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MATURATION NUCLÉAIRE ET CYTOPLASMIQUE DE L'OVOCYTE ÉQUIN: INFLUENCE DE LA CYSTEAMINE

IN VITRO NUCLEAR AND CYTOPLASMIC MATURATION OF THE EQUINE OOCYTE: INFLUENCE OF CYSTEAMINE

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To my parents

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PART 1 : INTRODUCTION

Part 1 : Introduction

1. Collection, evaluation and maturation of equine oocytes

Assisted Reproductive Technologies (ARTs) for the horse developed steadily over the last two decades. These techniques range from simple procedures such as artificial insemination to the complex production of clones. These technologies now enable salvation of otherwise lost genetics from either subfertile or even dead animals.

Further use and development of these techniques highly depends on availability of oocytes and their manipulation. These embryotechnologies were first developed in the bovine. Thanks to the virtually unlimited availability of bovine abattoir oocytes, research rapidly led to efficacious *in vitro* maturation (IVM) of oocytes and their subsequent *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of the early embryos produced, allowing *in vitro* embryo production.

The limited interest of the horse industry for these technologies as well as the scarcity of abattoir oocytes available for research and the technical difficulties for their recovery from follicles have seriously impeded development of ARTs in this species. This section will focus on the clinical aspects of the oocyte recovery, their assessment and their use in assisted reproduction in the mare.

This introductory chapter reviews the state-of-the-art in the different techniques using oocytes for in vitro horse embryo production.

1.1. Oocyte collection from abattoir ovaries (*ex vivo*)

1.1.1 Handling and transportation of ovaries

No study directly compares techniques, media and transportation conditions of ovaries from abattoirs to the laboratories. Media used for this purpose are crystalloids supplemented with antibiotics, phosphate buffered solution (PBS), a complete medium (TCM-199) or commercial embryo flush solutions (Hinrichs *et al.*, 1993; Alm and Torner, 1994; Del Campo *et al.*, 1995; Franz *et al.*, 2003; Preis *et al.*, 2004).

Storage periods for shipment ranging from 1 to 24 hours have been studied and it has been shown that storage up to 15 hours does not affect oocyte meiotic competence (Shabpareh *et al.*, 1993; Del Campo *et al.*, 1995; Guignot *et al.*, 1999). However, high maturation rates reported by labs where transportation duration was minimal, suggest that storage time for shipment could influence oocytes quality and their subsequent resuming of meiosis (Love *et al.*, 2003; Hinrichs *et al.*, 2005).

Transportation temperature has also been investigated. Based on a morphological study of the cumulus and the apoptosis of its cells, it has been suggested that ovaries transported between 20°C - 30°C and 35°C - 37°C, should ideally be processed within 3 hours and 2 hours respectively (Pedersen *et al.*, 2004). Another study showed that maturation rates (Metaphase I+II) after 24 hours were higher for oocytes collected immediately than for those retrieved after further storage of the ovaries for 15 to 18 hours at 4°C or at room temperature (Love *et al.*, 2003). This study also suggests that 4°C is probably too low a temperature for an optimal storage of ovaries except when delay between ovaries collection and oocytes retrieval cannot be avoided.

The optimal solution might be found in harvesting oocytes at the slaughterhouse (Love *et al.*, 2003). The oocytes can then be placed under maturation conditions in a portable incubator and undergo IVM during their transport to the lab. Due to its technical difficulties (collection facilities in the abattoir, transportation under controlled temperature and atmosphere), this method is not widely spread but could potentially optimize maturation rates for abattoir oocytes (Caillaud *et al.*, 2008).

1.1.2 Oocyte collection from excised ovaries (ex vivo)

Oocyte harvesting can be technically challenging in the equine. In order to preserve oocytes quality, each step requires a constant concern for conditions of hygiene, temperature, pH and medium quality. Oocytes can be aspirated (Desjardins *et al.*, 1985; Shabpareh *et al.*, 1993) with a 18G needle and an aspiration system (+/- 1 bar) or a vacuum pump (100-150mm Hg). The ovary can be sliced to gain access to more follicles. The equine oocyte is tightly fixed on a short, broad-based cumulus hillock with cumulus cell expansions into the underlying thecal pad (Hawley *et al.*, 1995). They are therefore difficult to detach from the follicular wall. Consequently, recovering of equine oocytes requires incision of the follicles, scraping of the follicle wall with curette and flushing to detach the oocyte and its surrounding cells. Highest collection rates, approximating 80%, have been achieved using this technique (Del Campo *et al.*, 1995). Most of the oocytes collected by this technique maintain cells immediately surrounding the oocyte intact and are imbedded in sheets of granulosa cells, forming Oocyte-Cumulus Complexes (COCs) (Hinrichs *et al.*, 1993). Conversely, aspirated oocytes often only maintain immediately surrounding cells (Hinrichs, 1991). The ovary is then sliced in small pieces to gain access to the follicles deeper in the ovarian stroma. Alternatively, the follicles can be aspirated and scraped simultaneously using the tip of an injection needle. Despite the scraping and the flushing, oocyte recovery remains rather low (3-5 oocytes/ovary), as average number of antral follicles present on an equine ovary is 6 (Hinrichs, 1991; Del Campo *et al.*, 1995). This also reflects in a ten-fold increase in required time and personnel for harvesting of equine versus bovine oocytes (Galli *et al.*, 2007). All these technical difficulties and the scarce availability of abattoir ovaries represent major limitations for fundamental research on equine oocyte and consequently for all the technologies that depend on its success.

1.2. Oocyte collection by ovum pick up (*in vivo*)

Ovaries recovered from slaughterhouses provide a material with an obvious lack of repeatability, delay in time between collection and placement in culture medium and lack of information about the stage of cycle and follicular growth and the age of the donor mare.

Embryo technologies in species such as cattle and pigs rapidly developed to the point of commercial application. By contrast, their development and application in the equine is mainly only accessible to a minority of performing horses. Consequently, abattoir derived oocytes are almost exclusively of interest for research purposes.

Most oocytes that are transferred for clinical purposes are collected *in vivo* from preovulatory follicles. Oocytes collections are scheduled based on the induction of the ovulation. This can be achieved by administration of hCG or a GnRH analog to the donor (Carnevale, 2004). Crude Equine Gonadotropin (CEG) which is not commercially available can also be used for research purposes (Duchamp *et al.*, 1987).

Follicles have been punctured and oocytes collected by laparotomy (Vogelsang *et al.*, 1988), colpotomy (Hinrichs and Kenney, 1987), and flank puncture (Vogelsang *et al.*, 1983; Palmer *et al.*, 1986). The surgical approaches were rapidly considered too invasive and flank or ultrasound guided punctures became the most common methods of oocyte collection. Flank puncture is done by manual palpation of the ovary without the aid of an ultrasound machine. The ovary is manipulated per rectum and gently pulled against the flank wall. A trocard is placed through the flank musculature against the preovulatory follicle. A large gauge needle is inserted through the canula into the antral cavity. The follicular fluid is aspirated and the follicular antrum is lavaged several times with PBS supplemented with heparin. As there is no visual control, this technique can only be successfully used for large follicles.

Transvaginal ultrasound-guided aspiration as performed in the bovine (McKinnon *et al.*, 1988; Bruck *et al.*, 1992), was modified for the equine (Bracher *et al.*, 1993; Cook *et al.*, 1993; Duchamp *et al.*, 1994; Carnevale and Ginther, 1995; Kanitz *et al.*, 1995; Meintjes *et al.*, 1995; Hinrichs *et al.*, 1998) and is now yielding collection rates ranging from 51 to 86% (Cook *et al.*, 1993; Bezard *et al.*, 1995; Meintjes *et al.*, 1995; Goudet *et al.*, 1997a; Scott *et al.*, 2001).

Practically, the donor mare is sedated and a sector transducer is positioned within the anterior vagina, lateral to the posterior cervix. The ovary is manipulated transrectally and positioned against the transducer. A needle is advanced through a needle guide and the vaginal and follicular walls are punctured. Follicular fluid is aspirated and the antral cavity is flushed several times with heparinised PBS or an embryo flush solution. Scraping of the follicular wall can be achieved by rotating the needle during the aspiration. COCs are searched in follicular fluid and flushes under stereomicroscope. Follicles larger than 25mm can be

punctured using a single lumen needle. Follicles smaller than 25mm can be punctured with a double lumen needle allowing a continuous flush of their antral cavity as described by Duchamp and collaborators (Duchamp *et al.*, 1995).

1.3. Oocyte Evaluation

Ex vivo or *in vivo* collected oocytes can be denuded. However they are usually surrounded by a multilayered cumulus investment. Granulosa and cumulus cells are similar in texture. The innermost cumulus cells have numerous projections penetrating the zona pellucida and establishing close contact with the oolemma (Grondahl *et al.*, 1995). COCs can be characterized based on their degree of expansion (Hinrichs *et al.*, 1993; Goudet *et al.*, 1997a; Hinrichs and Williams, 1997), which is correlated to the nuclear maturation of the oocyte (Zhang *et al.*, 1989; Goudet *et al.*, 1997a; Gable and Woods, 2001). In general, immature oocytes are associated with a compact cumulus. Expanded cumuluses are usually associated with atretic follicles or the preovulatory stages. Oocytes from atretic follicles are known to have a greater ability to resume meiosis and progress from germinal vesicle (GV) stage to metaphase of the second meiotic division (MII) (Hinrichs, 1991). It is well established that follicles are responsible for meiotic arrest of the oocyte (Pincus and Enzemann, 1935) and this has also been confirmed in the horse (Hinrichs *et al.*, 1995). As they undergo atresia, follicles lose their capacity to maintain that meiotic arrest (Gougeon and Testart, 1986; Blondin and Sirard, 1995). This explains the higher maturation rates for expanded than compact COCs obtained in several studies (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000; Galli *et al.*, 2007).

The cumulus cells directly surrounding the oocyte are called the corona radiata. They are often last to expand resulting in a typical image of a cellular ring around the oocyte. Compared to the COC, the oocyte is small and very often difficult to image as its surrounding cumulus cells effectively prevent its detailed observation. These cells play an essential role in oocyte maturation and early embryonic development. Except for the Intra Cytoplasmic Sperm Injection (ICSI), their presence is required for all the techniques involving oocyte transfer, making morphological assessment of the oocyte difficult to perform. However, it has been shown that ooplasm aspect is correlated to follicular viability assessed by histological appearance of the mural granulosa. Oocytes with a homogeneous ooplasm are significantly

associated with viable follicles and compact COCs. Conversely, oocytes with a granulous, polarized ooplasm, corresponding to an uneven distribution of lipid droplets and organelles, are associated with expanded COCs and slightly or moderately atretic follicles (Hinrichs and Williams, 1997).

1.4. Oocytes in vitro Maturation (IVM)

Oocyte maturation involves nuclear and cytoplasmic events by which the oocyte acquires its developmental competence. The final maturation of the oocyte has been associated with 6 consecutive stages of nuclear and cytoplasmic changes. These changes have been described by Grondahl and co-workers (Grondahl *et al.*, 1995) and can be summarized as : (1) the central spherical nucleus stage ; (2) presence of a spherical nucleus located at the periphery of the ooplasm ; (3) a peripheral oocyte nucleus in stage II ; (4) oocyte nuclear breakdown also termed Germinal Vesicle Break Down (GVBD) ; (5) metaphase I ; and (6) metaphase II with metaphase chromosome peripherally located and a polar body within the perivitelline space. Observation of the polar body under the stereomicroscope is difficult but it can occasionally be seen in the perivitelline space. A specific DNA staining, such as Hoechst 33258 can be used on decoronised oocytes allowing chromatin examination and classification into: (1) Germinal Vesicle (GV), (2) Dense chromatin, (3) Metaphase I, (4) Metaphase II, (5) Degenerated (Hinrichs *et al.*, 1993; Goudet *et al.*, 1998b).

Most IVM media used for horse oocyte are derived from those used in the bovine species. Since the first equine oocyte IVM (Fulka and Okolski, 1981), a variety of media comprising synthetic tissue culture media such as TCM-199 (Willis *et al.*, 1991; Dell'Aquila *et al.*, 1997b; Galli *et al.*, 2001), or Ham’s F10 (Okolski *et al.*, 1991; Willis *et al.*, 1991; Shabpareh *et al.*, 1993) have been investigated. Most media now are based on TCM-199, equine maturation medium I (EMMI) and with addition of foetal calf serum, various hormones such as LH, FSH, oestradiol (Carnevale *et al.*, 2004b) or EGF (Goudet *et al.*, 1998a; Lorenzo *et al.*, 2002). A few studies have investigated the effects of co-cultures for IVM (Hinrichs *et al.*, 1995; Li *et al.*, 2001; Choi *et al.*, 2002b; Tremoleda *et al.*, 2003). These gave a better understanding of the control mechanisms of the meiotic arrest within the follicle but failed to show a significant improvement in oocyte maturation.

IVM duration has also been extensively investigated (Fulka and Okolski, 1981; Zhang *et al.*, 1989; Del Campo *et al.*, 1990; Willis *et al.*, 1991; Del Campo *et al.*, 1995; Bezard *et al.*, 1997). After 30 hours of maturation under a controlled atmosphere (39°C and 5% CO2), 50% to 80% oocytes reach the metaphase II stage (Scott *et al.*, 2001). It has also been shown that further duration of IVM does not improve maturation rates (Shabpareh *et al.*, 1993). Optimum maturation duration depends on the initial degree of maturation of the oocytes, which is correlated to the aspect of their COC. Maximum maturation rates for oocytes retrieved from expanded and compact COCs were obtained after 24h and 32h respectively for Hinrichs and co-workers (Hinrichs *et al.*, 1993) and 24h and 30h for Zhang and collaborators (Zhang *et al.*, 1989).

Fertilization of an oocyte requires both its nuclear and cytoplasmic maturation. Nuclear maturation can resume spontaneaously when oocytes are removed from their inhibitory follicular environment. Nuclear maturation is achieved when metaphase II stage of meiosis is reached. This can be readily evaluated by specific DNA staining. Conversely, to date there is no reliable marker of cytoplasmic maturation. The ultimate criterion of oocyte competence is its ability to be fertilized and develop into a viable embryo. The lack of an efficient *in vitro* embryo production (IVP) system has limited oocyte maturation assessment to the nuclear maturation. Although some studies investigated cytoplasmic changes observed during nuclear maturation, such as migration of cortical granules or mitochondria (Grondahl *et al.*, 1995; Goudet *et al.*, 1997a; Aguilar *et al.*, 2002; Carneiro *et al.*, 2002), they did not measure their significance to developmental competence of the oocyte. Cytoplasmic maturation depends on the accumulation of proteins and mRNA (Grondahl *et al.*, 1993). As already mentioned, once oocytes are removed from their follicular environment they are relieved of its meiotic inhibitory effect and they condense their chromatin. This stops transcription and subsequent protein production. Consequently, proteins and mRNA storage should obviously be complete before meiotic resumption and germinal vesicle breakdown is induced (Sirard, 2001). Based on this observation, bovine oocytes have been cultured under conditions that temporally maintain meiotic arrest but allow cytoplasmic maturation, which translated into an improved developmental competence (Fouladi Nashta *et al.*, 1998; Hashimoto *et al.*, 2002). A similar approach has been considered in the horse (Franz *et al.*, 2003) but the effect on cytoplasmic maturation has only been studied on small numbers (Hinrichs *et al.*, 2006; Hinrichs *et al.*, 2007). A better understanding of the control mechanisms of the meiotic arrest might certainly

lead to new IVM strategies based on a better synchronisation of nuclear and cytoplasmic maturation events *in vitro*.

2. Use of equine oocytes in assisted reproduction

Development of ARTs in the horse has been slow compared to other species, but significantly progressed over the last decade. However, the techniques involved require specific equipment and expertise, which are most often only accessible in specialised centres.

Conventional IVF failed to give consistent results in the equine and reports of successes with IVF are very few. There are only two live foals reported to be born following conventional IVF and both derived from *in vivo* matured oocytes (Palmer *et al.*, 1991; Bezard *et al.*, 1992). Failure of IVF is probably related to inefficient sperm capacitation (Alm *et al.*, 2001), changes occurring with the zona pellucida (Dell'Aquila *et al.*, 1999; Hinrichs *et al.*, 2002) or to incomplete or imperfect *in vitro* oocytes maturation (Li *et al.*, 2001). To date, calcium ionophore A23187 is the most efficient sperm capacitation treatment, inducing acrosome reaction and sperm penetration (Zhang *et al.*, 1990; Alm *et al.*, 2001; Hinrichs *et al.*, 2002) although heparin was shown to give similar results in one study (Alm *et al.*, 2001) and a recent paper reports even higher penetration rates (60%) after capacitation with modified Whittens and hyperactivation with procaine (McPartlin *et al.*, 2009) . In order to overcome the barrier of inefficient IVF, research focused on either in vivo or in vitro techniques to produce embryos such as intra-follicular and intra-oviductal oocyte transfer, and ICSI.

2.1. Intra-Follicular Oocyte Transfer (IFOT)

IFOT is based on the injection of one or more oocytes into the preovulatory follicle of a recipient mare where the oocytes can find the conditions to complete their maturation. When ovulation occurs, transferred oocytes are ovulated at the same time as the recipient’s preovulatory oocyte. IFOT has been first unsuccessfully attempted in the baboon, bovine and porcine, probably due to damage caused to the follicle during manipulations (Fleming *et al.*, 1985; Hinrichs and Digiorgio, 1991). Thanks to the large size of the ovary, its specific anatomy with a peripheral medulla and a surrounding fibrous tunique, the mare appears to be a unique model for the study of IFOT. Technically, the procedure is similar to that of the ovum pick up. The recipient mare is sedated and a sector transducer is positioned within the

anterior vagina, lateral to the posterior cervix. The ovary is manipulated transrectally and positioned against the transducer. A needle is advanced through a needle guide and the vaginal and follicular walls are punctured. A few millilitres of follicular fluid are aspirated, the oocytes are injected into the follicle and the needle is flushed with some follicular fluid. Hinrichs and Di Giorgio (Hinrichs and Digiorgio, 1991) developed the technique and obtained 12 embryos in excess after transfer of 135 immature oocytes into preovulatory follicles. The same technique has been used for a study of the follicular fluid contents during the oocyte maturation but there was no attempt to produce embryos (Goudet *et al.*, 1997b). Surprisingly, no further studies about IFOT in the mare have been reported probably due to rather poor results of early reports.

2.2. Intra-oviductal Oocyte Transfer (OT)

The technique involves surgical transfer of one or more oocytes from a donor mare into the oviduct of an inseminated recipient. Subsequent fertilization and early embryo development occur within the recipient's oviduct.

Alternatively, sperm and oocyte can be transferred together into the recipient's oviduct. This technique called: « Gamete Intra-Fallopian Transfer » (GIFT) has been shown to give pregnancy rates similar to those obtained by OT (Carnevale and Ginther, 1995; Coutinho da Silva *et al.*, 2002b). However, fresh sperm appears to be more efficient than frozen-thawed semen for GIFT (Coutinho da Silva *et al.*, 2002a). Oocyte transfer and GIFT are essentially used for research purposes and for mares affected with oviductal, uterine or cervical pathologies that preclude embryo transfer as an option to obtain a live foal from these mares. Cyclic and non-cyclic mares have been used as recipients with similar pregnancy rates (Carnevale *et al.*, 2005) and xenogenous transfer into sheep oviducts has also been reported (Wirtu *et al.*, 2004). However, most oocyte transfers involve injection of oocytes into the oviduct of a cyclic recipient mare, whose preovulatory oocyte has been aspirated. Oocyte transfer in the horse was first described by Mac Kinnon and co-workers (McKinnon *et al.*, 1988), but their blastocyst rate and those from the subsequent studies (Zhang *et al.*, 1989; Ray *et al.*, 1994) remained low. Carnevale and Ginther (Carnevale and Ginther, 1995) first reported higher embryo production rates following transfer of *in vivo* matured oocytes. More recent studies transferring a single oocyte collected from a preovulatory follicle 24 hours after

an injection of hCG and matured *in vitro* for another 16 to 20 hours before transfer also showed high embryo production rates reaching approximately 85% (Hinrichs *et al.*, 1997) and commercial programmes using OT for mares with reproductive abnormalities are now available (Carnevale *et al.*, 2001). Intra-oviductal oocyte transfer can nowadays be considered a viable clinical alternative to embryo transfer in selected mares (Hinrichs *et al.*, 2002; Carnevale, 2004). Birth of a foal following transfer of *in vitro* matured oocytes collected from ovaries after the death of the donor mare was first reported in 2003 (Carnevale *et al.*, 2003). The same authors reported embryo development rates of 15% following transfer of oocytes (n=191) recovered from commercial mares post-mortem (Carnevale *et al.*, 2004a).

Reported embryo yields following intra-oviductal transfer of *in vitro* matured oocytes vary: 7% (2/29) (Scott *et al.*, 2001), 15% (11/73), 18% (13/73) (Preis *et al.*, 2004), 7% (2/29) (Zhang *et al.*, 1989). These results remain lower than those obtained after transfer of preovulatory, *in vivo* matured oocytes, that range from 73% to 83% (Carnevale and Ginther, 1995; Hinrichs *et al.*, 1998; Scott *et al.*, 2001; Carnevale *et al.*, 2004b). Age of the donor is a major factor affecting success of OT (Carnevale, 2004). More morphological abnormalities were observed in oocytes from older mares (Carnevale *et al.*, 1999) and significantly more oocytes collected from young donor mares can develop into embryonic vesicles (Carnevale and Ginther, 1995).

2.3. Intra-Cytoplasmic Sperm Injection (ICSI)

ICSI was developed as an alternative to bypass the inadequacy conventional IVF in the equine and fertilize equine oocytes *in vitro* (Squires *et al.*, 2003; Hinrichs, 2005; Galli *et al.*, 2007). Since the first report of a pregnancy established after ICSI in the horse (Squires *et al.*, 1996), it has produced several foals after injection of both *in vivo* and *in vitro* matured oocytes (Mc Kinnon *et al.*, 2000; Li *et al.*, 2001; Galli *et al.*, 2002)

Briefly, a single motile spermatozoon is immobilized by crushing its tail. It is then injected into the cytoplasm of a denuded metaphase II oocyte. This overcomes the barrier of capacitation and acrosome reaction and even permits the use of sperm with poor motility and performances *in vivo* (Lazzari et al., 2002). Early studies rapidly reported high fertilization rates (40% vs 5% for conventional IVF) (Dell'Aquila *et al.*, 1997a; Dell'Aquila *et al.*, 1997b;

Grondahl *et al.*, 1997). Embryo yields following ICSI with either frozen-thawed or fresh semen have been shown to be similar (Choi *et al.*, 2002a) as long as a motile sperm is selected (Lazzari *et al.*, 2002). In another study, oocytes successfully developed to blastocysts after being injected with immotile spermatozoa isolated from semen that had undergone two freeze-thaw cycles (Choi *et al.*, 2006a). Enhancement of further development of the zygote to the blastocyst stage with oocyte activators has been tested but most only increased parthogenesis (Li *et al.*, 2000).

The culture of 2-cell embryos to the blastocyst stage when they can be transferred into a recipient's uterus is another major limiting step in IVP. Many culture systems for early cleavage stage embryos have been investigated, including: co-cultures with oviduct cells (Battut *et al.*, 1991), Vero cells (Dell'Aquila *et al.*, 1997b), cumulus cells (Li *et al.*, 2001), granulosa cells (Rosati *et al.*, 2002), and culture in defined media : G1.2 (Choi *et al.*, 2002a), DMEM-F12 and CZB (Choi *et al.*, 2004), and modified SOF (Galli *et al.*, 2002). In most of these systems, however, the percentage of 2-cell embryos developing to blastocysts remained rather low, ranging from 4 to 16%. ICSI early embryos can alternatively be transferred into the oviduct of a recipient mare (Squires *et al.*, 1996) or a temporary recipient sheep, which remains the most successful system for producing equine blastocysts with a blastocyst rate of 45% (Galli *et al.*, 2002; Lazzari *et al.*, 2002). However, Choi and collaborators (Choi *et al.*, 2006b; Choi *et al.*, 2006c) recently developed a culture system using DMEMF/F-12 medium claimed to have achieved blastocyst development rates (27-38%) similar to those obtained after transfer in both the mare and the sheep (approximately 36%) (Galli *et al.*, 2007).

2.4. Conclusion

These *in vivo* techniques involve transfer of oocytes previously matured *in vitro* or not in surrogate mares. They require availability of recipients, synchronisation between donor and recipient mares, surgery or specific equipment and skills. However, they potentially represent valuable alternatives to IVF procedures which can be poorly efficient (conventional IVF) or technically demanding (ICSI).

3. Use of cysteamine in IVM

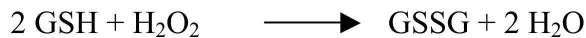
3.1. Glutathione

Glutathione(GSH), a tripeptide thiol (γ-glutamyl-cysteine-glycine) is the major non-protein sulfydryl compound in mammalian cells that plays an important role in protecting the cell from oxidative damage (Meister and Tate, 1976; Meister and Anderson, 1983). GSH cellular content is regulated by the γ-glutamyl cycle. The reactions involved account for the synthesis and degradation of GSH, and the balance of GSH transport out of the cells and that of γ-glutamyl amino acids into the cells. GSH is synthesised within the cell in two steps. First, γ-glutamylcysteine synthetase catalyses synthesis of γ-glutamyl-cysteine from glutamate and cysteine. Then, glycine is added to the C-terminal of γ-glutamyl-cysteine in the presence of GSH synthetase (Meister and Tate, 1976).

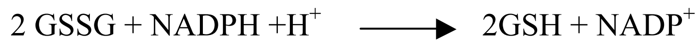
The first step of GSH synthesis appears to determine the upper concentration of cellular GSH because of a feedback inhibition by GSH (Richman and Meister, 1975). It has been shown that GSH concentration also depends on availability of cysteine for synthesis (Meister, 1983; Furnus and de Matos, 1999). Conversely, buthionine sulfoximine (BSO) binds to and inhibits γ-glutamylcysteine synthetase and thus induces GSH depletion (Griffith and Meister, 1979; Meier and Issels, 1995).

Glutathione is present in the cell either under its reduced (GSH) or oxidized form (GSSG). In the presence of GSH, glutathione peroxidase (GPX) catalyses the reduction of hydrogen peroxide to form H_2O and GSSG. GSSG is reduced by glutathione reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) which maintains a high ratio (about 100:1) of GSH to GSSG in the cell (Meister, 1983). GSH peroxidase and GSH catalase promote removal of hydrogen peroxide and dismutation product of superoxide radicals (Fridovich, 1978).

