic measures and, while not an entirely accurate system, this may be used as a selection tool for oocytes of higher competence (Dell'Aquila *et al.*, 2003). However, as is done in many species, some laboratories have chosen to use only Cp cumuli oocytes for their experiments, believing Ex oocytes to have lower developmental competence due to this atretic follicular origin; showing pre-maturational changes such as chromatin condensation as a result (Choi *et al.*, 2004a). To clarify the situation in horses, various studies were conducted that concluded that

Ex oocytes appear to mature in similar, or even greater proportions, than Cp oocytes, and have further demonstrated a more rapid maturation rate and readiness to activate; likely due to being "aged" in comparison to Cp oocytes. In addition, Ex oocytes had a markedly higher rate of male pronucleus formation after ICSI than the Cp oocytes (Dell'Aquila *et al.*, 2003; Choi *et al.*, 2004a).

In one such study, the Ex oocytes were later transferred to recipient mares and 85% cleaved and developed to 16-cell stage within 96 hours of ET, which can be considered a similar rate achieved in normal *in vivo* developed embryos (Dell'Aquila *et al.*, 2003). It is also widely accepted that Cp oocytes need a longer *in vitro* maturation period to reach metaphase II of meiosis than Ex oocytes; 30 hours *vs*. 24 hours, respectively (Choi *et al.*, 2004a). The disagreement among some laboratories as to the correlation between meiotic competence and cumulus morphology is likely the result of subjective morphological analysis under low magnification and is therefore dependent upon the individual operators. As atresia and apoptosis are more objective measures, they can be considered as stronger indicators of meiotic competence; however, further research is needed into the effect of granulosa cell apoptosis on the embryonic and foetal development after ICSI in the mare (Dell'Aquila *et al.*, 2003).

2.3.4.3.3: Donor mare influence

The failure of cleavage after ICSI fertilisation is likely multifactorial and has not been adequately defined yet in equine studies. As this technique is primarily applied in aged, sub-fertile mares, and stallions with poor sperm quality or limited supply, it follows that lower embryo production rates are achieved as a result. In various species, maternal aging causes a decline in reproductive capacity, due to age-associated alterations like mitochondrial changes and oxidative stress. It is widely accepted that fertility declines with increasing age in mares, and ages >20 years are associated with a high prevalence of early embryonic loss in all reproductive technologies. The primary issue in this case is a decrease in the quality of the oocyte and therefore the developmental competence, with recent studies highlighting a frequent misalignment in the chromosomes of metaphase II in aged oocytes. As outlined in a previous section, nuclear and cytoskeleton rearrangements are important elements of oocyte activation and spindle organisation after fertilisation, with microtubule and chromatin remodelling necessary for genomic union and early embryonic development. The microtubules can be generated by the oocyte independently in the horse and helps assemble maternal chromosomes, activate the oocyte, and reposition the two pronuclei. If aging of

these oocytes causes changes in the actin and tubulin patterns, this could be an important adverse factor on the fertilisation rates in these mares (Ruggeri *et al.*, 2015).

Ruggeri *et al.* (2015) conducted a study with the aim of clarifying senescence-related changes that occur in aged oocytes, connected to decreased development and fertilisation rates after ICSI. They hypothesise that changes occur in the cytoskeleton due to aging of the oocytes *in vitro*, and that it is affected by the age of the donor mare. More precisely, they hoped to (1) identify specific cellular alterations that occur *in vitro*; (2) determine the potential cause of the developmental failure post-ICSI; (3) establish connecting factors between oocyte aging and cleavage failure; and (4) compare oocytes from young and old mares that failed to cleave, to identify any cytoskeleton and chromosomal changes. Overall, the ICSI zygotes that did not cleave demonstrated an abnormal organisation of the microtubules and defective centrosome reconstruction, that was responsible for meiotic arrest and the failure of embryonic development. Cellular aging happens once metaphase II is reached, and this study observed cellular changes in the oocytes cultured *in vitro* for up to 48 hours after ICSI; in particular, an increase in microtubule foci that originated from the fragmentation of defective meiotic spindles were recorded. Any such instability can lead to fragmentation in the spindle and defective centrosome reformation (Ruggeri *et al.*, 2015).

