EQUINE EMBRYOS PRODUCED IN VITRO: HOW MUCH DO THEY MISS A MARE?

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Live as if you were to die tomorrow.

Learn as if you were to live forever.

Mahatma Gandhi
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACTB</td>
<td>beta actin</td>
</tr>
<tr>
<td>AI</td>
<td>artificial insemination</td>
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<tr>
<td>ART</td>
<td>artificial reproductive technologies</td>
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<tr>
<td>BEX2</td>
<td>brain expressed X-linked 2</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>COC</td>
<td>cumulus oocyte complex</td>
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<tr>
<td>Cq value</td>
<td>quantification cycle value</td>
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<tr>
<td>CZB medium</td>
<td>Chatot-Ziomek-Bavister medium</td>
</tr>
<tr>
<td>DMEM-F12</td>
<td>Dulbecco's modified Eagle medium: nutrient mixture F-12</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate buffered saline</td>
</tr>
<tr>
<td>ET</td>
<td>embryo transfer</td>
</tr>
<tr>
<td>FABP3</td>
<td>fatty acid binding protein 3</td>
</tr>
<tr>
<td>FAF-BSA</td>
<td>fatty acid-free bovine serum albumin</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H2A/I</td>
<td>histone H2A type 1-C</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>heat shock protein 90kDa alpha, class A member 1</td>
</tr>
<tr>
<td>ICSI</td>
<td>intracytoplasmic sperm injection</td>
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<tr>
<td>IVC</td>
<td>in vitro culture</td>
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<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
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<tr>
<td>IVM</td>
<td>in vitro maturation</td>
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<tr>
<td>IVP</td>
<td>in vitro production</td>
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<tr>
<td>KSOM</td>
<td>simplex optimization medium with elevated K⁺ concentration</td>
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<tr>
<td>MII</td>
<td>metaphase of the second meiotic division</td>
</tr>
<tr>
<td>MCM7</td>
<td>minichromosome maintenance complex component 7</td>
</tr>
<tr>
<td>MEM</td>
<td>Eagle's minimal essential medium</td>
</tr>
<tr>
<td>MOBKL3</td>
<td>mps one binder kinase activator-like 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
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<td>OPU</td>
<td>ovum pick-up</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pFSH</td>
<td>porcine follicle-stimulating hormone</td>
</tr>
<tr>
<td>pLH</td>
<td>porcine luteinizing hormone</td>
</tr>
<tr>
<td>POU5F1</td>
<td>POU domain, class 5, transcription factor 1</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPL32</td>
<td>ribosomal protein L32</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>SDHA</td>
<td>succinate dehydrogenase complex, subunit A</td>
</tr>
<tr>
<td>SOF</td>
<td>synthetic oviduct fluid</td>
</tr>
<tr>
<td>SSH</td>
<td>suppression subtractive hybridization</td>
</tr>
<tr>
<td>SR</td>
<td>serum replacement</td>
</tr>
<tr>
<td>TALP</td>
<td>Tyrode’s albumin-lactate-pyruvate</td>
</tr>
<tr>
<td>TCM199</td>
<td>tissue culture medium 199</td>
</tr>
<tr>
<td>TUBA4A</td>
<td>tubulin, alpha 4a</td>
</tr>
<tr>
<td>UBC</td>
<td>ubiquitin C</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 Artificial Reproductive Technologies in the Horse

Up to the end of the nineteenth century, the vast majority of horses were bred by natural cover. The introduction of artificial reproductive technologies (ART) started with the first reported equine pregnancy produced by artificial insemination (AI), which is the placement of stallion sperm into a mare’s uterus (Heape, 1898). Nowadays, the majority of mares are impregnated by AI using fresh, cooled or frozen-thawed sperm, thereby providing the opportunity for genetically valuable stallions to produce more offspring. On the female side, genetic selection can be accelerated by recovering an embryo from the uterus of a valuable donor mare and subsequent embryo transfer (ET) to a recipient mare, which carries the foal to term (for review see Stout, 2006). The first successful ET in horses was achieved in 1974 (Oguri and Tsutsumi, 1974) and ET is now a routine procedure in practice (Scherzer et al., 2008). In both of these techniques, early embryonic development occurs in the female reproductive tract, i.e. in vivo. This is in contrast to in vitro production (IVP) of embryos, in which fertilization and early development occur under laboratory conditions outside the mare. In vitro production of equine embryos only evolved recently and is the subject of this thesis.

1.2 Early Equine Embryonic Development In Vivo

As for all mammalian oocytes, the equine oocyte is arrested in the prophase of the first meiotic division during foetal development, and only a few hundred selected oocytes ever reach the second metaphase (MII) stage prior to ovulation (King et al., 1987; Pierson, 1992). Fertilization of the mature oocyte occurs at the ampulla-isthmus junction of the oviduct, where the developing embryo remains during its subsequent cleavage divisions (Betteridge et al., 1982; McKinnon et al., 1992; Weber et al., 1996). After 5 days at the ampulla-isthmus junction, transport through the oviductal isthmus occurs rapidly and the late morula or early blastocyst enters the uterus through the utero-tubal junction 144-156 h after ovulation (Oguri and Tsutsumi, 1972; Weber et al., 1996; Battut et al., 1997) (Figure 1). Unfertilized eggs on the other hand are retained in the oviduct (Van Niekerk and Gerneke, 1966). This selective oviductal transport is linked to the stage specific production of prostaglandin E2 by the equine
conceptus, an example of extremely early embryo-maternal communication or interaction (Weber et al., 1991).

Figure 1 Equine embryonic development in vivo. Fertilization (1,2) and early cleavage (3,4,5) occur at the ampulla-isthmic junction. After rapid transport through the istmus, the morula (6) or early blastocyst (7) reaches the uterus. A glycoprotein capsule is formed between the trophectoderm and the zona pellucida, which is subsequently shed from the rapidly expanding blastocyst (8).
Another exceptional feature of the equine is the formation of an acellular glycoprotein capsule. This structure was first described over a century ago (Bonnet, 1889; Krölling, 1937), but most of the research has been performed in the past 40 years (Marrable and Flood, 1975; Betteridge et al., 1982; Flood et al., 1982). Upon arrival of the embryo in the uterus, the capsule is formed between the trophectoderm and the zona pellucida of the equine blastocyst and it surrounds the conceptus until around day 21 of gestation (Betteridge et al., 1982; Enders and Liu, 1991) (Figure 2). Embryonic tertiary coats are described in several species (Betteridge, 1989; Denker, 2000). The glycoprotein capsule as present in equids shows the most similarities with the neozona in rabbits (Betteridge, 1989; Denker, 2000).

![Figure 2 Equine embryonic capsule in vivo. This figure displays the capsule of two in vivo derived blastocysts in a different stadium of the embryonic development. A: Slightly collapsed hatched horse blastocyst surrounded by a loose, overlarge capsule; B: Tight capsule between the trophectoderm and the zona pellucida of an expanding blastocyst.](image)

Up to day 16, the spherical equine embryo is very mobile and migrates through the uterus. This migration is necessary to obtain maternal recognition of the pregnancy. While migrating through the uterus, the equine conceptus signals its presence and prevents cyclical luteolysis (Allen, 2000). The strong, elastic capsule has been suggested to protect the preimplantation embryo during this migratory phase (Betteridge et al., 1982). The capsule also appears to be
involved in subsequent fixation (day 16-17) and orientation of the embryo (Oriol, 1994). Throughout embryonic development, the capsule is thought to function as a ‘mailbox’, incorporating endometrial components and transporting these to the developing embryo (Herrler and Beier, 2000). In this way, it can be involved in the early embryo-maternal communication. Even though the precise role is not entirely clear, the capsule has been shown to be essential for the continuance of pregnancy (McKinnon et al., 1989; Stout et al., 2005). The equine trophoblast is the primary source of the capsular material (Albihn et al., 2003). However, absence of normal capsule formation in vitro implies an essential role of the maternal environment (Hinrichs et al., 1990a; Tremoleda et al., 2003). It remains to be determined which aspects of the uterine surroundings and which mechanisms are involved in the formation of this intriguing component of the equine embryo.

Endometrial secretions (‘histotrophe’) are particularly important in the horse, because of the exceptionally long pre-implantation period, during which the developing embryo totally depends on these secretions for its nutrition (Stewart et al., 2000). In this regard, uterocalin, a component of the uterine secretions, might be involved in capsule formation. Uterocalin, a 19 kDa protein, was first isolated using SDS-PAGE of equine embryonic capsules (Stewart et al., 1995). Structural analysis classifies uterocalin as a member of the lipocalin family and suggests that the primary function of uterocalin is to act as a carrier of biologically important lipids and as a source of essential amino acids for the developing conceptus (Suire et al., 2001; Kennedy, 2004). Substantial concentrations of uterocalin have been associated with the capsule, and passage through the capsule to the developing conceptus has been evidenced by the presence of uterocalin on the trophoblast cells (Crossett et al., 1996; Ellenberger et al., 2008). Furthermore, uterocalin is positively charged. This facilitates its binding to the negatively charged sialic acid residues of the capsule (Oriol et al., 1993; Crossett et al., 1998). Uterocalin is secreted by the endometrial glands in a progesterone dependant way during both dioestrus and early pregnancy (Stewart et al., 1995). Interestingly, the high concentrations of uterocalin in the uterus, which are prevailing during early pregnancy (day 6-day 23), coincide exactly with the period during which the equine embryo is surrounded by the capsule (Crossett et al., 1998). In summary, structural, functional and temporal associations have been made between the
maternal uterocalin and the embryonic capsule in the horse, but whether uterocalin effectively supports the capsule formation remains to be determined.

The examples mentioned above show the important interaction between the developing embryo and the maternal genital tract. In vivo derived horse embryos have been exposed to the maternal genital tract and are supposed to have developed normally. Such equine embryos represent therefore interesting study material and can be used as a gold standard, to which in vitro produced embryos that have been cultured in the absence of the maternal genital tract can be compared. Unfortunately, in vivo derived horse embryos are relatively difficult to obtain. From the zygote to the early morula stage, they remain for a relatively long stay in the oviduct and can only be harvested surgically. After its arrival in the uterus, a horse blastocyst can be recovered atraumatically by uterine flushing. But even then, generally only one embryo can be obtained per mare, since the mare is mono-ovulatory and responds only moderately to superovulatory treatments (for review see McCue, 1996; Stout, 2006). In the experiments described in this thesis, equine in vivo embryos were flushed at day 7, at which time they have reached the blastocyst stage. These in vivo derived blastocysts represented the gold standard for comparison with the in vitro produced blastocysts. When equine embryos are produced and cultured in vitro, the absence of these maternal interactions have morphological and developmental consequences, which will be discussed in the next chapters of this thesis.

1.3 EARLY EQUINE EMBRYONIC DEVELOPMENT IN VITRO

The in vitro production (IVP) of equine embryos consists of several important steps.

First of all, oocytes need to be collected. In the living mare, the mature oocyte can be obtained from a preovulatory follicle by aspiration using a long needle placed through the flank of the sedated standing mare (Vogelsang et al., 1983; Palmer et al., 1986; Hinrichs et al., 1990b). Alternatively, several follicles can be aspirated through ultrasound guided transvaginal ovum pick up (OPU) (Brück et al., 1992; Cook et al., 1993; Bezar et al., 1995; Meintjes et al., 1995; Goudet et al., 1997; Galli et al., 2001). Post mortem, immature oocytes can be collected from ovaries through either aspiration (Desjardins et al., 1985; Shabpareh et al., 1993) or scraping of
the follicles (Del Campo et al., 1995; Dell’Aquila et al., 2001). Next, all immature oocytes must undergo maturation in vitro (IVM). Finally, the mature oocytes must be fertilized. Conventional in vitro fertilization (IVF), which implies the co-incubation of mature COCs with capacitated sperm, is largely unsuccessful in horses. Therefore, intracytoplasmic sperm injection (ICSI), a micromanipulation technique during which a single sperm cell is injected into the cytoplasm of a mature oocyte, is the method of choice. These fertilized oocytes are then cultured in vitro (IVC) up to the blastocyst stage, which can be transferred to the uterus of a recipient mare (ET). Specific problems during these various steps of the IVP of equine embryos have hindered rapid progress towards large scale equine IVP and will be covered in the subjoined paragraphs. The general protocol that was followed throughout the thesis is described in Addendum 1.

1.3.1 Oocyte collection

Oocytes available for research are scarce, since the access to abattoir ovaries is limited. In some countries, such as the USA, all horse abattoirs have been closed, and in these countries all oocytes must be collected using the time consuming technique of in vivo collection through OPU (McPartlin et al., 2007). Furthermore, the very tight connection between the equine oocyte and the follicle wall requires scraping or vigorous flushing of the follicle to recover the oocyte (Hawley et al., 1995).

Figure 3 Ex vivo collection of horse oocytes by means of aspiration. The follicular fluid is aspirated (-100 mm Hg) (A), the follicles are scraped with the aspirating needle and simultaneously flushed (B) with heparin (25IU/ml). After aspiration of the superficial follicles, the ovary is cut to reach the follicles inside (C).
Oocytes can be recovered *ex vivo* either by incising the follicle and scraping the wall with a bone curette or by aspiration of the follicular contents (*Figure 3*). An experiment conducted in our laboratory comparing scraping with aspiration, indicated that the scraping technique is associated with more cumulus cells surrounding the recovered oocytes, when compared to aspiration (*Table 1*). Our observations are in agreement with Dell’Aquila et al. (2001), who reported 53% of the aspirated oocytes to have only a partial cumulus, while this was only 15% for the scraped oocytes. The presence of several layers of cumulus cells favors the classification of the oocytes into expanded (*Figure 4A*) versus compact (*Figure 4B*) cumulus oocyte complexes (COCs) (Hinrichs, 2010b). It is useful to be able to make an accurate distinction between the expanded and the compact COCs, because the optimal maturation time is different for both populations (Hinrichs et al., 1993). In other species, expanded COCs are discarded, as they mostly originate from atretic follicles and are associated with a low developmental capacity. In contrast, the equine expanded COCs have been shown to result in similar blastocyst rates, when compared to compact COCs (Galli et al., 2007; Hinrichs, 2010a). When oocytes are collected by aspiration, a significant proportion of oocytes is only surrounded by a few layers of corona cells, which complicates an accurate classification. Despite the difficult classification of oocytes collected by aspiration, the aspiration technique is the preferred technique for oocyte recovery in our laboratory. This preference is largely based on the higher oocyte recovery rate (73%) for aspiration compared to scraping (51%) in our laboratory, and by the fact that aspiration is up to 4 times less time consuming (Using the aspiration methodology developed in our laboratory, a trained technician can collect the same number of oocytes in one hour as two technicians using scraping technique in two hours). It should however be pointed out that in order to obtain as much oocytes as possible, the follicle wall is scraped repeatedly with the point of the aspirating needle, and thus our aspiration technique is in fact a combination of the scraping and the aspiration method. As a whole, oocyte recovery in the horse is far more time consuming than it is in other species and for example over 10-fold more time consuming than oocyte recovery in cattle (Galli et al., 2007). Galli et al. (2007) give the example of 4 technicians needing 3-4 hours to collect 100 equine oocytes, while 2 technicians can collect the same number of bovine oocytes in only 30-40 minutes.
Table 1  

**Oocyte collection technique.** Oocytes were classified based on the expansion and extensiveness of the surrounding cumulus cells. Oocytes were categorised as expanded COCs, compact COCs and oocytes with only a few layers from the corona radiata. Recovery rates for each category of oocytes were compared for two different collection techniques: scraping of the follicle wall with a bone curette and follicular aspiration.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Expanded</th>
<th>Compact</th>
<th>Corona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scraping</td>
<td>200</td>
<td>50 (25%)</td>
<td>105 (52.5%)</td>
<td>45 (22.5%)</td>
</tr>
<tr>
<td>Aspiration</td>
<td>430</td>
<td>68 (16%)</td>
<td>169 (39%)</td>
<td>193 (45%)</td>
</tr>
</tbody>
</table>

**Figure 4**  

**Immature equine cumulus oocyte complexes (magnification 100x).**  
A: expanded COC;  
B: compact COC

1.3.2 *In vitro* maturation

In equine, compared to bovine, a larger proportion of degenerate oocytes can be identified after IVM of abattoir-derived oocytes (Galli et al., 2007). This post mortem degeneration affects around 30% of the collected oocytes and substantially decreases the maturation rate. Maturation of horse oocytes is judged after IVM and removal of the cumulus cells by assessing the extrusion of the polar body (*Figure 5*), which indicates progression to metaphase II (MII).
The mature MII oocyte is characterized by an extruded polar body (arrow).