These reactions can be summarized by the following equations:



$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$$



$$2\ GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

3.2. Low molecular weight thiol compounds and GSH

As mentioned before, GSH synthesis is limited by the availability of cysteine. Except for hepatocytes, which are able to synthesize cysteine from methionine via the cystathionine pathway, eukaryotic cells depend on extracellular medium for the supply of cysteine (Meier and Issels, 1995). Direct supplementation of medium with cysteine is usually avoided as cysteine is easily oxidized to cystine in the culture medium (Bannai, 1984) and can exhibit a high degree of toxicity (Nishiuch *et al.*, 1976).

Low molecular thiols such as β-mercaptoethanol (Ishii *et al.*, 1981), N-acetylcysteine, cysteamine (Meier and Issels, 1995) can promote cystine uptake. These compounds share a free SH group as a common molecular structure. Modification of this free thiol group by chemical reaction to form a disulfide bond or the addition of a phosphate moiety to the thiol group abolishes the potential of the compound to promote cystine uptake (Meier and Issels, 1995).

Effects of extracellular thiols on cystine uptake can be summarized in two steps: 1) formation of cysteine and cysteine mixed disulfides (e.g., cysteine-cysteamine) and 2) uptake of cysteine and/or the mixed disulfide via cellular transport systems (Ishii *et al.*, 1981; Meier and Issels, 1995). Within the same species, activity of these transport systems vary from one cellular type to the other (Meier and Issels, 1995). Oocytes are deficient in their capacity to take up Cystine (cysteine-cysteine). Cysteine is utilized by the oocyte, but it is easily oxidised to cystine in the culture medium. Thiol compounds react with cystine to form a mixed disulfide. The mixed disulfide is taken up by the cells, where it is reduced to produce a thiol compound and cysteine. Thiol compounds do not accumulate in the cells and escape to the medium to react with cystine. Thiol compounds are repeatedly taken up by the oocytes in the form of mixed disulfide with cysteine and return to the medium in their reduced form. Extracellular cysteine

may also be taken up by the oocytes by a transporter system (ASC) shared with other amino acids (Alanine, Serine, Cysteine) but at a very low rate as cysteine in the culture medium is rapidly oxidized to cystine. A schematic diagram of the action of low molecular weight thiols is proposed in Figure 1.

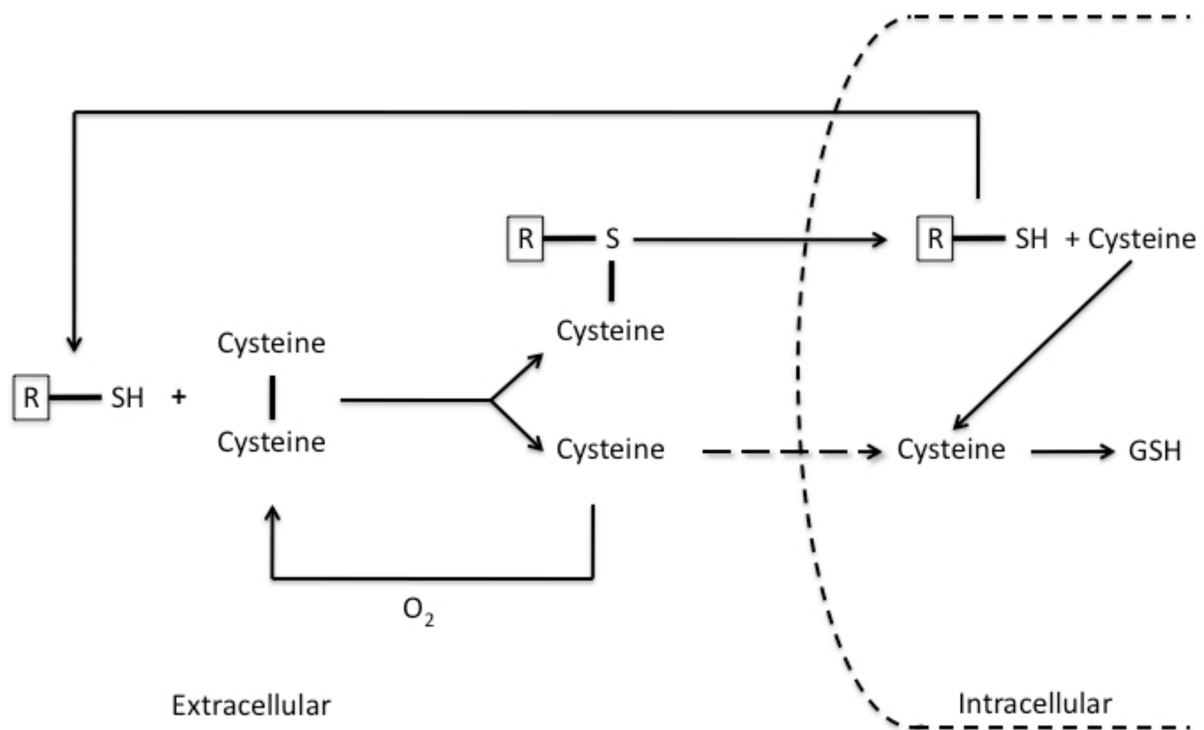


Figure 1: Proposed action of thiol compounds on cystine and cysteine utilization by oocytes. Adapted from Ishi and collaborators (Ishii *et al.*, 1981).

3.3. GSH and IVM

Under *in vitro* culture conditions, oxidative modifications of cell components via increased Reactive Oxygen Species (ROS), which are responsible for DNA damage, oxidative alterations of proteins and lipid peroxidation (Johnson and Nasr-Esfahani, 1994), represent a major culture induced stress (Noda *et al.*, 1991; Goto *et al.*, 1993). Anti-oxidant systems can attenuate the deleterious effects of oxidative stress by scavenging reactive oxygen species (Del Corso *et al.*, 1994).

Low molecular weight thiols compounds have been shown to have beneficial effects on lymphocyte viability and enhance various cell reactions (Fanger *et al.*, 1970). Addition of cysteine or β-mercaptoethanol to culture media for lymphocytes has been shown to increase intracellular GSH levels and prevent its decrease during proliferation (Zmuda and Friedenson, 1983).

GSH synthesis during oocyte maturation has been reported in mouse (Calvin *et al.*, 1986), hamster (Perreault *et al.*, 1988), pig (Yoshida *et al.*, 1993), sheep (de Matos *et al.*, 2002a), bovine (de Matos *et al.*, 1996) and equine (Luciano *et al.*, 2006). GSH concentration is highly correlated with the presence of cumulus cells (Sawai *et al.*, 1997) however, denuded oocytes GSH synthesis has also been shown to be stimulated by thiol compounds, although to a lesser extend than COCs (de Matos *et al.*, 1997; Yamauchi and Nagai, 1999). GSH contents increase during maturation of oocytes in the ovary and peak at the metaphase II stage (Perreault *et al.*, 1988) but drops rapidly in early embryos (Zuelke *et al.*, 2003). It has been suggested that GSH content in oocytes may serve as a reservoir protecting the zygote and the early embryos from oxidative damage before genomic activation and de novo GSH synthesis occur (Furnus *et al.*, 1998; de Matos and Furnus, 2000). Moreover, GSH has been shown to play a role during fertilization by promoting male pronucleus formation (Yamauchi and Nagai, 1999). Quite naturally, effects of supplementation of IVM media with low molecular weight thiols have been studied in various species.

The following sections review the use of cysteamine supplementation for IVM media, its effects on and various parameters that can modulate those.

3.3.1 Addition of cysteamine to the IVM medium : dose effect

Most commonly, IVM media have been supplemented with a dose of 100 μM of cysteamine in cattle, (de Matos *et al.*, 1995; de Matos *et al.*, 1996; de Matos *et al.*, 1997; de Matos *et al.*, 2002b; Oyamada and Fukui, 2004; Balasubramanian and Rho, 2007), buffalo (Gasparrini *et al.*, 2003; Anand *et al.*, 2008), goats (Rodriguez-Gonzalez *et al.*, 2003b; Urdaneta *et al.*, 2003; Zhou *et al.*, 2008), horses (Luciano *et al.*, 2006), pigs (Kobayashi *et al.*, 2006; Kobayashi *et al.*, 2007; Song and Lee, 2007), mice (de Matos *et al.*, 2003; Chen *et al.*, 2005), cats (Bogliolo *et al.*, 2001) and dogs (Hossein *et al.*, 2007). However, other concentrations of cysteamine,

ranging from 50 to 500 μM, have been tested in different species. A summary of the different concentrations used in various species is given is Table 1.

Species	**Dose of Cysteamine (μM)**	**References**
Bovine	100	de Matos *et al.*, 1995; de Matos *et al.*, 1996; de Matos *et al.*, 1997; de Matos *et al.*, 2002b; Oyamada and Fukui, 2004; Balasubramanian and Rho, 2007
Buffalo	50	Gasparrini *et al.*, 2003; Singhal *et al.*, 2008
	100	Gasparrini *et al.*, 2000
	50, 100, 200	Anand *et al.*, 2008
Ovine	50, 100, 200	de Matos *et al.*, 2002a
Caprine	100	Rodriguez-Gonzalez *et al.*, 2003a
	100, 200, 400	Urdaneta *et al.*, 2003
	50, 100, 200, 300	Zhou *et al.*, 2008
Equine	100	Luciano *et al.*, 2006
Porcine	100	Kobayashi *et al.*, 2006; Kobayashi *et al.*, 2007; Song and Lee, 2007
	150	Yamauchi and Nagai, 1999; Bing *et al.*, 2001
	50, 500	Grupen *et al.*, 1995
Canine	50, 100, 200	Hossein *et al.*, 2007
Mice	100	Chen *et al.*, 2005
	0, 25, 50, 100, 200	de Matos *et al.*, 2003

Table 1: Summary of the different concentrations used in various species.

Effects of one given concentration of cysteamine supplementation not only depend on the species but also on the maturation medium. These differences between media might be explained by the presence and the quantities of different factors that can help protect the cell from oxidative damage such as citrate (Holm *et al.*, 1999) and pyruvate (Morales *et al.*, 1999). Moreover, as most media use TCM-199, it is important to bear in mind that whether the cystine it contains (83.2 µM) is sufficient to sustain oocyte IVM of different species is unknown (Zhou *et al.*, 2008).

3.3.2 Addition of cysteamine to the IVM medium : effect on nuclear maturation rates

Cysteamine supplementation during IVM, was reported to improve nuclear maturation rates in dogs (Hossein *et al.*, 2007), in mice (Chen *et al.*, 2005), in goats (Urdaneta *et al.*, 2003), and in pigs (Bing *et al.*, 2001). However, other studies in goats (Zhou *et al.*, 2008), pigs (Yamauchi and Nagai, 1999; Kobayashi *et al.*, 2006; Song and Lee, 2007), horses (Luciano *et al.*, 2006), buffalos (Singhal *et al.*, 2008) and cattle (Oyamada and Fukui, 2004; Balasubramanian and Rho, 2007) could not show any increase in nuclear maturation rates.

3.3.3 Addition of cysteamine to the IVM medium : effect on GSH oocyte content

Cysteamine has been shown to increase intracellular GSH synthesis in bovine (Takahashi *et al.*, 1993; de Matos *et al.*, 1995; de Matos *et al.*, 1997; de Matos *et al.*, 2002b; Oyamada and Fukui, 2004), buffalo (Gasparrini *et al.*, 2003), canine (Hossein *et al.*, 2007), goats (Rodriguez-Gonzalez *et al.*, 2003a; Zhou *et al.*, 2008), mice (de Matos *et al.*, 2003), horses (Luciano *et al.*, 2006), sheep (de Matos *et al.*, 2002a) and pigs (Yamauchi and Nagai, 1999; Kobayashi *et al.*, 2007). This increase in GSH content has been consistent in all reports regardless the species. In sheep, a concomitant decrease in hydrogen peroxide has also been demonstrated (de Matos *et al.*, 2002a), illustrating the scavenging effect of GSH synthesis stimulation on ROS.

3.3.4 Addition of cysteamine to the IVM medium : effect on male pronucleus formation

Effect of cysteamine supplementation during IVM on male pronucleus formation following either conventional IVF or ICSI has not been extensively investigated. However, addition of cysteamine to the IVM medium has been shown to improve male pronucleus formation in goats (Rodriguez-Gonzalez *et al.*, 2003a; Urdaneta *et al.*, 2003), buffalos (Anand *et al.*, 2008) and pigs (Grupen *et al.*, 1995; Yamauchi and Nagai, 1999; Bing *et al.*, 2001). Only two studies, one in pigs (Kobayashi *et al.*, 2006) and one in cattle (Balasubramanian and Rho, 2007), reported a lack of effect of cysteamine on male pronucleus formation.

3.3.5 Addition of cysteamine to the IVM medium : effect on cleavage rates

Effect of cysteamine supplementation to IVM media on cleavage rates is controversial. A few studies have reported an increase in cleavage rates following IVM with cysteamine in goats (Rodriguez-Gonzalez *et al.*, 2003a), pigs (Grupen *et al.*, 1995), cattle (Oyamada and Fukui, 2004), and buffalos (Anand *et al.*, 2008; Singhal *et al.*, 2008). Conversely, many other reports in various species, including species where an increase has been reported, have failed to observe any effect of cysteamine supplementation on cleavage rates. This lack of effect has been reported in pigs (Kobayashi *et al.*, 2006; Kobayashi *et al.*, 2007; Song and Lee, 2007), cattle (de Matos *et al.*, 1995; Balasubramanian and Rho, 2007), buffalos (Gasparrini *et al.*, 2003), sheep (de Matos *et al.*, 2002a), horses (Luciano *et al.*, 2006) and mice (de Matos *et al.*, 2003; Chen *et al.*, 2005).

3.3.6 Addition of cysteamine to the IVM medium : effect on blastocyst development

In very few studies in pigs (Kobayashi *et al.*, 2007; Song and Lee, 2007), and mice (Chen *et al.*, 2005), blastocyst development has not been shown to be affected by cysteamine supplementation. Conversely, many authors reported increased blastocyst development following cysteamine supplementation to the IVM medium. This has been observed in cattle (Takahashi *et al.*, 1993; de Matos *et al.*, 1995; de Matos *et al.*, 2002b; Oyamada and Fukui,

2004; Balasubramanian and Rho, 2007), buffalos (Gasparrini *et al.*, 2003; Anand *et al.*, 2008; Singhal *et al.*, 2008), sheep (de Matos *et al.*, 2002a), goats (Zhou *et al.*, 2008), pigs (Grupen *et al.*, 1995; Kobayashi *et al.*, 2006) and mice (de Matos *et al.*, 2003). In these studies, although cleavage rates are not necessarily affected further development of the blastocysts obtained has been positively influenced by cysteamine supplementation.

3.4. Conclusion

Low molecular weight thiol compounds can increase cysteine uptake by oocytes during IVM. This subsequently can increase GSH content, which is a major anti-oxidant system that protect the cells against the deleterious effects of oxidative stress by scavenging reactive oxygen species. Beneficial effects of thiol compounds supplementation to IVM media have been observed in many species but they appear to be highly species and dose dependant.

PART 2 : RESEARCH OBJECTIVES

Part 2 : Research Objectives

Research on IVP in the equine is impeded by the limited availability of mature oocytes as the mare is mono-ovulating and superovulation is still difficult. Transvaginal ultrasound guided follicular aspiration has become an alternative source for mature and immature equine oocytes. Despite recent improvement in IVM of equine oocytes, success rates of IVM in that species remain low in all culture media tested compared to other species such as cows, goats and sows. However, most studies have focused on the percentage of oocytes reaching the metaphase II stage (nuclear maturation) but few concentrated on the final oocyte competence as measured by its ability to develop into a blastocyst and further evolve into a successful pregnancy. Blastocyst production rate is influenced not only by oocyte fertilization, culture environment (IVC) but also by oocyte maturation conditions.

The addition of GSH synthesis precursors, such as cysteamine, has been shown to improve IVP in various species by protecting the oocyte and early embryo against ROS. Very little information on the use of thiol compounds in the equine is available and there is no published report of the effect of IVM medium supplementation with cysteamine on subsequent *in vivo* embryo production.

The aims of the present work were :

(1) To investigate the influence of supplementation with 100 µM of cysteamine on conventional IVF success rate;
(2) To determine what *in vivo* technique could best bypass the lack of an efficient conventional IVF procedure;
(3) To investigate the influence of supplementation with 100 µM of cysteamine on *in vitro* nuclear and cytoplasmic maturation by specific DNA staining and to evaluate the ability of oocytes to undergo *in vivo* fertilization.

PART 3 : SYNOPSIS OF EXPERIMENTS

Part 3 : Synopsis of experiments

1. Influence of IVM medium cysteamine supplementation on conventional IVF

Introduction

During growth and maturation of the oocyte within the ovary, intracellular GSH content increases as the oocyte approaches the time of ovulation (Perreault *et al.*, 1988; Yoshida *et al.*, 1993; de Matos and Furnus, 2000). The addition of GSH in IVM media improves the rate of male pronuclear formation (de Matos *et al.*, 1995; de Matos and Furnus, 2000). Glutathione synthesis can be increased, and thus the intracellular concentration of GSH, by the addition of low molecular weight thiols to IVM medium, such as cysteamine and ß-mercaptoethanol (Takahashi *et al.*, 1993). This has increased the success of bovine (de Matos *et al.*, 1995; de Matos *et al.*, 1996), ovine (de Matos *et al.*, 1999) and porcine (Grupen *et al.*, 1995; Bing *et al.*, 2001) embryo development. However, the effect of IVM medium cysteamine supplementation on conventional IVF in the equine has not been reported.

In vitro embryo production is the final result of a three step process: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of the embryo to a stage that is transferable to the uterus (Dell'Aquila *et al.*, 1996; Hinrichs *et al.*, 2002). Equine spermatozoa have been incubated in solutions containing an ionophore or heparin, however it is unclear as to which solution is optimal. An *in vitro* fertilization rate of 30% was achieved in a technique where the spermatozoa were prepared with the calcium ionophore A23187 (Palmer *et al.*, 1991; Bezard *et al.*, 1992). This technique was used to produce the only two live foals following an IVF procedure to date (Palmer *et al.*, 1991). Heparin has also been tested as a capacitation treatment (Dell'Aquila *et al.*, 1996) and that technique gave similar results to those obtained with calcium ionophore (Alm *et al.*, 2001). Heparin also increased the IVF rate in cows (Handrow *et al.*, 1989; Farlin *et al.*, 1993) so it may also be useful in equine IVF.

Aim of the study

This study was designed to investigate the influence of supplementation with 100 µM of cysteamine of IVM medium on the ability of oocytes to undergo conventional *in vivo* fertilization.

Material and methods

The study was conducted in spring using 30 cycling Welsh pony mares from 200 to 300 kg. Ovarian activity was monitored daily using transrectal ultrasound scanning.

Follicular puncture and oocyte recovery

When a preovulatory follicle ≥ 33 mm was observed, the mares were injected i.v. with 25mg of Crude Equine Gonadotropin (CEG), to induce ovulation. Transvaginal ultrasound-guided aspiration of follicles ≥ 5 mm was performed 24 hours after CEG injections. Follicles were punctured routinely using a transvaginal aspiration technique as previously described (Duchamp *et al.*, 1994).

Culture of cumulus oocyte complexes

After recovery the cumulus oocyte complexes (COCs) were isolated and classified according to cumulus morphology into expanded cumulus or compact cumulus (Goudet *et al.*, 1998b) as illustrated in Figure 2. COCs with expanded cumulus were discarded. Compact COCs were individually matured at 38,5°C in a humidified atmosphere of 95% air and 5% CO_2 for 30 h in 20µl of maturation medium under mineral oil (M8410 Sigma, France). Two maturation media were used. Medium 1 contained Medium 199 with Earle's salt (M4530, Sigma, France) supplemented with 20% inactivated fetal calf serum (FCS, F4135, Sigma, France) and with 50ng/ml Epidermal Growth Factor (EGF, E4127, Sigma, France) as described by Goudet et al. (Goudet *et al.*, 2000a). Medium 2 was the same base with the addition of cysteamine at 100µM (M9768, Sigma, France).

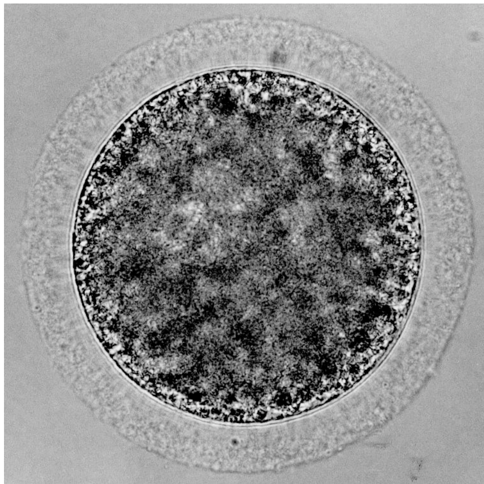


Fig 2 –A : immature oocyte

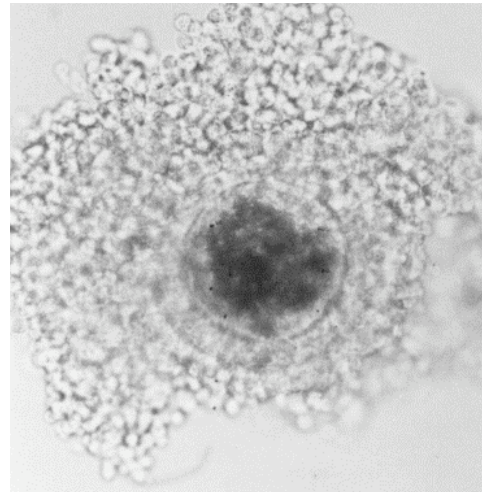


Fig 2-B : Compact Cumulus

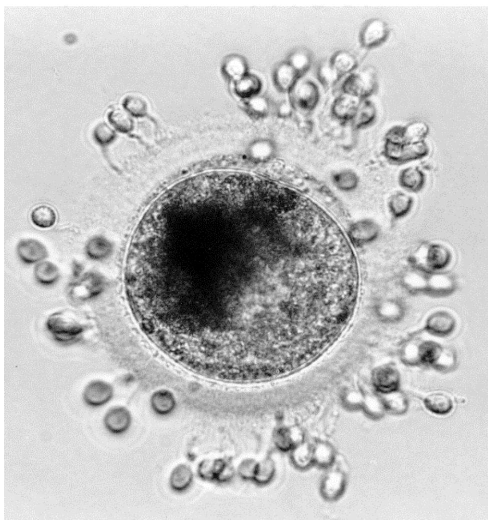


Fig 2-C : Expanded Cumulus

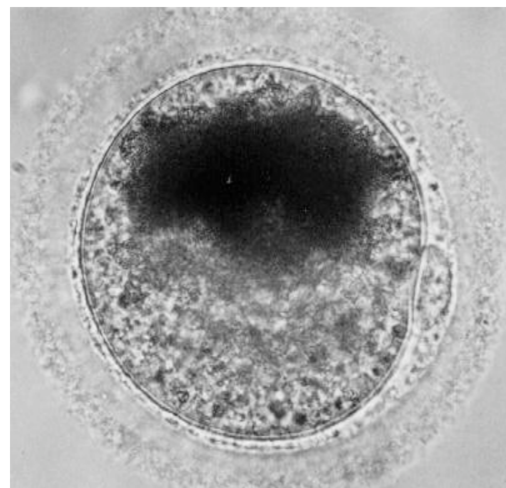


Fig 2-D : Metaphase II + Polar body

Figure 2 : classification of COCs as described by Goudet and collaborators (Goudet *et al.*, 1998b).

Influence of spermatozoa media and cysteamine

Oocytes were rinsed in IVF modified TALP (Tyrode's medium added with albumine lactate and pyruvate) medium and incubated with thawed spermatozoa in 20µl of the medium under mineral oil. TALP medium contained 100mM NaCl, 3.1mM KCl, 0.3mM NaH_2PO_4 $2H_2O$, 2.1mM $CaCl_2$, 0.4mM $MgCl_2$ $6H_2O$, 25mM $NaHCO_3$, 6mg/ml fatty acid-free albumin fraction V (Sigma), 4mg/ml sodium lactate (Sigma), 0.11mg/ml sodium pyruvate (Sigma). Cryopreserved semen was used, one ejaculate from three stallions pooled after thawing in a 37°C waterbath for 30 seconds and washed by centrifugation at 600g for 5 minutes, resuspended in medium and then centrifuged again. The sperm pellet was resuspended in IVF medium (group A) or Hank's solution supplemented with BSA (1%) and Hepes buffer (20mM) (Palmer *et al.*, 1991) (group B) and adjusted to $16X10^6$ spermatozoa/ml. In group A, spermatozoa were supplemented with 1µg/ml heparin. In group B, spermatozoa were incubated with 6µM of Ionophore A23187 (free acid, Sigma) at 37°C for 5 minutes (Palmer *et al.*, 1991), centrifuged at 600g for 5 minutes and resuspended in IVF medium. The sperm suspension was added to the oocytes for a final concentration of $4X10^5$ spermatozoa/ml. Oocytes were incubated with spermatozoa for 24 hours at 38.5°C in a humidified atmosphere (95% air and 5% CO_2), and examined for penetration.

Results

131 oocytes were evaluated for evidence of sperm penetration and fertilization. Overall, 8 of them (6%) were penetrated. We observed one oocyte with a decondensed sperm head, six oocytes with 2 pronuclei or subsequent stages of mitosis, and one 2 cell-embryo. Four oocytes were penetrated by spermatozoa treated with heparin (Group A), and four oocytes were penetrated by spermatozoa treated with ionophore (Group B). Of the 8 penetrated oocytes, 4 came from maturation medium 1, and 4 came from maturation medium 2.

Discussion

In the mare, few studies have reported fertilization rates after standard IVF procedures (Palmer *et al.*, 1991; Dell'Aquila *et al.*, 1996; Alm *et al.*, 2001; McPartlin *et al.*, 2009). Failure of IVF is probably related to inefficient sperm capacitation (Alm *et al.*, 2001), changes occurring with the zona pellucida (Dell'Aquila *et al.*, 1999; Hinrichs *et al.*, 2002) or to incomplete or imperfect *in vitro* oocytes maturation (Li *et al.*, 2001). To date, only two foals were born from IVF (Palmer *et al.*, 1991) and there is no published report of equine pregnancies following both *in vitro* maturation and conventional IVF. In our study, both techniques (ionophore or heparin) yielded 6% of IVF and results were similar for both oocytes matured with or without cysteamine. This percentage is low when compared to data published by Palmer (Palmer *et al.*, 1991) and Dell'Aquila (Dell'Aquila *et al.*, 1996). However these results have not been shown to be repeatable and the IVF rates we observed, although rather low, are more comparable to others reported in the horse (Choi *et al.*, 1994; Dell'Aquila *et al.*, 1997a). Although, it seems likely that cysteamine did not significantly improve IVF rates under our conditions, our general success rates for IVF procedures may be too low for us to conclude definitely about the effect of cysteamine.