Furthermore, actin vesicles were observed in the oocytes from older mares after fertilisation via ICSI. It is suggested that these components could indicate a deleterious process within these oocytes. While the exact role of actin vesicles in equine zygote development is not known, there was a marked increase in their presence in oocytes from older mares compared to the oocytes from younger mares. It can be considered a sign of remodelling within the cytoskeleton, to compensate for the oocyte's inability to organise the cytoskeleton and chromosomes before and after ICSI is performed. The elevated presence in older mare oocytes may indicate a suboptimal environment within the cytoplasm for zygote-, and later embryo development, due to their advanced age, triggering a cascade of events that results in failure of these processes (Ruggeri *et al.*, 2015).

4.4.3.5: Temperature of ICSI procedure

Early experiments in mice ICSI found no difference in embryo survival rates when the ICSI was performed at low temperatures and at room temperature. Some researchers use a heated stage in conventional equine ICSI programs, but despite the suspicion that temperature may affect the integrity of the meiotic spindle, no prior studies had been done on the effect of temperature on equine oocytes during ICSI. In humans, the ICSI process is extremely

temperature sensitive, with even minor temperature changes outside of 37.8°C during manipulation causing damage to the spindle microtubules and decreasing fertilisation and pregnancy rates. In a study by Choi *et al.* (2004a), no significant difference was found in equine oocyte survival rate and embryo development between low and high temperatures; however, it must be noted that if any chromosomal anomalies did occur from microtubule damage, these may not be seen until further stages in development that were not realised in the present study.

2.3.4.4: Recipient mare management

Claes *et al.* (2018) conducted a study to evaluate the factors affecting embryonic loss and pregnancy rates after ET of cryopreserved ICSI embryos. The factors taken into consideration included: the age and reproductive history of the donor mare; the age, reproductive status and management of the recipient mare; the time required for the ICSI embryo to reach blastocyst stage; and the season, year and method of ET used. From the data recorded, it appears that donor mare fertility, and the day of ET post-ovulation in the recipient, are the most likely to impact pregnancy rates. Embryos from known infertile donor mares were much less likely to result in pregnancy, with oocyte quality as the probable background factor. Furthermore, pregnancy rates were lower in recipients who were transferred embryos on day 6 post-ovulation, indicating an asynchrony of uterine environment and embryo stage of development. It is generally accepted in conventional ET programs that the recipient's ovulation window is between one day before and four days after the donor mare. From this study, it appears that for *in vitro* produced embryos, a less advanced uterine environment is more compatible for development; as Claes *et al.* (2018) found that most ICSI embryos were only at an equivalent stage of 5- or 6-day old embryos at the time of their transfer, a clear developmental delay when compared to *in vivo* produced embryos. It can therefore be concluded that ICSI embryos are best transferred to recipients on day 4 or day 5 post-ovulation to provide the best environment for development and therefore chance for pregnancy.

2.3.4.5: ICSI method used

Early studies on mouse oocytes reported a significantly higher survival rate in the Piezo method *versus* conventional ICSI (80% *vs*. 16%, respectively), and higher blastocyst formation rates overall with the Piezo method. Furthermore, it has been shown that the Piezo drill causes less damage to the oolemma and oocyte cytoplasm, as well as more rapid sperm