However, nuclear maturation which is characterized by the extrusion of a polar body, does not necessarily reflect the cytoplasmic maturation of the oocyte. During cytoplasmic maturation, proteins, RNA, substrates and nutrients are accumulated, rendering the oocyte competent for further development (Watson, 2007). This process is crucial for further embryonic development and it is greatly influenced by the oocyte environment in vivo or in vitro. Therefore, the maturation conditions can have a major influence on the blastocyst rate. Replacement of Tissue Culture Medium199 (TCM199) by Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) for equine IVM has been shown to result in higher cleavage and blastocyst rates without affecting the proportion of MII oocytes (Galli et al., 2007). For this reason, DMEM-F12-based maturation medium is used in our laboratory.

1.3.3 Fertilization

Conventional in vitro fertilization (IVF), which implies the co-incubation of mature COCs with capacitated sperm, is largely unsuccessful in horses, primarily because it is difficult to adequately stimulate horse sperm to penetrate the zona pellucida in vitro (Alm et al., 2001; Roasa et al., 2007). Only two foals produced by means of conventional IVF have been born to date (Palmer et al., 1991). Even though acceptable in vitro fertilization rates were recently
reported following hyperactivation of stallion sperm with procaine (McPartlin et al., 2009), this procedure has not yet been repeated in other laboratories.

To circumvent the problem of sperm activation in vitro, intracytoplasmic sperm injection (ICSI) is now used for the IVP of equine embryos. This technique, using micromanipulation to inject a single sperm cell into the cytoplasm of a mature oocyte (Figure 6), was initially developed in Belgium for treatment of male factor infertility in humans (Palermo et al., 1992). The first ICSI foal was produced by Squires et al. (1996), who injected in vitro matured oocytes and transferred these oocytes to the oviduct of recipient mares.

**Figure 6 Intracytoplasmic sperm injection in the horse (magnification 300x).** The mature oocyte is immobilized with the polar body at the 6 o’clock position (arrow) by aspiration with a holding pipette at 9 o’clock. An immobilized sperm cell is injected using a fine injection pipette at 3 o’clock.

After this initial success, several laboratories tried to implement the ICSI technique, with variable results. In 2002, the piezo drill, a device that creates minute vibrations of the injection pipette, was introduced for equine ICSI and, at the same time, increased cleavage rates and more consistent results were reported (Choi et al., 2002, Galli et al., 2002). This has led to general use of the piezo drill, even though a causal relationship with the improved results has never been proven. Various aspects can explain the positive effect of piezo assisted ICSI. In mice, the piezo drill was applied for ICSI and resulted in the first ICSI offspring (Kimura and
Advantages compared to conventional ICSI include a higher probability of oolemma breakage and reduced damage to the oocyte. Moreover, the piezo might cause increased permeabilization of the sperm membrane, which could facilitate oocyte activation (Choi et al., 2002). The best results are obtained when a small amount of mercury is present in the tip of the injection pipette (Ediz and Olgac, 2004). Mercury reduces the lateral oscillations of the pipette, which reduces oocyte damage during the piercing (Ediz and Olgac, 2005). On the other hand, mercury is a cumulative neurotoxin. Furthermore, piezo assisted micromanipulation in mice has been associated with DNA damage (Yu et al., 2007). The possible influence of the injection method on subsequent embryonic developmental competence, including the advantages and disadvantages, needs to be investigated further. In this respect, it might be interesting to consider other sperm injection technologies, like laser-assisted ICSI. During laser-assisted ICSI, a hole is made in the zona pellucida prior to sperm injection. This causes less oocyte disturbance than conventional ICSI. In human, the laser has been shown to be advantageous for patients with fragile oolemmas or patients with high rates of oocyte degeneration after conventional ICSI (Abdelmassih et al., 2002; Rienzi et al., 2004).

1.3.4 In vitro culture

As fertilized oocytes reside in the oviduct under physiological circumstances, initially sperm injected horse oocytes were transferred to a recipient mare’s oviduct (Squires et al., 1996; Cochran et al., 1998; McKinnon et al., 2000), with all the disadvantages associated with the required surgery. Therefore, efforts were made to develop in vitro conditions to culture the embryos up to the blastocyst stage, allowing transcervical transfer into the uterus. Firstly, co-culture of embryos with different types of cells was performed. In vivo derived embryos were cultured with oviduct cells (Battut et al., 1991) or uterine cells (Ball et al., 1993) and IVP embryos with Vero cells (Guignot et al., 1998), cumulus cells (Li et al., 2001) or granulosa cells (Rosati et al., 2002). Then, several defined media, like G1.2 (Choi et al., 2002) and CZB (Choi et al., 2004) were evaluated, but blastocyst rates remained variable and below 20%. Eventually, important progress was obtained by the finding that using DMEM-F12 as an equine embryo culture medium yielded blastocyst development rates of up to 34% (Choi et al., 2006b).
Originally developed for cell culture, this medium contains a high glucose concentration when compared to regular embryo culture media. Contrary to other species, equine embryos apparently benefit from high glucose concentrations during early development (Herrera et al., 2008). Despite these advances, the percentage of injected oocytes reaching the blastocyst stage in clinical practice is only 10%, as recently published by one of the most experienced groups in the field (Barbacini et al., 2010) and temporary culture in sheep oviducts still appears to yield better results than total IVP (Lazzari et al., 2010), illustrating further the necessity for optimizing IVC conditions for equine embryos.

1.3.5 Embryo transfer and foals

Several ICSI foals have been born to date (Squires et al., 1996; Cochran et al., 1998; McKinnon et al., 2000; Li et al., 2001; Hinrichs et al., 2007; Galli et al., 2007). Pregnancy rates after transfer of IVP embryos are comparable to those obtained after transfer of *in vivo* derived embryos (Galli et al., 2007; Hinrichs, 2010b; Farin et al., 2010). In cattle, initial pregnancy rates are also similar for *in vivo* and *in vitro* produced embryos, but subsequent survival is compromised by considerable embryonic and fetal losses and the occurrence of the large offspring syndrome (Willadsen et al., 1991; Walker et al., 1996; Niemann and Wrenzycki, 2000). The large offspring syndrome, as observed in cattle after IVP and cloning, has not been reported in the horse. As the application of ICSI in horses has only evolved recently and the number of foals born to date is limited, data on possible long term influences are scarce. Initially, abnormal pregnancies without an embryo proper were reported (Hinrichs et al., 2007). However, normal development has been reported recently and this improved development has been contributed to more consistent IVC conditions (Hinrichs, 2010b). An evaluation of 14 cloned foals revealed the need for intensive support during the first week, but if the foals survived this critical period, further development appeared to be normal (Johnson et al., 2010). As the number of IVP foals is still limited, further research is necessary to evaluate long term consequences. Moreover, the ability of IVP embryos to develop to normal foals remains the method of choice to evaluate the quality of *in vitro* embryo production in horses: it is the ultimate proof that the equine embryos which are produced after ICSI are not parthenogenetic or chromosomally abnormal blastocysts.
Despite an initially slow progress, IVP of equine embryos has evolved rapidly in the last decade. Nowadays the combination of OPU, IVM, ICSI, IVC and ET is clinically available (Colleoni et al., 2007). Since only a few normal sperm cells are required, ICSI can be applied for subfertile stallions and even for semen which has been frozen-thawed, diluted and frozen again or for sexed semen (Lazzari et al., 2002; Choi et al., 2006a; Squires et al., 2008). ICSI has also been shown to be helpful in case of problems on the female side such as advanced age of the breeding mare, degenerative endometrosis and cervical laceration (Colleoni et al., 2007). Moreover, ICSI can be beneficial for creating offspring from valuable animals post mortem (Hinrichs, 2005).

1.4 WHAT TO LOOK FOR WHEN EVALUATING EQUINE EMBRYOS: IN VITRO VERSUS IN VIVO

Even though the capability of establishing pregnancies is comparable for IVP and in vivo derived equine embryos (Colleoni et al., 2007), differences between both types of embryos remain present. Compared to their in vivo counterparts, equine IVP embryos display several morphologic and developmental aberrations.

The kinetics of development differ, being slower in vitro than in vivo (Tremoleda et al., 2003; Pomar et al., 2005; Rambags et al., 2005). Most equine in vivo embryos recovered 7 days after ovulation have reached the blastocyst stage and are larger and contain more cells than their in vitro counterparts 7 days after ICSI, which are predominantly still at the morula stage (Pomar et al., 2005). In order to compare embryos at the same stage, Tremoleda et al. (2003) and Rambags et al. (2005) used day 7 in vivo embryos and day 9-10 IVP embryos. Figure 7 represents an equine IVP blastocyst 9 days after ICSI and an in vivo derived blastocyst flushed 7 days after ovulation.
Further morphological analysis using specific stains has revealed higher incidences of apoptotic (Tremoleda et al., 2003; Pomar et al., 2005) and chromosomally abnormal cells (Rambags et al., 2005) in IVP (compared to in vivo) equine embryos. Moreover IVP embryos exhibit a disturbed microfilament distribution. A very intriguing abnormality is the failure of normal capsule formation; even though capsular glycoproteins are formed in vitro, they fail to coalesce into the distinct continuous capsule seen around in vivo embryos (Tremoleda et al., 2003). Two hypotheses have been formulated to explain this phenomenon (Oriol et al., 1993; Tremoleda et al., 2003). A first explanation is the simple dispersal of the glycoproteins in the culture medium, impeding the critical concentration for assembling to be reached. Another possibility is the failure of hydration and cross-linking of the capsular glycoproteins in the absence of a uterine component. Recently, a positive effect of temporary transfer to the mare’s uterus for 2-3 days on capsule formation of equine IVP blastocysts has been shown (Choi et al., 2009). Further experiments are needed to reveal what is the mechanism behind this observation.

The importance of the early embryo-maternal interaction is illustrated above. When equine embryos are produced in vitro, and therefore in the absence of the maternal tract, they differ markedly from their in vivo counterparts. In order to bridge this difference, the IVP process

Figure 7 Equine blastocysts (magnification 200x). An in vitro produced blastocyst cultured for 9 days after ICSI (A) and an in vivo derived blastocyst recovered 7 days after ovulation (B).
needs to be optimized. This requires fundamental insight into the aberrations in vitro and sensitive evaluation of the impact of environmental changes. A valuable approach to achieve this, is the assessment of the impact of the embryonic environment on gene expression levels. It is commonly accepted that the suboptimal in vitro culture environment of embryos exerts negative short-term effects, such as aberrations in genes involved in development and metabolism up to the blastocyst stage. Even long-term effect with implications for the resulting offspring have been described (Khosla et al., 2001; Fleming et al., 2004). These genetic aberrations have been related to the use of suboptimal culture media (Fleming et al., 2004). Differential gene expression in IVP versus in vivo derived embryos has been described in several species, including cattle (Mohan et al., 2004; Corcoran et al., 2006; Goossens et al., 2007), pigs (Magnani and Cabot, 2008) and mice (Fernández-González et al., 2009). In these species, the expression level of specific genes has also been used to evaluate the effect of different embryo culture media. The expression level of developmentally important genes can also be used to assess the effect of specific components, like serum or cytokines in the embryo culture medium (McElroy et al. 2008; Chin et al. 2009; Purpera et al. 2009). Some recent studies examined the expression of specific genes in horse embryos, including the pluripotency marker POU5F1 (Choi et al., 2009) and the embryonic receptors for estrogen and progesterone (Rambags et al., 2008). However, large studies, evaluating gene expression in horse embryos produced in vivo and in vitro, have not yet been reported. The genetic techniques used in this thesis, namely SSH and RT-qPCR, are illustrated in Addendum 2 and 3.

REFERENCES


68. McKinnon A.O., Lacham-Kaplan O., Trounson A.O. 2000. Pregnancies produced from fertile and infertile stallions by intracytoplasmic sperm injection (ICSI) of single frozen-


CHAPTER 2

AIMS OF THE THESIS
The general aim of this research is to test the hypothesis that development is altered in \textit{in vitro} produced blastocysts as compared to \textit{in vivo} derived equine blastocysts, with gene expression and capsule formation as a parameter, and that this altered development can be influenced by the mare’s uterine environment, more specifically by uterocalin.

In order to test this general hypothesis, in a first part of the thesis we needed to optimize the procedure for \textit{in vitro} embryo production in the horse. This was achieved by evaluating different methods for ICSI (3.1) and by confirming the developmental competence of \textit{in vitro} produced equine blastocysts after embryo transfer (3.2) (CHAPTER 3).

Next, we continued by investigating the aforementioned general hypothesis, which was subdivided in three specific aims, namely:

1. To develop a reliable method for evaluating gene expression in equine blastocysts (CHAPTER 4).
2. To identify genes which are differentially expressed between \textit{in vivo} derived and \textit{in vitro} produced equine blastocysts (CHAPTER 5).
3. To examine the influence of the maternal environment on embryonic development, and more specifically, to determine the influence of uterocalin on equine embryonic capsule development and gene expression (CHAPTER 6).
CHAPTER 3

THE *IN VITRO* PRODUCTION OF EQUINE EMBRYOS

3.1 A DIFFERENT APPROACH TO THE *IN VITRO* PRODUCTION OF HORSE EMBRYOS: A PILOT STUDY OF LASER-ASSISTED VERSUS PIEZO DRILL ICSI


3.2 BIRTH OF THE FIRST ICSI-FOAL IN THE BENELUX

3.1 A DIFFERENT APPROACH TO THE *IN VITRO* PRODUCTION OF HORSE EMBRYOS: A PILOT STUDY OF LASER-ASSISTED VERSUS PIEZO DRILL ICSI

### 3.1.1 Abstract

Intracytoplasmic sperm injection (ICSI) is the method of choice for the *in vitro* production of equine embryos. Conventional ICSI has been associated with mechanical damage to the oocyte due to deformation of the zona and exposure of the oolemma to negative pressure during injection. The introduction of the less traumatic and more efficient piezo drill-assisted ICSI yielded higher cleavage rates and more consistent results. This device is also associated with disadvantages such as the use of mercury and its association with DNA damage in the mouse. The *in vitro* production of equine embryos would profit from further optimization in order to be efficiently applicable on large scale like in cattle. This has led us to explore an alternative method avoiding oocyte trauma, namely laser-assisted ICSI, which involves creating a hole in the zona pellucida prior to ICSI. In this pilot study both the piezo drill and the laser were compared for ICSI in the horse. A higher cleavage rate was achieved in the laser group, but no significant influences on subsequent blastocyst development were observed.

### 3.1.2 Introduction

Conventional *in vitro* fertilization (IVF) is of limited success in the horse, mainly because stallion spermatozoa have difficulties in penetrating the zona pellucida *in vitro* (Roasa et al., 2007, McPartlin et al., 2009). Therefore ICSI has been introduced as an alternative to circumvent this problem and it is used routinely for the *in vitro* production of equine embryos. Conventional ICSI is characterized by the crushing of a sperm tail on the bottom of the petri dish, thus causing immobilization of the spermatozoon, followed by mechanical breakage of the zona pellucida and the oolemma with a beveled injection pipette and subsequent injection of the immobilized sperm into the cytoplasm of a mature oocyte. The first successful pregnancy after ICSI in horses was announced in 1996 (Squires et al., 1996) and was followed by a period in which different laboratories tried to implement the technique, but unfortunately with variable results. In 2002,
the piezo drill was introduced for ICSI in horses and at the same time, increased cleavage rates were reported during in vitro production of equine embryos (Choi et al., 2002; Galli et al., 2002). Whether the introduction of the piezo drill had a causal relationship with the increased cleavage rates was not clear, since no comparative studies have been published on this topic. Acceptable results with the piezo led to a generalized use of the device for ICSI in horses.