These poor results reflect the overall poor results of conventional IVF in the equine. Moreover cytoplasmic maturation assessment requires the observation of embryo development, which cannot be evaluated by IVF. This confirms the need to further investigate other means to assess the effects of cysteamine supplementation.

2. Efficiency of embryonic development after Intra-Follicular and Intra-Oviductal transfer of *in vitro* and *in vivo* matured horse oocytes

Introduction

The first study failed to show an effect of cysteamine supplementation in IVM media at 100µM on conventional IVF results. However, due to the poor results of conventional IVF in general, and in our study in particular, it would be hazardous to conclude that cysteamine supplementation has no effect on cytoplasmic maturation. The ultimate indicator of effective cytoplasmic maturation of the oocyte is the ability of the oocyte to produce an embryo and an ongoing pregnancy. ICSI is now providing fertilization rates (40%) (Dell'Aquila *et al.*, 1997a; Dell'Aquila *et al.*, 1997b; Grondahl *et al.*, 1997) that make it a valuable and repeatable means of producing embryos *in vitro,* allowing cytoplasmic maturation assessment. Unfortunately, ICSI technology requires specific equipment and expertise, which is very often not available. As an alternative to IVF and ICSI, it was decided to explore which *in vivo* fertilization option was best.

Transfer of an immature oocyte into the preovulatory follicle of an inseminated recipient mare (Intra-Follicular Oocyte Transfer, IFOT) has produced embryos but the success rate was low (Hinrichs and Digiorgio, 1991; Goudet *et al.*, 1997b). Collection of embryos following intra-follicular transfer of *in vitro* matured oocytes would assess their cytoplasmic maturation as shown by their ability to produce an embryo. To our knowledge no intra-follicular transfer of oocytes previously matured *in vitro* has been reported. Similarly, oocyte transfer (OT) into the oviduct of an inseminated recipient mare was investigated. Recent studies transferring a single oocyte collected from a preovulatory follicle after induction of ovulation showed high embryo production rates (Hinrichs *et al.*, 1997) and commercial programmes using OT for mares with reproductive abnormalities are now available (Carnevale *et al.*, 2001). Intra-oviductal oocyte transfer can nowadays be considered a viable clinical alternative to embryo transfer in selected mares (Hinrichs *et al.*, 2002; Carnevale, 2004). In addition, OT can be used to assess the quality of oocytes after IVM by their ability to undergo *in vivo* fertilization (Carnevale, 2004; Carnevale *et al.*, 2005).

The aims of this present study were (1) to test if *in vitro* matured oocytes would complete

their maturation and yield embryos following transfer into the preovulatory follicle of an inseminated recipient mare and (2) to compare OT following IVM, IFOT following IVM, and IFOT immediately after oocyte collection as alternative techniques where ICSI is not available.

Materials and methods

The study was conducted in spring using 30 cycling Welsh pony mares from 200 to 300 kg. Ovarian activity was monitored daily and follicular puncture and oocyte recovery was performed as described in study 1.

Culture of Cumulus Oocyte Complexes

After recovery, the cumulus oocyte complexes (COCs) were isolated and classified according to cumulus morphology into expanded cumulus and compact cumulus (Goudet *et al.*, 1998b) as in study 1. The same classification was used for post IVM examination. Collected oocytes were either matured *in vitro* for 30 hours or prepared for immediate transfer.

When matured *in vitro*, COCs were individually matured at 38,5°C in a humidified atmosphere of 95% air and 5% CO_2 for 30 h in 20µl of maturation medium under mineral oil (M8410 Sigma, Saint-Quentin Fallavier, France). Maturation medium contained Medium 199 with Earle's salt (M4530, Sigma, France) supplemented with 20% inactivated fetal calf serum (FCS, F4135, Sigma, France) and with 50ng/ml Epidermal Growth Factor (EGF, E4127, Sigma, France) as described by Goudet et al. (Goudet *et al.*, 2000b). Following IVM, no selection based on appearance was performed and intact COCs were assigned to either OT or Post-IVM IFOT group.

Oocytes from sessions that failed to yield more than 3 oocytes were cultured similarly and prepared for post IVM examination. They were rinsed in PBS and COCs were stripped with small glass pipettes, stained with 1 µg/ml bis-benzamide (Hoechst 33342; Sigma, France), evaluated with a fluorescence microscope. Oocytes were classified according to the stage of nuclear maturation, membrane integrity and the ooplasm aspect as "germinal vesicle", "dense chromatin", "metaphase I", "metaphase II" or "degenerate" as previously described by Goudet et al. (Goudet *et al.*, 1998b). Figure 3 illustrates maturation stages after

DNA staining with bis-benzamide.

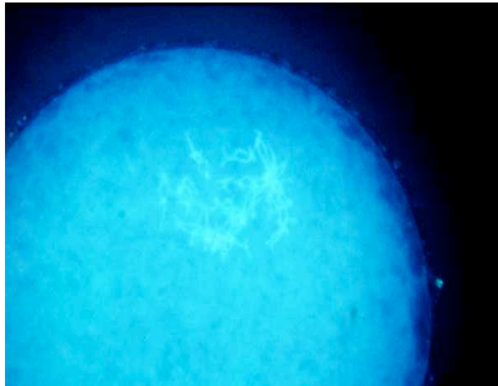


Fig 3-A : Germinal Vesicle

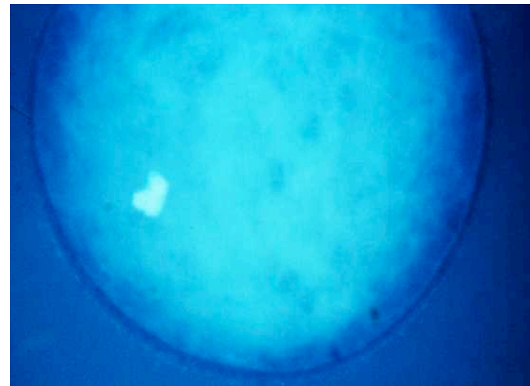


Fig 3-B : Dense Chromatin

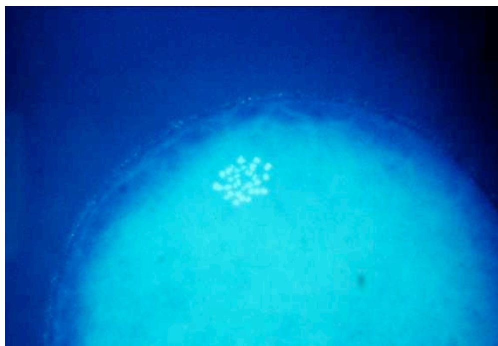


Fig 3-C : Metaphase I

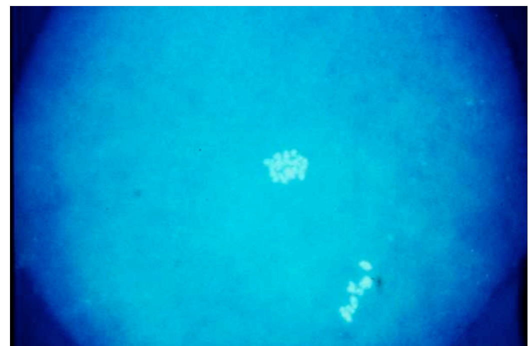


Fig 3-D : Metaphase II

Fig 3 : Chromatin configuration. Equine oocytes after DNA staining with bis-benzamide (X400)

Preparation of oocytes before transfer

Oocytes for intra-follicular transfer were rinsed three times in 500 µl of PBS and loaded in 1ml of PBS into a 1ml syringe for transfer after IVM and in 0.2 to 0.5 ml of PBS with 1mL/L heparin (Heparin 5 IU/ml; Sanofi, France) for immediate transfer.

Oocytes for intra-oviductal transfer were rinsed three times in 500 µl of PBS after IVM and stored in PBS at 38.5°C. The system to aspirate the oocytes consisted of a blunted sterile glass pipette connected to a piece of tube, which was in turn connected to a 1ml syringe. The whole

system was kept at 38.5°C. Oocytes were loaded just prior to the oocyte transfer, in a minimal volume of PBS between two bubbles of air.

Intra-Follicular Oocyte Transfer (IFOT)

Intra-follicular oocyte transfer was performed as previously described by Goudet et al (Goudet *et al.*, 1997b). Briefly, the preovulatory follicle was punctured using a single lumen needle (length, 600mm; outer diameter, 1.1mm, 17G; Thiebaud Frères, Jouvernex Margencel, France), with a specific attention to the needle going through as much ovarian stroma as possible before reaching the follicle. Then, 5 ml of follicular fluid were aspirated from the preovulatory follicle. The 1ml syringe containing the oocytes in PBS was connected to the 17 gauge needle, the oocytes were injected and the needle flushed with 4.5ml of follicular fluid. Immediately after transfer, the catheter and needle were checked to ensure that no oocytes remained logged in them. Recipient mares were injected with their own oocytes, either just after collection (Immediate Intra-Follicular Oocyte Transfer) or following IVM (Post-IVM Intra-Follicular Oocyte Transfer). Recipient mares were inseminated 24 hours after CEG injection, using fresh semen of a stallion with a known normal fertility: 400 X 10^6 spermatozoa in 10ml of extender (INRA96®; IMV Technologies, France).

Intra-oviductal Oocyte Transfer (OT)

Oocytes from preovulatory follicles were aspirated and discarded. Following their ovum pick up session, mares were fasted for 24 hours. Recipient mares were inseminated 5 h prior to surgery, using fresh semen of a stallion with a known normal fertility: 400 X 10^6 spermatozoa in 10ml of extender (INRA96®; IMV Technologies, France). Surgical transfers of the recipient mare's own oocytes occurred 30 hours after the oocyte recoveries. The recipient mare was treated with antibiotics (about 6 hours before surgery) (4,000,000 IU streptomycin, 4g procaine penicilline i.m., Intramicine®, Ceva, France). Animals were sedated with detomidine (3-5 µg/kg; 0.1ml/animal i.v. Domosedan®; Pfizer, France) for surgical preparation of the mare for the standing flank laparotomy. An injection of butorphanol tartrate 4mg (13-20 µg/kg; 0.4ml/animal i.v. Torbugesic®; Fort Dodge, Southampton, UK) for analgesia was followed by a second injection of detomidine (6-10 µg/kg, 0.2ml/animal i.v. Domosedan®). A cuffed urinary catheter was placed to allow continuous emptying of the bladder during the surgery in order to limit discomfort. After local anesthesia (lidocaïne, Laocaïne®, Schering Plough, France), a flank approach was used to gain access to the ovary. The skin incision was directed vertically or at a slight oblique and extended 10 to 15 cm. The

muscles of the flank, external and internal abdominal oblique muscles and the transversus abdominis muscle were bluntly dissected in the direction of their fibres. The peritoneum was perforated and the opening was manually dilated. After the ovary was exteriorised and the pipette was inserted into the oviduct. Oocytes were gently injected into the oviduct. The different layers were closed using absorbable suture material (Vicryl® 0; Johnson and Johnson, UK) in an interrupted pattern. Immediately after surgery, a second insemination with 200 X 10^6 spermatozoa in 10ml of semen extender (INRA96®, IMV Technologies, France) stored at 15°C was performed.

Embryo collection

Embryo collection was performed 10 days after surgery by three successive uterine lavage with saline at 38°C. Collection fluid was filtered and embryos were isolated under a stereomicroscope.

Statistical analysis

To compare maturation rates and embryo yields, a chi-square test was performed. Significance was established at $p < 0.05$.

Results

Oocyte collection

Following the 31 puncture sessions, a total of 158 oocytes were obtained from 284 immature follicles resulting in a recovery rate of 55,6%. Global collection rate was 5.1 immature oocytes per puncture session (158/31).

Maturation rates

Out of the 158 oocytes, 47 were used for immediate IFOT, 109 oocytes were matured *in vitro* and two oocytes, which were degenerated, were not cultured. Out of these 109 *in vitro* matured oocytes, 55 were transferred into preovulatory follicles (IFOT), 40 were used for intra-oviductal oocyte transfer and 14 were used for analysis. Table 2 shows the percentage of nuclear maturation of the non-transferred oocytes.

Table 2: Evaluation of nuclear maturation after culture

	Nuclear stage				
	Metaphase II	Metaphase I	Dense chromatin	Germinal vesicle	Degenerated
Total	71.3% (10/14)	0% (0/14)	7.2% (1/14)	7.2% (1/14)	14.3% (2/14)

Embryo collection rates

Table 3 shows the results of embryo collection. When embryo collections yielded one or more embryos, one of the embryos was assumed to result from the fertilization of the oocyte from the preovulatory follicle. Recovery rates of embryos in excess compared to the number of ovulations were calculated. Recovery rates after OT (32.5%, 13/40) were significantly different from the 5.5% (3/55) observed in the Post-IVM IFOT group (p=0.001) and the 12.8% (6/47) of the Immediate IFOT group (p=0.026).

Table 3 : Results of collected embryos

	No of transfers	No of pregnant	No of transferred oocytes	No of embryos recovered	No of embryos recovered in excess	Embryo Collection Rate
Immediate-IFOT	10	4	47	10	6	12.8% (6/47) [a]
Post-IVM IFOT	8	4	55	7	3	5.5% (3/55) [a]
OT	6	5	40	13	13	32.5% (13/40) [b]

[a, b] Values with a different superscript are statistically different ($p<0.05$)

Following IFOT, 3 mares showed a preovulatory follicle that became diffusely echogenic and

showed ultrasonographic signs of clotting. Table 4 shows the results of embryo collections without these 3 transfers where mares failed to ovulate. Embryo collection rates were recalculated considering only successful transfers. Recovery rates after OT (32.5%, 13/40) were significantly different from the 5.8% (3/52) observed in the Post-IVM IFOT group (p=0.001) and the 14.6% (6/41) of the Immediate IFOT group (p=0.058).

	No of transfers	No of pregnant	No of transferred oocytes	No of embryos recovered	No of embryos recovered in excess	Embryo Collection Rate
Immediate-IFOT	8	4	41	10	6	14.6% (6/41) [a,b]
Post-IVM IFOT	7	4	52	7	3	5.8% (3/52) [a]
OT	6	5	40	13	13	32.5% (13/40) [b,c]

Table 4 : Results of collected embryos for successful transfers*

[a, b, c] Values with a different superscript are statistically different ($p<0.05$)

*: transfers were considered successful when ovulation occurred and transferred oocytes were released.

Table 5 shows the per transfer distribution of number of transferred oocytes and embryos collected in excess in both Immediate and Post-IVM groups.

	Immediate IFOT		**Post-IVM IFOT**	
Transfer number	No of transferred oocytes	No of embryos recovered in excess	No of transferred oocytes	No of embryos recovered in excess
1	4	2	5	0
2	2	0*	9	0
3	3	0	3	0*
4	10	2	7	0
5	8	1	4	0
6	4	1	9	1
7	4	0*	14	2
8	6	0	4	0
9	2	0	-	-
10	4	0	-	-
Total	47	6	55	3

Table 5: Per transfer distribution of number of transferred oocytes and embryos collected in excess in both Immediate and Post-IVM groups

*: refers to transfers where no ovulation occurred and a Corpus Hemorrhagicum was observed.

Discussion

Our oocyte collection rate of 55.6% after puncture is similar to those reported in the literature (Bogh *et al.*, 2002; Galli *et al.*, 2007), although lower collection rates have also been reported (Mari *et al.*, 2005). The maturation rate of 71% we observed is similar to those previously reported ranging from 40% to 70% in the equine (Goudet *et al.*, 1997a; Bogh *et al.*, 2002; Hinrichs *et al.*, 2005; Galli *et al.*, 2007). One of the 14 oocytes matured *in vitro* had initially been prepared for one of the immediate IFOT. However, it was found in the syringe after the transfer and subsequently matured *in vitro*.

Intra-follicular oocyte transfer has been first unsuccessfully attempted in different species, probably due to damage caused to the follicle during manipulations (Fleming *et al.*, 1985). Thanks to the large size of the ovary, its specific anatomy with a peripheral medulla and a surrounding fibrous tunique, the mare appears to be a unique model for the study of IFOT. Hinrichs and Di Giorgio (Hinrichs and Digiorgio, 1991) developed the technique of IFOT in the horse and obtained embryos in excess after transferring immature oocytes into preovulatory follicles. It was subsequently used by Goudet et al (Goudet *et al.*, 1997b) to study follicular contents and maturation rates. Surprisingly, no further studies about IFOT in the mare have been reported probably due to rather poor results of early reports. The recovery of excess embryos following intra-follicular transfer of oocytes matured *in vitro* in our study shows that they were ovulated, fertilized in the recipient's oviduct before progressing to the uterus. This is the first report of embryonic development following intra-follicular transfer of *in vitro* matured oocytes. Embryo collection rates following immediate transfer of ovum pick up oocytes of 12.8% (6/45) did not differ significantly from those observed following transfer of *in vitro* matured oocytes (5.5%; 3/55). This could mean that *in vitro* maturation of oocytes for 30 hours under our conditions and subsequent passage into the preovulatory follicle of a recipient for 0 to 18 hours is comparable to *in vivo* maturation within a preovulatory follicle. Alternatively, this could mean that although no statistical difference could be shown in our experiment, IVM oocytes suffer some inadequate maturation when compared to *in vivo* matured oocytes or that their expulsion at the time of ovulation is impaired. This is supported by Hinrichs and DiGiorgio's observation (Hinrichs and Digiorgio, 1991) who reported that only 3 recent oocytes out of 12 transferred could be recovered from one euthanized mare's

oviduct 3 days after transfer. Inadequate IVM can yield under- or overmature oocytes which will not undergo normal fertilization and embryo development (Hinrichs and Digiorgio, 1991).

Although echogenic spots have been visualized in some follicles prior to a normal ovulation (Carnevale *et al.*, 1988), echogenic spots and fibrous strands are more commonly associated with hemorrhagic, anovulatory follicles (Ghinter. O.J, 1986; Goudet *et al.*, 1997b). Hemorrhagic follicles have been reported with a spontaneous occurrence of 4.7% (12/255) in the mare (Ginther and Pierson, 1989). We observed 3 anovulatory hemorrhagic follicles following a total of 18 intra-follicular transfers (16,6%), this is comparable to the 15,8% (3/19) Hinrichs and Di Giorgio observed (Hinrichs and Digiorgio, 1991), but statistically ($p<0.05$) different from the 66,6% previously reported by Goudet et al. (Goudet *et al.*, 1997b) who punctured the same follicles twice during the oestrus phase. These results suggest that IFOT procedure increases the occurrence of hemorrhagic follicles probably due to transfer induced damage as invoked by Fleming et al (Fleming *et al.*, 1985). When the 3 transfers with anovulatory hemorrhagic follicles were not considered, embryo collection rates showed that although a trend was still observable ($p=0.058$), OT was no longer statistically different from IFOT immediately after oocyte recovery. One limitation of IFOT seems to be the increased occurrence of anovulatory hemorrhagic follicles, which appears to be related to puncture technique and repetition. In order to minimize trauma to the follicle, attempts with thinner needles were performed in a preliminary study. However, these needles tended to bend and did not allow proper puncture of the follicles. Different approaches for the puncture, either more superficial or going through as much ovarian tissue as possible before reaching the preovulatory follicle should be compared to see if they can reduce occurrence of hemorrhagic follicles. Intra-follicular transfer of immature oocytes is a relatively easy to perform and inexpensive procedure compared to surgical intra-oviductal oocyte transfer. It is less invasive as it avoids the need for surgery and the donor mare can easily be used as her own recipient to yield numerous embryos. Improving the technique, and thus reducing the risk of anovulation, might make the IFOT a viable option to increase the number of embryos of selected mares as superovulation treatments are yet not very efficient in the mare (Dippert and Squires, 1994; Bezard *et al.*, 1995; Alvarenga *et al.*, 2001b). It could also be used to assess the quality of oocytes after IVM by their ability to undergo *in vivo* fertilization as suggested by Hinrichs and DiGiorgio (Hinrichs and Digiorgio, 1991).

Intra-oviductal oocyte transfer is now considered a viable clinical alternative to embryo transfer in selected mares (Hinrichs *et al.*, 2002; Carnevale, 2004). We collected 13 embryos on day 10 after transfer of 40 *in vitro* matured oocytes resulting in a collection rate of 32.5%. This is higher than rates of 7% (2/29) (Scott *et al.*, 2001), 15% (11/73) and 18% (13/73) (Preis *et al.*, 2004) previously reported but comparable to Zhang's results who collected 7 embryos following transfer of 29 *in vitro* matured abattoir oocytes (24.1%) (Zhang *et al.*, 1989). However, even when expressed in relation to our *in vitro* maturation rate of 71%, the embryo yield of 45.8% remains lower than embryo collection rates or pregnancies obtained following transfer of preovulatory *in vivo* recovered oocytes ranging from 73 to 83% (Carnevale and Ginther, 1995; Hinrichs *et al.*, 1998; Carnevale *et al.*, 2004b). The recovery rate of embryos in excess following OT we observed is statistically different from that observed after IFOT, either immediately after oocyte recovery or post IVM. IVM oocytes were matured for 30 hours before transfer either to the preovulatory follicle or the oviduct. Oocytes were readily available for fertilization in the OT group. Those from the Post-IVM IFOT group were released into the oviduct within 18 hours of transfer. A possible explanation for the poor recovery rate of embryos in the Post-IVM IFOT group could be that oocytes were over-matured when ovulation and fertilization could occur. More regular rectal examinations to more accurately determine the exact timing of ovulation could help supporting this hypothesis.

Our results show that, in comparison to IFOT, OT is the most reliable *in vivo* alternative to *in vitro* fertilization where ICSI technology is not available.

Conclusion

Our results support previous work that immature oocytes can be matured within the follicle of a mare *in vivo*, and be ovulated, fertilized and yield excess embryos following IFOT. In addition, we show for the first time that *in vitro*-matured oocytes can be used for IFOT and result in excess embryos. General success rate of IFOT was low but tended to be higher when immature oocytes were transferred. Improvement of the technique could turn it into an inexpensive, easy to perform procedure, which could be an answer to the relative inefficiency of superovulation treatments in the mare.

We also established that, when ICSI is not an option, intra-oviductal oocyte transfer is to be preferred to IFOT as an *in vivo* alternative to bypass the inadequacy of conventional *in vitro* fertilization and to assess oocyte developmental competence.

3. Influence of cysteamine on *in vitro* maturation and *in vivo* fertilization of equine oocytes

Introduction

Research on IVM in mares is impeded by the limited availability of mature oocytes and early embryos for study. Superovulation treatments are not very efficient in the mare (Dippert and Squires, 1994; Bezard *et al.*, 1995; Alvarenga *et al.*, 2001a). Ovaries recovered from slaughterhouses provide a material with an obvious lack of repeatability, delay in time between collection and placement in culture medium and lack of information about the stage of cycle and follicular growth. Transvaginal ultrasound guided follicular aspiration has become an alternative source for mature and immature equine oocytes since it has been first described by Bruck et al. in 1992 (Bruck *et al.*, 1992). Immature oocytes collected by transvaginal ultrasound guided follicular aspiration can undergo IVM. However, success rates of IVM of equine oocytes (40% to 70%) (Squires, 1996; Goudet *et al.*, 1997a; Bogh *et al.*, 2002) remain low in all culture media tested compared to other species such as cows, goats and sows, (≥ 90%) (Goudet *et al.*, 2000a).

In order to improve the IVM maturation rates and quality of equine oocytes, the addition of compounds that can protect the cell from oxidative damage has been used such as glutathione (GSH) (Grupen *et al.*, 1995; de Matos *et al.*, 1996). During growth and maturation of the oocyte within the ovary, intracellular GSH content increases as the oocyte approaches the time of ovulation (Perreault *et al.*, 1988; Yoshida *et al.*, 1993; de Matos and Furnus, 2000). The addition of GSH to IVM medium also improves the rate of male pronuclear formation (de Matos *et al.*, 1995; de Matos and Furnus, 2000). As already mentioned, GSH synthesis can be increased and thus the intracellular concentration of GSH by the addition of low molecular weight thiols to IVM medium, such as cysteamine and ß-mercaptoethanol (Takahashi *et al.*, 1993). This has improved *in vitro* maturation rates in the canine (Hossein *et al.*, 2007) but not in the bovine (de Matos *et al.*, 1995) or the porcine (Grupen *et al.*, 1995), nevertheless, it has increased the success of bovine (de Matos *et al.*, 1995; de Matos *et al.*, 1996), ovine (de Matos *et al.*, 1999) and porcine (Grupen *et al.*, 1995; Bing *et al.*, 2001) embryo development. However, the influence of cysteamine on IVM and subsequent *in vivo* fertilization rates has

not been reported in mares.

The assessment of the success of IVM and IVF techniques is difficult because no reliable feature of cytoplasmic maturation has been defined in the mare whereas nuclear maturation is readily assessed by nuclear fluorescent microscopy. There have been few papers about the ultrastructure (Grondahl *et al.*, 1995; Tremoleda *et al.*, 2001; Carneiro *et al.*, 2002) and the biochemical changes (Goudet *et al.*, 1998a; Goudet *et al.*, 1998b) of oocytes during cytoplasmic maturation in the mare (Neumann *et al.*, 1995; Goudet *et al.*, 1997a). Therefore, the best current indicator of effective cytoplasmic maturation of the oocyte remains the ability of the oocyte to produce an embryo.

In order to circumvent the inadequacy of conventional IVF, other methods to produce embryos from oocytes have been investigated, such as intra cytoplasmic sperm injection (ICSI) (Dell'Aquila *et al.*, 1997a; Dell'Aquila *et al.*, 1997b; Choi *et al.*, 2002a; Galli *et al.*, 2002), transfer of an immature oocyte into the preovulatory follicle of an inseminated recipient mare (Intra-Follicular Oocyte Transfer, IFOT) (Hinrichs and Digiorgio, 1991; Goudet *et al.*, 1997b) and oocyte transfer to oviducts of a recipient mare (McKinnon *et al.*, 1988; Zhang *et al.*, 1989; Ray *et al.*, 1994; Carnevale and Ginther, 1995; Hinrichs *et al.*, 1997; Scott *et al.*, 2001; Hinrichs *et al.*, 2002; Carnevale, 2004; Preis *et al.*, 2004; Carnevale *et al.*, 2005).

Our second study showed that when ICSI is not an option, intra-oviductal oocyte transfer is to be preferred to IFOT as an *in vivo* alternative to bypass the inadequacy of conventional *in vitro* fertilization and to assess oocyte developmental competence.