plasma membrane dissolution (Salgado *et al.*, 2018). A study in 2018 was conducted to compare the formation of blastocysts between the conventional and Piezo ICSI methods, observing significant differences in sperm structure remodelling and the morphokinetics of oocyte activation. The conventional method showed a delay in oocyte activation that may be the result of the manner of sperm immobilisation in this technique; a more limited disruption in the plasma membrane may cause a slower release of sperm factors necessary for oocyte activation. With regards to sperm chromatin processing, differences were also observed between the two methods, and progression of metaphase II by oocytes was notably slower in the conventional ICSI group. A comparison of blastocyst development between methods revealed that conventionally fertilised embryos had a lower nucleus number, higher nuclear fragmentation, and delayed blastocyst formation compared to the Piezo method. This study is one of the first to consider nuclear number and fragmentation as measurements of zygote quality, and through another experiment, these researchers demonstrated a link between the percentage of fragmented nuclei and proportion of apoptotic nuclei (Salgado *et al.*, 2018).

This study also found that the Piezo drill method had no impact on the acrosome of sperm, but still achieved 40% blastocyst formation rates in these zygotes, therefore concluding that, in the horse, it is not necessary to remove the acrosome in order for embryonic development to occur. However, it was observed that when the acrosome was shed into the cytoplasm post-ICSI, it did so almost fully intact and visibly affected the sperm chromatin decondensation in some oocytes. It follows that this could affect the synchrony of sperm chromatin processing and therefore affect embryo quality. This study found that the above phenomenon was more likely to occur in conventional ICSI *versus* Piezo methods (Salgado *et al.*, 2018).

Overall, Salgado *et al.* (2018) noted significant differences between the two ICSI methods. The conventional method showed a delay in sperm chromatin decondensation, a decreased acrosomal loss, and a delay in oocyte meiotic resumption. The delay in activation appears to be due to the less effective manual immobilisation of the sperm, with this delay being connected to lower embryo quality at blastocyst stage, despite similar rates being achieved in the two methods. It must be noted, however, that this study was conducted in horses already established as fertile, and therefore, such results may not be reproducible in clinical practice with sub-fertile animals. Ultimately, the study concluded that the Piezo drill appears to be the more efficient method to improve embryo quality in the horse. Since mares can be considered as good models for studying human follicular and oocyte biology, due to their

similarity to women in follicle development, oocyte maturation dynamics and pattern of reproductive aging, the results of this study may be beneficial enough to human ICSI technology to warrant further research (Salgado *et al.*, 2018).

2.3.5: Effect of ICSI on offspring

One of the most important factors for the success of ICSI as a commercial enterprise in equine breeding, is the quality of the offspring produced. In several species, the use of assisted reproduction techniques has been associated with a higher risk of perinatal abnormalities. In humans, there have been abnormalities of birthweight reported in single children born through ART methods, as well as an increased risk of placental complications and pre-eclampsia in the mothers. Similar findings have been reported in cattle after IVEP, with placental anomalies and large offspring syndrome (LOS) particularly prevalent. LOS in sheep and cattle has been associated with prolonged gestation periods, increased birth weights, and notable changes in placental weight area and gene expression patterns after ART in these species. While foals produced via nuclear transfer have been reported as having umbilical and forelimb abnormalities, no foals born through ICSI have been reported with LOS to date; however, little data has been collected and examined on neonatal foal morphometrics born through OPU/ICSI/IVC. Consequently, a study was conducted on a group of American Quarter horses to examine differences in gestation length, neonatal morphometrics and placental morphology between foals born through natural means, ET and ICSI techniques (Valenzuela *et al.*, 2018).

In this study, the ICSI oocytes were obtained via OPU and cultured in M199 medium with 10% foetal bovine serum (FBS). After ICSI was performed, the zygotes were cultured in Dulbecco's modified Eagle's medium with 10% FBS, or in Global medium, a commercial human embryo culture medium, supplemented with glucose. Embryos selected for transfer were those that were observed as forming blastocysts on day 7-10 post-ICSI. Of the mares that had a normal foaling, all placentas were examined within 6.5 hours of birth, and the foals weighed and measured within the first 8 hours of delivery. Data from any irregular foalings, for example, in retained foetal membranes (RFM) cases, were excluded from the study. The resulting data recorded no significant difference in gestation lengths between the different technique groups, or in any placental measurements taken. The foals were born without any obvious abnormalities and were all similar in birth weights and morphometric values, apart from the length of the upper hind limb. This factor was measured as the distance between the tuber coxae point of the buttocks to the cranial surface of the patella, which