Possible explanations for the positive effect of piezo-assisted over conventional ICSI include the higher probability of oolemma breakage, the reduction of damage to the oocyte since mechanical suction of the oolemma is replaced by a single pulse and the increased permeabilization of the sperm membrane facilitating oocyte activation (Yanagida et al., 1998; Choi et al., 2002). The piezo produces pulses on the injection pipette resulting in micro-oscillations of the blunt pipette of \( \geq 0.1 \mu m \) at \( \leq 40 \mu m s^{-1} \), which allows efficient and precise penetration of the zona pellucida and the oolemma (Ediz and Olgac, 2004; Yoshida et al., 2007). However, research to reveal the exact reason has not been performed yet. Another aspect which needs further investigation is the favorable effect of mercury during piezo-assisted ICSI (Ediz and Olgac, 2005). It leads to significant improvement of the success rate, but its toxicity implies an important drawback (Ediz and Olgac, 2004). Another possible disadvantage that should be considered, is that piezo-assisted micromanipulation has been associated with DNA damage in mice (Yu et al., 2007).

An alternative advanced approach could be offered by laser-assisted ICSI. Drilling a hole in the zona pellucida by means of a diode laser prior to ICSI can avoid the mechanical compression and oocyte distortion as it occurs in the initial phase of zona penetration during conventional ICSI (Rienzi et al., 2001). This less traumatic approach has been described in human to be beneficial in case of patients which suffered of high rates of oocyte degeneration or fragile oolemmas after conventional ICSI (Abdelmassih et al., 2002; Rienzi et al., 2004), although a subsequent study reported no advantage (Richter et al., 2006).

In horses the in vitro production of embryos happens on a small scale and in most laboratories piezo-assisted ICSI is preferred. However, a direct comparison of different ICSI methods has not been performed yet. The aim of this technical study was to introduce a new method for horse
ICSI and to evaluate some of the advantages and disadvantages of piezo- versus laser-assisted ICSI in the horse.

### 3.1.3 Materials and Methods

#### 3.1.3.1 General procedure for the in vitro production of equine embryos

Horse embryos were produced in vitro as described in Addendum 1. Briefly, oocytes were aspirated from ovaries that were recovered at the slaughterhouse. The oocytes were matured in vitro during 28h in DMEM-F12 based medium, containing 10% serum replacement, 10 µg/ml pFSH and 2 µg/ml pLH (Stimufol, ULg FMV PhR, Sart-Tilman, Belgium) in 5% CO₂ in air (Galli et al., 2007). In this experiment, only compact cumulus-oocyte-complexes (n=253) were used. After denudation, oocytes with visible polar bodies were selected and fertilized by means of piezo- (Prime Tech, Ibaraki, Japan) or laser- (XYClone, Hamilton Thorne, Beverly, USA) assisted ICSI. Six replicates were performed, in half of them piezo-assisted ICSI preceded laser-assisted ICSI and in the other half the sequence was reversed. Injected oocytes were cultured in vitro up to the blastocyst stage in DMEM-F12 with 10% fetal calf serum at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂. On day 2.5, cleavage was evaluated, the embryos that did not cleave were removed and half of the medium was changed. Part of the cleaved embryos resulting from the piezo-assisted ICSI was used for another experiment (CHAPTER 6) (Table 1). On day 6, half of the medium was refreshed again and on day 9 blastocyst formation was evaluated.

#### 3.1.3.2 Piezo-assisted ICSI

Piezo-assisted ICSI was performed with a blunt injection pipette (piezo-6-25, Humagen, Charlottesville, VA, USA). A small amount of mercury was put in the tip of the pipette. A progressive motile sperm was immobilized by piezo pulses and subsequently the zona and the oolemma were penetrated with a piezo intensity setting of 5 and 4 respectively and a speed of 4 and 3 respectively, after which the sperm was injected into the ooplasm.
3.1.3.3 Laser-assisted ICSI

A beveled injection pipette (MIC-50-35, Humagen, Charlottesville, VA, USA) was used and one progressively motile sperm cell was immobilized by crushing its tail. A hole was drilled in the zona pellucida using the laser. In all replicates maximal power was used. In a preliminary experiment, 250 µs pulses were given, but this resulted in cytoplasm leakage. Therefore, this was reduced to 200 µs in the first replicate. Because some cytoplasm leakage remained, this was further reduced to 150 µs in the other replicates. Several adjacent small holes were made until the zona was fully penetrated. The oolemma was penetrated mechanically in conformity with conventional ICSI without the ‘squeezing’ of the oocyte.

3.1.3.4 Statistical analysis

The cleavage and blastocyst rates in both groups were compared by means of a Pearson Chi-square test (SPSS 16.0, SPSS Inc., Headquarters, Chicago, Illinois, US). The mean injection times were compared using a t-test. A p-value <0.05 was considered significant.

3.1.4 RESULTS

Of the 96 oocytes which were injected using laser, 74 cleaved (cleavage rate: 77%). The cleavage rate of the piezo injected oocytes was a little lower (65%), a difference which was significant (p=0.0421). In contrast, in the piezo drill-assisted group, 11.3 % of the cleaved oocytes developed to blastocysts, and 6.8 % for the laser-assisted ICSI, but this was not significant (p>0.05). The mean time for ICSI, including the handling of the oocytes before and after the procedure, was longer for the laser-assisted ICSI (4.0 minutes/oocyte) than for the piezo-assisted ICSI (2.9 minutes/oocyte). This difference was not significant. The details on development are listed in Table 1 and summarized in Figure 1.
Figure 1 Developmental competence of equine embryos after piezo- versus laser-assisted ICSI. For both groups the mean cleavage rate (cleaved per injected) and the blastocyst rate (blastocyst per cleaved) are displayed as well as the corresponding standard errors. * significant difference (p=0.0421).

Table 1 Development of equine embryos following piezo- versus laser-assisted ICSI. For the six conducted replicates, cleavage and blastocyst development was recorded for the oocytes fertilized by piezo (P) and laser (L). Half of the cleaved embryos resulting from piezo-assisted ICSI was destined to another experiment. The number of cleaved embryos used in this experiment are listed under ‘used P’.

<table>
<thead>
<tr>
<th></th>
<th>ICSI P</th>
<th>ICSI L</th>
<th>Cleaved P (% of injected)</th>
<th>Cleaved L (% of injected)</th>
<th>Used P</th>
<th>Blastocyst P (% of cleaved)</th>
<th>Blastocyst L (% of cleaved)</th>
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<tr>
<td>1</td>
<td>53</td>
<td>18</td>
<td>31 (58%)</td>
<td>11 (61%)</td>
<td>13</td>
<td>1 (7%)</td>
<td>1 (9%)</td>
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<tr>
<td>2</td>
<td>20</td>
<td>23</td>
<td>16 (80%)</td>
<td>19 (83%)</td>
<td>7</td>
<td>1 (14%)</td>
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<td>3</td>
<td>20</td>
<td>22</td>
<td>10 (50%)</td>
<td>16 (72%)</td>
<td>5</td>
<td>1 (20%)</td>
<td>2 (12.5%)</td>
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<tr>
<td>4</td>
<td>35</td>
<td>16</td>
<td>26 (74%)</td>
<td>12 (75%)</td>
<td>15</td>
<td>0 (0%)</td>
<td>1 (8.3%)</td>
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<tr>
<td>5</td>
<td>23</td>
<td>12</td>
<td>14 (61%)</td>
<td>12 (100%)</td>
<td>8</td>
<td>2 (25%)</td>
<td>1 (8.3%)</td>
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<tr>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5 (83%)</td>
<td>4 (80%)</td>
<td>5</td>
<td>1 (20%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
<td>96</td>
<td>102 (65%)</td>
<td>74 (77%)</td>
<td>53</td>
<td>6 (11.3%)</td>
<td>5 (6.8%)</td>
</tr>
</tbody>
</table>
Figure 2 Day 2.5 embryo resulting from laser-assisted ICSI. After laser-assisted ICSI the hole in the zona pellucida (arrow) was still apparent at the cleavage stage. This resulted in blastomere leakage in some embryos.

The hole in the zona after laser-assisted ICSI was persistent in later stages of development. This resulted in what we describe as blastomere leakage at day 2.5 (Figure 2) and prominent hatching at the blastocyst stage (Figure 3) when compared to the piezo group. However, this became less frequent when the pulse duration was diminished.

Figure 3 Blastocyst resulting from laser-assisted ICSI (magnification 150x). A substantial part of the blastocyst has hatched through the hole in the zona. This hatching was more prominent when compared to the blastocysts resulting from piezo-assisted ICSI.
3.1.5 DISCUSSION

This study was performed to compare the piezo drill and the laser during ICSI in the horse. The developmental competence of the embryos produced in both ways is comparable. A smaller cleavage rate is observed in the group where the piezo was used. The obtained cleavage rate of 65% is nevertheless comparable to results of 62-79%, reported in literature (Galli et al., 2007). It must be remarked that the 65% cleavage rate was lower than average results (75%) with the piezo in concurrent experiments (unpublished data). This may be related to the fact that the injection pipette had to be changed for both procedures, which resulted in small variations in the angle of the injection pipette. For piezo-assisted injection this angle is critical, since the holding pipette, the oolemma and the injection pipette need to be in the same plane. In one replicate the angle appeared suboptimal during the piezo-assisted injection, resulting in a few more piezo pulses required to penetrate the oocyte and subsequently a reduced cleavage rate (50%). The precise alignment of both pipettes (holding and injection) in the 3 dimensional aspect of this technique appears to be less critical when laser-assisted ICSI is performed. This could be possibly explained by the fact that the laser acts in a vertical plane, acting on the full height of the zona at a particular point. Subsequently, the injection in the horizontal plane becomes less critical.

Generally, the laser is very easy to handle and allows precise working. However, the sperm needs to be captured and the oolemma is penetrated in the same way like in conventional ICSI. Combined with the fact that several adjacent holes need to be made to penetrate the zona pellucida, the laser-assisted ICSI remains rather time consuming when compared to piezo-assisted ICSI where the penetration of the zona is followed fluently by the penetration of the oolemma. Concerning the oolemma breakage, this study represents a comparison between piezo-assisted and conventional ICSI, since the laser does not interact at this level. The suction of the oolemma and the ooplasm into the injection pipette during conventional ICSI is considered to be more traumatic than the oolemma breakage through piezo drill. Whether this or the other piezo associated advantages as previously described contributed to the tendency of a higher blastocyst rate is not clear. Anyhow, no significant differences were obtained.
Another peculiarity is the apparent hole in the zona after laser-assisted ICSI. Although the blastomere leakage and the early hatching were reduced by minimizing the laser settings, it remained present in some embryos. This might be associated with an increased chance of monozygotic twinning and it was suggested that the zona pellucida can be thinned instead of fully penetrated by the laser prior to ICSI when applying it to human embryos (Moser et al., 2004). Also, we did not attempt reducing the power of the laser, which might be worth exploring as an option with the laser method.

While interpreting these results, it must be taken into account that the lab had no prior experience with the laser, while piezo-assisted ICSI had been performed routinely for a year. The ease of laser use permitted these preliminary results, indicating the laser as a valid alternative for equine ICSI. This opens new avenues for research into the molecular effects and events that result from these methods.

### 3.1.6 CONCLUSIONS

The *in vitro* production of equine embryos is based upon ICSI. To minimize oocyte damage associated with conventional ICSI, piezo- or laser-assisted ICSI can be used. In this study both techniques were compared and present specific advantages and disadvantages, but no major differences in embryonic development were observed. Further exploration of these methods is required to define their value for equine ICSI.

### REFERENCES


3.2 BIRTH OF THE FIRST ICSI-FOAL IN THE BENELUX

3.2.1 ABSTRACT

This manuscript describes the creation of a foal using conventional intracytoplasmic sperm injection (ICSI) and embryo transfer. Oocytes were aspirated from ovaries from slaughtered mares. After in vitro maturation, the oocytes were fertilized by ICSI and cultured in vitro for 9 days. Two embryos reached the blastocyst stage and they were transferred to the uterus of a synchronized mare. Six days later a single embryonic vesicle was diagnosed by ultrasound. After a normal pregnancy a healthy foal was born the 27th of October 2009. Parentage testing via microsatellite genotyping confirmed that the foal originated from the transferred embryo.

3.2.2 INTRODUCTION

Conventional in vitro fertilization (IVF), which implies culture of matured oocytes with capacitated sperm, is not efficient in horses. Only two IVF foals, produced from in vivo matured oocytes, have been born up till now (Palmer et al., 1991). It is not yet completely clear why the in vitro fertilization of horse oocytes is so difficult. One theory states that it may be due to a defective capacitation of stallion sperm, which impairs the normal hyperactivation process of the sperm (McPartlin et al., 2009). Hyperactivation is a typical motility pattern which is exhibited by capacitated sperm and it is generally characterized by an increased lateral head displacement and beat asymmetry. Hyperactivation is believed to be necessary for the sperm cell to penetrate the equine zona pellucida (McPartlin et al., 2009). To circumvent this problem of defective hyperactivation and fertilization in vitro, ICSI has been used for the in vitro production (IVP) of equine embryos. This technique involves injecting a single sperm cell into the cytoplasm of a mature oocyte using a fine glass needle. It was initially developed for treatment of male factor infertility in humans and the first ICSI baby was born in Belgium in 1992 (Palermo et al., 1992).

The first ICSI foals resulted from in vitro matured equine oocytes, which were surgically transferred to the oviduct after ICSI (Squires et al., 1996; Cochran et al., 1998; McKinnon et al.,
Subsequently also embryos which were cultured in vitro up to the blastocyst stage and transferred to the uterus resulted into healthy foals (Li et al., 2001; Hinrichs, 2005; Galli et al., 2007). Although ICSI is now commercially available for infertility treatment of both mares and stallions, only a few laboratories perform this practice. In this case report, the birth of the first ICSI foal in the Benelux is described.

### 3.2.3 Materials and Methods

Equine ovaries were collected in the abattoir and all follicles larger than 5 mm were aspirated with a vacuum pump (−100 mm Hg), scraped with the aspirating needle and flushed with heparin in phosphate buffered saline (PBS) (25IU/ml). The oocytes were matured during 26 hours in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) based medium in an atmosphere containing 5% CO₂ (Galli et al., 2007). After removal of the surrounding cumulus cells by means of gentle pipetting, the oocytes with an extruded polar body were fertilized by conventional ICSI. Frozen sperm from a stallion of proven fertility was thawed, centrifuged at 750 x g during 40 minutes over a 90%/45% Percoll® gradient, washed with calcium free Tyrode’s Albumin-Lactate-Pyruvate (TALP) solution and centrifuged again at 400x g for 10 minutes. The sperm pellet was resuspended in Synthetic Oviductal Fluid (SOF) medium and stored at 38.5 °C in 5% CO₂. During ICSI the oocytes were kept in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered SOF medium and the sperm in 9% polyvinylpyrrolidone in PBS. All manipulations were performed on a heated plate (38.5 °C) of an inverted microscope. A progressively motile sperm cell was immobilized by crushing the tail on the bottom of the scale and injected into the cytoplasm of a mature oocyte. The injected oocytes were cultured in groups of 10-20 embryos in 20µl droplets of DMEM-F12 with 10% fetal calf serum at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂. On day 2.5 after fertilization, the embryos which were not cleaved were removed and half of the medium was changed. On day 6 again half of the medium was changed and on day 9 the embryonic development was evaluated and blastocysts were selected. Two blastocysts were washed in Emcare Holding Medium® and put in a 2 ml tube filled with Emcare Holding Medium®. During the 3 hour transport to the embryo transfer centre, this tube was kept in 50 ml preheated saline and surrounded by 15 l infusion
bags at 38.5 °C in an isothermal box. Upon arrival, the embryos were washed again in Emcare Washing Medium® and they were both transferred to the uterus of a recipient mare at day 6 post ovulation. Due to recipient availability, both embryos were transferred to the same mare.