Aims of the study:

The broad aim of this study was to improve IVM and IVF procedures in the mare. The specific aim was to investigate if there is an influence of supplementation with 100 µM of cysteamine on *in vitro* nuclear and cytoplasmic maturation by the ability of oocytes to undergo *in vivo* fertilization.

Material and methods

Animals, ovarian activity monitoring, follicular puncture oocyte recovery, were similar to what has been described in study 1 and 2.

After recovery the cumulus oocyte complexes (COCs) were isolated and classified according to cumulus morphology into expanded cumulus or compact cumulus (Goudet *et al.*, 1998b). COCs with expanded cumulus were discarded. Compact COCs were individually matured at 38,5°C in a humidified atmosphere of 95% air and 5% CO_2 for 30 h in 20µl of maturation medium under mineral oil (M8410 Sigma, France). Two maturation media were used. Medium 1 contained Medium 199 with Earle's salt (M4530, Sigma, France) supplemented with 20% inactivated fetal calf serum (FCS, F4135, Sigma, France) and with 50ng/ml Epidermal Growth Factor (EGF, E4127, Sigma, France) as described by Goudet et al. (Goudet *et al.*, 2000a). Medium 2 was the same base with the addition of cysteamine at 100µM (M9768, Sigma, France).

Oocyte intra-oviductal transfer

Depending on puncture session yields, oocytes were transferred or analysed after IVM. If the puncture session yielded ≥10 oocytes, 10 oocytes were prepared for oocyte transfer. Any additional oocytes were prepared for post IVM examination. When puncture sessions yielded ≤10 oocytes, they were all prepared for post IVM examination. Oocytes for intra-oviductal transfer were either prepared and transferred surgically in recipients'oviduct as described in study 2 or stained for nuclear maturation assessment as described in study 1 and 2.

Embryo collection and maternity testing

Embryo collection was performed 10 days after surgery as previously described. Embryos were rinsed 10 times in saline and kept at -20°C until maternity test. For maternity testing (Guerand *et al.*, 1997), DNA of each embryo was extracted and typed by radio active PCR amplification using 10 characterized micro-satellites (HMS 1, 2, 5, 6, 7, 8 and HG 3, 4, 6 and 10).

Statistical analysis

To compare maturation rates and embryo yields, a chi-square test was performed. Significance was established at $p < 0.05$.

Results

Oocytes collection

A total of 237 oocytes were obtained from 479 immature follicles resulting in a recovery rate of 48,5%. 108 oocytes were maturated in medium 1 (Control group) and 124 in medium 2 (Cysteamine group).

Maturation rates

Five oocytes out of the 327 collected were damaged or lost and were not cultured.

Table 6 shows the percentage of nuclear maturation of the non-transferred oocytes.

Overall maturation rate was 52%. Nuclear maturation was not statistically different ($p>0.05$) between oocytes cultured with or without cysteamine (55% and 47% respectively).

	Nuclear stage				
IVM medium	Metaphase II	Metaphase I	Dense chromatin	Germinal vesicle	Degenerated
Control	47% (24/51)	12% (6/51)	12% (6/51)	6% (3/51)	23% (12/51)
Cysteamine	55% (37/67)	10% (7/67)	8% (5/67)	3% (2/67)	24% (16/67)
Total	52% (61/118)	11% (13/118)	9% (11/118)	4% (5/118)	24% (28/118)

Table 6: Evaluation of nuclear maturation after culture

Oocyte transfers

Six transfers were performed for a total of 57 oocytes in each group. Three oocytes were found in the pipette after one surgery in the control group. Only 7 oocytes from the cysteamine group were transferred during the next surgery to keep groups even.

Embryo collection

Five embryos were obtained from 57 transferred oocytes (9%) in the cysteamine group and ten embryos from 57 transferred oocytes (17%) in the control group (Table 7). Those two percentages were not significantly different ($p>0.05$).

IVM medium	No of recipients	No of pregnant	No transferred oocytes	No of embryos Total*	Embryos from the recipient	Embryos from the donors
Control	6	6	57	15	5	10
Cysteamine	6	5	57	10	5	5
Total			114	25	10	15

Table 7: Number of collected embryos and result of maternity testing.

* N embryos total refers to the embryos emerging from the oocytes of both the donor and the recipient mares.

Discussion

The overall maturation rate of 52% we observed is similar to previous reports, which vary from 40% to 70% in the equine (Squires, 1996; Goudet *et al.*, 1997a; Bogh *et al.*, 2002). Higher values are usually observed when oocytes are matured in groups (Shabpareh *et al.*, 1993; Dell'Aquila *et al.*, 1996). Ideally, groups of 10 to 20 oocytes are cultured, which suits best conditions where a large number of oocytes are available. However oocytes were matured individually in our study so maturation conditions would be the same regardless the number of oocytes yielded by the ovum pick up session. Hinrichs et al (Hinrichs *et al.*, 2005) recently reported meiotic resumption rates over 70% after individual maturation, with an actual metaphase II rate around 50% which is comparable to our results. Maturation rates as assessed by the percentage of oocytes reaching metaphase II were not statistically different with or without cysteamine supplementation in our study. This is consistent with other reports in various species where cysteamine supplementation failed to affect metaphase II rates (Yamauchi and Nagai, 1999; Oyamada and Fukui, 2004; Kobayashi *et al.*, 2006; Luciano *et al.*, 2006; Balasubramanian and Rho, 2007; Song and Lee, 2007; Zhou *et al.*, 2008)

A concentration of 100 µM of cysteamine was used in the present study. Reported concentrations in various species range from 50 to 500 µM with highly variable results (de Matos *et al.*, 1995; Grupen *et al.*, 1995; Yamauchi and Nagai, 1999; Bing *et al.*, 2002; de Matos *et al.*, 2002a; Gasparrini *et al.*, 2003; Whitaker and Knight, 2004). Grupen et al (Grupen *et al.*, 1995), who compared concentrations of 50 and 500 µM of cysteamine in IVM medium for porcine oocytes found that both increased synchronous pronuclear formation whereas only the higher concentration significantly enhanced embryonic development. They suggested that cysteamine in excess allows cytoplasmic accumulation of thiol compounds during IVM which may persist in the cleaving embryo and assist mitotic cell division. Conversely, Gasparrini et al (Gasparrini *et al.*, 2000) working on buffalo oocyte, found that supplementation with 50 µM of cysteamine significantly improved *in vitro* embryo production whereas concentrations of 100 and 200 µM did not affect cleavage rate compared to control group. Similar results were observed in a recent study where cysteamine was added to IVM and IVC medium (Anand *et al.*, 2008). It appears effect of cysteamine supplementation to IVM medium is highly species and concentration dependant. The

inadequacy of the chosen concentration may explain that equine embryo production has not been increased by the cysteamine under our conditions as opposed to what has been observed in other species such as bovine (Takahashi *et al.*, 1993; de Matos *et al.*, 1995; de Matos *et al.*, 1996); porcine (Grupen *et al.*, 1995; Bing *et al.*, 2001); ovine (de Matos *et al.*, 2002a); and buffalo (Gasparrini *et al.*, 2003). However, our results are in agreement with Luciano et al. (Luciano *et al.*, 2006) who recently demonstrated that the addition of 100 µM of cysteamine during *in vitro* maturation of equine oocytes does not influence GSH synthesis.

Alternatively, contents of IVM medium itself should be considered as a potential reason for the absence of effect of cysteamine supplementation in our study. We can hypothesize that some substances present in the IVM medium can interfere with GSH synthesis. Bing et al (Bing *et al.*, 2001) suggested that FSH and estradiol both affected GSH levels in porcine oocytes which in turn increased maturation rates. It has also been suggested that gonadotropins regulate ovarian GSH synthesis (Luderer *et al.*, 2001). Although our maturation medium is not supplemented with gonadotropins or estradiol, factors contained in fetal calf serum or EGF might have an effect on GSH synthesis.

In regards to our overall maturation rate of 52% following IVM, it can be considered that only roughly 59 metaphase II oocytes were actually transferred. Consequently, 15 blastocysts were obtained from 59 genuinely fertilizable oocytes (26%), which is similar to what we (Daels *et al.*, 2001) and others (Carnevale *et al.*, 2005) obtained in previous studies where preovulatory *in vivo* recovered oocytes were transferred. The global results, 15 embryos from 114 transferred oocytes giving a 13 % recovery rate can be compared to those reported in other studies. From 29 oocytes aspirated during diestrus stage Scott et al. (Scott *et al.*, 2001) obtained 2 pregnancies on day 16 (7%) following *in vitro* maturation and *in vivo* fertilization. Zhang et al. (Zhang *et al.*, 1989) obtained 7 embryos from 29 oocytes from slaughterhouse matured *in vitro* and fertilized *in vivo*. Hinrichs et al. (Hinrichs *et al.*, 2002) obtained a high *in vivo* fertilization rate of 77% after *in vitro* maturation of abattoir oocytes. The recipient mares were euthanatized 40 to 44 hours after transfer and oocytes were recovered from the oviduct. When comparing the percentage of embryos per transferred oocytes, 18% of the oocytes recovered from the oviduct had undergone cleavage (2 cells or more). The discrepancy between the fertilization rate and the relatively low embryo recovery rate in all studies combined may suggest that the percentage of embryos able to reach the blastocyst stage is probably lower. This may be due to a blockage of embryonic development of the embryos and/or to an intra-fallopian embryonic reduction. This gives a new light to overall low results

obtained following uterine collection. This confirms that in the absence of a reliable marker of cytoplasmic maturation, the ultimate test to appreciate the quality of a gamete following *in vitro* culture is by obtaining at least a blastocyst.

Conclusion

Under our conditions, the addition of 100 µM of cysteamine to a classic culture medium does not improve equine oocyte maturation or embryonic development after oocyte transfer. Considering its beneficial effects in many other species, supplementation with cysteamine to different IVM media should be further investigated; ideally combining different concentrations and ICSI in order to determine an optimal concentration and its effects on cleavage rates and early embryonic development.

PART 4 : GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

Part 4 : General discussion, conclusion and perspectives

In vivo matured oocytes represent a gold standard for ARTs. The mare is mono ovulating and to date, there is no efficient superovulation treatment available. This translates into a very short supply of *in vivo* matured oocytes, which has seriously limited the development of ARTs in comparison to other species. Research requires large numbers of oocytes, which cannot be covered by *in vivo* matured oocytes. In that context, the quality and the competence of *in vitro* matured oocytes is a keystone to assisted reproduction techniques. However, most studies have focused on the percentage of oocytes reaching the metaphase II stage (nuclear maturation) but few concentrated on the final oocyte competence as measured by its ability to develop into a blastocyst and further establish an ongoing pregnancy. This depends not only on the embryo production conditions (IVM, IVF, IVC), but also on the intrinsic quality of the oocyte. Aspiration of immature follicles permits retrieval of more oocytes and IVM skips the final selection of oocytes during the final steps of folliculogenesis. *In vitro* processing of oocytes thus permits the maturation of oocytes otherwise destined to undergo atresia. It can be speculated that, at the time of aspiration, some oocytes have already started this maturation process while some others have not. This could explain the population heterogeneity of aspirated oocytes and the subsequent variable response to IVM. This explains the need of perfectly adapted conditions of IVM to ensure an optimal oocyte competence acquisition. Unfortunately, to date there is no marker of oocyte cytoplasmic maturation available. Such a marker could prove very usefull in assessing oocyte development competence. This could possibly allow adapting IVM conditions to specific requirements of oocytes, based on their stage of development at the time of retrieval. Recent improvement in IVM conditions have recently been shown to improve cleavage rates and blastocyst development after ICSI, despite their lack of significant effect on maturation rates (metaphase II) (Galli *et al.*, 2007). This clearly illustrates the crucial importance of the quality of IVM on the further oocyte competence and the need to further investigate IVM conditions.

Under *in vitro* culture conditions, oxidative alterations of cell components via increased ROS represent a major culture induced stress. In order to improve the IVM maturation rates and quality of oocytes, the addition of compounds that can protect the cell from oxidative damage has been investigated. The addition of GSH synthesis precursors, such as cysteamine, a thiol

compound, to IVM media has been shown to improve IVP in various species. Very little information on the use of thiol compounds in the equine was available.

Our first objective was to investigate if there is an influence of supplementation with 100 µM of cysteamine on conventional IVF success rate. In our first experiment, oocytes were matured *in vitro* in a medium supplemented with cysteamine and then submitted to conventional IVF. The IVF rates we observed (6%), although rather low, were comparable to others reported in the horse (Choi *et al.*, 1994; Dell'Aquila *et al.*, 1997a). Although, it seems likely that cysteamine did not significantly improve IVF rates under our conditions, our general success rates for IVF procedures may be too low for us to conclude definitely about the effect of cysteamine. This illustrates how the lack of an efficacious and reproductible conventional IVF procedure is a major limitation in assessing oocyte development competence in the equine.

Our second objective was to determine what *in vivo* technique could best bypass the lack of an efficient conventional IVF procedure. Although ICSI is now providing fertilization rates (40%) (Dell'Aquila *et al.*, 1997a; Dell'Aquila *et al.*, 1997b; Grondahl *et al.*, 1997) that make it a valuable and repeatable means of producing embryos *in vitro*, it requires specific equipment and expertise, which are not easily available in all laboratories. As an alternative to conventional IVF and ICSI, it was decided to explore which *in vivo* fertilization option was to be preferred. Oocyte transfer following IVM, intra-follicular oocyte transfer following IVM, and intra-follicular oocyte transfer immediately after oocyte collection were tested as alternative techniques to ICSI. Embryo collection rates were compared and that following oocyte transfer was significantly higher than that following both intra-follicular oocyte transfer techniques. We concluded that when ICSI is not an option, intra-oviductal oocyte transfer is to be preferred to IFOT as an *in vivo* alternative to bypass the inadequacy of conventional *in vitro* fertilization and to assess oocyte developmental competence.

Our final aim was to investigate if there is an influence of supplementation with 100 µM of cysteamine on *in vitro* nuclear and cytoplasmic maturation by specific DNA staining and the ability of oocytes to undergo *in vivo* fertilization. Oocytes were retrieved by ultrasound guided aspiration, submitted to IVM with or without cysteamine supplementation and were either stained with Hoechst for nuclear maturation assessment or transferred into recipients'oviduct. Embryo collection rates following oocyte transfer, were compared to assess cytoplasmic maturation and oocyte development competence. Maturation rates after

DNA staining were not statistically different with or without cysteamine supplementation in our study. This is consistent with other reports in various species where cysteamine supplementation failed to affect metaphase II rates (Yamauchi and Nagai, 1999; Oyamada and Fukui, 2004; Kobayashi *et al.*, 2006; Luciano *et al.*, 2006; Balasubramanian and Rho, 2007; Song and Lee, 2007; Zhou *et al.*, 2008). This is also further supported by another study where the same dose of cysteamine failed to increase GSH content of equine oocytes (Luciano *et al.*, 2006). Effect of cysteamine has been shown to be highly species and dose dependant. The dose used in this study was 100 µM. Although this has been used most commonly in various species, the inadequacy of the chosen concentration may explain that equine embryo production has not been increased by cysteamine under our conditions. Alternatively, contents of IVM medium itself should be considered as a potential reason for the absence of effect of cysteamine supplementation in our study. We can hypothesize that some substances present in the IVM medium can interfere with GSH synthesis. Bing et al (Bing *et al.*, 2001) suggested that FSH and estradiol both affected GSH levels in porcine oocytes which in turn increased maturation rates. It has also been suggested that gonadotropins regulate ovarian GSH synthesis (Luderer *et al.*, 2001). Although our maturation medium is not supplemented with gonadotropins or estradiol, factors contained in fetal calf serum or EGF might have an effect on GSH synthesis. These differences between media might also be explained by the presence and the amount of different factors that can help protect the cell against oxidative stress such as citrate (Holm *et al.*, 1999) and pyruvate (Morales *et al.*, 1999). Moreover, it is important to remember that TCM-199, which is used in most media, contains cystine (83.2 µM) and cysteine (0.11mg/L) and that whether it is sufficient to sustain oocyte IVM of different species remains unknown (Zhou *et al.*, 2008).

Under our conditions, the addition of 100 µM of cysteamine to a classic culture medium does not improve equine oocyte nuclear maturation or embryonic development after oocyte transfer. Considering its beneficial effects in many other species, where it has been shown to be highly on species and concentration dependant, supplementation with cysteamine to different IVM media should be further investigated. Ideally, different concentrations of cysteamine should be tested and development competence should be assessed after OT and after ICSI in order to determine the effect on early embryonic development and pregnancy rates.

Success rates of IVM of equine oocytes (40% to 70%) (Squires, 1996; Goudet *et al.*, 1997a; Bogh *et al.*, 2002) remain low in all culture media tested compared to other species such as cows, goats and sows, (≥ 90%) (Goudet *et al.*, 2000a). Since very high maturation rates have been achieved in most species, it has been assumed that IVM could no longer be a limiting step and research has focused on the next steps of IVP such as IVF, ICSI or IVC. However, most studies use the percentage of oocytes reaching the metaphase II stage to assess oocyte maturation. Fertilization of an oocyte requires both its nuclear and cytoplasmic maturation. Despite their apparent maturation it is likely that post-IVM oocytes do not necessarily achieve their cytoplasmic maturation and gain their development competence. This incomplete or faulty cytoplasmic maturation only gets to be revealed once oocytes are fertilized and embryo development appears to be impaired.

This work illustrates that although IVM appears to be successful in most species there is still a crucial need for improvement. Improving IVM conditions, and gaining a better understanding of the oocyte competence acquisition is a necessary step to improve the overall *in vitro* embryo production, in all species in general and in the equine in particular.

PART 5 : REFERENCES

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PART 6 : SCIENTIFIC PAPERS

Part 6 : Scientific papers

EFFICIENCY OF EMBRYONIC DEVELOPMENT AFTER INTRA-FOLLICULAR AND INTRA-OVIDUCTAL TRANSFER OF *IN VITRO* AND *IN VIVO* MATURED HORSE OOCYTES

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Abstract

In vivo techniques, such as intra-oviductal Oocyte Transfer (OT) and Intra-Follicular Oocyte Transfer (IFOT) can be considered as alternatives to bypass the lack of efficient superovulation treatments and the inadequacy of conventional *in vitro* fertilization techniques in the horse. We compared embryo production following transfer of *in vivo* recovered oocytes (1) into a recipient's oviduct or (2) into her preovulatory follicle either immediately after ovum pick up or (3) after *in vitro* maturation. Recipients were inseminated with fresh semen of a stallion with a known normal fertility. Ten days after surgery, rates of embryos collected in excess to the number of ovulations were calculated and compared for each group. Embryo collection rates were 32.5% (13/40), 5.5% (3/55) and 12.8% (6/47) for OT, post-IVM and immediate IFOT respectively. OT significantly yielded more embryos than immediate and post-IVM IFOT did. We also showed that *in vitro* matured oocytes could succesfully be used for IFOT. Our results also suggest that improvement of the IFOT technique could turn it into an inexpansive and easy to perform procedure that could be an answer to the inefficiency of superovulation treatments in the mare.

Keywords: horse, oocyte, transfer, intra-follicular, IVM

1. Introduction

Conventional *in vitro* fertilization (IVF) has not been successful in the mare, and a repeatable IVF technique has not yet been developed, possibly due to the lack of an efficient *in vitro* capacitation system for stallion spermatozoa [1]. To overcome the limitation of conventional IVF procedures, other methods to produce embryos from oocytes, either *in vivo* or *in vitro*, have been investigated. Among these, intra cytoplasmic sperm injection (ICSI) [2-5] has recently permitted efficient equine *in vitro* blastocyst production [6-8]. However, ICSI requires specific equipment and skills and the spermatozoon is still arbitrary chosen as, to date, no selection criteria for the male gamete have been established. Transfer of an immature oocyte into the preovulatory follicle of an inseminated recipient mare (Intra-Follicular Oocyte Transfer, IFOT) has produced embryos but the success rate was low [9,10]. These early studies used immature oocytes. Collection of embryos following intra-follicular transfer of *in vitro* matured oocytes would assess their cytoplasmic maturation as shown by their ability to produce an embryo. To our knowledge no intra-follicular transfer of oocytes previously matured *in vitro* has been reported. Similarely, oocyte transfer (OT) into the oviduct of an inseminated recipient mare was investigated. Early studies showed a rather low blastocyst development [11-13]. Carnevale and Ginther [14] first reported higher embryo production rates following transfer of *in vivo* matured oocytes. More recent studies transferring a single oocyte collected from a preovulatory follicle 24 hours after an injection of hCG and matured *in vitro* for another 16 to 20 hours before transfer also showed high embryo production rates [15] and commercial programmes using OT for mares with reproductive abnormalities are now available [16]. Intra-oviductal oocyte transfer can nowadays be considered a viable clinical alternative to embryo transfer in selected mares [17,18]. Birth of a foal following transfer of *in vitro* matured oocytes collected from ovaries after the death of the donor mare was first reported in 2003 [19]. The same authors reported embryo development rates of 15% following transfer of oocytes (n=191) recovered from commercial mares post-mortem [20].

In addition, OT can be used to assess the quality of oocytes after IVM by their ability to undergo *in vivo* fertilization [17,21]. However, using one oocyte from a preovulatory follicle, OT as it is generally actually performed, suffers the same limitation as embryo transfer to produce more than one embryo at a time. In the absence of an efficient superovulation treatment in the mare [22], transfer of immature oocytes into a preovulatory

follicle or transfer of *in vitro* matured oocytes either into a preovulatory follicle or into a recipient's oviduct are options to be considered. Unfortunately, although it is a non-surgical procedure, IFOT is poorly documented in the literature and reports of OT have been published by various laboratories and under various conditions, making comparisons between results and choosing among these as substitutive techniques to ICSI or embryo transfer difficult.

2. Aims of the study

The first aim of this study is to test if *in vitro* matured oocytes would complete their maturation and yield embryos following transfer into the preovulatory follicle of an inseminated recipient mare.

The second aim is to compare OT following IVM, IFOT following IVM, and IFOT immediately after oocyte collection as alternative techniques where ICSI is not available.

3. Materials and methods

The study was conducted in spring using 30 cycling Welsh pony mares from 200 to 300 kg. All the procedures have been approved by the Local Committee of Health Guide for Care and Use of Laboratory Animals. Ovarian activity was monitored daily using transrectal ultrasound scanning using an Aloka 210 with a 5MHz linear probe (Société Bernard, Nantes, France).

3.1. Follicular puncture and oocyte recovery

Mares with a preovulatory follicle ≥ 33 mm were injected i.v. with 25mg of Crude Equine Gonadotropin (CEG), produced in our lab [23], to induce ovulation. Transvaginal

ultrasound-guided aspiration of follicles ≥ 5 mm was performed immediately after CEG injections. Follicles were punctured routinely using a transvaginal aspiration technique as previously described [24]. Transrectal ultrasound was performed to detect ovulation 48 hours after CEG injection in all mares.

3.2. Culture of Cumulus Oocyte Complexes

After recovery, the cumulus oocyte complexes (COCs) were isolated and classified according to cumulus morphology into expanded cumulus and compact cumulus [25]. The same classification was used for post IVM examination. Collected oocytes were either matured *in vitro* for 30 hours or prepared for immediate transfer.

When matured *in vitro*, COCs were individually matured at 38,5°C in a humidified atmosphere of 95% air and 5% CO_2 for 30 h in 20µl of maturation medium under mineral oil (M8410 Sigma, Saint-Quentin Fallavier, France). Maturation medium contained Medium 199 with Earle's salt (M4530, Sigma, France) supplemented with 20% inactivated fetal calf serum (FCS, F4135, Sigma, France) and with 50ng/ml Epidermal Growth Factor (EGF, E4127, Sigma, France) as described by Goudet et al. [26]. Following IVM, no selection based on appearance was performed and intact COC's were assigned to either OT or Post-IVM IFOT group.

Oocytes from sessions that failed to yield more than 3 oocytes were cultured similarely and prepared for post IVM examination. They were rinsed in PBS and COCs were stripped with small glass pipettes, stained with 1 µg/ml bis-benzamide (Hoechst 33342; Sigma, France) and evaluated with a fluorescence microscope. Oocytes were classified according to the stage of nuclear maturation, membrane integrity and the ooplasm aspect as "germinal vesicle", "dense chromatin", "metaphase I", "metaphase II" or "degenerate" as previously described by Goudet et al. [25].

3.3. Preparation of oocytes before transfer

Oocytes for intra-follicular transfer were rinsed three times in 500 µl of PBS and loaded in 1ml of PBS into a 1ml syringe for transfer after IVM and in 0.2 to 0.5 ml of PBS with 1mL/L heparin (Heparin 5 IU/ml; Sanofi, France) for immediate transfer.

Oocytes for intra-oviductal transfer were rinsed three times in 500 µl of PBS after IVM and stored in PBS at 38.5°C. The system to aspirate the oocytes consisted of a blunted sterile glass pipette connected to a piece of tube, which was in turn connected to a 1ml syringe. The whole system was kept at 38.5°C. Oocytes were loaded just prior to the oocyte transfer, in a minimal volume of PBS between two bubbles of air.

3.4. Intra-Follicular Oocyte Transfer (IFOT)

Intra-follicular oocyte transfer was performed as previously described by Goudet et al [10]. Briefly, the preovulatory follicle was punctured using a single lumen needle (length, 600mm; outer diameter, 1.1mm, 17G; Thiebaud Frères, Jouvernex Margencel, France), with a specific attention to the needle going through as much ovarian stroma as possible before reaching the follicle. Then, 5 ml of follicular fluid were aspirated from the preovulatory follicle. The 1ml syringe containing the oocytes in PBS was connected to the 17 gauge needle, the oocytes were injected and the needle flushed with 4.5ml of follicular fluid. Immediately after transfer, the catheter and needle were checked to ensure that no oocytes remained logged in them. Recipient mares were injected with their own oocytes, either just after collection (Immediate Intra-Follicular Oocyte Transfer) or following IVM (Post-IVM Intra-Follicular Oocyte Transfer). Recipient mares were inseminated 24 hours after CEG injection, using fresh semen of a stallion with a known normal fertility: 400 X 10^6 spermatozoa in 10ml of extender (INRA96®; IMV Technologies, France).