proved significantly higher in ET and ICSI foals than those conceived and carried naturally. It is possible that the recipient mares chosen to carry the embryos were larger than the donor mare and sire used, resulting in positive effects on neonatal size. Previous studies in ET programmes have found that recipient mare height and parity can be connected to foal height, and therefore, are likely to be contributing factors in the present study. In other species, there have been reports of marked effects of ART on placental gene expression, however, this study recorded no such differences in the expression of genes related to angiogenesis, growth and nutrient transport, or metabolism (Valenzuela *et al.*, 2018).

In cattle and sheep, LOS has been linked to the use of serum supplementation in *in vitro* culture media, and since then, the use of serum has been largely avoided in IVEP of these species. Yet, no evidence of LOS was observed in this study, despite the addition of 10% FBS to the culture media during IVC at both oocyte and embryo stages. It is worth noting that, in general, excessively large foals are a rare occurrence in equine. The mare appears to regulate the size of the foetus she carries, a phenomenon clearly demonstrated in experiments where large-breed equine embryos were transferred into small-breed recipient mares; the resulting foals were born in relative proportional size to the recipient pony mothers. Ultimately, this study concluded that no significant adverse changes can be detected in connection with the use of ET and ICSI methods in equine breeding, and they remain a viable choice as clinical tools in the reproduction of sport horses (Valenzuela *et al.*, 2018).

3. CONCLUSION

The sphere of equine breeding has expanded over the last few decades to include many assisted reproductive technologies; what began with artificial insemination has progressed to the *en masse* production of embryos *in vitro*. While the limited availability of oocytes has kept the horse at the back of the ART race, the development of transvaginal OPU in this species has allowed for the expansion of the reproduction field to include sophisticated techniques like ICSI; procedures made all the more essential by the failures of conventional IVF to produce viable equine embryos. Improvements in the efficiency of IVEP is continuous and remains a primary focus in the advancement of all equine ARTs. Since its adaptation from cattle to horse breeding, transvaginal OPU technology has paved the way for advancements in IVEP and ICSI production of viable equine offspring. The ultrasound-guided approach has provided a minimally invasive and safe procedure, that can be repeated in mares all year round, without detrimental effects on fertility or general health status. It negates the need for hormonal stimulation and circumvents the physiological inability of superovulation in the horse to produce numerous oocytes per reproductive cycle. The unique histological nature of the equine ovary initially proved challenging, and as such, many modifications were made to the original system designed in cattle. Additional improvements, such as needle guiding systems, have since allowed the procedure to emerge from the specialised laboratory scene to the sphere of general equine practitioners; an important step in allowing for the expansion of IVEP to a wider breeder audience. Many biological and technical factors govern the success of OPU oocyte recovery, and particular emphasis is placed on the difference between collection of immature and dominant oocytes protocols. The maturation level of the oocyte within the dominant follicle dictates a precise timing of ICSI that must be adhered to for embryo production success. Conversely, immature follicle oocytes are in meiotic arrest and can be kept in culture for much longer periods of time until ICSI is performed, allowing for a looser protocol schedule. Their presence in higher numbers ultimately yields a higher rate of total blastocyst development than dominant follicle puncture, and they have the additional advantage of being present outside the breeding season for collection.

Since the introduction of IVF into the reproduction sphere in the 1970s, it has become an integral part of domestic animal obstetrics; especially in bovine, where it has emerged as a keystone of commercial dairy cattle IVEP. It has furthermore provided an essential foundation for the development and improvement of IVM/IVC media and conditions.