3.2.4 Results

Of the 52 collected oocytes, 27 (52%) extruded the first polar body at 26 h of maturation and were subsequently injected with a spermatozoon (Figure 1).

![Figure 1 Intracytoplasmic sperm injection (ICSI).](image)

This resulted in 23 cleaved embryos (Figure 2), of which 2 (8.7%) reached the blastocyst stage at day 9 (Figure 3).

![Figure 2 Cleaved equine embryo (day 2.5). Figure 3 Equine IVP blastocyst (day 9).](image)
On December 18th 2008 the embryos were transferred to the uterus of a mare that had ovulated 6 days before. Six days after transcervical transfer one single embryonic vesicle of 0.7 cm was diagnosed by rectal ultrasound. The 12th of January 2009 one embryonic heart beat was detected and regular ultrasound examinations revealed a normal development of a singleton conceptus. The recipient mare was transported at 10 months of pregnancy to the Veterinary Faculty at Merelbeke, where she gave birth the 27th of October 2009. A chestnut mare foal of 44 kg was born without complications and was called SMICSI (Figure 4,5).

*Figure 4 Birth of SMICSI.*

*Figure 5 SMICSI 3 days old.*
A DNA profile, based on 12 microsatellite markers, was determined for the foal, the sperm donor and the recipient mare. DNA profile comparison assigned the sperm donor as the father and excluded the receptor mare as the biological mother of the foal. The filly is now over one month old and is developing normally.

3.2.5 DISCUSSION

The IVP of equine embryos has lagged behind that of other large domestic animals. This can be explained by the scarce availability of equine oocytes, the inefficiency of conventional IVF and the suboptimal culture conditions and low blastocyst rates. However, after the introduction of ICSI, the IVP of equine embryos developed into a reasonably efficient and repeatable technique (Hinrichs, 2005). Especially the use of the piezo drill, a device which causes minute vibrations of the injection pipette during ICSI, seemed to be less traumatic for the oocyte and resulted in a rapid evolution in recent years (Choi et al., 2002; Galli et al., 2007).

Nowadays the combination of ovum pick up, in vitro maturation, ICSI, in vitro culture and embryo transfer is not only used for research purposes, but also clinically. Since only a few normal sperm cells are required, it can be applied for subfertile stallions, for semen which has been frozen-thawed, diluted and frozen again and for sexed semen (Lazzari et al., 2002; Choi et al., 2006; Squires et al., 2008). It has also been successful in case of problems on the female side like advanced age, degenerative endometriosis and cervical laceration (Colleoni et al., 2007). Moreover, ICSI can be beneficial in creating offspring of valuable animals post mortem. These prosperous results are only achieved in a few laboratories in the world. In general the technique is labour intensive, expensive and not yet optimally efficient. Problems concerning superovulation in horses and a very tight connection between the equine oocyte and the follicle wall imply only limited oocyte collection. Moreover the IVP process is still suboptimal in horses when compared to other species. The application of IVP of equine embryos on a larger scale, like it happens in cattle, has not been achieved yet in the horse (Blanco et al., 2009).

In this study a cleavage rate of 85% was obtained, which is comparable to the results in other recent publications and which is rather good when it is considered that the ICSI procedure was
performed in the conventional way without the use of the piezo drill. However, only 2 out of 23 injected oocytes (8.7%) reached the blastocyst stage, a fairly low number when compared to other reports of 15.2% (Colleoni et al., 2007) and 23% (Hinrichs et al., 2007). Possible explanations might include differences in oocyte source and culture conditions as well as in the ICSI technique and experience. Even though spectacular blastocyst rates up to 38% have been obtained during in vitro culture of equine embryos in DMEM/F12 (Hinrichs et al., 2005), there are still quantitative and qualitative differences when (temporary) in vivo culture is performed. Recent studies illustrated a higher percentage of equine ICSI embryos developing to the compact morula or blastocyst stage in sheep oviducts (56%) when compared to culture in vitro (20%) (Lazzari et al., 2010). Another intriguing finding was that aberrant expression of a pluripotency gene in IVP blastocysts when compared to in vivo derived embryos could be normalized by 2-3 days culture in an equine uterus (Choi et al., 2009). Studying gene expression can reveal fundamental differences between equine in vivo and in vitro embryos (Smits et al., 2009). When compared to in vivo derived equine blastocysts, IVP horse embryos have been shown to be retarded in the kinetics of development, with more apoptosis and higher levels of chromosomal abnormalities (Tremoleda et al., 2003; Pomar et al., 2005; Rambags et al., 2005). Despite these differences, pregnancy rates after intra-uterine transfer of IVP equine blastocysts are comparable to those after transfer of their in vivo counterparts and the possibility of freezing IVP embryos at rather early stages provides the opportunity of successful cryopreservation (Galli et al., 2007).

### 3.2.6 Conclusion

Although the IVP of equine embryos has evolved only recently and is not yet optimally efficient when compared to other species, it provides important possibilities for research and clinical application. This first ICSI foal of the Benelux is an important step in the improvement of the IVP processes, which opens possibilities for more research in the domain of equine embryonic development and valuable applications of the technique.
REFERENCES


DETERMINATION OF A SET OF RELIABLE REFERENCE GENES FOR RT-qPCR IN IN VIVO DERIVED AND IN VITRO PRODUCED EQUINE BLASTOCYSTS

4.1 **Abstract**

**Background**

Application of reverse transcription quantitative real-time polymerase chain reaction is very well suited to reveal differences in gene expression between *in vivo* and *in vitro* produced embryos. Ultimately, this may lead to optimized equine assisted reproductive techniques. However, for a correct interpretation of the real-time PCR results, all data must be normalized, which is most reliably achieved by calculating the geometric mean of the most stable reference genes. In this study a set of reliable reference genes was identified for equine *in vivo* and fresh and frozen-thawed *in vitro* embryos.

**Findings**

The expression stability of 8 candidate reference genes (*ACTB, GAPDH, H2A/I, HPRT1, RPL32, SDHA, TUBA4A, UBC*) was determined in 3 populations of equine blastocysts (*fresh in vivo, fresh and frozen-thawed in vitro* embryos). Application of geNorm indicated *UBC, GAPDH, ACTB* and *HPRT1* as the most stable genes in the *in vivo* embryos and *UBC, RPL32, GAPDH* and *ACTB* in both *in vitro* populations. When *in vivo* and *in vitro* embryos were combined, *UBC, ACTB, RPL32* and *GAPDH* were found to be the most stable. *SDHA* and *H2A/I* appeared to be highly regulated.

**Conclusions**

Based on these results, the geometric mean of *UBC, ACTB, RPL32* and *GAPDH* is to be recommended for accurate normalization of quantitative real-time PCR data in equine *in vivo* and *in vitro* produced blastocysts.

4.2 **Background**

Conventional IVF has been rather unsuccessful in horses. To overcome this barrier ICSI has been introduced and has resulted in transferable blastocysts that were used for research and
commercial purposes (Galli et al., 2007; Hinrichs et al., 2007; Stokes et al., 2009). The ability of \textit{in vitro} produced blastocysts to establish normal pregnancies is comparable to that of the \textit{in vivo} derived ones and, contrary to other species, \textit{in vitro} produced equine blastocysts tolerate freezing better than \textit{in vivo} produced ones and they are able to establish pregnancies after thawing (Galli et al., 2007). Despite these successes, there is a great variability in oocyte quality and culture conditions resulting in a low percentage of blastocyst formation \textit{in vitro} with great variations amongst laboratories. Compared to their \textit{in vivo} counterparts, \textit{in vitro} blastocysts are retarded in the kinetics of development, are smaller with fewer cells, show more apoptosis and higher levels of chromosomal abnormalities (Tremoleda et al., 2003a; Rambags et al., 2005; Pomar et al., 2005). Moreover intra-uterine transfer of \textit{in vitro} produced embryos can give rise to abnormal pregnancies with the development of a trophoblastic vesicle without an embryo proper (Hinrichs et al., 2007). Understanding the fundamental difference between equine \textit{in vivo} versus \textit{in vitro} embryos may prove beneficial in the development of equine assisted reproductive techniques.

RT-qPCR is a highly specific and sensitive tool to compare mRNA expression levels of specific genes (Bustin et al., 2005). Not only the RNA quality, the RT, the reagents and the protocol are critical factors, the analytical method can also influence the results dramatically (Bustin et al., 2004; Bustin et al., 2005). All data must be normalized for technical differences between samples. The method of choice consists in normalization to internal reference genes, which should be constitutively expressed without influence of the experimental treatment. Since there is no universal reference gene with a constant expression in all tissues, optimal reference genes need to be selected for each system (Kubista et al., 2006). The use of a single reference gene or of multiple unstable reference genes may lead to erroneous normalization (Dheda et al., 2005). Therefore, the geometric mean of carefully selected genes is recommended for reliable normalization (Vandesompele et al., 2002).

Reference genes have been validated in preimplantation embryos of cattle (Goossens et al., 2005), mice (Mamo et al., 2007), pigs (Kuijk et al., 2007) and rabbits (Mamo et al., 2008). However, gene stability in mammalian embryos turned out to be species specific, which implies
the need of reference gene selection for the study of equine embryos. Gene expression studies in equine species are relatively scarce. Real-time PCR has been conducted on equine conceptuses to evaluate expression of progesterone and oestrogen receptors and on equine blastocysts to identify POU5F1 expression, but in these studies only one reference gene, β-actin, was used (Rambags et al., 2008; Choi et al., 2009). While sets of reference genes have been identified for equine skin (Bogaert et al., 2006) and lymphocytes (Capelli et al., 2008), the most reliable reference genes in equine embryos have not been studied before.

The aim of this study was to identify a set of stable reference genes for equine in vivo derived embryos and fresh and frozen-thawed in vitro embryos.

4.3 METHODS

The in vivo blastocyst collection procedures used were approved by the ethics committee of the faculty of veterinary medicine (reference number EC 2007/009). Cycling mares were followed up by transrectal ultrasound. When the follicle size exceeded 35 mm in the presence of an edematous uterus, 3000 IU hCG (Chorulon, Intervet, Belgium) was injected intravenously. The next day the mare was inseminated with fresh semen. The time of ovulation was determined by daily ultrasonography of the genital tract. If the mare did not ovulate within 48 hours after AI, insemination was repeated. The mares’ uterus was flushed 7 days after ovulation and recovered embryos were washed in DPBS (14190, Gibco, Invitrogen, Belgium) and conserved individually at -80 °C in lysis buffer (10 % RNasin Plus RNase inhibitor (Promega, The Netherlands), 5 % dithiothreitol (Promega, The Netherlands), 0.8 % Igepal CA-630 (Sigma, Belgium) in RNase free water) until further analysis.

For the production of fresh in vitro blastocysts, ovaries were recovered at the slaughterhouse and follicles ≥ 5 mm were aspirated with a vacuum pump at a negative pressure of 100 mm Hg. The follicle wall was scraped with the aspirating 16 gauge needle and flushed with 0.5 % (v/v) heparin (H 1027, Sigma, Belgium) in DPBS (14190, Gibco, Invitrogen, Belgium). Oocytes were matured for 28 h in DMEM-F12 based medium (Galli et al., 2007) at 38.5 °C in 5% CO₂ in air. The MII oocytes were fertilized by conventional ICSI as described by Tremoleda et al. (2003b).
Frozen-thawed sperm was used from the same fertile stallion that was used for production of *in vivo* embryos of this experiment. *In vitro* culture was performed for 8.5 to 9.5 days in 20 µl drops of DMEM-F12 (D 2906, Sigma, Belgium) with 10% FCS (F 9665, Sigma, Belgium) or 5% FCS and 5% SR (10828-028, Gibco, Invitrogen, Belgium) at 38.5 °C in 5% CO₂, 5% O₂ and 90% N₂. Blastocysts were stored individually at -80 °C in lysis buffer until further analysis.

The frozen-thawed *in vitro* embryos were produced in Italy (Galli et al., 2007). Briefly, oocytes were recovered from abbatoir ovaries by scraping and washing all the follicles between 5 and 30 mm. Oocytes were washed in HEPES buffered TCM199 and transferred for 24 h into DMEM-F12 based maturation medium as previously described (Galli et al., 2007). After removal of all cumulus cells, the oocytes with a polar body were fertilized by ICSI with frozen-thawed sperm from stallions of proven fertility. Injected oocytes were cultured up to day 8 in 20 µl drops of modified SOF (from day 6 half SOF: half DMEM-F12) at 38.5 °C in 5% CO₂, 5% O₂ and 90% N₂. Embryos that reached the blastocyst stage were loaded individually into 0.25 ml straws, frozen with a slow cooling protocol (0.5 °C/min up to -32°C) in glycerol and stored in liquid N₂. The embryos were shipped to Belgium in dry ice and upon arrival they were immediately transferred into lysis buffer with a minimum of medium and conserved at - 80 °C until further analysis.

In all groups only blastocysts which were not hatched and had good morphological characteristics were retained.

For each of the 3 groups, 8 embryos were analysed separately. Total RNA was extracted from single embryos with the PicoPure RNA-isolation Kit (Arcturus, Mountain View, CA, USA), treated with RQ1 DNase (Promega, The Netherlands) and purified over a spin column (Microcon YM-100, Millipore, Belgium). After minus RT control with primers for *GAPDH* to check for contaminating genomic DNA, RNA was concentrated by precipitation with 3M sodium acetate and ethanol. RNA amplification and conversion into cDNA was performed by means of the WT-Ovation RNA Amplification System (NuGEN, USA) according to the manufacturers’ instructions and the cDNA was purified again over a spin column. Amplified cDNA samples were diluted 10-20 times, depending on the yield, in 10 mM Tris HCL pH 8.0 and stored at - 80 °C.
Eight reference genes were selected based on previous studies (Goossens et al., 2005; Bogaert et al., 2006; Capelli et al., 2008). The selected genes (\textit{ACTB}, \textit{GAPDH}, \textit{H2A/I}, \textit{HPRT}, \textit{RPL32}, \textit{SDHA}, \textit{TUBA4A} and \textit{UBC}) belong to different functional classes, which reduces the chance of co-regulation.

Primers for \textit{ACTB}, \textit{HPRT1}, \textit{RPL32}, \textit{TUBA4A} and \textit{UBC} were provided by Bogaert and colleagues (2006). The other primers were designed by means of Primer3 software (http://frodo.wi.mit.edu/primer3/), based on horse sequences found in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/). Primers were selected over intron-exon boundaries, tested using a BLAST analysis against the NCBI database and verified using MFold (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi). The optimal primer annealing temperatures were determined on cDNA of equine mixed tissues. A melt curve analysis followed by agarose gel electrophoresis was performed to test for primer-dimer formation and specificity of the amplicons. All primers are listed in \textit{Table 1}.

All reactions were executed in duplicate for 8 embryos in each of the three groups and a blank was included in each run.

The diluted cDNA (2.5 µl), 0.33 µM of both forward and reverse primers and 4µl of RNAse free water were added to 7.5 µl of KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems) to a final volume of 15 µl. The reactions were performed on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Belgium).

The PCR program started with an initial denaturation at 95°C for 3 minutes to activate the DNA polymerase. Then 45 cycles were performed with a denaturation step at 95°C for 20 seconds followed by an annealing/extension step at the primer specific annealing temperature for 40 seconds, during which fluorescence was measured. A dilution series with pooled cDNA from all 24 embryos was included for each gene to acquire PCR efficiencies based on a relative standard curve. Calculation of the \textit{C}ₜ values, PCR efficiencies, correlation coefficients and analysis of the melting curves was performed by means of iCycler iQ Optical System Software Version 3.0a. The standard errors of the PCR efficiencies were obtained by the qBasePlus software (http://www.qbaseplus.com/).
Table 1 Primers. For each reference gene, the NCBI GenBank accession number, the sequence of both forward and reverse primer, the size of the amplicon and the optimal primer annealing temperature are listed. The RTPrimerDB ID (http://rtprimerdb.org/) is also reported, except for the primers for which there is not yet an official reference sequence available in the database (*).