3.5. Intra-oviductal Oocyte Transfer (OT)

Oocytes from preovulatory follicles were aspirated and discarded. Following their ovum pick up session, mares were fasted for 24 hours. Recipient mares were inseminated 5 h prior to surgery, using fresh semen of a stallion with a known normal fertility: 400 X 10^6 spermatozoa in 10ml of extender (INRA96®; IMV Technologies, France). Surgical transfers of the recipient mare's own oocytes occurred 30 hours after the oocyte recoveries. The recipient mare was treated with antibiotics (about 6 hours before surgery) (4,000,000 IU streptomycin, 4g procaine penicilline i.m., Intramicine®, Ceva, France). Animals were sedated with detomidine (3-5 µg/kg; 0.1ml/animal i.v. Domosedan®; Pfizer, France) for surgical preparation of the mare for the standing flank laparotomy. An injection of butorphanol tartrate 4mg (13-20 µg/kg; 0.4ml/animal i.v. Torbugesic®; Fort Dodge, Southampton, UK) for analgesia was followed by a second injection of detomidine (6-10 µg/kg, 0.2ml/animal i.v. Domosedan®). A cuffed urinary catheter was placed to allow continuous emptying of the bladder during the surgery in order to limit discomfort. After local anesthesia (lidocaïne, Laocaïne®, Schering Plough, France), a flank approach was used to gain access to the ovary. The skin incision was directed vertically or at a slight oblique and extended 10 to 15 cm. The muscles of the flank, external and internal abdominal oblique muscles and the transversus abdominis muscle were bluntly dissected in the direction of their fibres. The peritoneum was perforated and the opening was manually dilated. After the ovary was exteriorised and the pipette was inserted into the oviduct. Oocytes were gently injected into the oviduct. The different layers were closed using absorbable suture material (Vicryl® 0; Johnson and Johnson, UK) in an interrupted pattern. Immediately after surgery, a second insemination with 200 X 10^6 spermatozoa in 10ml of semen extender (INRA96®, IMV Technologies, France) stored at 15°C was performed.

3.6. Embryo collection

Embryo collection was performed 10 days after surgery by three successive uterine lavage with saline at 38°C. Collection fluid was filtered and embryos were isolated under a stereomicroscope.

3.7. Statistical analysis

To compare maturation rates and embryo yields, a chi-square test was performed. Significance was established at $p < 0.05$.

4. RESULTS

4.1. Oocyte collection

Following the 31 puncture sessions, a total of 158 oocytes were obtained from 284 immature follicles resulting in a recovery rate of 55,6%. Recovery rates ranged from 0% (0/7) to 100% (6/6). Global collection rate was 5.1 immature oocytes per puncture session (158/31).

4.2. Maturation rates

Out of the 158 oocytes, 47 were used for immediate IFOT, 109 oocytes were matured *in vitro* and two oocytes, which were degenerated, were not cultured. Out of these 109 *in vitro* matured oocytes, 55 were transferred into preovulatory follicles (IFOT), 40 were used for intra-oviductal oocyte transfer and 14 were used for analysis. Table 1 shows the percentage of nuclear maturation of the non-transferred oocytes.

4.3. Embryo collection rates

Table 2 shows the results of embryo collection. When embryo collections yielded one or more embryos, one of the embryos was assumed to result from the fertilization of the oocyte from the preovulatory follicle. Recovery rates of embryos in excess compared to the number of ovulations were calculated. Recovery rates after OT (32.5%, 13/40) were significantly different from the 5.5% (3/55) observed in the Post-IVM IFOT group ($p=0.001$) and the 12.8% (6/47) of the Immediate IFOT group ($p=0.026$).

Following IFOT, 3 mares showed a preovulatory follicle that became diffusely echogenic and showed ultrasonographic signs of clotting. Table 3 shows the results of embryo collections without these 3 transfers where mares failed to ovulate. Embryo collection rates were recalculated considering only successful transfers. Recovery rates after OT (32.5%, 13/40) were significantly different from the 5.8% (3/52) observed in the Post-IVM IFOT group ($p=0.001$) and the 14.6% (6/41) of the Immediate IFOT group ($p=0.058$).

Table 4 shows the per transfer distribution of number of transferred oocytes and embryos collected in excess in both Immediate and Post-IVM groups.

5. Discussion

Our oocyte collection rate of 55.6% after puncture is similar to those reported in the litterature [27,28], although lower collection rates have also been reported [29]. The maturation rate of 71% we observed is similar to those previously reported ranging from 40% to 70% in the equine [6,27,28,30]. One of the 14 oocytes matured *in vitro* had initially been prepared for one of the immediate IFOT. However, it was found in the syringe after the transfer and subsequently matured *in vitro*.

Intra-follicular oocyte transfer has been first unsuccessfully attempted in different species, probably due to damage caused to the follicle during manipulations [31]. Thanks to the large size of the ovary, its specific anatomy with a peripheral medulla and a surrounding fibrous tunique, the mare appears to be a unique model for the study of IFOT. Hinrichs and Di

Giorgio [9] developed the technique of IFOT in the horse and obtained embryos in excess after transferring immature oocytes into preovulatory follicles. It was subsequently used by Goudet et al [10] to study follicular contents and maturation rates. Surprisingly, no further studies about IFOT in the mare have been reported probably due to rather poor results of early reports. The recovery of excess embryos following intra-follicular transfer of oocytes matured *in vitro* in our study shows that they were ovulated, fertilized in the recipient's oviduct before progressing to the uterus. This is the first report of embryonic development following intra-follicular transfer of *in vitro* matured oocytes. Embryo collection rates following immediate transfer of ovum pick up oocytes of 12.8% (6/45) did not differ significantly from those observed following transfer of *in vitro* matured oocytes (5.5%; 3/55). This could mean that *in vitro* maturation of oocytes for 30 hours under our conditions and subsequent passage into the preovulatory follicle of a recipient for 0 to 18 hours is comparable to *in vivo* maturation within a preovulatory follicle. Alternatively, this could mean that although no statistical difference could be shown in our experiment, IVM oocytes suffer some inadequate maturation when compared to *in vivo* matured oocytes or that their expulsion at the time of ovulation is impaired. This is supported by Hinrichs and DiGiorgio's observation [9] who reported that only 3 recent oocytes out of 12 transferred could be recovered from one euthanized mare's oviduct 3 days after transfer. Inadequate IVM can yield under- or overmature oocytes which will not undergo normal fertilization and embryo development [9].

Although echogenic spots have been visualized in some follicles prior to a normal ovulation [32], echogenic spots and fibrous strands are more commonly associated with hemorrhagic, anovulatory follicles [10,33]. Hemorrhagic follicles have been reported with a spontaneous occurence of 4.7% (12/255) in the mare [34]. We observed 3 anovulatory hemorrhagic follicles following a total of 18 intra-follicular transfers (16,6%), this is comparable to the 15,8% (3/19) Hinrichs and Di Giorgio observed [9], but statistically ($p<0.05$) different from the 66,6% previously reported by Goudet et al. [10] who punctured the same follicles twice during the oestrus phase. These results suggest that IFOT procedure increases the occurence of hemorrhagic follicles probably due to transfer induced damage as invoked by Fleming et al [31]. When the 3 transfers with anovulatory hemorrhagic follicles were not considered, embryo collection rates showed that although a trend was still observable ($p=0.058$), OT was no longer statistically different from IFOT immediately after oocyte recovery. One limitation of IFOT seems to be the increased occurrence of anovulatory hemorrhagic follicles which appears to be related to puncture technique and repetition. In

order to minimize trauma to the follicle, attempts with thinner needles were performed in a preliminary study. However, these needles tended to bend and did not allow proper puncture of the follicles. Different approaches for the puncture, either more superficial or going through as much ovarian tissue as possible before reaching the preovulatory follicle should be compared to see if they can reduce occurrence of hemorrhagic follicles. Intra-follicular transfer of immature oocytes is a relatively easy to perform and inexpansive procedure compared to surgical intra-oviductal oocyte transfer. It is less invasive as it avoids the need for surgery and the donor mare can easily be used as her own recipient to yield numerous embryos. Improving the technique, and thus reducing the risk of anovulation, might make the IFOT a viable option to increase the number of embryos of selected mares as superovulation treatments are yet not very efficient in the mare [22,35,36]. It could also be used to assess the quality of oocytes after IVM by their ability to undergo *in vivo* fertilization as suggested by Hinrichs and DiGiorgio [9].

Intra-oviductal oocyte transfer is now considered a viable clinical alternative to embryo transfer in selected mares [17,18]. Intra-oviductal and intra-uterine single inseminations of recipients during oocyte transfer result in similar embryo production rates when fresh good quality semen is used [14,37,38]. We collected 13 embryos on day 10 after transfer of 40 *in vitro* matured oocytes resulting in a collection rate of 32.5%. This is higher than rates of 7% (2/29) [39], 15% (11/73) and 18% (13/73) [40] previously reported but comparable to Zhang's results who collected 7 embryos following transfer of 29 *in vitro* matured abattoir oocytes (24.1%) [13]. However, even when expressed in relation to our *in vitro* maturation rate of 71%, the embryo yield of 45.8% remains lower than embryo collection rates or pregnancies obtained following transfer of preovulatory *in vivo* recovered oocytes ranging from 73 to 83% [14,37,38]. The recovery rate of embryos in excess following OT we observed is statistically different from that observed after IFOT, either immediately after oocyte recovery or post IVM. IVM oocytes were matured for 30 hours before transfer either to the preovulatory follicle or the oviduct. Oocytes were readily available for fertilization in the OT group. Those from the Post-IVM IFOT group were released into the oviduct within 18 hours of transfer. A possible explanation for the poor recovery rate of embryos in the Post-IVM IFOT group could be that oocytes were over-matured when ovulation and fertilization could occur. More regular rectal examinations to more acurately determine the exact timing of ovulation could help supporting this hypothesis.

Our results show that, in comparison to IFOT, OT is the most reliable *in vivo* alternative to *in vitro* fertilization where ICSI technology is not available.

6. Conclusion

Our results support previous work that immature oocytes can be matured within the follicle of a mare *in vivo*, and be ovulated, fertilized and yield excess embryos following IFOT. In addition, we show for the first time that *in vitro*-matured oocytes can be used for IFOT and result in excess embryos. General success rate of IFOT was low but tended to be higher when immature oocytes were transferred. Improvement of the technique could turn it into an inexpensive, easy to perform procedure which could be an answer to the relative inefficiency of superovulation treatments in the mare.

We also established that, when ICSI is not an option, intra-oviductal oocyte transfer is to be preferred to IFOT as an *in vivo* alternative to bypass the inadequacy of conventional *in vitro* fertilization and to assess oocyte developmental competence.

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Table 1: Evaluation of nuclear maturation after culture

	Nuclear stage				
	Metaphase II	Metaphase I	Dense chromatin	Germinal vesicle	Degenerated
Total	71.3% (10/14)	0% (0/14)	7.2% (1/14)	7.2% (1/14)	14.3% (2/14)

Table 2 : Results of collected embryos

	No of transfers	No of pregnant	No of transferred oocytes	No of embryos recovered	No of embryos recovered in excess	Embryo Collection Rate
Immediate-IFOT	10	4	47	10	6	12.8% (6/47) [a]
Post-IVM IFOT	8	4	55	7	3	5.5% (3/55) [a]
OT	6	5	40	13	13	32.5% (13/40) [b]

[a, b] Values with a different superscript are statistically different ($p<0.05$)

Table 3 : Results of collected embryos for successful transfers*

	No of transfers	No of pregnant	No of transferred oocytes	No of embryos recovered	No of embryos recovered in excess	Embryo Collection Rate
Immediate-IFOT	8	4	41	10	6	14.6% (6/41) [a,b]
Post-IVM IFOT	7	4	52	7	3	5.8% (3/52) [a]
OT	6	5	40	13	13	32.5% (13/40) [b,c]

[a, b, c] Values with a different superscript are statistically different ($p<0.05$)

*: transfers were considered successful when ovulation occurred and transferred oocytes were released.

Table 4: Per transfer distribution of number of transferred oocytes and embryos collected in excess in both Immediate and Post-IVM groups

	Immediate IFOT		**Post-IVM IFOT**	
Transfer number	No of transferred oocytes	No of embryos recovered in excess	No of transferred oocytes	No of embryos recovered in excess
1	4	2	5	0
2	2	0*	9	0
3	3	0	3	0*
4	10	2	7	0
5	8	1	4	0
6	4	1	9	1
7	4	0*	14	2
8	6	0	4	0
9	2	0	-	-
10	4	0	-	-
Total	47	6	55	3

*: refers to transfers where no ovulation occurred and a Corpus Hemorrhagicum was observed.

INFLUENCE OF CYSTEAMINE ON *IN VITRO* MATURATION, *IN VITRO* AND *IN VIVO* FERTILIZATION OF EQUINE OOCYTES

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ABSTRACT

The effect of cysteamine on *in vitro* nuclear and cytoplasmic maturation of equine oocytes collected by transvaginal ultrasound guided follicular aspiration was assessed. Oocytes were matured *in vitro* with (cysteamine group) or without (control group) cysteamine. The nuclear stage after DNA Hoechst staining, penetration rates after 2 different IVF techniques (IVF media with ionophore and Hepes buffer with heparin) and the embryo yield following oocyte intra-oviductal transfer were used as a criterion for assessing nuclear and cytoplasmic maturation, respectively. Contrary to the data described in other domestic species, there was no effect of cysteamine on *in vitro* nuclear maturation, *in vitro* fertilization or *in vivo* embryonic development under our conditions.

Ovum pick up yields (52%) and maturation rates (control group: 47% and cysteamine group: 55%) were similar to those previously reported. From 57 oocytes transferred to the oviduct in each group, the number of embryos collected was 10 (17%) in the control group and 5 in the cysteamine group (9%). Those two percentages were not statistically different ($p>0.05$). No effect of IVF technique was seen on the success rate (6%) in each group.

Keywords : cysteamine, GIFT, OPU, oocyte, horse, IVF

INTRODUCTION

In vitro embryo production is the final result of a three step process: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of the embryo to a stage that is transferable to the uterus (Dell'Aquila et al. 1996, Hinrichs et al. 2002). In the mare, few studies have reported fertilization rates after standard IVF procedures (Alm et al. 2001, Dell'Aquila et al. 1996, Palmer et al. 1991) and there is no published report of equine pregnancies following both *in vitro* maturation and conventional IVF. This limited success is due to the restricted availability of oocytes and consequent lack of selection, poor understanding of oocyte maturation, fertilization and early embryo development. Research on IVM in mares is impeded by the limited availability of mature oocytes and early embryos for study. Superovulation treatments are not very efficient in the mare (Alvarenga et al. 2001, Bezard et al. 1995, Dippert and Squires 1994). Ovaries recovered from slaughterhouses provide a material with an obvious lack of repeatability, delay in time between collection and placement in culture medium and lack of information about the stage of cycle and follicular growth. Transvaginal ultrasound guided follicular aspiration has become an alternative source for mature and immature equine oocytes since it has been first described by Bruck et al. in 1992 (Bruck et al. 1992). Immature oocytes collected by transvaginal ultrasound guided follicular aspiration can undergo IVM. However, success rates of IVM of equine oocytes (40% to 70%) (Bogh et al. 2002, Goudet et al. 1997a, Squires 1996) remain low in all culture media tested compared to other species such as cows, goats and sows, (≥ 90%) (Goudet et al. 2000).

In order to improve the IVM maturation rates and quality of equine oocytes, the addition of compounds that can protect the cell from oxidative damage have been used such as glutathione (GSH) (de Matos et al. 1996, Grupen et al. 1995). During growth and maturation of the oocyte within the ovary, intracellular GSH content increases as the oocyte approaches the time of ovulation (Perreault et al. 1988, Yoshida et al. 1993, de Matos and Furnus 2000). The addition of GSH also improves the rate of male pronuclear formation (de Matos et al. 1995, de Matos and Furnus 2000). Glutathione synthesis can be increased and thus the intracellular concentration of GSH by the addition of low molecular weight thiols to IVM medium, such as cysteamine and ß-mercaptoethanol (Takahashi et al. 1993). This has

improved *in vitro* maturation rates in the canine (Hossein et al. 2007) but not in the bovine (de Matos et al. 1995) or the porcine (Grupen et al. 1995), nevertheless, it has increased the success of bovine (de Matos et al. 1995, de Matos et al. 1996), ovine (de Matos et al. 1999) and porcine (Bing et al. 2001, Grupen et al. 1995) embryo development. However, the influence of cysteamine on IVM and IVF rates has not been reported in mares.

IVF has not been successful in the mare possibly due to the lack of an efficient *in vitro* capacitation system for stallion spermatozoa. Equine spermatozoa have been incubated in solutions containing an ionophore and heparin, however it is unclear as to which solution is optimal. An *in vitro* fertilization rate of 30% was achieved in a technique where the spermatozoa prepared with the calcium ionophore A23187 (Bezard et al. 1992, Palmer et al. 1991, Zhang et al. 1990). This technique was used to produce the only two live foals following a conventional IVF procedure to date (Palmer et al. 1991). Another method in which spermatozoa were treated with heparin has been used in the horse (Dell'Aquila et al. 1996) and they obtained a 29% penetration rate which is similar to the overall 28,4% penetration rate observed by Alm et al*.* (Alm et al. 2001).

The assessment of the success of IVM and IVF techniques is difficult because no reliable feature of cytoplasmic maturation has been defined in the mare whereas nuclear maturation is readily assessed by nuclear fluorescent microscopy. There have been few papers about the ultrastructure (Grondahl et al. 1995, Tremoleda et al. 2001, Carneiro et al. 2002) and the biochemical changes (Goudet et al. 1998b, Goudet et al. 1998a) of oocytes during cytoplasmic maturation in the mare (Neumann et al. 1995, Goudet et al. 1997a). Therefore, the best current indicator of effective cytoplasmic maturation of the oocyte is the ability of the oocyte to produce an embryo.

In order to circumvent the inadequacy of conventional IVF, other methods to produce embryos from oocytes have been investigated, such as intra cytoplasmic sperm injection (ICSI) (Choi et al. 2002, Dell'Aquila et al. 1997a, Dell'Aquila et al. 1997b, Galli et al. 2002), which has recently permitted efficient equine in vitro blastocyst production (Hinrichs et al. 2005, Choi et al. 2006b, Choi et al. 2006a). Transfer of an immature oocyte into the preovulatory follicle of an inseminated recipient mare (Intra-Follicular Oocyte Transfer, IFOT) has also been investigated (Hinrichs and Digiorgio 1991, Goudet et al. 1997b). This method has produced embryos but the success rate was low therefore the oocyte transfer was

investigated. Three early studies showed blastocyst development after oocyte transfer to oviducts of a recipient mare ranging from 8% (n=26) (Ray et al. 1994), 13% (n=15) (McKinnon et al. 1988) to 17% (n=29) (Zhang et al. 1989). However, these studies used *in vivo* matured oocytes aspirated immediately before ovulation and transferred within one hour to the recipient mare's oviduct. In 1995, Carnevale and Ginther (Carnevale and Ginther 1995) reported an 83% rate (n=12) when transferring *in vivo* matured oocytes. Similarely, Hinrichs et al. (Hinrichs et al. 1997) showed a 75% rate (n=8) after transferring a single oocyte collected 24 hours after an injection of hCG and matured *in vitro* for another 16 to 20 hours before transfer. These results make this procedure a viable clinical alternative to embryo transfer in selected mares (Hinrichs et al. 2002, Carnevale 2004). In addition this method can be used to assess the quality of oocytes after IVM and IVF by their ability to undergo *in vivo* fertilization (Carnevale et al. 2005, Carnevale 2004) .

Aims of the study:

The broad aim of this study was to improve IVM and IVF procedures in the mare. The specific aims were to 1) to investigate the effect of the incubation of spermatozoa in two different solutions on the success of IVF, 2) to investigate if there is an influence of supplementation with 100 $\mu M\ L^{-1}$ of cysteamine on *in vitro* nuclear and cytoplasmic maturation by the ability of oocytes to undergo *in vitro* and *in vivo* fertilization.

MATERIALS AND METHODS

The study was conducted in spring using 30 cycling Welsh pony mares from 200 to 300 kg. All the procedures have been approved by the Local Committee of Health Guide for Care and Use of Laboratory Animals. Ovarian activity was monitored daily using transrectal ultrasound scanning using an Aloka 210 with a 5MHz linear probe (Société Bernard, Nantes, France).

Follicular puncture and oocyte recovery

When a preovulatory follicle ≥ 33 mm was seen, the mares were injected i.v. with 25mg of Crude Equine Gonadotropin (CEG), produced in our lab (Duchamp et al. 1987), to induce ovulation. Transvaginal ultrasound-guided aspiration of follicles ≥ 5 mm was performed 24 hours after CEG injections. When immature oocytes are collected ex vivo, maturation rates are highest when oocytes are collected with that timing (Goudet et al. 1997a, Goudet et al. 1998b). Follicles were punctured routinely using a transvaginal aspiration technique as previously described (Duchamp et al. 1994).

Culture of cumulus oocyte complexes

After recovery the cumulus oocyte complexes (COCs) were isolated and classified according to cumulus morphology. into expanded cumulus and compact cumulus (Goudet et al. 1998b). COCs with expanded cumulus were discarded. Compact COCs were individually matured at 38,5°C in a humidified atmosphere of 95% air and 5% CO_2 for 30 h in 20µl of maturation medium under mineral oil (M8410 Sigma, Saint-Quentin Fallavier, France). Two maturation media were used. Medium 1 contained Medium 199 with Earle's salt (M4530, Sigma, France) supplemented with 20% inactivated fetal calf serum (FCS, F4135, Sigma, France) and with 50ng/ml Epidermal Growth Factor (EGF, E4127, Sigma, France) as described by Goudet et al. (Goudet et al. 2000). Medium 2 was the same base with the addition of cysteamine at 100µM L^{-1} (M9768, Sigma, France).

Experiment 1: IVF – Influence of spermatozoa media and cysteamine

In the first experiment, influence of cysteamine on IVF was tested.

Oocytes for IVF were rinsed in IVF modified TALP medium and incubated with thawed spermatozoa in 20µl of the medium under mineral oil. TALP medium contained 100mM NaCl, 3.1mM KCl, 0.3mM NaH_2PO_4 $2H_2O$, 2.1mM $CaCl_2$, 0.4mM $MgCl_2$ $6H_2O$, 25mM $NaHCO_3$, 6mg/ml fatty acid-free albumin fraction V (Sigma), 4mg/ml sodium lactate (Sigma), 0.11mg/ml sodium pyruvate (Sigma). Cryopreserved semen was used, one ejaculate from three stallions pooled after thawing in a 37°C waterbath for 30 seconds and washed by centrifugation at 600g for 5 minutes, resuspended in medium and then centrifugated again. The sperm pellet was resuspended in IVF medium (group A) or Hank's solution supplemented with BSA (1%) and Hepes buffer (20mM) (Palmer et al. 1991) (group B) and adjusted to $16X10^6$ spermatozoa/ml. In group A, spermatozoa were supplemented with 1µg/ml heparin. In group B, spermatozoa were incubated with 6µM of Ionophore A23187 (free acid, Sigma) at 37°C for 5 minutes (Palmer et al. 1991), centrifuged at 600g for 5 minutes and resuspended in IVF medium. The sperm suspension was added to the oocytes for a final concentration of $4X10^5$ spermatozoa/ml. Oocytes were incubated with spermatozoa for 24 hours at 38.5°C in a humidified atmosphere (95% air and 5% CO_2), and examined.

For the examinations oocytes were rinsed in PBS. COCs were stripped with small glass pipettes and stained with 1 µg/ml bis-benzamide (Hoechst 33342; Sigma, France) and evaluated with a fluorescence microscope. Oocytes were classified as penetrated or not penetrated. Oocytes not penetrated were classified according to the stage of nuclear maturation, membrane integrity and the ooplasm aspect as "germinal vesicle", "dense chromatin", "metaphase I", "metaphase II" or "degenerate" as previously described by Goudet et al. (Goudet et al. 1998b).

Experiment 2 : Oocyte intra-oviductal transfer

In the second experiment, depending on puncture session yields, oocytes were transferred or analysed after IVM.

If the puncture session yielded ≥10 oocytes, 10 oocytes were prepared for oocyte transfer. Any additional oocytes were prepared for post IVM examination. When puncture sessions

yielded ≤10 oocytes, they were all prepared for post IVM examination. Oocytes for intra-oviductal transfer were rinsed three times in 500 µl of PBS after IVM and stored in PBS at 38.5°C. The system to aspirate the oocytes consisted of a blunted sterile glass pipette connected to a piece of tube which was in turn connected to a 1ml syringe. The whole system was kept at 38.5°C. The oocytes were loaded just prior to the oocyte transfer, in a minimal volume of PBS between two bubbles of air.

Oocyte transfer

On the day of oocyte recovery from the donor mare, the recipient mare scanned with a follicle ≥33mm was injected with 25mg of CEG and fasted for 24 hours. Recipient mares were inseminated 5 h prior to surgery, using fresh semen of a stallion with a known normal fertility: 400 X 10^6 spermatozoa in 10ml of extender (INRA96®; IMV Technologies, France). Surgical transfers of oocytes occurred 30 hours after the oocyte recoveries. The recipient mare was treated with antibiotics (about 6 hours before surgery) (4,000,000 IU streptomycin, 4g procaine penicilline i.m., Intramycine®, Ceva, France). Animals were sedated with detomidine (3-5 µg/kg; 0.1ml/animal i.v. Domosedan®; Pfizer, France) for surgical preparation of the mare for the standing flank laparotomy. An injection of butorphanol tartrate 4mg (13-20 µg/kg; 0.4ml/animal i.v. Torbugesic®; Fort Dodge, Southampton, UK) for analgesia was followed by a second injection of detomidine (6-10 µg/kg, 0.2ml/animal i.v. Domosedan®). A cuffed urinary catheter was placed to allow continuous emptying of the bladder during the surgery in order to limit discomfort. After local anesthesia (lidocaïne, Laocaïne®, Schering Plough, France), a flank approach was used to gain access to the ovary. The skin incision was directed vertically or at a slight oblique and extended 10 to 15 cm. The muscles of the flank, external and internal abdominal oblique muscles and the transversus abdominis muscle were bluntly dissected in the direction of their fibres. The peritoneum was perforated and the opening was manually dilated. After the ovary was exteriorised and the pipette was inserted into the oviduct. Oocytes were gently injected into the oviduct. The different layers were closed using absorbable suture material (Vicryl® 0; Johnson and Johnson, UK) in an interrupted pattern. Immediately after surgery, a second insemination with 200 X 10^6 spermatozoa in 10ml of semen extender (INRA96®, IMV Technologies, France) stored at 15°C was performed.

Embryo collection and maternity testing

Embryo collection was performed 10 days after surgery by three successive uterine lavage with saline at 38°C. Collection fluid was filtered and embryos were isolated under a stereomicroscope. Embryos were rinsed 10 times in saline and kept at -20°C until maternity test. For maternity testing (Guerand et al. 1997), DNA of each embryo was extracted and typed by radio active PCR amplification using 10 characterized micro-satellites (HMS 1, 2, 5, 6, 7, 8 and HG 3, 4, 6 and 10).