Adhering to the same pattern of adapting a bovine technique to the application in horses, many attempts were made to establish a reliable IVF protocol in equine over the last few decades; with a disappointing total of just two foals born though this ART to date. The exact reasons for the shortfalls in this process have been hypothesised by many researchers over the years, yet they remain elusive for the most part, and embryo production rates have varied widely between, and even within, laboratories. It was initially presumed to be an issue with the oocyte's zona pellucida hardening *in vitro* that prevented the penetration of the sperm, however, manual disrupting of the ZP resulted in increased polyspermy and ultimately failed to achieve improved embryo production rates. Suspicion then fell on the failure of the sperm to capacitate under *in vitro* conditions; by initiating capacitation using calcium ionophores, some oocytes could be fertilised, but the overall blastocyst number remained low. By comparing the blastocyst formation rates of *in vivo*- and *in vitro* fertilised IVM oocytes, it was clear that there is some step occurring in the oviduct that was missing from the IVF protocol.

The use of oviductal cell models in IVC in other species has proven highly beneficial for embryo production, and while some improvement was observed in equine IVF rates, it was not on a comparable level to that seen in other species. Extensive research into oviductal microenvironment, questioned the role that the oviduct-sperm binding and sperm reservoir has on sperm capacitation, and therefore fertilisation in the horse; with some studies suggesting sperm binding was not obligatory for either process *in vivo*. In other species, it is widely accepted that oviductal fluid contains essential proteins and factors necessary for fertilisation; some of these have been identified and successfully applied to improve IVEP rates in these species. However, when various experiments were conducted to establish similar proteins in the horse, they yielded disappointing results, and these key influential factors have yet to be identified in equine oviductal fluid. Furthermore, follicular fluid has been established as containing factors influential in sperm capacitation in humans and other animals, but the equivalent element remains elusive in the horse.

Overall, it is clear that many elements of the *in vivo* fertilisation mechanism in the horse are still unknown and appear unique to this species. Exploring the shortfalls of IVF elucidates the still limited understanding of many *in vivo* mechanisms in the horse, and the necessity of discovering these unknowns to improve all techniques for IVEP. Ultimately, further research is needed into these various unidentified factors if an efficient IVF protocol is to be successfully established in the horse. Recent studies into the epigenetics of ARTs, suggest

that IVF may have a physiological relevance over ICSI technology and therefore, research will continue to try develop IVF as a commercially viable technique in equine.

Despite reports of ICSI use in other animals, it has only been commercially implemented in equine breeding for a number of reasons; primarily the failure of IVF in the horse, and the highly specialised nature and expense of ICSI failing to achieve economically viable results in other species – most notably in bovine, where attempts to establish a standard protocol have been futile. For humans, ICSI has become the first choice ART procedure, even over IVF nowadays, and its development in the human medicine field paved the way for its rapid adaptation into equine breeding; with the same equipment and even same pipette sizes suitable in both cases. Thanks to improvements made in IVEP and the development of OPU, ICSI is achieving acceptable embryo and pregnancy results in current commercial equine programmes, making the ART an increasingly popular option. The many advantages of ICSI, such as embryo production year-round, the ability to cryopreserve said embryos for later use, and maximising the use of limited or valuable semen straws, further strengthen its significance as a commercial ART option. However, it is highly specialised and therefore limited to central facilities that oocytes must be shipped to under a strict transport protocols; all elements that result in ICSI being a very expensive ART for everyday breeders, and ultimately limiting the procedure to use within a small field of highly valuable horses.