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>Ta (°C)</th>
<th>RTprimerDB ID</th>
<th>Cq range</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>AF035774</td>
<td>CCAGCAGATGAAGATCAAG</td>
<td>88</td>
<td>60</td>
<td>7848</td>
<td>16.3-23.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTGGACAATGAGGCCAGAAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>XM_001496020.1</td>
<td>CAGAACATCATCCCTGCTTC</td>
<td>187</td>
<td>59</td>
<td>7849</td>
<td>14.2-24.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATGCCTGCTCACCACCTTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2A/I</td>
<td>XM_001497311.2</td>
<td>ATATTCAGGCGTGCTGCT</td>
<td>105</td>
<td>60</td>
<td>*</td>
<td>23.5-37.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTTGGGTTCAAAGCGTTTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT1</td>
<td>AY372182</td>
<td>GGCAAAAAATGCAAACCTT</td>
<td>163</td>
<td>57</td>
<td>7850</td>
<td>17.6-25.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAAGGCGATATCCTACGACAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL32</td>
<td>XM_001492042.2</td>
<td>AGCCATCTACTCGCGGTCA</td>
<td>149</td>
<td>60</td>
<td>*</td>
<td>16.8-25.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCCAATGCGCTCTGGGTTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDHA</td>
<td>XM_001490889</td>
<td>TCCATCGCATAAGGCAAAG</td>
<td>159</td>
<td>59</td>
<td>7851</td>
<td>20.7-33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGTGGAACTGAACGAACTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUBA4A</td>
<td>XM_001491910.2</td>
<td>GCCCTACAACCTCCATCTGA</td>
<td>78</td>
<td>60</td>
<td>*</td>
<td>16.6-27.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATGGGCTTGGTCCACCCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBC</td>
<td>AF506969</td>
<td>GCAAGACCACACCTCGGA</td>
<td>206</td>
<td>60</td>
<td>7874</td>
<td>15.5-23.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTAACAGGCCACCCCTTGAGAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The expression stabilities were evaluated using the geNorm software for Microsoft Excel (Vandesompele et al., 2002). This program ranks the genes based on the internal control gene stability parameter M. Stepwise exclusion of the gene with the highest M value and recalculation results in a ranking of the reference genes. Lower M values represent higher
expression stabilities. Furthermore the minimum number of genes required for the calculation of a reliable normalization factor is determined.

### 4.4 RESULTS AND DISCUSSION

For the recovery of the *in vivo* embryos (n=8), 13 uterine flushes were performed in a population of 8 mares (recovery rate: 61.5 %). To collect the fresh *in vitro* embryos (n=8), 123 ovaries were recovered in 5 experiments, which gave rise to a total number of 365 oocytes. Maturation was completed and ICSI was performed in 209 oocytes (57 %) and 74 % of these injected oocytes cleaved. Of the cleaved oocytes 5.8 % reached the blastocyst stage. The 8 *in vitro* blastocysts which were used for genetic analysis were produced in 2 of those 5 batches where the blastocyst percentage was 7.3 %.

Frozen embryos were produced in experiments for quality control purposes. They were frozen on day 8 at the early blastocyst or blastocyst stage.

Prior to the start of the experiment, PCR was tested on one of the frozen-thawed embryos to evaluate possible effects of remnants of the cryoprotectant. Both undiluted and 25x diluted cDNA resulted in clear electrophoretic bands of expected size for *UBC* and *ACTB*. Only a minimal amount of cryoprotectant accompanying the embryo was transferred to and diluted in the lysis buffer. Importantly Cq-values of the fresh and frozen-thawed embryos remained in the same range.

Because only a small quantity of RNA can be extracted from one embryo, an RNA pre-amplification step was included in the protocol. Ribo-SPIA, the isothermal messenger RNA amplification method used, has been shown to be accurate and reproducible (Dafforn et al., 2004). High sensitivity permits quantification of low abundance transcripts and there is a good correlation in differential gene expression between amplified and nonamplified cDNA (Dafforn et al., 2004).
Dilution series of all candidate reference genes gave PCR-efficiencies between 97.1 % and 100 % and linear correlation coefficients that varied from 0.973 to 1.000. In table 2 the efficiencies and their standard errors are listed.

One in vivo and one fresh in vitro embryo consistently resulted in C_q-values around 40. Probably the amplification went wrong and both samples were deleted from further analysis.

The selection of appropriate reference genes can be achieved by evaluating RT-qPCR data with statistical algorithms. The uniformity in gene ranking between geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) has been shown to be high (Cappelli et al., 2008; Willems et al., 2006).

\textit{Table 2 PCR efficiency and the respectively standard error.} Results of the reference gene stability, as determined by geNorm, are shown in Figure 1, 2, 3 and 4. When all three groups of embryos were included, the most stable genes were UBC, ACTB, RPL32 and GAPDH. The M values of these genes range between 0.6 and 0.9, which indicates relatively good stability.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Efficiency (%)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>98.1</td>
<td>0.01</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100</td>
<td>0.021</td>
</tr>
<tr>
<td>H2A/I</td>
<td>100</td>
<td>0.067</td>
</tr>
<tr>
<td>HPRT1</td>
<td>100</td>
<td>0.037</td>
</tr>
<tr>
<td>RPL32</td>
<td>100</td>
<td>0.071</td>
</tr>
<tr>
<td>SDHA</td>
<td>99.3</td>
<td>0.033</td>
</tr>
<tr>
<td>TUBA4A</td>
<td>100</td>
<td>0.017</td>
</tr>
<tr>
<td>UBC</td>
<td>97.1</td>
<td>0.017</td>
</tr>
</tbody>
</table>
Figure 1 Average expression stability values of equine in vivo and in vitro embryos. The average stability values of the control genes were calculated with geNorm. When in vivo embryos and fresh and frozen-thawed in vitro embryos were combined, UBC, ACTB, RPL32 and GAPDH were found to be the most stable.

Figure 2 Average expression stability values of equine in vivo embryos. The average stability values of the control genes were calculated with geNorm. In the population of the equine in vivo embryos UBC, GAPDH, ACTB and HPRT1 were found to be the most stable.
Figure 3 Average expression stability values of fresh equine in vitro embryos. The average stability values of the control genes were calculated with geNorm. In the population of the fresh equine in vivo embryos UBC, RPL32, GAPDH and ACTB were found to be the most stable.

Figure 4 Average expression stability values of frozen-thawed equine in vitro embryos. The average stability values of the control genes were calculated with geNorm. In the population of the fresh equine in vivo embryos UBC, RPL32, GAPDH and ACTB were found to be the most stable.
The order of the 4 most stable genes, *UBC*, *RPL32*, *GAPDH* and *ACTB*, is identical in both *in vitro* groups. This is an interesting finding as there was a difference in embryo culture conditions and as the cryopreservation could have affected the results. Three of those 4 genes, *UBC*, *GAPDH* and *ACTB*, represent the most stable genes in the *in vivo* embryos. In murine embryos the order of stability of reference genes has also been found to be similar in both *in vitro* and *in vivo* embryos (Mamo et al., 2007). In the latter study however, the stability measure values M were described to be higher in the *in vitro* samples compared to the *in vivo* samples, which was not the case in the equine embryos. *SDHA* and *H2A/I* generally turned out to be highly regulated. These results differ from those in embryos from other species and from those in other equine tissues. *SDHA*, which was highly regulated in equine embryos, appeared to be very stable in bovine embryos (Goossens et al., 2005) and equine lymphocytes (Cappelli et al., 2008). This is also the case for *H2A/I*, which was preferred as a reference gene in embryos of mice (Mamo et al., 2007) and rabbits (Mamo et al., 2008). *ACTB* on the other hand appears fairly stable in equine embryos, although it is highly regulated in bovine embryos (Goossens et al., 2005). This again demonstrates the necessity to validate the genes according to the species and the tissue type.

The use of the geometric mean of several internal control genes as a normalization factor (NF) is more accurate than the use of a single reference gene. Inclusion of unstable genes on the other hand negatively influences the NF and the number of reference genes used to calculate this NF is a trade-off between practical considerations and accuracy. The optimal number of genes was determined with geNorm by means of the pairwise variations \(V_{n/n+1}\) between the sequential normalization factors (NF\(_n\) and NF\(_{n+1}\)) after successive inclusion of less stable reference genes (*Figure 5*). The value of the pairwise variations reduces until 0.207 for \(V_{3/4}\). This suggests that the inclusion of a fourth reference gene contributes to the stability. Therefore it is recommended to use the 4 most stable genes, *UBC*, *ACTB*, *RPL32* and *GAPDH*. The high values of \(V_{6/7}\) and \(V_{7/8}\) represent the instability of *H2A/I* and *SDHA*, which is also clear in the steep rise in *Figure 1*. 
Figure 5 Determination of the optimal number of control genes for normalization. The optimal number of control genes for normalization was calculated by geNorm. The value of the pairwise variations reduces until 0.207 for V₃/₄, which indicates that the inclusion a fourth reference gene contributes to the stability. Therefore the average of the 4 most stable genes is recommended to determine a reliable NF.

In conclusion, the geometric mean of ACTB, UBC, RPL32 and GAPDH is recommended for normalization of RT-qPCR data in experiments with equine in vivo and in vitro blastocysts.

REFERENCES


differential staining and effect of medium calcium concentrations during culture.

*Theriogenology* 68 521-529.


IN VIVO DERIVED HORSE BLASTOCYSTS SHOW
TRANSCRIPTIONAL UPREGULATION OF DEVELOPMENTALLY
IMPORTANT GENES COMPARED TO IN VITRO PRODUCED
HORSE BLASTOCYSTS

Reproduction Fertility and Development in press.
5.1 ABSTRACT

In vitro produced (IVP) equine blastocysts can give rise to successful pregnancies, but their morphology and developmental rate differ from that of in vivo derived equine blastocysts. The aim of this study was to evaluate this difference at the genetic level. Therefore suppression subtractive hybridization (SSH) was used to construct a cDNA library enriched for transcripts preferentially expressed in in vivo derived equine blastocysts compared to in vitro produced ones. Of the 62 different genes identified in this way, 6 genes involved in embryonic development (BEX2, FABP3, HSP90AA1, MOBKL3, MCM7 and ODC) were selected for the confirmation of this differential expression by means of reverse transcription quantitative real-time PCR (RT-qPCR). For 5 of these genes the higher expression in vivo was proven by RT-qPCR to be significant (FABP3 and HSP90AA1) or highly significant (ODC, MOBKL3 and BEX2), confirming the results of the SSH. For MCM7 the difference was not significant. In conclusion 5 genes which are transcriptionally upregulated in in vivo derived equine blastocysts as compared to IVP blastocysts have been identified. Because of their possible importance in embryonic development, the expression of these genes can be used as a marker to evaluate in vitro embryo production systems in the horse.

5.2 INTRODUCTION

Compared to other species the in vitro production (IVP) of equine embryos has been hampered by the scarce availability of oocytes and by the inefficiency of conventional in vitro fertilization (IVF). The introduction of intracytoplasmic sperm injection (ICSI) induced a rapid progress over the last decade and transferable equine in vitro blastocysts have been successfully produced for research as well as for commercial purposes (Galli et al., 2007; Hinrichs et al., 2007; Smits et al., 2010). The IVP of equine embryos is a very valuable technique to study early stages of embryo development, which used to be quite inaccessible because of their location in the oviduct. Moreover in vitro embryos can be used to optimize the different steps of the IVP process itself. Further research is required to obtain large scale clinical applications like in cattle (Blanco et al., 2009), but promising results illustrating the great value of IVP in the horse have been published.
recently (Colleoni et al., 2007). The combination of OPU and IVP represents in some cases the only opportunity to obtain foals from valuable subfertile mares and stallions. It can be used for mares with reproductive disorders or for aged mares and since the requirements for sperm which is selected for ICSI are minimal, it is also applicable for stallions with poor sperm (Colleoni et al., 2007). In this way IVP provides the production of offspring from genetically valuable horses, which is of considerable benefit to the horse breeding industry.

Even though the capability of establishing pregnancies is comparable for IVP and in vivo derived equine embryos (Colleoni et al., 2007), differences between both types of embryos remain present. Compared to their in vivo counterparts equine IVP embryos have fewer cells with more apoptosis and chromosomal abnormalities (Tremoleda et al., 2003; Pomar et al., 2005; Rambags et al., 2005). In in vivo derived equine blastocysts a distinct glycoprotein capsule is formed between the trophectoderm and the zona pellucida. In vitro produced blastocysts have a deficient capsule formation and show only scattered patches of glycoproteins (Tremoleda et al., 2003). Suboptimal culture conditions are a possible cause of this aberrant differentiation since even temporary exposure of IVP embryos to an in vivo environment can have a major influence on embryonic development. Lazzari et al. (2010) reported that 56 % of cleaved equine in vitro embryos were developing to compact morulae and blastocysts after temporary culture in sheep oviducts, which differed significantly from the 20 % which was achieved after total in vitro culture. Another example was the finding that impaired differentiation of the equine trophectoderm in vitro, which was determined by aberrant expression of the pluripotency marker POU5F1 when compared to equine in vivo blastocysts, could be normalized by culturing the IVP blastocysts in vivo for 2-3 days in an equine uterus (Choi et al., 2009).

In vivo derived horse embryos are relatively difficult to obtain, because the mare does not respond adequately to superovulatory treatments and because the horse embryo arrives only in the uterus at day 6.5, which limits the time period during which equine embryos can be obtained by non-surgical flushing (Scherzer et al., 2008). As such, horse embryos are mostly flushed during a natural cycle at day 7, at which time they have developed to the blastocyst or expanded blastocyst stage. Such horse blastocysts represent the gold standard for early equine
embryonic development. Comparing IVP and *in vivo* derived horse blastocysts at the genetic level can reveal fundamental differences responsible for the observed morphological and developmental changes. In other mammalian species the expression level of specific genes has been used to evaluate the effect of different embryo culture media as well as the effect of adding specific components like serum or cytokines to these media on embryonic differentiation (McElroy et al., 2008; Chin et al., 2009; Purpera et al., 2009). In the horse, the expression of genes involved in cumulus expansion has been used to assess different oocyte maturation conditions (Dell’Aquila et al., 2004). Evaluation of horse embryos by means of RT-qPCR may provide the basis for improving the culture conditions for horse embryos.

Little is known about gene expression in equine embryos. At the start of this project, no commercial horse microarray was available and annotation of the horse genome was poor. Microarray is very useful if one wants to investigate a known panel of genes but it is not able to detect novel or unknown genes. Therefore we decided to use suppression subtractive hybridization (SSH), a powerful method for the detection of differentially expressed genes when prior knowledge is limited (Diatchenko et al., 1996). This technique provides a selective amplification of target cDNA fragments which are specifically expressed in one of the two cDNA populations and in this way can be applicable for the comparison of the gene expression in *in vivo* versus *in vitro* embryos. Results of this SSH however need to be confirmed by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), a highly sensitive and specific tool, which can evaluate and quantify the difference in expression of the genes of interest selected from the SSH.