Statistical analysis

To compare maturation rates and embryo yields, a chi-square test was performed. Significance was established at $p < 0.05$.

RESULTS

Experiment 1: IVF – Influence of spermatozoa media and cysteamine

We incubated 131 oocytes that were evaluated for evidence of sperm penetration and fertilization. Overall, 8 of them (6%) were penetrated. We observed one oocyte with a decondensed sperm head, six oocytes with 2 pronuclei or subsequent stages of mitosis, and one 2 cell-embryo. Four oocytes were penetrated by spermatozoa treated with heparin (Group A), and four oocytes were penetrated by spermatozoa treated with ionophore (Group B). Of the 8 penetrated oocytes, 4 came from maturation medium 1, and 4 came from maturation medium 2.

Experiment 2: Oocyte intra-fallopian transfer

Oocytes collection

Recovery rates in experiment 2 are given in Table I.

A total of 237 oocytes were obtained from 479 immature follicles resulting in a recovery rate of 48,5%. 108 oocytes were maturated in medium 1 (Control group) and 124 in medium 2 (Cysteamine group).

Maturation rates

Five oocytes out of the 327 collected were damaged or lost and were not cultured.

Table II shows the percentage of nuclear maturation of the non-transferred oocytes.

Overal maturation rate was 52%. Nuclear maturation was not statistically different ($p>0.05$) between oocytes cultured with or without cysteamine (55% and 47% respectively).

Oocyte transfers

Six transfers were performed for a total of 57 oocytes in each group. Three oocytes were found in the pipette after one surgery in the control group. Only 7 oocytes from the cysteamine group were transferred during the next surgery to keep groups even.

Embryo collection

3 oocytes were found in the pipette. Five embryos were obtained from 57 transferred oocytes (9%) in the cysteamine group and ten embryos from 57 transferred oocytes (17%) in the control group (Table III). Those two percentages were not significantly different ($p>0.05$).

DISCUSSION

The overal maturation rate of 52% we observed is similar to previous reports, which vary from 40% to 70% in the equine (Bogh et al. 2002, Goudet et al. 1997a, Squires 1996). Higher values are usually observed when oocytes are matured in groups (Dell'Aquila et al. 1996, Shabpareh et al. 1993). Ideally, groups of 10 to 20 oocytes are cultured, which suits best conditions where a large number of oocytes are available. However oocytes were matured individually in our study so maturation conditions would be the same regardless the number of oocytes yielded by the ovum pick up session. Hinrichs et al (Hinrichs et al. 2005) recently reported meiotic resumption rates over 70% after individual maturation, with an actual metaphase II rate around 50% which is comparable to our results.

A concentration of 100 µM L^{-1} of cysteamine was used in the present study. Reported concentrations in various species range from 50 to 500 µM L^{-1} with highly variable results (Whitaker and Knight 2004, de Matos et al. 1995, de Matos et al. 2002, Gasparrini et al. 2003, Grupen et al. 1995, Yamauchi and Nagai 1999, Bing et al. 2002). Grupen et al (Grupen et al. 1995), who compared concentrations of 50 and 500 µM L^{-1} of cysteamine in IVM medium for porcine oocytes found that both increased synchronous pronuclear formation whereas only the higher concentration significantly enhanced embryonic development. They suggested that cysteamine in excess allows cytoplasmic accumulation of thiol compounds during IVM which may persist in the cleaving embryo and assist mitotic cell division. Conversely, Gasparrini et al (Gasparrini et al. 2000) working on buffalo oocyte, found that supplementation with 50 µM of cysteamine significantly improved *in vitro* embryo production whereas concentrations of 100 and 200 µM L^{-1} did not affect cleavage rate compared to control group. Similar results were observed in a recent study where cysteamine was added to IVM and IVC medium (Anand et al. 2008). It appears effect of cysteamine supplementation to IVM medium is highly species and concentration dependant. The inadequacy of the chosen concentration may explain that equine embryo production has not been increased by the cysteamine under our conditions as opposed to what has been observed in other species such as bovine (de Matos et al. 1995, de Matos et al. 1996, Takahashi et al. 1993); porcine (Bing et al. 2001, Grupen et al. 1995); ovine (de Matos et al. 2002); and buffalo (Gasparrini et al. 2003). However, our results are in agreement with Luciano et al. (Luciano et al. 2006) who recently demonstrated that the

addition of 100 μM L^{-1} of cysteamine during *in vitro* maturation of equine oocytes does not influence GSH synthesis.

Alternatively, contents of IVM medium itself should be considered as a potential reason for the absence of effect of cysteamine supplementation in our study. We can hypothesize that some substances present in the IVM medium can interfere with GSH synthesis. Bing et al (Bing et al. 2001) suggested that FSH and estradiol both affected GSH levels in porcine oocytes which in turn increased maturation rates. It has also been suggested that gonadotropins regulate ovarian GSH synthesis (Luderer et al. 2001). Although our maturation medium is not supplemented with gonadotropins or estradiol, factors contained in fetal calf serum or EGF might have an effect on GSH synthesis.

In our study, both techniques (ionophore or heparin) yielded 6% of IVF. This percentage is low when compared to data published by Palmer (Palmer et al. 1991) and Dell'Aquila (Dell'Aquila et al. 1996). However these results have not been shown to be repeatable and the IVF rates we observed, although rather low, are more comparable to others reported in the horse (Choi et al. 1994, Dell'Aquila et al. 1997a). Nevertheless the general success rates for IVF procedures may be too low for us to conclude definitely about the effect of cysteamine.

In regards to our overall maturation rate of 52% following IVM, it can be considered that only roughly 59 metaphase II oocytes were actually transferred. Consequently, 15 embryos were obtained from 59 genuinely fertilizable oocytes (26%), which is similar to what we (Daels et al. 2001) and others (Carnevale et al. 2005) obtained in previous studies where preovulatory *in vivo* recovered oocytes were transferred. The global results, 15 embryos from 114 transferred oocytes giving a 13 % recovery rate can be compared to those reported in other studies. From 29 oocytes aspirated during diestrus stage Scott et al. (Scott et al. 2001) obtained 2 pregnancies on day 16 (7%) following *in vitro* maturation and *in vivo* fertilization. Zhang et al. (Zhang et al. 1989) obtained 7 embryos from 29 oocytes from slaughterhouse matured *in vitro* and fertilized *in vivo*. Hinrichs et al. (Hinrichs et al. 2002) obtained a high *in vivo* fertilization rate of 77% after *in vitro* maturation of abattoir oocytes. The recipient mares were euthanasied 40 to 44 hours after transfer and oocytes were recovered from the oviduct. When comparing the percentage of embryos per transferred oocytes, 18% of the oocytes recovered from the oviduct had undergone cleavage (2 cells or more). The discrepancy between the fertilization rate and the relatively low embryo recovery rate in all studies

combined may suggest that the percentage of embryos able to reach the blastocyst stage is probably lower. This may be due to a blockage of embryonic development of the embryos and/or to an intra-fallopian embryonic reduction. This gives a new light to overall low results obtained following uterine collection. This confirms that in the absence of a reliable marker of cytoplasmic maturation, the ultimate test to appreciate the quality of a gamete following *in vitro* culture is by obtaining at least a blastocyst.

CONCLUSION

Regardless of sperm treatment (heparin vs ionophore), IVF rates were low with oocytes matured in either of two types of solutions. Under our conditions, the addition of 100 $\mu M\ L^{-1}$ of cysteamine to a classic culture medium does not improve equine oocyte maturation or embryonic development after oocyte transfer. Considering its beneficial effects in many other species, supplementation with cysteamine to different IVM media should be further investigated; ideally combining different concentrations and ICSI in order to determine an optimal concentration and its effects on sperm penetration and early embryonic development.

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Table I: Oocytes recovery rate

Donor mare	No of OPUs	No of follicles	No of oocytes	Recovery rate
1	2	46	21	45,7%
2	2	11	6	54,5%
3	2	16	11	68,8%
4	2	18	5	27,8%
5	1	9	4	44,4%
6	2	27	12	44,4%
7	1	16	13	81,3%
8	1	8	2	25,0%
9	1	14	12	85,7%
10	2	36	20	55,6%
11	2	12	6	50,0%
12	2	19	7	36,8%
13	1	14	8	57,1%
14	2	23	12	52,2%
15	1	13	5	38,5%
16	2	20	11	55,0%
17	1	7	5	71,4%
18	2	21	5	23,8%
19	1	4	1	25,0%
20	2	13	4	30,8%
21	1	6	3	50,0%
22	2	9	4	44,4%
23	2	14	9	64,3%
24	2	29	16	55,2%
25	2	6	3	50,0%
26	1	3	1	33,3%
27	2	17	8	47,1%
28	1	11	6	54,5%
29	2	16	6	37,5%
30	1	6	2	33,3%
Total		479	237	48,5%

Table II: Evaluation of nuclear maturation after culture

	Nuclear stage				
IVM medium	Metaphase II	Metaphase I	Dense chromatin	Germinal vesicle	Degenerated
Control	47% (24/51)	12% (6/51)	12% (6/51)	6% (3/51)	23% (12/51)
Cysteamine	55% (37/67)	10% (7/67)	8% (5/67)	3% (2/67)	24% (16/67)
Total	52% (61/118)	11% (13/118)	9% (11/118)	4% (5/118)	24% (28/118)

Table III: Number of collected embryos and result of maternity testing.

IVM medium	N of recipients	N of pregnant	N transferred oocytes	N of embryos Total*	Embryos from the recipient	Embryos from the donors
Control	6	6	57	15	5	10
Cysteamine	6	5	57	10	5	5
Total			114	25	10	15

* N embryos total refers to the embryos emerging from the oocytes of both the donor and the recipient mares.

REPRODUCTION ASSISTEE DANS L'ESPECE EQUINE : COLLECTE, EVALUATION, MATURATION ET UTILISATIONS D'OVOCYTES EQUINS.

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RESUME :

Malgré le faible nombre d'ovocytes équins disponibles pour la recherche, des techniques de reproduction assistée récemment développées dans cette espèce permettent maintenant de produire des embryons en utilisant des étalons et des juments subfertiles ou même de sauvegarder leur potentiel génétique après leur mort. Cette revue de littérature décrit les aspects cliniques de la collecte d'ovocytes ex vivo ou in vivo, leur évaluation, leur maturation in vitro et leur utilisation pour le transfert intra-folliculaire ou salpyngien et l'injection intra-cytoplasmique de spermatozoïde.

Assisted reproduction in the equine: collection, evaluation, maturation and use of equine oocytes.

SUMMARY

Despite the paucity of equine oocytes available for research, recently developped assisted reproduction techniques can now be offered to produce embryos from subfertile stallions and mares or to salvage their genetics after their death. This paper describes the clinical aspects of oocytes collection either *ex vivo* or *in vivo*, their evaluation and *in vitro* maturation and discusses their uses for intra-follicular and intra-oviductal oocyte transfer and intra-cytoplasmic sperm injection.

INTRODUCTION

L’acronyme « ART » pour : « Assisted Reproduction Technologies », ou en Français : « Techniques de Reproduction Assistées » (TRA), reprend des procédures allant de la simple conduite d’une saillie à la production de clones. Les techniques de reproduction assistée permettent de nos jours de préserver le potentiel génétique d’animaux subfertiles ou même morts. Ces dernières années, ces techniques ont été utilisées avec succès dans des cas cliniques où l’obtention d’une gestation n’était pas possible.

Le succès et le développement de ces techniques reposent sur la maîtrise de la collecte et les manipulations d’ovocytes. Les premiers pas et les premiers grands succès ont été rapportés dans l’espèce bovine. Bénéficiant d’un intérêt marqué par le secteur bovin et d’une disponibilité quasi illimitée d’ovocytes provenant d’ovaires prélevés en abattoirs, les recherches ont rapidement débouché sur des techniques efficaces de production *in vitro* d’embryons reprenant la maturation *in vitro* (MIV) des ovocytes, la fécondation (FIV) et la culture *in vitro* d’embryons.

L’industrie du cheval, en refusant encore dans bien des cas le recours à ces biotechnologies, et la bien moindre disponibilité d’ovaires d’abattoirs n’ont pas permis un essors aussi rapide des techniques de reproduction assistée dans cette espèce.

Cet article s’intéresse aux aspects cliniques de la récolte d’ovocytes, leur évaluation et leur usage en reproduction assistée chez la jument.

COLLECTE, EVALUATION ET MATURATION DES OVOCYTES

Collecte des ovocytes *ex-vivo*

Manipulation et transport des ovaires prélevés à l'abattoir

Bien qu'aucune étude ne compare les méthodes et les milieux de transport des ovaires équins depuis l'abattoir jusqu'au laboratoire, différents milieux et conditions de transport ont été explorés. Parmi les milieux de transport utilisés, on retrouve des solutions cristalloïdes supplémentées en antibiotiques, des solutions commerciales destinées au flush d'embryons, un milieu complet de culture cellulaire (TCM-199) et du PBS (phosphate-buffered saline) (Hinrichs *et al.*, 1993; Alm et Torner, 1994; Del Campo *et al.*, 1995; Franz *et al.*, 2003; Preis *et al.*, 2004).

Le délai de transport des ovaires jusqu'au laboratoire ne semble pas affecter significativement la compétence méiotique des ovocytes pour autant qu'il reste inférieur à 15 heures (Shabpareh *et al.*, 1993; Del Campo *et al.*, 1995; Guignot *et al.*, 1999). Néanmoins, des taux de maturation relativement élevés ont été rapportés par des laboratoires pour lesquels le délai entre l'abattage et la récolte d'ovocytes est court, ce qui suggère que la compétence des ovocytes pourrait malgré tout être affectée par ce délai (Love *et al.*, 2003; Hinrichs *et al.*, 2005).

La température à laquelle sont conservés les ovaires pendant leur transport a également fait l'objet d'études. Une étude qui a évalué la morphologie du cumulus oophorus et l'apoptose des cellules de la granulosa suggère que les ovaires ne devraient pas être conservés plus de 3 heures entre 20 et 30°C afin d'éviter l'apoptose des cellules de la granulosa et pas plus de 2 heures à 35-37°C pour éviter les modifications morphologiques du cumulus oophorus (Pedersen *et al.*, 2004). Une autre étude a montré que les taux de maturation (Metaphase I+II) après 24h étaient supérieurs pour des ovocytes collectés immédiatement à l'arrivée des ovaires au laboratoire que pour des ovocytes collectés après conservation des ovaires pendant

15 à 18h à température ambiante ou à 4°C (Love *et al.*, 2003). Les résultats de cette même étude laissent penser que 4°C est sans doute une température trop basse pour une bonne conservation des ovaires et que leur réfrigération devrait être envisagée uniquement lorsque la durée de conservation est prolongée.

Une solution optimale serait probablement de réaliser la récolte des ovocytes sur le site même de l'abattoir (Love *et al.*, 2003) et de les placer immédiatement dans les conditions de MIV dans un incubateur portable pour les ramener au laboratoire. Cette méthode encore peu utilisée permettrait sans doute d'obtenir des taux de maturation optimaux, mais comporte des difficultés techniques évidentes (lieu et matériel de récolte des ovocytes sur place, transport des ovocytes à température et atmosphère contrôlées) (Caillaud *et al.*, 2008).

Collecte d'ovocytes à partir d'ovaires prélevés à l'abattoir (ex vivo)

La récolte des ovocytes équins présente certaines difficultés techniques. L'ensemble des étapes doit être réalisé avec un souci constant des conditions d'hygiène, de température, de pH et de milieu dans lequel se trouvent les ovocytes afin de les préserver au maximum. Les ovocytes peuvent être récoltés par aspiration (Desjardins *et al.*, 1985; Shabpareh *et al.*, 1993) à l'aide d'une aiguille 18G et d'un système d'aspiration (+/- 1 bar) ou d'une pompe à vide (100-150mm Hg). L'ovaire peut également être tranché afin d'augmenter le nombre de follicules accessibles. L'ovocyte équin présente la particularité d'être fermement ancré sur un cumulus oophorus épais et trapus et dont les cellules présentent des extensions s'engrenant dans la couche de la thèque (Hawley *et al.*, 1995). Cette caractéristique rend difficile l'extraction de l'ovocyte hors de sa cavité. Ceci explique que la technique impliquant un grattage de la paroi interne du follicule après incision semble donner les meilleurs taux de collectes avec des valeurs d'environ 80% (Del Campo *et al.*, 1995). Cette technique permet de récupérer des ovocytes dont la majorité ont encore un Complexe Ovocyte-Cumulus (COC) intact (Hinrichs *et al.*, 1993) alors que ceux obtenus par aspiration seule ne présentent souvent plus que les cellules de la corona radiata qui forment un anneau ceignant l'ovocyte (Hinrichs, 1991). Après incision des follicules visibles, leur paroi est grattée à l'aide d'une curette. L'ovaire est ensuite disséqué en petits fragments afin d'accéder aux follicules qui se trouvent à l'intérieur du parenchyme ovarien. Alternativement, un grattage avec le biseau de l'aiguille

peut être réalisé en cours d'aspiration des follicules. Malgré cela, le taux global de récolte d'ovocytes équins reste relativement faible (3-5 oocytes/ ovaire) étant donné que l'ovaire d'une jument comporte en moyenne 6 follicules antraux (Hinrichs, 1991; Del Campo *et al.*, 1995). En outre, ceci se traduit par une augmentation du temps et du personnel nécessaires pour la collecte d'ovocytes. Galli (Galli *et al.*, 2007) considère qu'en terme de temps et de personnel, la collecte d'ovocytes équins est 10 fois plus ardue que celle d'ovocytes bovins.

Toutes ces difficultés techniques constituent un frein au développement de la recherche fondamentale sur l'ovocyte équin et, naturellement, sur toutes les technologies qui en découlent.

Collecte d'ovocytes par ovum pick up (*in vivo*)

Les ovocytes collectés à partir d'ovaires d'abattoirs représentent par définition une population très hétérogène et souffrent entre autre, d'un manque d'informations concernant l'âge des juments, leur phase du cycle et leur état gestatif.

Contrairement aux animaux de rente, l'application des biotechnologies pour la production d'embryons équins est limitée aux animaux souffrant de troubles de la fertilité ou aux sportifs de haut niveau. Il n'y a donc dans cette espèce, en dehors des fins de recherche que peu d'intérêt réel à produire des embryons à partir d'ovaires prélevés en abattoirs.

Pour les utilisations plus cliniques, les ovocytes sont souvent collectés *in vivo* après initiation de la maturation folliculaire et ovocytaire chez une jument en oestrus. Celle-ci peut être induite par l'administration de préparations commerciales contenant de l'hCG (effet LH), ou un analogue de GnRH (induction d'un pic endogène de LH). A des fins de recherche, des extraits hypophysaires (Crude Equine Gonadotropin, CEG) non disponibles commercialement, peuvent également être utilisés (Duchamp *et al.*, 1987).

Différentes approches visant à ponctionner des follicules et collecter leur ovocyte, telles que : la laparotomie (Vogelsang *et al.*, 1988), la colpotomie (Hinrichs et Kenney, 1987), et la ponction à l'aveugle par le flanc (Palmer *et al.*, 1986) ont été envisagées. Les deux premières techniques chirurgicales ont assez rapidement été abandonnées pour des raisons évidentes. La ponction par le flanc nécessite très peu de matériel. Pratiquement, un trocard est placé dans la

fosse sous-lombaire de la jument, l'ovaire est manipulé par voie trans-rectale et conduit en regard du trocard, une aiguille est passée au travers du trocard et le follicule est ponctionné et rincé à plusieurs reprises à l'aide de PBS (Phosphate Buffered Saline) hépariné. Cette technique ne permet malheureusement pas de visualiser le follicule et l'aiguille d'aspiration et ne donne des résultats satisfaisants que pour la ponction de follicules de grande taille. La ponction échoguidée par voie transvaginale est-elle devenue la technique de choix. La technique adaptée à partir de celle utilisée chez le bovin (McKinnon *et al.*, 1988; Bruck *et al.*, 1992) a ensuite été modifiée et utilisée par de nombreuses équipes (Bracher *et al.*, 1993; Cook *et al.*, 1993; Duchamp *et al.*, 1994; Carnevale et Ginther, 1995; Kanitz *et al.*, 1995; Meintjes *et al.*, 1995; Hinrichs *et al.*, 1998) et permet maintenant d'obtenir des taux de collectes qui vont de 51 à 86% (Cook *et al.*, 1993; Bezard *et al.*, 1995; Meintjes *et al.*, 1995; Goudet *et al.*, 1997; Scott *et al.*, 2001).

Pratiquement, la jument est tranquillisée et une sonde échographique sectorielle est introduite dans le vagin antérieur jusqu'au niveau du col (fornix). L'ovaire est manipulé à travers la paroi rectale et positionné en regard de la sonde et une aiguille est avancée le long de la sonde et introduite dans la cavité antrale au travers de la paroi du vagin. Le liquide folliculaire est aspiré, et le follicule est rincé à plusieurs reprises. Des mouvements de rotation de l'aiguille sont réalisés en cours d'aspiration pour gratter la paroi folliculaire afin d'en détacher l'ovocyte. Le liquide folliculaire et les liquides de rinçage sont récoltés et immédiatement transmis en salle de culture pour rechercher l'ovocyte. Les follicules de plus de 25mm sont ponctionnés à l'aide d'une aiguille à simple voie et vidangés et remplis à plusieurs reprises. Les follicules de moins de 25mm sont ponctionnés à l'aide d'une aiguille à double voie comme décrit par Duchamp et al (Duchamp *et al.*, 1995).

Evaluation des ovocytes collectés

Après collecte *ex vivo* ou *in vivo*, les ovocytes peuvent être dénudés mais ils sont généralement entourés d'une quantité plus ou moins abondante de cellules de la corona radiata et du cumulus oophorus. Les couches cellulaires les plus internes présentent des projections qui traversent la zone pellucide pour établir un contact intime avec la membrane de l'ovocyte (Grondahl *et al.*, 1995). Ces cellules qui entourent l'ovocyte forment avec celui-

ci l'ensemble complexe ovocyte-cumulus. Les COC's peuvent être caractérisés en fonction de leur degré d'expansion (Hinrichs *et al.*, 1993; Goudet *et al.*, 1997; Hinrichs et Williams, 1997) et celui-ci est directement corrélé au degré de maturation nucléaire de l'ovocyte (Zhang *et al.*, 1989; Goudet *et al.*, 1997; Gable et Woods, 2001). Les ovocytes immatures sont associés à des COC's compacts. Les COC's expansés sont associés à des follicules en cours d'atrésie. Ils présentent une meilleure aptitude à la reprise de la méiose après maturation *in vitro* (Hinrichs, 1991). Il est depuis longtemps établi que les follicules sont responsables du blocage de la méiose de l'ovocyte (Pincus et Enzemann, 1935). En s'atrésiant les follicules perdent leur capacité à maintenir ce bloquage (Gougeon et Testart, 1986; Blondin et Sirard, 1995), ceci explique une reprise spontanée de la méiose des ovocytes en leur sein.

Les couches de cellules de la corona radiata et de la granulosa qui entourent l'ovocyte en limitent l'examen morphologique. En dehors de la technique d'injection intra-cytoplasmique d'un spermatozoïde (Intra Cytoplasmic Sperm Injection, ICSI), pour l'ensemble des procédures impliquant un transfert de l'ovocyte, ces couches cellulaires sont nécessaires au bon déroulement des événements de fécondation et de développement embryonnaire précoce. Aussi, l'examen de l'ovocyte lui même reste-t-il assez difficile et superficiel à moins de le dénuder mécaniquement ou à l'aide de trypsine. Néanmoins, l'aspect de l'ooplasme est corrélé à la viabilité des follicules, évaluée sur base de l'aspect des noyaux, le contact entre les cellules de la granuleuse et la membrane basale. Les ovocytes présentant un ooplasme homogène sont davantage associés à des COC's compacts et à des follicules viables. Inversément, un ooplasme d'aspect granuleux et polarisé, correspondant à une distribution irrégulière de gouttelettes lipidiques et d'organelles intra-cellulaires, est davantage associé à des follicules atrétiques et à des COC's expansés (Hinrichs et Williams, 1997).

Maturation *in vitro* des ovocytes (MIV)

La maturation ovocytaire est le résultat d'un double processus de maturation, au niveau du cytoplasme d'une part et au niveau du noyau d'autre part. Les modifications morphologiques associées à la maturation de l'ovocyte ont été décrites en 6 étapes par Grondahl et collaborateurs (Grondahl *et al.*, 1995). Successivement, l'ovocyte présente : (1) un noyau sphérique au centre du cytoplasme, (2) un noyau sphérique à la périphérie de l'ooplasme, (3)

un noyau aplati en périphérie de l'ooplasme, (4) la rupture du noyau de l'ovocyte, aussi appelé : vésicule germinale d'où le terme de rupture de la vésicule germinale (Germinal Vesicle Break Down, GVBD), (5) metaphase I, (6) metaphase II caractérisée par la localisation périphérique des chromosomes métaphasiques et la présence d'un globule polaire dans l'espace périvitellin. Le globule polaire est généralement difficile à observer sous la loupe mais il arrive qu'occasionnellement il puisse être distingué dans l'espace périvitellin. La coloration spécifique de l'ADN des ovocytes dénudés par le bis benzamide ou Hoechst 33258 permet la classification des configurations de la chromatine en (1) vésicule germinale, (2) chromatine dense, (3) metaphase I, (4) métaphase II, (5) dégénéré (Hinrichs *et al.*, 1993; Goudet *et al.*, 1998).

Les milieux utilisés pour la MIV d'ovocytes équins dérivent principalement du secteur bovin. Depuis la première MIV d'ovocytes équins (Fulka et Okolski, 1981), sur base des milieux déjà utilisés chez le bovin, la recherche s'est orientée vers des milieux de culture cellulaire synthétiques tels que le TCM-199 (Willis *et al.*, 1991; Dell'Aquila *et al.*, 1997; Galli *et al.*, 2001), ou le Ham's F10 (Okolski *et al.*, 1991; Willis *et al.*, 1991; Shabpareh *et al.*, 1993). Actuellement, la plupart des milieux utilisent comme base le TCM-199, le EMMI et des solutions de sels auxquels sont ajoutées du sérum de veau fœtal (FCS) et des hormones telles que la LH, la FSH, le benzoate d'oestradiol (Carnevale *et al.*, 2004) ou l'EGF (Goudet *et al.*, 1998; Lorenzo *et al.*, 2002). Quelques études (Hinrichs *et al.*, 1995; Li *et al.*, 2001; Choi *et al.*, 2002; Tremoleda *et al.*, 2003) ont investigué les cocultures pour la MIV des ovocytes équins mais elles ont davantage apporté d'informations sur les mécanismes d'inhibition de la méiose au sein du follicule que de bénéfice réel en terme de qualité de maturation.