Initially, ICSI was deemed controversial, as it essentially bypasses significant physiological selection processes of fertilisation, whereby the most suitable sperm was selected out by mechanisms in the oviduct to fertilise the oocyte. As a result, the method of sperm selection used *in vitro* has become an important area of focus in ICSI procedures, with swim-up and density gradient methods being the primary choice in animal *in vitro* fertilisation techniques. Extensive research has been done into the nature of oocyte activation and mechanisms of nuclear and cytoplasmic remodelling in oocytes after ICSI fertilisation. It is clear from the various experiments, that a 'sperm factor' is necessary for oocyte activation and early embryonic development, and that aspects of this mechanism have yet to be fully understood in equine. It remains plausible that some step occurs under *in vivo* conditions, that is required for correct oocyte activation and/or sperm factor processing, that is still missing from ICSI protocols and is responsible for the failure of these mechanisms in the fertilised oocytes.

Other factors play important roles in the success of ICSI, namely the quality and morphology of oocytes used, the IVM/IVM conditions, and the ICSI method performed. To date, two different ICSI methods have been developed – conventional ICSI, and the more recently developed Piezo-driven ICSI. Studies have concluded that the Piezo method is more efficient

for production of good quality embryos in equine. In addition, donor and recipient mare management are significant factors for this ART. Donor mare age in particular plays an important role in the success of embryo production; the main limiting factor for ICSI is oocyte quality – if this is the source of infertility in the mare, then ICSI is not a solution in this case. It is widely accepted that oocyte quality declines as mare age increases, and, as this technique is employed mostly in older sub-fertile mares, this factor is an important consideration for choosing ICSI. Furthermore, studies on the blastocysts produced by ICSI have revealed a delay in their maturation compared to *in vivo* produced embryos, and as such, the day of implantation into recipient mares is of chief concern. As they are developmentally behind *in vivo* produced embryos, ICSI embryos should be transferred to recipients on day 4 or day 5 post-ovulation specifically, to allow for the best chance of pregnancy. Even if performed precisely, ICSI still has significant rates of early embryonic loss that exceeds those recorded in conventional flushed embryos, and more research is necessary into the reasons for this embryonic loss.

Overall, based on embryo production and pregnancy rates achieved, ICSI represents a viable alternative for conventional ET in the horse, even sparking the emergence of a new niche market for the sale of cryopreserved embryos. Its growing commercial popularity and production of high-quality offspring ultimately ensures that it will continue to advance in both efficiency and availability in the coming years. While research into developing IVF continues in the horse, it is questionable whether such a programme will be able to compete with the successes of ICSI and conventional ET already established.

4. SUMMARY

Assisted reproduction technologies have become the principle focus of maximising the efficiency and quality of the production of many domestic animals. Advancements over the previous century have been monumental in establishing the many techniques available to commercial breeders today. In equine reproduction, the development of a transvaginal OPU procedure opened the door into IVEP in this species, which has been heavily limited previously, when compared to other animals. This thesis reviews the literature of many researchers regarding the techniques of OPU, IVF and ICSI; focusing on the history and development of these methods in other species, their adaptation to equine reproduction, and ultimately the emergence of OPU-ICSI in the wake of the failure to establish a successful IVF protocol in the horse. Exploring the shortfalls of IVF elucidates the still limited understanding of many *in vivo* fertilisation mechanisms in the horse, and the necessity of discovering these unknowns to improve all techniques for IVEP.

By looking at the OPU, IVF and ICSI processes in detail, influential factors emerge that affect the success of each procedure, and these can be analysed to further improve ARTs in the future. The continuous evolution of equine ARTs have ensured their position as a keystone of modern sport horse breeding; not only offering the opportunity to preserve valuable genetics, and generate offspring from elite individuals who cannot reproduce through natural means, but also expanding to become a convenient method of breeding normally fertile horse year-round. OPU-ICSI programmes offer a way to circumvent many physiological limitations in the horse, to maximise embryo/offspring production and bypass many fertility issues in both mares and stallions. Since the introduction of each advanced technique, the breeding of sport horses has markedly moved away from traditional breeding management, culminating in the emergence of a niche market for ICSI produced cryopreserved embryos. While *in vivo* fertilisation rates cannot be equally matched, the efficiency of equine IVEP is constantly improving, and its growing popularity ensures its continued development and advancement in the future.

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