The aim of this study was to evaluate gene expression in equine blastocysts at the transcriptional level. SSH was used to identify genes which were expressed at a higher level in *in vivo* derived equine blastocysts when compared to IVP equine blastocysts. Six of these genes were selected for the confirmation and quantification of this difference in expression by RT-qPCR. The choice of these genes was based upon their involvement in embryonic development in other species.
5.3 MATERIALS AND METHODS

5.3.1 Collection of embryos

The procedures performed on the mares were approved by the Ethics Committee of the Faculty of Veterinary Medicine (EC 2007/009). The *in vivo* embryos were collected by uterine flushing of inseminated mares 7 days after detection of ovulation as described previously (Smits et al., 2009). The *in vitro* embryos were produced following the protocol as previously described by Smits et al. (2010). Briefly slaughterhouse oocytes were matured *in vitro* during 26h in DMEM-F12 based medium in an atmosphere containing 5% CO₂ (Galli et al., 2007). MII oocytes were fertilized by conventional ICSI and cultured *in vitro* during 9-9.5 days in DMEM-F12 with 10% fetal calf serum at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂.

In order to compare similar developmental stages, rather than embryos of equal chronological age, the *in vitro* blastocysts were collected later than the *in vivo* derived blastocysts. This choice was based upon previous research on equine *in vivo* and *in vitro* produced embryos. Tremoleda et al. (2003) and Rambags et al. (2005) described day 7 IVP embryos to be smaller, to contain fewer cells and to be retarded in the kinetics of development when compared to their *in vivo* derived counterparts of the same age (day 7). In a subsequent study by Pomar et al. (2005) *in vivo* embryos were collected 7 days after ovulation and *in vitro* embryos 9 days after ICSI and in a recent publication by Choi et al. (2009) similarities in cell count and embryonic diameter were found between IVP blastocysts on day 10 and the smallest *in vivo* derived blastocysts (day 7). In both populations in our study the blastocyst stage was characterized by a blastocoele cavity surrounded by a layer of trophoblast cells. Early expansion was displayed by a thinned zona pellucida. However, considering rapid expansion *in vivo*, blastocysts in the *in vivo* group were collected rather early and variation was minimized through regular ultrasound to detect ovulation (1-2 times daily). For the SSH 10 *in vivo* embryos and 12 *in vitro* embryos were collected. The number of *in vitro* embryos exceeded the number of *in vivo* embryos to provide sufficient cDNA in the driver group, i.e. the *in vitro* embryos, in order to achieve hybridization of common strands with the *in vivo* embryos. The RT-qPCR was performed with 8 *in vivo* and 8 *in vitro* embryos.
5.3.2 SSH

To identify the genes that were preferentially expressed in the *in vivo* embryos, 10 *in vivo* embryos were pooled and composed the tester population. The driver population consisted of 12 pooled *in vitro* embryos. In both groups total RNA was extracted with the Pico Pure RNA Isolation Kit (Arcturus, Mountain View, CA, USA), treated with RQ1 DNase (Promega, The Netherlands) and purified over a spin column (Microcon YM-100, Millipore, Belgium). After concentration by precipitation with ammonium acetate and 95% ethanol, conversion to and amplification of cDNA was performed with the SMART PCR cDNA Synthesis Kit (Takara Bio Inc., France).

The SSH was accomplished with the PCR-Select cDNA Subtraction Kit (Takara Bio Inc., France) following the manufacturer’s instructions. Briefly, Rsa I digestion of the cDNA and adaptor ligation to the tester population was followed by 2 hybridizations and 2 PCR amplifications. This resulted in enrichment of the differentially expressed high- and low-abundance tester sequences. These were cloned into a T/A cloning vector (Invitrogen, Belgium) and transformed into competent DH5α E. coli cells (Invitrogen, Belgium). The DNA-inserts were sequenced with the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Belgium) on the Applied Biosystems 3710xl DNA Analyzer and identified by BLAST analysis against the NCBI gene database (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/). Functional and pathway analysis of the identified genes was performed by means of Ingenuity software (http://www.ingenuity.com/products/pathways_analysis.html).

5.3.3 RT-qPCR

In both the *in vivo* and the *in vitro* group 8 embryos were analysed individually. The RNA extraction, purification, concentration, amplification and conversion into cDNA were performed as described previously (Smits et al., 2009).

The primers (Integrated DNA Technologies, Belgium) were designed by means of Primer3 software (Rozen and Skaletsky 2000), based on horse RNA and DNA sequences found in the NCBI GenBank. To distinguish genomic DNA amplification, to provide specificity and to avoid
secondary structures in the primer region, primers were respectively selected over intron-exon boundaries, tested using a BLAST analysis against the NCBI database and characterized with MFold (Zuker, 2003). Primers were tested and optimal annealing temperature was determined on cDNA of equine mixed tissues. The amplicons were run on a 2% agarose gel and confirmed by nucleotide sequencing. All primers are listed in Table 1.

All RT-qPCR reactions were performed in duplicate with the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, USA) as previously described in Smits et al. (2009).

A blank, a melting curve and a 5- or 10-fold serial dilution series of pooled amplified embryonic cDNA were included for each gene to check for contamination and specificity and to acquire PCR efficiencies (Table 1) based on a relative standard curve. All Cq values were converted into raw data using these PCR efficiencies and normalized by dividing them by their respective normalization factor. This normalization factor was determined per embryo by calculating the geometric mean of the validated reference genes ACTB, UBC, RPL32 and GAPDH (Smits et al., 2009). For each gene the difference in expression level between the in vivo derived embryos and the in vitro produced ones was analyzed by means of a Mann Whitney test and p-values smaller than 0.05 were considered statistically significant.

Table 1 Primers RT-qPCR (Equus caballus). For each gene the GenBank accession number, the primer sequences, the size of the amplicon, the primer annealing temperature and the qPCR efficiency with its respective standard error are listed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Sequence 5‘→3’</th>
<th>Amplicon size (bp)</th>
<th>Ta (°C)</th>
<th>Efficiency (%)</th>
<th>Standard error</th>
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<td>AAGCTGGTGAATGCTGTGTG AACTGCCCGCAAAACTATGAC</td>
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<td>97.2</td>
<td>0.018</td>
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5.4 RESULTS

5.4.1 Collection of equine blastocysts

For the recovery of the 10 in vivo blastocysts for the SSH 20 uterine flushes were performed (recovery rate: 50 %), the 8 blastocysts for RT-qPCR were recovered in 13 flushes (recovery rate: 61.5 %). These were the same blastocysts as those, used in CHAPTER 4.

To produce the 12 in vitro blastocysts needed for the SSH 466 mature oocytes were injected and 44 % cleaved. Of the cleaved oocytes 5.8 % reached the blastocyst stage. To produce the 8 in vitro blastocysts necessary for RT-qPCR 123 ovaries were recovered in the course of 5 replicates, which gave rise to a total of 365 oocytes. ICSI was performed in 209 mature oocytes (57 %) and 74 % of these injected oocytes cleaved. Of the cleaved oocytes 5.8 % reached the blastocyst stage. The 8 in vitro blastocysts used for genetic analysis were derived from 2 out of 5 replicates, with a mean blastocyst percentage of 7.3 %. Parallel experiments revealed mean cell counts of around 500 and the developmental competence of the in vitro blastocysts was shown by embryo transfer of 2 in vitro blastocysts resulting in a foal (Smits et al., 2010).

5.4.2 SSH

A total of 84 clones was sequenced, but some genes were represented by more than one clone. This lead to the identification of 62 different genes preferentially expressed in the in vivo blastocysts when compared to the in vitro produced blastocysts. Table 2 represents the accession numbers of the SSH sequences and the respective genes which were identified through BLAST.

Analysis of these genes by means of Ingenuity Pathway Analysis revealed the most important functions in which these genes were involved (Figure 1). A majority of genes identified play a role in protein synthesis, including many ribosomal genes, and in energy production, including many mitochondrial genes. Furthermore several of the genes with higher expression in in vivo embryos could be mutually linked in a functional network. The networks are added in Figure 2.
Table 2 Genes upregulated in in vivo derived blastocyst when compared to in vitro produced blastocysts as determined by SSH (Equus caballus). This table describes the 62 genes that were expressed at a higher level in the in vivo equine blastocysts than in the in vitro equine blastocysts as determined by SSH. For each gene the NCBI accession number (AN) of the sequence resulting from the SSH is listed. This sequence was identified through BLAST analysis and the details on the homologous gene which was found in the NCBI database are also included in the table. Finally the query coverage and the % of homology between both sequences are described. * Predicted sequence, Ecab: Equus caballus, sim: similar to

<table>
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<th>AN SSH</th>
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<th>Gene</th>
<th>GenelD</th>
<th>Query coverage (%)</th>
<th>Max identity (%)</th>
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<td>Ecb hypothetical LOC100066131 (LOC100066131)</td>
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Figure 1 Functions genes SSH. The genes upregulated in in vivo equine blastocysts resulting from SSH analysis were divided into functional classes by means of Ingenuity Pathway Analysis software. The 13 most significant functions ($p<0.003$) are listed in this figure. The legend of the $X$ axis is $-\log (p\text{-value})$. This $p$-value is calculated through Ingenuity Pathway analysis by means of a right tailed Fisher’s Exact test and determines the probability that the association between the genes in the dataset and the function is explained by chance alone.
Figure 2 Network classification according to Ingenuity Pathway Analysis. The genes resulting from the SSH were mutually linked in several networks as determined by Ingenuity Pathway Analysis (http://www.ingenuity.com/products/pathways_analysis.html), namely **A**: energy production, nucleic acid metabolism, small molecule biochemistry **B**: organ development, gene expression, cancer **C**: cardiovascular system development and function, hair and skin development and function, organ development and **D**: organismal development, cell death, nervous system development and function. The genes resulting from the SSH are colored grey, the other genes involved in the networks are white. The genes selected for RT-qPCR are indicated with a red circle. Solid lines imply direct relationships between proteins; dotted lines imply indirect interactions.

5.4.3 RT-qPCR

Of the 62 genes identified, 6 which were previously shown to be involved in embryo development in other species were selected for further evaluation by RT-qPCR. For 5 (brain expressed X-linked 2 (BEX2), fatty acid binding protein 3 (FABP3), heat shock protein 90kDa alpha, class A member 1 (HSP90AA1), mps one binder kinase activator-like 3 (MOBKL3), and ornithine decarboxylase (ODC)) of the 6 genes that were selected from the SSH, the higher expression in the *in vivo* derived embryos could be confirmed with RT-qPCR. Both FABP3 and HSP90AA1 were significantly more expressed in the *in vivo* derived embryos compared to the *in vitro* produced embryos (p<0.05). For ODC, MOBKL3 and BEX2 this difference was highly significant (p<0.005). For the sixth gene (minichromosome maintenance complex component 7 (MCM7) the mean expression was higher in the *in vivo* population than in the *in vitro* group, but this was not statistically significant. The results are summarized in Figure 3.
Figure 3 Differential gene expression in vivo versus in vitro as determined by RT-qPCR. This figure presents the mean expression of 6 genes as determined by RT-qPCR in in vivo derived and in vitro produced equine blastocysts. The standard errors are indicated by bars. For BEX2, FABP3, HSP90AA1, MOBKL3 and ODC the mean expression was significantly higher in the in vivo derived embryos. *significant (p<0.05) **highly significant (p<0.005)

5.5 DISCUSSION

Until recently the equine zygote and cleaving embryo were relatively inaccessible as a research specimen without using surgery or slaughter (Betteridge, 2007), but since the establishment of ICSI in the horse, it is possible to produce horse embryos outside the genital tract. As such, IVP of equine embryos represents a valuable tool for research as well as for clinical purposes. However, IVP embryos show marked differences when compared to embryos derived in vivo. Research in other species revealed the influence of different steps in the IVP process on the embryonic expression of developmentally important genes. Not only the fertilization through IVF (Giritharan et al., 2007) or ICSI (Fernández-González et al., 2008), but also the IVC conditions (Niemann and Wrenzycki, 2000; Rizos et al., 2002, Goossens et al., 2007) affect gene expression levels. Even simple modifications of the embryo culture media, like the presence or absence of
serum, can profoundly influence the gene expression, which is reflected in the developmental competence and the quality of the IVP embryos and even in long-term consequences (McElroy et al., 2008; Fernández-González et al., 2009). Therefore the expression of specific genes has been monitored to evaluate how different fertilization techniques and culture media can influence embryonic differentiation.

In horses very little is known about gene expression in early embryos, with just a few recent papers focusing on this topic (Rambags et al., 2008; Choi et al., 2009; Smits et al., 2009). Comparative analysis of embryonic gene expression in the presence or absence of the maternal genital tract is however a very valuable technique to get more insight into embryo-maternal interaction in the horse and as such it can provide the basis for the optimisation of culture conditions for horse embryos. Since the expression of specific genes can be stage specific, care was taken to use similar developmental stages in the \textit{in vivo} and the \textit{in vitro} group. For the reasons stated earlier, suppression subtractive hybridization (SSH) was used for the detection of differentially expressed genes. In this study the genes expressed at a higher level in \textit{in vivo} derived equine blastocysts (tester) when compared to IVP equine blastocysts (driver) were selectively amplified. This comparison, which has been shown to be valuable in other species, has not yet been performed in the horse.

Ingenuity Pathway Analysis revealed the networks and functional categories in which these 62 differentially expressed genes are involved (\textit{Figure 1}, \textit{Figure 2}). Remarkable and on the other hand logical is their implication in embryonic development, which shows clearly from the different networks. Furthermore both the networks and the function categories illustrate the involvement of these genes in energy production, nucleic acid metabolism and small molecule biochemistry. The most important functional role appeared to be protein synthesis. This is in agreement with the upregulation of protein synthesis genes in bovine \textit{in vivo} blastocysts when compared to \textit{in vitro} blastocysts as determined by SSH (Mohan et al., 2004). In another comparison of bovine \textit{in vivo} versus \textit{in vitro} blastocysts using microarrays the authors suggested that phenotypic differences between both types of embryos might be associated with inefficient transcription and translation in IVP embryos when compared to \textit{in vivo} derived
embryos (Corcoran et al., 2006). The differentially expressed genes involved in protein synthesis contained several ribosomal proteins. Knockdown studies in zebrafish and allelic inactivation in mice illustrated the importance of ribosomal proteins in embryonic development (Panić et al., 2006; Chakraborty et al., 2009).

The occurrence of false positive results in SSH analysis, as in microarray analysis, requires confirmation of the differential expression by a very sensitive and specific method, RT-qPCR. Six genes were selected from the SSH results based upon evidence of their involvement in embryonic development in other species. For 5 of these genes the higher expression in in vivo derived equine blastocysts when compared to IVP equine blastocysts was proven by RT-qPCR to be significant (FABP3 and HSP90AA1) or highly significant (ODC, MOBKL3 and BEX2), confirming the results of the SSH. Subsequently the biological relevance of the RT-qPCR results will be discussed for ODC, HSP90AA1, BEX2, MOBKL3, FABP3 and MCM7.

Ornithine decarboxylase (ODC) is an enzyme which decarboxylates L-ornithine to putrescine, the first step in the biosynthetic pathway of polyamines, which are regulators of cell growth and differentiation. Polyamines stimulate DNA repair and they prevent DNA damage by stabilizing the chromatin structure and by acting as antioxidant as they scavenge reactive oxygen species (ROS) (Pendeville et al., 2001). Several studies illustrate the importance of polyamines, proline, a major substrate for the polyamine synthesis, and ODC, the key regulatory enzyme in this synthesis, throughout embryonic and fetal development in different species (Wu et al., 2008; Gao et al., 2009; Lopez-Garcia et al., 2009). The essential role of ODC in embryonic development has been demonstrated by the lethality of Odc-deficient murine embryos, caused by a deficient expansion of the inner cell mass (ICM) of these embryos, which was found to be mediated through increased apoptosis in the ICM (Pendeville et al., 2001). The lower expression of ODC in equine in vitro blastocysts could be a possible factor influencing the indistinct separation between the ICM and the trophectoderm as observed in in vitro produced horse blastocysts. A possible way to improve embryonic expression of ODC was investigated in a study of pig parthenotes: by means of addition of exogenous polyamines to the embryo culture medium, the mRNA expression of ODC could be enhanced and this improved porcine
embryonic development to the blastocyst stage, with increased cell counts and decreased apoptosis (Cui and Kim 2005). Although the exact molecular mechanisms are not clear, the authors state that the induced transcription of ODC through the exogenous polyamines might regulate cell cycle and/or apoptosis related gene expression in the embryos, resulting in enhanced embryo viability. This anti-apoptotic effect of ODC was also found during oocyte maturation and appeared to be mediated through the suppression of ROS since an increase of ROS was demonstrated in ODC-deficient oocytes (Zhou et al., 2009).