La durée de maturation nécessaire a également fait l'objet de nombreuses investigations (Fulka et Okolski, 1981; Zhang *et al.*, 1989; Del Campo *et al.*, 1990; Willis *et al.*, 1991; Del Campo *et al.*, 1995; Bezard *et al.*, 1997). Il est communément admis maintenant que l'on peut espérer un taux de maturation jusqu'au stade de métaphase II de l'ordre de 50 à 80% après 30 heures d'incubation sous atmosphère contrôlée (39°C et 5% de CO2) (Scott *et al.*, 2001) et qu'une durée prolongée d'incubation n'améliore pas ce résultat (Shabpareh *et al.*, 1993). Les durées de maturation optimales dépendent du degré de maturation initial des ovocytes qui est corrélé à l'aspect de leur COC. Les taux de maturation maximum pour des ovocytes issus de COC's expansés et compacts ont été obtenus respectivement après 24h et 32h de MIV pour Hinrichs et collaborateurs (Hinrichs *et al.*, 1993) et 24h et 30h pour l'équipe de Zhang (Zhang *et al.*, 1989).

Pour qu'un ovocyte puisse être fécondé, il doit achever sa maturation nucléaire et cytoplasmique. La maturation cytoplasmique repose principalement sur l'accumulation de protéines et d'ARNm dans l'ovocyte (Grondahl *et al.*, 1993). La maturation nucléaire repose sur la reprise de la méiose et le développement jusqu'au stade de métaphase II, ce qui peut être facilement évalué par des colorations spécifiques de l'ADN. En l'absence de technique de FIV conventionnelle efficace, la plupart des études portant sur la MIV des ovocytes se sont intéressées à leur maturation nucléaire, d'avantage qu'à leur maturation cytoplasmique pour laquelle le seul critère fiable est leur aptitude à produire un embryon. Lorsque des modifications cytoplasmiques au cours de la maturation, comme par exemple la migration des granules corticaux ou des mitochondries on été décrites (Grondahl *et al.*, 1995; Goudet *et al.*, 1997; Aguilar *et al.*, 2002; Carneiro *et al.*, 2002), leur importance en matière de compétence ovocytaire n'a pas été explorée.

Lorsque l'ovocyte est soustrait à l'environnement folliculaire, la méiose reprend spontanément, la chromatine des ovocytes se condense ce qui bloque la transcription ; il serait évidemment préférable que le stock de protéines et d'ARNm soit complet avant que la méiose ne reprenne (Sirard, 2001). C'est dans cette logique que des conditions de milieu qui maintiennent le blocage méiotique tout en maintenant la transcription avant culture d'ovocytes bovins permettent d'améliorer les taux d'embryons produits (Fouladi Nashta *et al.*, 1998; Hashimoto *et al.*, 2002). Cette même piste permettra peut être de mieux comprendre les mécanismes du blocage de la méiose de l'ovocyte équin et d'améliorer leur maturation cytoplasmique.

UTILISATIONS DES OVOCYTES : TECHNIQUES DE REPRODUCTION ASSISTEE

Au cours des dix dernières années, les techniques de reproduction assistée chez le cheval ont été largement développées. Cependant, la plupart de ces techniques requièrent des équipements spécifiques et une expertise particulière ce qui explique qu'elles restent principalement mises en œuvre dans des laboratoires spécialisés.

La fécondation *in vitro* (FIV) conventionnelle reste actuellement peu utilisable chez la jument et aucune technique reproductible de FIV n'a encore été mise au point. Les raisons invoquées concernent principalement l'absence d'une méthode efficace de capacitation des

spermatozoïdes équins (Alm *et al.*, 2001), les changements dans la zone pellucide (Dell'Aquila *et al.*, 1999; Hinrichs *et al.*, 2002) ou une maturation *in vitro* des ovocytes imparfaite (Li *et al.*, 2001). A ce jour, le seul traitement qui induise la capacitation, la réaction acrosomiale des spermatozoïdes équins et qui permette leur pénétration dans l'ovocyte est l'ionophore calcique A23187 (Zhang *et al.*, 1990; Alm *et al.*, 2001; Hinrichs *et al.*, 2002). C'est par ailleurs le traitement de capacitation utilisé pour produire les deux seuls poulains nés après FIV d'ovocytes maturés *in vivo* (Palmer *et al.*, 1991; Bezard *et al.*, 1992). Pour contourner ces difficultés, différentes approches pour produire des embryons à partir d'ovocytes soit *in vitro*, soit *in vivo* ont été explorées. Parmi celles-ci, le transfert intra-folliculaire d'ovocytes, le transfert intra-salpyngien d'ovocytes et l'injection intra-cytoplasmique de spermatozoïde.

Transfert d'Ovocyte Intra-Folliculaire (TOIF)

La technique consiste à injecter un ou plusieurs ovocytes dans le follicule préovulatoire d'une receveuse afin que ceux-ci trouvent les conditions favorables à leur maturation et soient ovulés en même temps que l'ovocyte du follicule préovulatoire. Le transfert intra-folliculaire a été initialement tenté chez le babouin, le bovin et le porc mais toujours sans succès, probablement suite aux dommages subis par le follicules lors des manipulations (Fleming *et al.*, 1985; Hinrichs et Digiorgio, 1991). Grâce à l'anatomie particulière de son ovaire présentant une médullaire périphérique et une tunique albuginée épaisse et résistante la jument constitue un modèle privilégié pour l'étude du TOIF. Pratiquement, la procédure s'apparente à celle décrite pour l'ovum pick up. Le follicule préovulatoire est ponctionné et quelques millilitres de liquide folluclaire sont aspirés. Les ovocytes sont injectés dans le follicule et le circuit est flushé avec le liquide folliculaire initialement prélevé. Hinrichs et Di Giorgio (Hinrichs et Digiorgio, 1991) ont développé la technique et ont été les premiers à obtenir des embryons équins après transfert d'ovocytes immatures dans des follicules préovulatoires. La technique a été ensuite utilisée pour l'étude endocrinologique de la liqueur folliculaire au cours de la maturation folliculaire et celle des taux de maturation ovocytaire (Goudet *et al.*, 1997). Plus récemment, des embryons ont été obtenus après TOIF d'ovocytes préalablement maturés *in vitro* (Deleuze *et al.*, 2009). Les taux rapportés d'embryons en excès par rapport aux ovocytes des receveuses varient de 12,8% (6 embryons en excès / 45 ovocytes

transférés) après transfert d'ovocytes immatures et 5,5% (3/55) après transfert d'ovocytes maturés 30h *in vitro* pour Deleuze et collaborateurs (Deleuze *et al.*, 2009) et 12 embryons en excès récoltés après transfert de 135 ovocytes immatures (8.9%) pour Hinrichs et Di Giorgio (Hinrichs et Digiorgio, 1991). Le TOIF semble augmenter l'incidence des follicules anovulatoires mais il reste cependant une procédure relativement simple et peu invasive qui pourrait être envisagée pour contourner l'absence de protocoles efficaces de superovulation chez la jument (Deleuze *et al.*, 2009).

Transfert d'Ovocytes intra-salpyngien (Oocyte Transfer, OT)

La technique consiste à transférer chirurgicalement un ou plusieurs ovocytes d'une donneuse dans l'oviducte d'une receveuse. Brièvement, une laparotomie par le flanc permet l'accès à l'ovaire et à l'oviducte, une pipette est introduite dans sa lumière et les ovocytes y sont déposés (Deleuze *et al.*, 2008). Les ovocytes peuvent être maturés *in vivo* et récoltés à partir d'un follicule préovulatoire ou bien être obtenus à partir de follicules en dioestrus et ensuite maturés *in vitro*. La receveuse est inséminée avant et après transfert des ovocytes afin que

la fécondation et le développement embryonnaire se déroulent dans l'oviducte et dans l'utérus. Alternativement, les spermatozoïdes peuvent être déposés dans l'oviducte en même temps que les ovocytes ; on parlera alors de GIFT pour « Gamete Intra-Falopian Transfer ». L'utilisation de sperme frais pour le GIFT donne de meilleurs résultats que le sperme congelé (Coutinho da Silva *et al.*, 2002), mais le transfert d'ovocytes seuls suivi de l'insémination de la receveuse est plus largement utilisé. L'application clinique majeure du transfert intra-salpyngien d'ovocytes concerne les juments qui souffrent de pathologies utérines, cervicales ou salpyngiennes qui ne leur permettent pas de produire un embryon qui pourrait être collecté et transféré dans l'utérus d'une jument receveuse. Bien qu'il ait été démontré que des juments cycliques et non-cycliques pouvaient être utilisées comme receveuses sans affecter le taux de gestation (Carnevale *et al.*, 2005), et que certaines études rapportent l'utilisation de brebis comme receveuses (Wirtu *et al.*, 2004), la plupart des transferts utilisent des juments cycliques dont l'ovocyte du follicule dominant a été retiré avant transfert afin d'éviter les manipulations hormonales des juments non-cyclées destinées à mimer la phase oestrale.

Le premier transfert d'ovocytes chez la jument a été rapporté par Mac Kinnon et collaborateurs (McKinnon *et al.*, 1988), mais leurs taux de blastocystes et ceux des études consécutives (Zhang *et al.*, 1989; Ray *et al.*, 1994) sont restés globalement bas. Des études plus récentes utilisant des ovocytes issus de follicules préovulatoires, maturés *in vivo* avec (Hinrichs *et al.*, 1997) ou sans (Carnevale et Ginther, 1995) maturation *in vitro* avant transfert ont permis d'obtenir des taux de blastocystes avoisinant les 85%. Le transfert d'ovocytes dans l'oviducte d'une receveuse peut actuellement être considéré comme une solution alternative au transfert d'embryon chez les juments infertiles (Hinrichs *et al.*, 2002; Carnevale, 2004). Le premier poulain issu du transfert d'ovocytes récoltés à partir d'ovaires après la mort de la jument donneuse et ensuite maturés *in vitro*, est né en 2003 (Carnevale *et al.*, 2003). Les mêmes auteurs rapportent un taux de développement embryonnaire de 15% (n= 191) après transfert d'ovocytes collectés post-mortem à partir de juments de grande valeur (Carnevale *et al.*, 2004). Les taux de développement embryonnaire après transfert intra-salpyngien d'ovocytes maturés *in vitro* rapportés dans la littérature sont variables : 7% (2/29) (Scott *et al.*, 2001), 15% (11/73) et 18% (13/73) (Preis *et al.*, 2004), 13% (15/114) (Deleuze *et al.*, 2008). Même les taux les plus élevés : 24,1% (2/29) (Zhang *et al.*, 1989) et 32,5% (13/40) (Deleuze *et al.*, 2009) restent plus faibles que ceux obtenus après transfert d'ovocytes maturés *in vivo* et collectés à partir de follicules préovulatoires qui varient de 73% à 83% (Carnevale et Ginther, 1995; Hinrichs *et al.*, 1998; Scott *et al.*, 2001; Carnevale *et al.*, 2004). Un facteur majeur affectant le taux de succès reste l'âge de la jument donneuse. Les ovocytes de juments donneuses âgées présentent davantage d'anomalies morphologiques (Carnevale *et al.*, 1999) et produisent significativement moins de vésicules embryonnaires (Carnevale et Ginther, 1995).

Injection Intra-Cytoplasmique de Spermatozoïde (Intra-Cytoplasmic Sperm Injection : ICSI)

Alors que les techniques conventionnelles de FIV restent décevantes dans l'espèce équine, l'ICSI s'est avérée une méthode efficace pour réaliser la fécondation *in vitro* des ovocytes équins (Squires *et al.*, 2003; Hinrichs, 2005; Galli *et al.*, 2007).

La technique repose sur la micromanipulation d'un spermatozoïde qui est injecté dans l'ooplasme d'un ovocyte dénudé en métaphase II. Pratiquement, l'ovocyte est immobilisé et à

l'aide d'un micro-injecteur, un spermatozoïde est déposé dans le cytoplasme. Cette technique élimine la difficulté de la capacitation du sperme équin, du passage de la zone pellucide de l'ovocyte et permet même l'usage de sperme de qualité médiocre ou d'étalons oligospermiques. Les résultats avec du sperme frais et du sperme congelé sont comparables (Choi *et al.*, 2002) pour autant qu'un spermatozoide mobile soit sélectionné pour l'injection intra-cytoplasmique (Lazzari *et al.*, 2002). Une fois la fécondation obtenue, l'ovocyte peut éventuellement être activé chimiquement. Ensuite, l'embryon doit encore être cultivé jusqu'au stade morula ou blastocyste afin de pouvoir être transféré dans l'utérus d'une jument receveuse. Cette étape de culture *in vitro* reste délicate et les jeunes embryons issus de l'ICSI peuvent alternativement être transférés dans l'oviducte d'une jument receveuse afin d'y poursuivre leur développement. La culture des embryons peut également être réalisée *in vivo* dans les oviductes de brebis avant d'être transférés dans l'utérus d'une jument receveuse (Hinrichs, 2005).

Depuis la naissance en 1996 d'un premier poulain né après ICSI (Squires *et al.*, 1996), la technique n'a cessé de s'améliorer et plusieurs poulains sont nés depuis en utilisant cette méthode (Squires *et al.*, 1996; Mc Kinnon *et al.*, 2000; Li *et al.*, 2001; Galli *et al.*, 2002). Des études récentes rapportent des taux de clivage après ICSI de 50-80% (Choi *et al.*, 2002). Malheureusement, seul un faible pourcentage de ces zygotes clivés poursuivent leur développement en culture jusqu'à former un blastocyste. Différents milieux de culture des jeunes embryons jusqu'au stade où ils peuvent être transférés ont été investigués : G1.2 (Choi *et al.*, 2002), DMEM-F12 et CZB (Choi *et al.*, 2004), et du SOF modifié (Galli *et al.*, 2002) mais les taux de développement jusqu'au stade blastocyste dans ces études sont restés faibles (4 à 16%). D'autres ont étudié les effets de co-cultures avec différents types cellulaires : des cellules Vero (Dell'Aquila *et al.*, 1997), des cellules épithéliales de l'oviducte (Battut *et al.*, 1991), des cellules de la granulosa (Rosati *et al.*, 2002), ou des cellules du cumulus (Li *et al.*, 2001) ; mais les taux de blastocystes sont restés faibles (4 à 16%) pour tous ces milieux. Par contre, la mise en culture des zygotes potentiels (soit les ovocytes après ICSI) *in vivo* dans l'oviducte d'une jument (Choi *et al.*, 2004) ou d'une brebis receveuse (Galli *et al.*, 2002; Lazzari *et al.*, 2002) permet un meilleur taux de développement embryonnaire (approximativement 36%) (Galli *et al.*, 2007), les meilleurs résultats ayant été obtenus après culture dans l'oviducte d'une brebis (45%) (Galli *et al.*, 2002; Lazzari *et al.*, 2002). Cependant, récemment un milieu de culture *in vitro* utilisant du DMEM-F12 sous atmosphère

mixte contrôlée a permis d'obtenir des taux de développement de blastocystes (27-38%) se rapprochant de ces résultats (Choi *et al.*, 2006; Choi *et al.*, 2006; Galli *et al.*, 2007).

CONCLUSION

La qualité de la maturation ovocytaire constitue un élément clé pour la mise en œuvre des techniques qui viennent d'être abordées. Les ovocytes maturés *in vivo* représentent les meilleurs candidats pour leur application. Malheureusement, la jument étant une espèce mono-ovulante chez qui la superovulation est décevante, ces ovocytes maturés *in vivo* restent peu nombreux et difficiles à obtenir. Le grand nombre d'ovocytes nécessaires à la recherche et à ses applications justifie les efforts pour améliorer les résultats de MIV. La compétence ovocytaire peut être mesurée par le taux de production de blastocystes après fécondation. Celui-ci dépend, non seulement du milieu de culture de l'embryon, mais aussi des conditions de maturation de l'ovocyte lui même. Les conditions de maturation *in vitro* de l'ovocyte sont déterminantes pour son aptitude ultérieure à voir se développer un embryon. Galli et collaborateurs (Galli *et al.*, 2007) ont récemment mis en évidence une amélioration significative de leurs taux de clivage et de développement des blastocystes après ICSI suite au changement de leur milieu de maturation sans amélioration significative de leurs taux de métaphase II. Ceci illustre l'importance de la compétence ovocytaire acquise durant la MIV.

Des progrès énormes ont été accomplis dans le domaine de la récolte et la manipulation des ovocytes équins. Ceci a conduit au développement de techniques de reproduction assistée et d'applications cliniques et commerciales qui permettent dorénavant d'obtenir des descendants d'étalons et de juments de faible fertilité ou de sauver leur potentiel génétique après leur mort.

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CYSTEAMINE SUPPLEMENTATION OF *IN VITRO* MATURATION MEDIA : A REVIEW.

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Content

Under *in vitro* culture conditions, oxidative modifications of cell components via increased Reactive Oxygen Species (ROS), represent a major culture induced stress. Anti-oxidant systems such as glutathione (GSH) can attenuate the deleterious effects of oxidative stress by scavenging ROS. It has been suggested that GSH content in oocytes may serve as a reservoir protecting the zygote and the early embryos from oxidative damage before genomic activation and *de novo* GSH synthesis occur.

Addition of low molecular weight compounds to culture media, such as cysteamine, can increase GSH levels by increasing cysteine uptake. Quite naturally, effects of supplementation of *in vitro* maturation (IVM) media with low molecular weight thiols have been studied in various species.

This paper reviews the use of cysteamine supplementation for IVM, its effects on maturation rates and further embryo development.

Glutathione

Glutathione (GSH), a tripeptide thiol (γ-glutamyl-cysteine-glycine) is the major non-protein sulfydryl compound in mammalian cells that plays an important role in protecting the cell from oxidative damage (Meister and Anderson 1983, Meister and Tate 1976). GSH cellular content is regulated by the γ-glutamyl cycle. The reactions involved account for the synthesis and degradation of GSH, and the balance of GSH transport out of the cells and that of γ-glutamyl amino acids into the cells. GSH is synthesised within the cell in two steps. First, γ-glutamylcysteine synthetase catalyses synthesis of γ-glutamyl-cysteine from glutamate and cysteine. Then, glycine is added to the C-terminal of γ-glutamyl-cysteine in the presence of GSH synthetase (Meister and Tate 1976).

The first step of GSH synthesis appears to determine the upper concentration of cellular GSH because of a feedback inhibition by GSH (Richman and Meister 1975). It has been shown that GSH concentration also depends on availability of cysteine for synthesis (Furnus and de Matos 1999, Meister 1983). Conversely, buthionine sulfoximine (BSO) binds to and inhibits γ-glutamylcysteine synthetase and thus induces GSH depletion (Griffith and Meister 1979, Meier and Issels 1995).

Glutathione is present in the cell either under its reduced (GSH) or oxidized form (GSSG). In the presence of GSH, glutathione peroxidase (GPX) catalyses the reduction of hydrogen peroxide to form H_2O and GSSG. GSSG is reduced by glutathione reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) which maintains a high ratio (about 100:1) of GSH to GSSG in the cell (Meister 1983). GSH peroxidase and GSH catalase promote removal of hydrogen peroxide and dismutation product of superoxide radicals (Fridovich 1978).

These reactions can be summarized by the following equations:

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

$$2\ GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Low molecular weight thiol compounds and GSH

As mentioned before, GSH synthesis is limited by the availability of cysteine. Except for hepatocytes, which are able to synthesize cysteine from methionine via the cystathionine pathway, eukaryotic cells depend on extra cellular medium for the supply of cysteine (Meier and Issels 1995). Direct supplementation of medium with cysteine is usually avoided as cysteine is easily oxidized to cystine in the culture medium (Bannai 1984) and can exhibit a high degree of toxicity (Nishiuch et al. 1976).

Low molecular thiols such as β-mercaptoethanol (Ishii et al. 1981), N-acetylcysteine, cysteamine (Meier and Issels 1995) can promote cystine uptake. These compounds share a free SH group as a common molecular structure. Modification of this free thiol group by chemical reaction to form a disulfide bond or the addition of a phosphate moiety to the thiol group abolishes the potential of the compound to promote cystine uptake (Meier and Issels 1995).

Effects of extracellular thiols on cystine uptake can be summarized in two steps: 1) formation of cysteine and cysteine mixed disulfides (e.g., cysteine-cysteamine) and 2) uptake of cysteine and/or the mixed disulfide via cellular transport systems (Meier and Issels 1995, Ishii et al. 1981). Within the same species, activity of these transport systems vary from one cellular type to the other (Meier and Issels 1995). Oocytes are deficient in their capacity to take up Cystine (cysteine-cysteine). Cysteine is utilized by the oocyte, but it is easily oxidised to cystine in the culture medium. Thiol compounds react with cystine to form a mixed disulfide. The mixed disulfide is taken up by the cells, where it is reduced to produce a thiol compound and cysteine. Thiol compounds do not accumulate in the cells and escape to the medium to react with cystine. Thiol compounds are repeatedly taken up by the oocytes in the form of mixed disulfide with cysteine and return to the medium in their reduced form. Extra cellular cysteine may also be taken up by the oocytes by a transporter system (ASC) shared with other amino acids (Alanine, Serine, Cysteine) but at a very low rate as cysteine in the culture medium is rapidly oxidized to cystine. A schematic diagram of the action of low molecular weight thiols is proposed in Figure 1.

GSH and *in vitro* maturation (IVM)

Under *in vitro* culture conditions, oxidative modifications of cell components via increased Reactive Oxygen Species (ROS), which are responsible for DNA damage, oxidative alterations of proteins and lipid peroxidation (Johnson and Nasr-Esfahani 1994), represent a major culture induced stress (Noda et al. 1991, Goto et al. 1993). Anti-oxidant systems can attenuate the deleterious effects of oxidative stress by scavenging reactive oxygen species (Del Corso et al. 1994).

Low molecular weight thiols compounds have been shown to have beneficial effects on lymphocyte viability and enhance various cell reactions (Fanger et al. 1970). Addition of cysteine or β-mercaptoethanol to culture media for lymphocytes has been shown to increase intracellular GSH levels and prevent its decrease during proliferation (Zmuda and Friedenson 1983).

GSH synthesis during oocyte maturation has been reported in mouse (Calvin et al. 1986), hamster (Perreault et al. 1988), pig (Yoshida et al. 1993), sheep (de Matos et al. 2002a), bovine (de Matos et al. 1996) and equine (Luciano et al. 2006). GSH concentration is highly correlated with the presence of cumulus cells (Sawai et al. 1997) however, denuded oocytes GSH synthesis has also been shown to be stimulated by thiol compounds, although to a lesser extend than COCs (Yamauchi and Nagai 1999, de Matos et al. 1997). GSH contents increase during maturation of oocytes in the ovary and peak at the metaphase II stage (Perreault et al. 1988) but drops rapidly in early embryos (Zuelke et al. 2003). It has been suggested that GSH content in oocytes may serve as a reservoir protecting the zygote and the early embryos from oxidative damage before genomic activation and *de novo* GSH synthesis occur (de Matos and Furnus 2000, Furnus et al. 1998). Moreover, GSH has been shown to play a role during fertilization by promoting male pronucleus formation (Yamauchi and Nagai 1999). Quite naturally, effects of supplementation of IVM media with low molecular weight thiols have been studied in various species.

The following sections review the use of cysteamine supplementation for IVM media, its effects on IVM and further embryo development.

Addition of cysteamine to the IVM medium : dose effect

Most commonly, IVM media have been supplemented with a dose of 100 μM of cysteamine in cattle, (Balasubramanian and Rho 2007, de Matos et al. 1997, de Matos et al. 1995, de Matos et al. 1996, de Matos et al. 2002b, Oyamada and Fukui 2004), buffalo (Gasparrini et al. 2003, Anand et al. 2008), goats (Rodriguez-Gonzalez et al. 2003b, Urdaneta et al. 2003, Zhou et al. 2008), horses (Luciano et al. 2006, Deleuze et al. 2008), pigs (Kobayashi et al. 2007, Kobayashi et al. 2006, Song and Lee 2007), mice (Chen et al. 2005, de Matos et al. 2003), cats (Bogliolo et al. 2001) and dogs (Hossein et al. 2007). However, other concentrations of cysteamine, ranging from 50 to 500 μM, have been tested in different species. A summary of the different concentrations used in various species is given is Table 1.

Effects of one given concentration of cysteamine supplementation not only depend on the species but also on the maturation medium. These differences between media might be explained by the presence and the quantities of different factors that can help protect the cell from oxidative damage such as citrate (Holm et al. 1999) and pyruvate (Morales et al. 1999). Moreover, as most media use TCM-199, it is important to bear in mind that whether the cystine it contains (83.2 μM) is sufficient to sustain oocyte IVM of different species is unknown (Zhou et al. 2008).

Addition of cysteamine to the IVM medium : effect on nuclear maturation rates

Cysteamine supplementation during IVM, was reported to improve nuclear maturation rates in dogs (Hossein et al. 2007), in mice (Chen et al. 2005), in goats (Urdaneta et al. 2003), and in pigs (Bing et al. 2001). However, other studies in goats (Zhou et al. 2008), pigs (Kobayashi et al. 2006, Song and Lee 2007, Yamauchi and Nagai 1999), horses (Luciano et al. 2006, Deleuze et al. 2008), buffalos (Singhal et al. 2008) and cattle (Balasubramanian and Rho 2007, Oyamada and Fukui 2004) could not show any increase in nuclear maturation rates.

Addition of cysteamine to the IVM medium : effect on GSH oocyte content

Cysteamine has been shown to increase intracellular GSH synthesis in bovine (de Matos et al. 1997, de Matos et al. 1995, de Matos et al. 2002b, Oyamada and Fukui 2004, Takahashi et al. 1993), buffalo (Gasparrini et al. 2003), canine (Hossein et al. 2007), goats (Rodriguez-Gonzalez et al. 2003a, Zhou et al. 2008), mice (de Matos et al. 2003), horses (Luciano et al. 2006), sheep (de Matos et al. 2002a) and pigs (Kobayashi et al. 2007, Yamauchi and Nagai 1999). This increase in GSH content has been consistent in all reports regardless the species. In sheep, a concomitant decrease in hydrogen peroxide has also been demonstrated (de Matos et al. 2002a), illustrating the scavenging effect of GSH synthesis stimulation on ROS.