Another group of proteins exhibiting an anti-apoptotic role during embryonic development are the heat shock proteins (HSP) (Esfandiari et al., 2007). Under physiological circumstances these molecular chaperones exhibit several of the functions indicated in Figure 1, like protein folding. Increased synthesis is induced in response to stressful conditions, including rapid cell growth and differentiation, toxicity and inflammation. Their important role in embryonic development starts at the early onset of zygotic genome activity (Bensaude et al., 1983; Christians et al., 1995). The supplementation of mouse in vitro culture media with mononclonal antibodies to HSP60, HSP70 or HSP90 has been shown to result in impaired embryonic development with reduced blastocyst formation and higher rates of apoptosis (Neuer et al., 1998; Esfandiari et al., 2007). This anti-apoptotic effect of HSPs is valuable for in vitro embryo production systems since suboptimal culture conditions can induce a higher degree of apoptosis when compared to conditions prevailing in the maternal genital tract. Therefore Esfandiari et al., (2007) suggest that overexpression of HSP might be beneficial for embryonic development. A similar conclusion was drawn in cattle in vivo embryos in which a positive correlation was found between HSP70 expression and embryo quality (Pretheeban et al., 2009), which is in agreement with our results. In the SSH several HSPs, namely HSP60, HSP90AA1 and HSPH1, were expressed at a higher level in the in vivo derived equine blastocysts when compared to the IVP blastocysts. For HSP90AA1 this difference was further evaluated by RT-qPCR and confirmed as significant. However, it must be noted that some authors interpret a higher HSP expression as a negative sign illustrating increased stress for the embryo (Chin et al., 2009).
One more group of genes which is intriguing with regard to embryonic development is the BEX family. It consists of 4 to 7 genes, according to the species, with BEX1 and BEX2 highly diverged from the others (Zhang, 2008). BEX1, BEX2 and BEX3 have been discovered in mice in an experiment where parthenogenetic and normal blastocysts were compared to identify new imprinted genes. The expression of BEX1 appeared to be upregulated in parthenogenetic blastocysts (Brown and Kay, 1999). However, Williams et al., (2002) found indications that this elevated expression did not result from genomic imprinting of the BEX1 gene since no difference in expression was observed between androgenotes (two paternal genomes), gynogenotes (two maternal genomes) and control embryos. On the other hand the BEX1 expression seemed to be dependent on the developmental stage and the cell type with an increased expression at the expanding blastocysts stage, which was moreover trophectoderm specific. Therefore a more likely explanation for the higher expression in the parthenogenotes as observed by Brown and Kay (1999) could be the combination of the effects of the trophectoderm specific expression of BEX1 and the differences in timing of trophectoderm differentiation between the different classes of embryos. In our study the expression of BEX2 was found to be higher in equine in vivo blastocysts than in IVP blastocysts. Possibly this might be explainable in a similar way since the differentiation of the equine trophectoderm, as indicated by loss of expression of the pluripotency marker POU5F1, has been shown to be impaired in IVP equine blastocysts (Choi et al., 2009). Recent studies however do support X-linked imprinting, as they describe a predominant expression in female embryos of BEX1 in mice (Kobayashi et al., 2010) and BEX1 and BEX2 in cattle (Bermejo-Alvarez et al., 2010). Bermejo-Alvarez et al. (2010) found a higher expression of BEX1 and BEX2 in female bovine blastocysts produced in vitro when compared to their male and parthenogenetic counterparts, suggesting preferential expression of the paternal allele. Even though little is known about X-chromosome inactivation during early embryonic development, several studies illustrate the importance of genes involved in imprinting for the long term phenotype as well as a substantial influence of the in vivo or in vitro environmental conditions on the expression of these genes (Young et al., 2001; Fernàndez-Gonzàlez et al., 2009). Further research is needed to elucidate the role and the mode of action of the BEX genes in horse embryos.
Another gene expressed at a significantly higher level in the \textit{in vivo} derived equine blastocysts is FABP3. The role of fatty acids in embryonic development is controversial. Compared to mice and human, oocytes and embryos of domestic animals contain high amounts of lipids. A hypothesis to explain this is that the lipids function as endogenous energy reserve during the time that the embryos remain unattached in the uterus, which is relatively long when compared to the early attachment in human and mice, which occurs immediately after hatching (Sturmey et al., 2009). Previous research indicates the ability of oocytes and early embryos to use fatty acids as energy substrates (Sturmey et al., 2009). Several studies illustrate the essential role of FABP during embryonic development (Gentili et al., 2004; Arai et al., 2005). However, abundance of fatty acids in the embryonic environment negatively influences the development. Supplementation of hyperlipidaemic sera to bovine embryo culture medium resulted in a reduction of blastocyst development (Leroy et al., 2010).

Mps One Binder kinase activator-like 3 (MOBKL3, MOB1) is also known as preimplantation protein 3 because of its expression during oocyte maturation and preimplantation embryo development following embryonic genome activation. Research in mice revealed a relatively high expression through the one cell stage, a decline at the two cell stage and a subsequent rise at either the eight cell or blastocyst stage, indicating a role in preimplantation embryogenesis (Temeles et al., 1994). In pigs the polymorphism of this gene was associated with litter size traits and suggested to be useful for marker assisted selection (Niu et al., 2006). In this study this preimplantation protein was expressed at a higher level in the \textit{in vivo} derived equine blastocysts when compared to the IVP blastocysts. Information about this gene in horses is absent, but an important role in the coordination of mitotic exit and cytokinesis has been found in yeast and mammalian homologues appear to play similar roles (Hergovich et al., 2008; Wilmeth et al., 2010).

Another gene involved in the guidance of the accurate execution of cell division is MCM7. For normal embryonic development it is important that complete duplication of the genome occurs exactly once per cell cycle (Blow and Laskey, 1988). This crucial event is ensured through DNA licensing by the assembly of a prereplication complex to the replication origins (Donaldson and
Blow, 1999). As a part of this complex the minichromosome maintenance (MCM) 2-7 helicase plays a central role in DNA replication initiation and elongation (Labib et al., 2000). The expression of MCM genes can serve as a sensitive marker for proliferation zones during embryogenesis (Ryu and Driever, 2006). A member of this MCM family, MCM7, which is involved in the oogenesis and the first embryonic cell cycle in mice (Sweich et al., 2007), was indicated by the SSH to be preferentially expressed in in vivo equine embryos when compared to their in vitro counterparts. However, evaluation of this differential expression by RT-qPCR revealed it to be not significant.

In conclusion 62 genes which were expressed at a higher level in in vivo derived equine blastocysts when compared to in vitro produced equine blastocysts were identified by means of SSH. Ingenuity Pathway analysis revealed the functional categories and networks in which these genes are involved and indicated an important role in protein synthesis. For 5 of these SSH sequences, namely ODC, HSP90AA1, BEX2, MOBKL3 and FABP3, the higher expression in vivo was confirmed by RT-qPCR. The evaluation of embryos at the genetic level has appeared to be valuable in other species. This study contains the first step of a similar approach in the horse, which may enable progress in the field of assisted reproductive techniques. Further research will be focused on environmental influences on the embryonic gene expression, finally aiming to improve knowledge and valuable clinical applications for the subfertile horse.

REFERENCES


maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim Reprod Sci* 98 39-55.


oviduct versus in vitro culture for different domestic species. Theriogenology 73 748-757.


INFLUENCE OF THE UTERINE ENVIRONMENT ON THE DEVELOPMENT OF *IN VITRO* PRODUCED EQUINE EMBRYOS

6.1 ABSTRACT

After an unusually long stay in the oviduct, the equine embryo passes through the utero-tubal papilla on day 6 after ovulation. Soon after its arrival in the uterus, the embryo becomes enveloped by a glycoprotein tertiary coat (the ‘capsule’); during the first 5 weeks of intrauterine development the conceptus is entirely dependent on endometrial secretions for its nutrition. The necessity for early interaction between the embryo and the oviductal and/or uterine environment in the horse is reflected by several striking differences between equine embryos that develop in vivo and those produced in vitro. Better understanding of the salient interactions may help to improve the efficiency of in vitro equine embryo production. In an initial experiment, cleavage-stage in vitro produced (IVP) equine embryos were transferred into the uterus of recently ovulated recipient mares to determine whether premature placement in this in vivo environment would improve subsequent development. In a second experiment, an important element of the uterine environment was mimicked by adding uterocalin, a major component of the endometrial secretions during early pregnancy, to the culture medium. Intra-uterine transfer of cleavage-stage equine IVP embryos yielded neither ultrasonographically detectable pregnancies nor day 7 blastocysts, indicating that the uterus is not a suitable environment for per-compact morula stage horse embryos. By contrast, exposure to uterocalin during IVP improved capsule formation, although it did not measurably affect development or expression of a panel of genes known to differ between in vivo and in vitro embryos. Nevertheless, the positive effect of uterocalin on capsule formation in IVP horse blastocysts illustrates that adding a specific endometrial protein to embryo culture medium can help embryos develop more physiologically; further studies are required to evaluate whether uterocalin serves purely as a carrier protein or more directly promotes improved capsule development.

6.2 INTRODUCTION

Early embryonic development in the horse is characterized by a number of peculiarities (Betteridge, 2007). Firstly, the equine embryo doesn’t enter the uterus via the prominent utero-
tubal papilla until as late as 144-156 h after ovulation (Battut et al., 1997). Moreover, unfertilized eggs are not capable of stimulating passage through the ampullary-isthmic junction and are instead retained in the oviduct. This selective oviductal transport has been shown to be a stage specific function of the production of prostaglandin E2 by day 4-5 equine conceptuses (Weber et al., 1991), and is a clear example of very early embryo-maternal interaction in the horse.

Another enigmatic feature exemplifying early embryo-maternal interaction in the horse is the formation of an acellular glycoprotein tertiary embryo coat (‘capsule’: Flood et al., 1982) very soon after the arrival of the horse embryo in the uterus; the capsule completely envelopes the equine conceptus until around day 21 of gestation (Betteridge, 1982). Although the precise functions of the capsule are not clear, it has been proposed to function as a ‘mailbox’, incorporating endometrial components such as signalling molecules and nutrients and transporting them to the embryo (Herrler & Beier, 2000), and to physically protect the mobile embryo and maintain its spherical shape while migrates around the uterus to signal its presence to its dam and prevent luteolysis (Ginther, 1985; Allen and Stewart, 2001; Stout and Allen, 2001). In addition, structural changes in the capsule are thought to be instrumental to the process of fixation and orientation of the conceptus within the mares’ uterus (Oriol, 1994).

Since a functional chorioallantoic placenta is not formed until as late as days 40-45 of gestation, equids have the longest pre-implantation period of all mammals studied to date (Allen and Stewart, 2001); moreover, during this prolonged pre-implantation period the embryo is entirely dependent on endometrial secretions (‘histotrophe’) for its nutrition. An undoubtedly important component of this histotrophe is the 19 kDa progesterone-dependent protein, uterocalin, that is secreted by the endometrial glands during both dioestrus and early pregnancy (Crossett et al., 1996). The marked drop in uterocalin production that coincides with the disappearance of the capsule at around day 21, and the fact that uterocalin is one of the most abundant proteins in the capsule (Quinn et al., 2007), suggests that there may be a functional correlation between uterocalin production and the presence of the capsule (Crossett et al., 1996). Moreover, detection of uterocalin in the trophoblast and yolk-sac fluid of equine
conceptuses implies passage through the capsule and absorption by the conceptus proper. On the basis of its structure, uterocalin has been classified as a member of the lipocalin family, which contains several transport proteins that bind small hydrophobic molecules (Crosset et al., 1996). Moreover, in depth structural analysis of uterocalin, suggests a putative function as a carrier of essential lipids and amino acids for the developing conceptus (Kennedy, 2004).

All of the above illustrate the importance of embryo-maternal interaction during early embryonic development in the horse. Furthermore, when equine embryos are produced \textit{in vitro}, and therefore in the absence of the maternal tract, they differ markedly from their \textit{in vivo} counterparts in terms of the kinetics of development, incidence of apoptotic cells, inner cell mass morphology and gene expression patterns (McKinnon, 1989; Hinrichs, 1990; Tremoleda et al., 2003; Pomar et al., 2005; Smits et al., 2010a). One of the more striking irregularities of IVP horse embryos is the failure of normal capsule formation (Tremoleda et al., 2003); even though capsular mucin-like glycoproteins are produced \textit{in vitro}, they fail to coalesce into the distinct continuous capsule observed around \textit{in vivo} equine embryos from the early blastocyst stage. The reason(s) for the failure of capsular glycoprotein coalescence \textit{in vitro} are not known but may involve simple dispersion of the glycoproteins into the culture medium, preventing attainment of the critical concentration required for capsule assembly, or failure of hydration and cross-linking of the capsular glycoproteins in the absence of a specific uterine component(s) (McKinnon, 1989; Hinrichs, 1990; Tremoleda et al., 2003). In either case, the presence of the mare’s uterus appears to be essential to the process of capsule formation; to confirm that the uterine environment and/or specific uterine components are central to capsule formation, we exposed IVP embryos to either the complete uterine environment or to the endometrial protein, uterocalin, which is known to contribute substantially to the substance of the capsule of day 10-18 blastocysts. A recent study demonstrated that temporary transfer of IVP horse blastocysts to the mare’s uterus for 2-3 days had a positive effect on capsule formation, as assessed by light microscopy (Choi et al., 2009). In this study, we wanted to further determine whether ‘premature’ transfer of day 2-3 embryos to the uterus could not only enhance capsule formation but also improve equine blastocyst development rates and quality, as compared to culture \textit{in vitro}. In this latter respect, while it is common practice to
culture embryos to the blastocyst stage prior to intra-uterine transfer in most domestic species, in human medicine premature intra-uterine transfer of day 2 and 3 embryos is a routine procedure that yields good results (Younis et al., 2009) and circumvents the potential downsides of prolonged in vitro culture, or the difficulty of transferring early embryos to the oviduct. If transfer of cleavage stage IVP embryos to the uterus of the mare was successful, it would considerably simplify the IVP process even if it didn’t have additional beneficial effects on embryonic development and capsule formation. To more specifically investigate the putative role of uterocalin in capsule formation and early development of equine embryos, recombinant uterocalin was added to culture medium for 5-10 days, and the effect on subsequent development was examined in terms of capsule formation and expression of a panel of genes known to be differentially expressed by in vivo versus IVP horse embryos.

6.3 MATERIALS AND METHODS

6.3.1 Experiment 1: Intra-uterine transfer of cleavage stage equine in vitro embryos

All animal procedures were approved by the ethics committee of the Faculty of Veterinary Medicine at Ghent University. In vitro embryos were produced as previously described by Smits et al. (2010b). Briefly, slaughterhouse oocytes were matured in vitro for 24h in a DMEM-F12 based medium in an atmosphere containing 5% CO₂ (Galli et al., 2007). MII oocytes were fertilized by conventional ICSI and cultured in vitro in DMEM-F12 with 10% fetal calf serum at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂. On day 2-3 after ICSI, cleaved embryos were transferred by means of a transcervical pipet (IMV Technologies, France) to the uterus of a recipient mare that had ovulated 2-3 days previously. A total of 99 cleaved embryos were transferred to the uterus of 12 synchronized mares (average of 8.25 embryos per mare). On the day of transfer, the recipient mare was injected intravenously with 1.1 mg/kg of the non-steroidal anti-inflammatory agent, flunixine meglumine (Emdofluxin®, Emdoka, Belgium) and daily per os treatment with 0.044mg/kg of the synthetic progestagen, altrenogest (Regumate®, Intervet, The Netherlands) was initiated and continued until evaluation for embryo development. Half of the mares were examined by per rectum uterine ultrasound 14 days after ICSI, and half were
subjected to embryo recovery by transcervical uterine lavage on day 7 after ICSI. Briefly, flushing was performed with 6 liters of Lactated Ringer’s solution using a Bivona-catheter (Minitüb, Germany) and the recovered fluid was passed through an EZ filter (Bioniche, Ireland). Any embryos recovered were stained with Hoechst 33342 (Molecular Probes, The Netherlands) to assess cell viability and number.