Addition of cysteamine to the IVM medium : effect on male pronucleus formation

Effect of cysteamine supplementation during IVM on male pronucleus formation following either conventional IVF or ICSI has not been extensively investigated. However, addition of cysteamine to the IVM medium has been shown to improve male pronucleus formation in goats (Rodriguez-Gonzalez et al. 2003a, Urdaneta et al. 2003), buffalos (Anand et al. 2008) and pigs (Bing et al. 2001, Grupen et al. 1995, Yamauchi and Nagai 1999). Only two studies, one in pigs (Kobayashi et al. 2006) and one in cattle (Balasubramanian and Rho 2007), reported a lack of effect of cysteamine on male pronucleus formation.

Addition of cysteamine to the IVM medium : effect on cleavage rates

Effect of cysteamine supplementation to IVM media on cleavage rates is controversial. A few studies have reported an increase in cleavage rates following IVM with cysteamine in goats (Rodriguez-Gonzalez et al. 2003a), pigs (Grupen et al. 1995), cattle (Oyamada and Fukui 2004), and buffalos (Anand et al. 2008, Singhal et al. 2008). Conversely, many other reports in various species, including species where an increase has been reported, have failed to observe any effect of cysteamine supplementation on cleavage rates. This lack of effect has

been reported in pigs (Kobayashi et al. 2007, Kobayashi et al. 2006, Song and Lee 2007), cattle (Balasubramanian and Rho 2007, de Matos et al. 1995), buffalos (Gasparrini et al. 2003), sheep (de Matos et al. 2002a), horses (Luciano et al. 2006, Deleuze et al. 2008) and mice (Chen et al. 2005, de Matos et al. 2003).

Addition of cysteamine to the IVM medium : effect on blastocyst development

In very few studies in pigs (Kobayashi et al. 2007, Song and Lee 2007), horse (Deleuze et al. 2008) and mice (Chen et al. 2005), blastocyst development has not been shown to be affected by cysteamine supplementation. Conversely, many authors reported increased blastocyst development following cysteamine supplementation to the IVM medium. This has been observed in cattle (Balasubramanian and Rho 2007, de Matos et al. 1995, de Matos et al. 2002b, Oyamada and Fukui 2004, Takahashi et al. 1993), buffalos (Gasparrini et al. 2003, Anand et al. 2008, Singhal et al. 2008), sheep (de Matos et al. 2002a), goats (Zhou et al. 2008), pigs (Grupen et al. 1995, Kobayashi et al. 2006) and mice (de Matos et al. 2003). In these studies, although cleavage rates are not necessarily affected further development of the blastocysts obtained has been positively influenced by cysteamine supplementation.

Conclusion

Low molecular weight thiol compounds can increase cysteine uptake by oocytes during IVM. This subsequently can increase GSH content, which is a major anti-oxidant system that protect the cells against the deleterious effects of oxidative stress by scavenging reactive oxygen species. Although it generally doesn’t affect nuclear maturation rates, IVM medium supplementation with cysteamine can improve pronucleus formation, cleavage rates and further embryo development, suggesting a beneficial effect on cytoplasmic maturation and oocyte competence. Beneficial effects of thiol compounds supplementation to IVM media have been observed in many species but they appear to be highly species and dose dependant.

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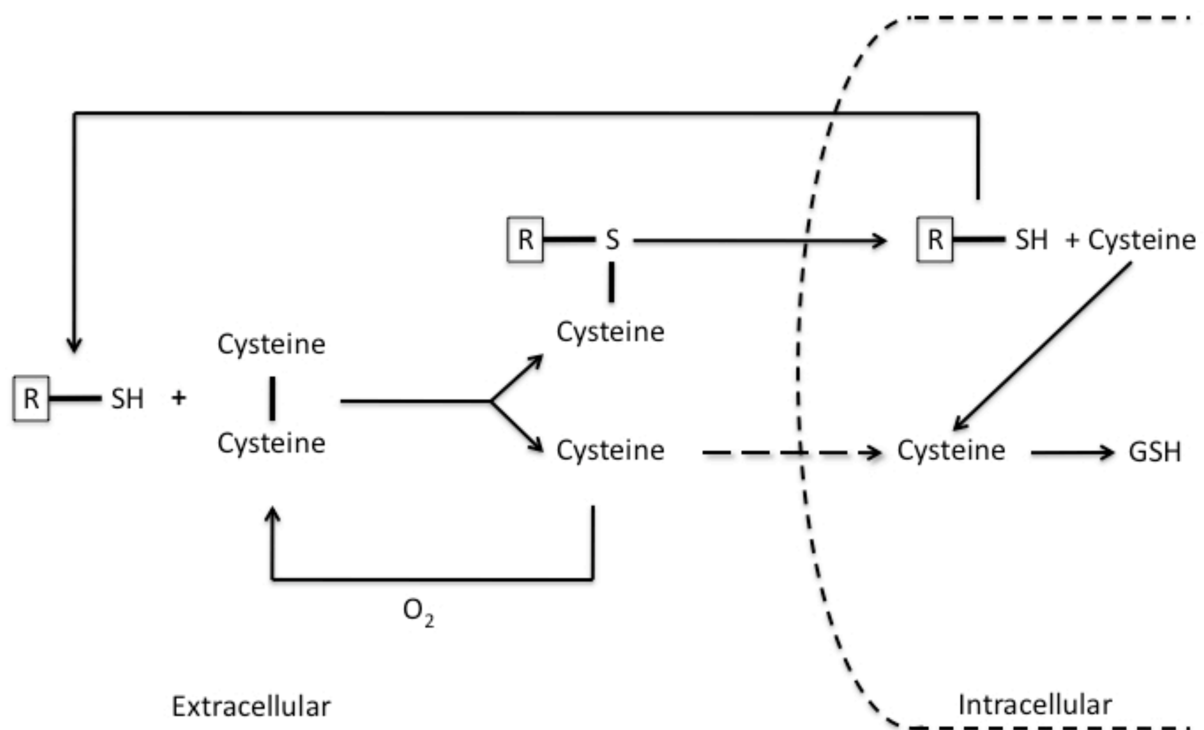

Figure 1: Proposed action of thiol compounds on cystine and cysteine utilization by oocytes.

Adapted from Ishi and collaborators (Ishii et al. 1981).

Species	Dose of Cysteamine (µM)	References
Bovine	100	(Balasubramanian and Rho 2007, de Matos et al. 1997, de Matos et al. 1995, de Matos et al. 1996, de Matos et al. 2002b, Oyamada and Fukui 2004)
Buffalo	50	(Gasparrini et al. 2003, Singhal et al. 2008)
	100	(Gasparrini et al. 2000)
	50, 100, 200	(Anand et al. 2008)
Ovine	50, 100, 200	(de Matos et al. 2002a)
Caprine	100	(Rodriguez-Gonzalez et al. 2003a)
	100, 200, 400	(Urdaneta et al. 2003)
	50, 100, 200, 300	(Zhou et al. 2008)
Equine	100	(Luciano et al. 2006, Deleuze et al. 2008)
Porcine	100	(Kobayashi et al. 2007, Kobayashi et al. 2006, Song and Lee 2007)
	150	(Bing et al. 2001, Yamauchi and Nagai 1999)
	50, 500	(Grupen et al. 1995)
Canine	50, 100, 200	(Hossein et al. 2007)
Mice	100	(Chen et al. 2005)
	0, 25, 50, 100, 200	(de Matos et al. 2003)

Table 1: Summary of the different concentrations used in various species.

PART 7 : SUMMARY

Part 7 : Summary

Research on *in vitro* embryo production (IVP) in the equine is impeded by the limited availability of mature oocytes as the mare is mono ovulating and superovulation is still difficult (Dippert and Squires, 1994; Bezard *et al.*, 1995; Alvarenga *et al.*, 2001b). Despite recent improvement in IVM of equine oocytes, success rates of IVM in that species remain low in all culture media tested compared to other species (Goudet *et al.*, 2000b). However, most studies have focused on the percentage of oocytes reaching the metaphase II stage (nuclear maturation) but few concentrated on the final oocyte competence as measured by its ability to develop into a blastocyst and further establish a pregnancy. Blastocyst production rate is influenced not only by culture environment but also by oocyte maturation conditions.

Under *in vitro* culture conditions, oxidative modifications of cell components via increased ROS represent a major culture induced stress (Johnson and Nasr-Esfahani, 1994). Anti-oxidant systems can attenuate the deleterious effects of oxidative stress by scavenging ROS (Del Corso *et al.*, 1994). Glutathione, a tripeptide thiol, is the major non-protein sulfydryl compound in mammalian cells that plays an important role in protecting the cell from oxidative damage (Meister and Tate, 1976; Meister and Anderson, 1983). It has been suggested that GSH content in oocytes may serve as a reservoir protecting the zygote and the early embryos from oxidative damage before genomic activation and de novo GSH synthesis occur (Furnus *et al.*, 1998; de Matos and Furnus, 2000). The addition of GSH synthesis precursors, such as cysteamine, a thiol compound, to IVM media has been shown to improve IVP in various species (Takahashi *et al.*, 1993; de Matos *et al.*, 1995; Grupen *et al.*, 1995; de Matos *et al.*, 2002a; de Matos *et al.*, 2002b; de Matos *et al.*, 2003; Gasparrini *et al.*, 2003; Oyamada and Fukui, 2004; Balasubramanian and Rho, 2007; Anand *et al.*, 2008; Singhal *et al.*, 2008; Zhou *et al.*, 2008). Very little information on the use of thiol compounds in the equine is available.

Conventional *in vitro* fertilization (IVF) has not been successful in the mare, and a repeatable IVF technique has not yet been developed (Alm *et al.*, 2001). To overcome the limitation of conventional IVF procedures, other methods to produce embryos from oocytes, either *in vivo* or *in vitro*, have been investigated. Among these, intra cytoplasmic sperm injection (ICSI) has permitted efficient equine *in vitro* blastocyst production (Galli *et al.*, 2002; Lazzari *et al.*,

2002; Choi *et al.*, 2006a; Choi *et al.*, 2006c). However, ICSI requires specific equipment and skills. Transfer of an immature oocyte into the preovulatory follicle of an inseminated recipient mare (Intra-Follicular Oocyte Transfer, IFOT) has produced embryos but the success rate was low (Hinrichs and Digiorgio, 1991). Similarly, oocyte transfer (OT) into the oviduct of an inseminated recipient mare was investigated (McKinnon *et al.*, 1988; Carnevale, 1996; Hinrichs *et al.*, 1997; Carnevale *et al.*, 2001; Carnevale *et al.*, 2003; Carnevale, 2004), and commercial programs using OT for mares with reproductive abnormalities are now available (Carnevale *et al.*, 2001). Unfortunately, IFOT is poorly documented in the literature and reports of OT have been published by various laboratories and under various conditions, making comparisons between results and choosing among these as substitutive techniques to ICSI or embryo transfer difficult.

The first aim of the present work was to investigate if there is an influence of supplementation with 100 μM of cysteamine on conventional IVF success rate. Cumulus oocytes complexes (COCs) retrieved by transvaginal ultrasound guided aspiration were matured *in vitro* with or without cysteamine supplementation and were then submitted to conventional IVF using either calcium ionophore or heparin as capacitation treatment for spermatozoa. A total of 131 oocytes were evaluated for evidence of sperm penetration. Both techniques (ionophore or heparin) yielded 6% of IVF and results were similar both for the cysteamine and the control group. This success rate of IVF is low compared to some published data (Palmer *et al.*, 1991; Dell'Aquila *et al.*, 1996; McPartlin *et al.*, 2009) but similar to what others reported in the literature (Choi *et al.*, 1994; Dell'Aquila *et al.*, 1997a). Although, it seems likely that cysteamine did not significantly improve IVF rates under our conditions, our general success rates for IVF procedures may be too low for us to conclude definitely about the effect of cysteamine.

As ICSI was not available to us, the second aim of this work was to determine what *in vivo* technique could best bypass the lack of an efficient conventional IVF procedure. We compared embryo production following transfer of *in vivo* recovered oocytes (1) into a recipient's oviduct or (2) into her preovulatory follicle either immediately after ovum pick up or (3) after *in vitro* maturation. Recipients were inseminated with fresh semen of a stallion with a known normal fertility. Ten days after transfer, rates of embryos collected in excess to the number of ovulations were calculated and compared for each group. Embryo collection rates were 32.5% (13/40), 5.5% (3/55) and 12.8% (6/47) for OT, post-IVM and immediate IFOT respectively. OT significantly yielded more embryos than immediate and post-IVM

IFOT did. These results show that, when ICSI is not an option, intra-oviductal oocyte transfer is to be preferred to IFOT, as an *in vivo* alternative, to bypass the inadequacy of conventional *in vitro* fertilization and to assess oocyte developmental competence.

After it was established that in comparison to IFOT, OT is the most reliable *in vivo* alternative to *in vitro* fertilization where ICSI technology is not available, this technique was used to assess the effect of cysteamine supplementation on nuclear maturation and oocyte competence. The third aim of this work was to investigate the influence of supplementation with 100 µM of cysteamine on *in vitro* nuclear and cytoplasmic maturation by specific DNA staining and the ability of oocytes to undergo *in vivo* fertilization after OT. Oocytes were collected by transvaginal ultrasound guided aspiration and matured *in vitro* with (cysteamine group) or without (control group) cysteamine. The nuclear stage after DNA Hoechst staining and the embryo yield following OT were used as a criterion for assessing nuclear and cytoplasmic maturation, respectively. Overall maturation rate was 52%, which is rates reported in the literature ranging from 40 to 70% in the equine (Goudet *et al.*, 1997a; Bogh *et al.*, 2002; Hinrichs *et al.*, 2005; Galli *et al.*, 2007). Nuclear maturation was not statistically different ($p>0.05$) between oocytes cultured with or without cysteamine (55% and 47% respectively). From 57 oocytes transferred to the oviduct in each group, the number of embryos collected was 10 (17%) in the control group and 5 in the cysteamine group (9%). Those two percentages were not statistically different ($p>0.05$). Contrary to the data described in other domestic species, there was no effect of cysteamine on *in vitro* nuclear maturation, or *in vivo* embryonic development under our conditions. Under our conditions, the addition of 100 µM of cysteamine to a classic culture medium does not improve equine oocyte maturation or embryonic development after OT. The same dose failed to increase GSH content in the equine (Luciano *et al.*, 2006). However, the effect of cysteamine supplementation is highly species and concentration dependant. The inadequacy of the chosen concentration may explain that equine embryo production has not been increased by the cysteamine under our conditions as opposed to what has been observed in many other species. Alternatively, we can hypothesize that some substances present in the IVM medium can interfere with GSH synthesis. This has been suggested for FSH and estradiol (Bing *et al.*, 2001) and, although our maturation medium is not supplemented with gonadotropins or estradiol, factors contained in fetal calf serum or EGF might also have an effect on GSH synthesis.

Considering its beneficial effects in many other species, supplementation with cysteamine to different IVM media should be further investigated in the equine. Ideally combining different concentrations and ICSI or OT in order to determine an optimal concentration and its effects on oocyte developmental competence.

PART 8: RESUME

Part 8 : Résumé

La jument est mono-ovulante et les protocoles de superovulation restent décevants chez les équins (Dippert and Squires, 1994; Bezard *et al.*, 1995; Alvarenga *et al.*, 2001b). Ceci se traduit par une faible disponibilité d'ovocytes matures qui limite le développement des technologies de production d'embryons dans cette espèce. Malgré des projets récents en matière de maturation *in vitro* (MIV) des ovocytes, les taux de maturation chez les équins restent inférieurs à ceux obtenus dans d'autres espèces (Goudet *et al.*, 2000b). Il est à noter que la plupart des études se sont concentrées sur le pourcentage d'ovocytes qui atteignent le stade de métaphase II (maturation nucléaire) négligeant la maturation cytoplasmique ou acquisition de la compétence ovocytaire, c'est-à-dire la capacité que doit acquérir l'ovocyte à être fécondé, à donner un blastocyste et à établir une gestation évolutive. Le taux de blastocystes produits *in vitro* dépend non seulement des conditions de culture mais aussi de la qualité de la maturation ovocytaire.

Dans les conditions de culture *in vitro*, les modifications des composantes cellulaires consécutives à l'augmentation des formes activées de l'oxygène (en Anglais: Reactive Oxygen Species ou ROS) constituent un facteur de souffrance cellulaire majeur (Johnson and Nasr-Esfahani, 1994). Différents systèmes anti-oxydants peuvent atténuer les effets délétères du stress oxydant en neutralisant les ROS (Del Corso *et al.*, 1994). Le Glutathion (GSH), un tripeptide porteur d'un groupement thiol (R-SH) constitue le principal agent non protéique, qui protège la cellule contre les dégâts induits par le stress oxydant (Meister and Tate, 1976; Meister and Anderson, 1983). Il a été suggéré que le GSH présent dans les ovocytes pourrait servir de réserve pour assurer la protection du zygote et des stades précoces de l'embryon. Ceci contre les souffrances dues au stress oxydant en attendant que l'activation du génome du jeune embryon soit effective et qu'il soit capable de produire son propre GSH pour se protéger (Furnus *et al.*, 1998; de Matos and Furnus, 2000). Il a été démontré, dans différentes espèces, que l'ajout de précurseurs de la synthèse de GSH, comme la cystéamine qui est un composé porteur d'un thiol, améliore la production *in vitro* d'embryons (Takahashi *et al.*, 1993; de Matos *et al.*, 1995; Grupen *et al.*, 1995; de Matos *et al.*, 2002a; de Matos *et al.*, 2002b; de Matos *et al.*, 2003; Gasparrini *et al.*, 2003; Oyamada and Fukui, 2004; Balasubramanian and Rho, 2007; Anand *et al.*, 2008; Singhal *et al.*, 2008; Zhou *et al.*, 2008).

A ce jour, peu d'informations quant à l'utilisation de ces composés thiols dans l'espèce équine sont disponibles.

La fécondation in vitro (FIV) conventionnelle s'est avérée peu efficace chez le cheval et on peut considérer que dans l'état actuel des choses aucune méthode de FIV efficace et reproductible n'est disponible dans cette espèce (Alm *et al.*, 2001). Afin de dépasser cette difficulté, d'autres techniques visant à produire *in vitro* ou *in vivo* des embryons à partir d'ovocytes ont été explorées. Parmi celles-ci, l'injection intra-cytoplasmique de spermatozoïde (ICSI) a permis de produire *in vitro* des taux corrects de blastocystes (Galli *et al.*, 2002; Lazzari *et al.*, 2002; Choi *et al.*, 2006a; Choi *et al.*, 2006c). Cependant, cette technique est très exigeante en termes de matériel et d'expertise ce qui limite malgré tout son utilisation. Après collecte, des ovocytes ont été réinjectés dans le follicule préovulatoire d'une jument receveuse inséminée, afin qu'ils soient libérés au moment de l'ovulation. Cette technique de transfert d'ovocyte intra-folliculaire (TOIF) a permis de produire des embryons mais le taux de succès est resté faible (Hinrichs and Digiorgio, 1991). Une autre approche, qui consiste à transférer un ou plusieurs ovocytes dans l'oviducte d'une jument receveuse qui est inséminée, a également été explorée et des programmes commerciaux utilisant cette technique de transfert intra-saplyngien d'ovocyte (OT) sont actuellement disponibles pour des juments souffrant de pathologies qui ne permettent pas de leur faire produire un embryon par d'autres méthodes (McKinnon *et al.*, 1988; Carnevale, 1996; Hinrichs *et al.*, 1997; Carnevale *et al.*, 2001; Carnevale *et al.*, 2003; Carnevale, 2004). Malheureusement, le TOIF est peu documenté dans la littérature et les publications portant sur le transfert d'ovocytes émanent de nombreuses équipes et les différences dans leur méthodologie rendent les comparaisons entre leurs résultats très difficiles. En l'absence d'une étude comparative des différentes techniques, il serait très imprudent de décider laquelle doit être privilégiée comme technique substitutive à l'ICSI.

Le premier objectif du présent travail est de déterminer l'effet de l'ajout de 100 µM de cystéamine au milieu de MIV sur les taux de succès de FIV conventionnelle. Les complexes cumulus-ovocytes (COCs) obtenus par ponction échoguidée ou ovum pick up (OPU) sont mis à maturer avec ou sans supplémentation du milieu avec de la cystéamine. Ils sont ensuite soumis à une technique conventionnelle de FIV utilisant un ionophore calcique ou de l'héparine comme agent de capacitation des spermatozoïdes. Les signes de pénétration par un spermatozoïde sont évalués sur 131 ovocytes. Les deux traitements du sperme (ionophore vs héparine) ont donné les mêmes taux de FIV (6%) et ces résultats sont identiques en présence

comme en absence de cystéamine dans le milieu de MIV. Ce taux de FIV est faible par rapport à certains résultats publiés dans la littérature (Palmer *et al.*, 1991; Dell'Aquila *et al.*, 1996; McPartlin *et al.*, 2009), mais similaire aux taux observés dans d'autres études (Choi *et al.*, 1994; Dell'Aquila *et al.*, 1997a). Bien qu'il semble que la cystéamine n'ait pas eu d'effet sur les taux de FIV, le faible taux de FIV global obtenu ne permet pas de tirer de conclusion définitive.

Comme la technologie de l'ICSI ne nous était pas accessible, le deuxième objectif poursuivi a été de déterminer quelle technique *in vivo* permettait de contourner au mieux l'absence d'une technique de FIV conventionnelle efficace. Nous avons comparé les taux de production d'embryons après transferts d'ovocytes, collectés par OPU, (1) dans l'oviducte de juments receveuses, ou dans le follicule préovulatoire de juments receveuses, soit (2) immédiatement après leur collecte par OPU soit (3) après MIV. Toutes les juments receveuses sont inséminées avec du sperme d'un étalon de fertilité connue. Les taux d'embryons collectés 10 jours plus tard, sont calculés après déduction du nombre d'ovulations des receveuses et comparés pour chacun des groupes. Les taux d'embryons sont de 32.5% (13/40), 5.5% (3/55) et 12.8% (6/47) pour les techniques de OT, post-MIV et TOIF immédiat respectivement. Le transfert d'ovocytes dans l'oviducte d'une receveuse donne significativement plus d'embryons que les transferts intra-folliculaires. Ces résultats montrent que lorsque l'ICSI n'est pas une option, la technique d'OT doit être préférée au TOIF comme alternative permettant de contourner la difficulté de l'absence d'une technique de FIV efficace et reproductible.

Le troisième objectif poursuivi a été d'évaluer l'effet de l'ajout de 100 µM de cystéamine au milieu de MIV sur la maturation nucléaire et cytoplasmique des ovocytes, respectivement par une coloration spécifique de l'ADN et par leur compétence à être fécondés et produire un embryon. Les ovocytes sont collectés par OPU et maturés *in vitro* avec (groupe cystéamine) ou sans (groupe contrôle) cystéamine. Le stade nucléaire est évalué sous microscopie à fluorescence après coloration au bis-benzamide. Le taux global de maturation nucléaire observé est de 52%. Cette valeur est cohérente avec les taux de MIV rapportés dans littérature qui varient de 40 à 70% dans l'espèce équine (Goudet *et al.*, 1997a; Bogh *et al.*, 2002; Hinrichs *et al.*, 2005; Galli *et al.*, 2007). Les taux de maturation ne sont pas significativement différents ($p>0,05$) entre les ovocytes maturés avec ou sans cystéamine (respectivement 55% et 47%). Les taux d'embryons produits sont utilisés pour évaluer la maturation cytoplasmique. Un total de 57 ovocytes est transféré dans les oviductes des receveuses pour chacun des

groupes. Les nombres d'embryons collectés sont de 10 (17%) pour le groupe contrôle et 5 pour le groupe cystéamine (9%). Ces deux pourcentages ne sont pas statistiquement différents ($p>0.05$). Contrairement à ce qui a été décrit dans les autres espèces, aucun effet de la supplémentation du milieu de MIV par la cystéamine n'a pu être mis en évidence dans nos conditions expérimentales.

En conclusion, l'ajout de 100 µM de cystéamine au milieu de MIV n'a apparemment pas amélioré les résultats de FIV conventionnelle. Cependant, le faible taux global de FIV ne permet pas de conclure avec certitude quant à l'effet, ou l'absence d'effet, de la cystéamine. Après qu'il ait été établi que lorsque l'ICSI n'est pas disponible, le transfert d'ovocytes dans l'oviducte est préférable au TOIF, cette technique a été utilisée pour évaluer l'effet de l'ajout de100 µM de cystéamine au milieu de MIV sur l'aptitude de l'ovocyte à être fécondé et donner un embryon. Dans nos conditions, cette supplémentation du milieu de MIV avec de la cystéamine n'améliore ni la maturation nucléaire, ni la maturation cytoplasmique de l'ovocyte équin. Cependant, les effets rapportés de la cystéamine dépendent pour beaucoup de l'espèce et de la dose utilisée. Il est possible que la dose de cystéamine testée, bien que largement utilisée dans d'autres espèces, ne permette pas d'améliorer la MIV des ovocytes équins. La même dose s'est avérée ne pas induire d'augmentation de la concentration en GSH dans les ovocytes équins (Luciano *et al.*, 2006). Il est également possible que certaines substances contenues dans le milieu de MIV interférent avec la synthèse de GSH. Ceci a été suggéré pour la FSH ou l'oestradiol (Bing *et al.*, 2001). Bien que notre milieu de maturation ne contienne ni gonadotrope, ni stéroïdes sexuels ajoutés, certains facteurs présents dans le sérum de veau foetal ou l'EGF pourraient également avoir un effet sur la synthèse de GSH.

Au vu de ses effets bénéfiques dans de nombreuses autres espèces, la cystéamine devrait faire l'objet d'études complémentaires. Idéalement en testant différentes doses et, grâce à l'ICSI et au OT, en mesurant leurs effets sur la concentration en GSH, les taux de clivage, la formation de blastocystes et la production d'embryons, afin de déterminer une éventuelle concentration optimale et ses effets sur la compétence de l'ovocyte.

PART 9 : INDEX OF ABBREVIATIONS

Part 9 : Index of abbreviations

ARTs	Assisted Reproductive Technologies
CEG	Crude Equine Gonadotropin
COC	Cumulus Oocyte Complex
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
FSH	Follicle-Stimulating Hormone
GIFT	Gamete Intra-Fallopian Transfer
GPX	Glutathione Peroxydase
GSH	Glutathione
GSSG	oxydized form of Glutathione
GV	Germinal Vesicle
ICSI	Intra-Cytoplasmic Sperm Injection
IFOT	Intra-Follicular Oocyte Transfer
IVC	In Vitro Culture
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
IVP	In Vitro embryo Production
LH	Luteinzing Hormone
M I	Metaphase I
M II	Metaphase II
mRNA	messenger Ribonucleic Acid
OT	intra-oviductal Oocyte Transfer
PBS	Phosphate Buffered Solution
ROS	Reactive Oxygen Species