6.3.2 Experiment 2: IVP in the presence of recombinant uterocalin

Production of recombinant uterocalin

A recombinant uterocalin clone and an anti-uterocalin-antibody were kindly provided by Professor MW Kennedy (University of Glasgow, UK). The recombinant uterocalin was purified mainly as described by Suire et al. (2001) using the Profinity™ IMAC Ni-Charged Resin (Bio-Rad), to produce a working concentration of 7.88 mg/ml.

Estimation of the physiological concentration of uterocalin

Since no absolute concentrations of uterocalin in the uterine environment are reported in the literature, the physiological concentration of uterocalin was estimated using a uterine secretion sample recovered from a day 7 pregnant mare. Sampling of uterine secretions was performed by means of aspiration through a pipette for deep intra-uterine insemination as described by Velazquez et al. (2010), while subsequent uterine lavage resulted in the recovery of an embryo, thereby confirming that the mare was pregnant. A dot blot technique was used to compare a dilution series of recombinant uterocalin with the recovered uterine secretion and indicated that the concentration of uterocalin in the uterine secretions was approximately 4 mg/ml.

In vitro production of equine blastocysts

In vitro embryos were produced as described for experiment 1, except that only oocytes with a compact cumulus complex were used, the maturation time was 28h and ICSI was performed using a Piezo Drill (Prime Tech Ltd., Ibaraki, Japan). The embryos were cultured in groups of 10-20 in 20 µl droplets of DMEM-F12 with 10% fetal calf serum at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂. On day 2.5, half of the medium was refreshed and the embryos that had not cleaved were
removed. On day 6, half of the medium was refreshed again and, in half of the culture droplets, 2.54 µl of the medium was replaced by the recombinant uterocalin solution, resulting in a final concentration of 1 mg/ml recombinant uterocalin. On day 9-9.5, the embryos that had reached the blastocyst stage were recovered for further analysis.

**Immunofluorescent staining of the capsule**

Immunofluorescent staining of the equine capsule was performed as described by Tremoleda et al. (2003) using the monoclonal anti-capsule antibody OC-1 (Oriol et al., 1993), which was kindly provided by Professor KJ Betteridge (University of Guelph, Canada). Day 9.5 blastocysts were fixed in 4% paraformaldehyde (P6118, Sigma-Aldrich, Bornem, Belgium) and stored at 4°C until analysis. Twelve blastocysts that had been cultured with uterocalin and 14 blastocysts from the control group were stained simultaneously. After permeabilisation by exposure to 0.5% (v/v) Triton X-100 for 30 minutes at room temperature, the blastocysts were washed 3 times in PBS containing 1 mg/ml PVP. Non-specific staining was blocked by incubation in 10% (v/v) goat serum (16210-064, Invitrogen, Merelbeke, Belgium) for 30 minutes at 37°C. The blastocysts were then washed again and incubated with the primary antibody (mouse monoclonal anti-capsule OC-1: 1/200 dilution) for 1.5h at 37°C. In both groups, a negative control blastocyst was incubated in 10% goat serum without primary antibody. After a washing step, incubation with the secondary antibody (goat-anti-mouse FITC: Molecular Probes, Leiden, The Netherlands), 1/100 dilution) was performed for 1h at 37°C, followed by another washing step. Nuclei were then stained by incubation with 2% propidium iodide (Molecular Probes, Leiden, The Netherlands) for 30 minutes at room temperature, after which the embryos were fixed in Dabco (Acros, Ghent, Belgium) on siliconized glass and enclosed under a cover slip supported by small vaseline bridges to prevent crushing of the embryos. All embryos were evaluated in one session using a Nikon C1 confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium) and identical settings. Subsequent fluorescence measurements were performed using Nikon EC-V1 FreeViewer software. Since some blastocysts were a little squeezed by the cover slip, intact capsules of 6 uterocalin blastocysts and 8 control blastocysts in similar condition were evaluated. For each
embryo, the total fluorescence of 3 areas in different, randomly selected, spots of the capsule were measured and the mean of these 3 measurements was recorded (Figure 1).

Figure 1 Measurement of the capsule fluorescence (Equus caballus). For each blastocyst capsular fluorescence was measured in 3 areas of at least 10 µm² as represented in the figure. Random places on different sides of the blastocyst showing an intact capsule were assessed. For each embryo the mean of these 3 measurements was calculated.

RT-qPCR

For both the uterocalin and the control group, 11 blastocysts were selected on day 9. After washing in DPBS, individual blastocysts were transferred to cryotubes with 2 µl lysis buffer, frozen in liquid nitrogen for 3 minutes and stored at -80°C. RNA-extraction was performed using the RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) and, after RT minus control, the RNA was converted into cDNA using the iScript™cDNA synthesis Kit (Bio-Rad, Nazareth Eke, Belgium). The expression of the 5 development ‘marker’ genes (BEX2, FABP3, HSP90AA1, MOBKL3 and ODC) was quantified by RT-qPCR as described by Smits et al. (2010a). Normalization of data was performed using UBC, ACTB, RPL32 and GAPDH as reference genes (Smits et al., 2009).
Statistical analysis

The blastocyst development rates for the uterocalin and control groups were compared using a Pearson Chi-square test (SPSS 16.0, SPSS Inc., Headquarters, Chicago, Illinois, US). Cell number and capsular fluorescence were compared between the groups using t-tests, while gene expression between the uterocalin and control groups was compared with a Mann-Whitney test using GraphPadInStat3. A p-value <0.05 was considered statistically significant.

6.4 RESULTS

6.4.1 Experiment 1: Intra-uterine transfer of cleavage stage IVP embryos

A total of 99 cleaved (2-8 cell stage) embryos were transferred to the uterus of 12 synchronized (day 2-3 after ovulation) mares (average of 8.25 embryos / mare). Six of these mares were subsequently examined for pregnancy by transrectal ultrasound on day 14 after ovulation, but no conceptus vesicles were detected. The uterus of the remaining six mares was flushed on day 7 after ovulation. Disappointingly, embryos were recovered from only 3 of the 6 mares and no mare yielded more than a single embryo (overall recovery rate 6%). Moreover, none of the 3 recovered embryos had developed to the blastocyst stage; instead all 3 were clearly degenerate (Figure 2).
Figure 2 Intra-uterine transfer of cleavage stage embryos (Equus caballus). After intra-uterine transfer of day 2-3 equine cleavage stage embryos and subsequent flushing on day 7 only degenerated embryos were recovered. The embryos were stained with Hoechst 33342.

6.4.2 Experiment 2: Addition of recombinant uterocalin to embryo culture medium

No significant differences in overall development were observed between embryos that had or had not been exposed to uterocalin during IVP (Table 1). In total, 60% of recovered oocytes reached the MII stage and 76% of sperm injected oocytes had cleaved 48h after ICSI. In the control group, 25 of 198 cleaved embryos developed to the blastocyst stage (12.6%); in the group cultured with uterocalin, 22 of 165 cleaved embryos (13.3%) reached the blastocyst stage (Figure 3 A, B). Mean cell counts and embryo diameters (± S.E.M.) were respectively 579 (± 40) and 247 (±15) µm for the blastocysts cultured with uterocalin (n=11) and 551 (± 47) and 270 (±12) µm for the control group (n=11) (p>0.05).
Table 1: Influence of uterocalin on development and capsule formation in equine in vitro produced blastocysts (Equus caballus). Addition of uterocalin during IVC did not affect blastocyst rate, cell count and embryonic diameter. Immunofluorescence of the embryonic capsule was significantly increased in the presence of uterocalin. Mean values and their respective standard errors are displayed.

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<td>Blastocyst percentage (%)</td>
<td>12.6 (±2.5)</td>
<td>13.3 (±3.5)</td>
<td>0.842</td>
</tr>
<tr>
<td>Mean cell count D9.5</td>
<td>551 (±47)</td>
<td>579 (±40)</td>
<td>0.660</td>
</tr>
<tr>
<td>Mean diameter (µm)</td>
<td>270 (±12)</td>
<td>247 (±15)</td>
<td>0.251</td>
</tr>
<tr>
<td>Fluorescence capsule</td>
<td>2123 (±117)</td>
<td>2745 (±208)</td>
<td>0.0312</td>
</tr>
</tbody>
</table>

By contrast, total fluorescence after immunofluorescent labeling of the embryos with the capsule specific antibody OC-1 (Oriol et al., 1993) was significantly higher in blastocysts that had been cultured in the presence of uterocalin (2745 ±208; n=6), than in those cultured in control medium (2123 ±117; n=8) (p=0.0312) (Table 1). Penetration of capsular glycoproteins into the transzonal channels was observed in both groups, and was more obvious in smaller blastocysts (Figure 3 C, D). In the larger blastocysts, the capsular material appeared to form a more or less continuous layer (Figure 3 E, F), although this did not extend over the part of the embryo that had herniated through the hole in the zona created during ICSI; instead the glycoprotein over the protruding trophectoderm was visible in patches in both groups (Figure 4). Interestingly, in the uterocalin group, an apparently continuous area of capsule associated with the trophectoderm was observed in an area where the zona had become loosened locally around one blastocyst and in a zona-free area of another blastocyst (Figure 5); similar findings were not observed in control embryos.
Figure 3 Day 9.5 blastocysts produced in vitro and cultured in the absence (A,C,E) or presence (B,D,F) of uterocalin (Equus caballus). A,B: Equine blastocysts before fixation. Small blastocysts display a thin capsular line (OC1-staining) and penetration of capsular material in the transzonal channels in both the embryos cultured in the absence (C) or presence (D) of uterocalin. Larger blastocysts present a more developed and continuous capsule in both the control (E) and the uterocalin (F) group, but still show penetration of the glycoproteins in the thinned zona. White scale bar = 10 µm.
Figure 4 Hatching blastocyst produced in vitro (*Equus caballus*). This immunofluorescent staining of the capsular OC-1 illustrates the capsular glycoproteins in the hatched part of an in vitro produced blastocyst of the control group (A) and the uterocalin group (B). No confluent capsule is apparent.
Figure 5 Confluent capsule adjacent to trophectoderm (Equus caballus). This day 9.5 horse blastocyst was cultured in the presence of uterocalin and capsule formation was assessed by immunofluorescent OC1-staining. Interruption in the zona pellucida reveals a confluent capsule which is attached to the trophectodermal surface. White scale bar = 10 µm.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is a highly specific and sensitive tool for comparing expression of mRNA for specific genes between experimental groups. The 5 genes analysed, $BEX2$, $FABP3$, $HSP90AA1$, $MOBKL3$ and $ODC$ were chosen as markers for developmental quality, because a previous study indicated downregulation of these genes in in vitro produced compared to in vivo derived equine blastocysts (Smits et al., 2010a). It was hypothesized that adding the endometrial protein, uterocalin, might induce an expression pattern in the in vitro embryos more closely resembling
that of in vivo embryos. In fact, no differences in expression levels were found between the blastocysts which were cultured with uterocalin (n=11) and the blastocysts from the control group (n=11) (Figure 6). In this experiment, qPCR efficiencies of ≥ 99% and correlation efficiencies ≥ 0.992 were obtained, indicating that the results were reliable.

Figure 6 Expression of BEX2, FABP3, HSP90AA1, MOBKL3 and ODC in blastocysts cultured with uterocalin and control blastocysts as determined by RT-qPCR (Equus caballus). The expression of these 5 genes was previously found to be downregulated in in vitro produced equine blastocysts when compared to in vivo derived equine blastocysts. However, this figure shows that the addition of uterocalin, an important protein in the uterine secretions during early pregnancy, to the in vitro culture medium from day 6 up to day 9 did not influence the expression levels of these genes. The error bars represent the S.E.M.

6.5 Discussion

The ability to efficiently produce equine embryos in vitro is of interest for both research purposes and the clinical treatment of (equine) infertility. Presently, acceptable rates of embryo production and levels of embryo quality can be obtained using intracytoplasmic sperm injection of in vitro matured oocytes followed by culture in DMEM/F12 supplemented with serum; however, there is still room for further optimisation of the in vitro culture process to improve
the overall efficiency of the procedure (Galli et al., 2007; Hinrichs et al., 2007; Blanco et al., 2009). Understanding the influence of the equine oviductal and uterine environments, and specific components thereof, on early embryonic development could lead to targeted adaptations of the in vitro environment to mimic aspects of the maternal environment identified as beneficial.

In this study, intra-uterine transfer of cleavage stage IVP equine embryos did not yield any pregnancies and, since no healthy blastocysts were recovered on day 7 (i.e. 4-5 days after transfer), it appears that the transferred cleavage stage embryos do not develop in the uterine environment. There are several possible explanations for this failure of development. Firstly, it is possible that closure of the cervix 2-3 days after ovulation was suboptimal when compared to the day 4-9 period during which commercial embryo transfers are usually performed. This could result in loss of embryos through the cervix soon after transfer. On the other hand, pregnancy after transfer of day 10 embryos to recipient mares on days 1 or 3 after ovulation has been described by Wilsher et al. (2010) and, although none of these embryo’s subsequently developed into normal pregnancies (none developed an embryo proper), it does illustrate that the uterus should be capable of mechanically retaining embryos introduced soon after ovulation. A second possible explanation is simple absence of intrinsic developmental potential of the transferred in vitro embryos. However, using identical preliminary steps, standard in vitro production yielded 5-10 % blastocysts in our hands, and transfer of one of these blastocysts has resulted in pregnancy and the birth of a live foal (Smits et al., 2010b); in short, at least some (5-10) of the cleaved embryos should have been capable of further development.

The remaining possible interpretation is that the mare’s uterus does not provide an adequate or appropriate environment for these early cleavage stage embryos. A similar failure to establish pregnancy following premature intrauterine transfer was reported for 292 day 2 mouse embryos (Goto et al., 1993), and for previous small studies that described the intra-uterine transfer of early in vivo derived horse embryos. For example, Weber et al. (1993) achieved no pregnancies following intra-uterine transfer of 7 day 2 horse embryos and while Allen and Rowson (1975) did describe a single pregnancy after transfer of 5 day 3 equine
embryos, the age of the embryo was estimated from daily examination for ovulation by transrectal palpation; the embryo that resulted in pregnancy could thus easily have been closer to 4 days. Indeed, day 4 equine embryos have been reported to be sufficiently mature to survive in the mare’s uterus (Peyrot et al., 1987).

In human medicine, cleavage stage embryos are routinely transferred to the uterus and, while the procedure is considered to entail both specific advantages and disadvantages when compared to blastocyst transfer, overall favourable pregnancy rates of 30-40% are common (Bromer & Seli, 2008; Papanikolaou et al., 2008). The reason for the marked differences in the success of intrauterine transfer of day 2-3 embryos in women and some primates compared with other domestic species might be due to marked anatomical differences. In the mare, there is a distinct, tightly closed uterotubal papilla, which presumably helps to maintain the marked differences in fluid composition observed in different parts of the oviduct and uterus in both horses and other domestic and laboratory species. This is in marked contrast to the lack of an anatomically distinct utero-tubal junction in women, in which the oviductal and uterine fluids appear to mix freely (Hunter, 1998).

During the initial intrauterine period, the equine embryo migrates continually, surrounded by a protective glycoprotein capsule and bathed in nourishing endometrial secretions, which contain several progesterone dependent proteins (Ellenberger et al., 2008). The addition of one of the major progesterone dominated proteins, uterocalin, to embryo culture medium had a positive effect on capsule formation around IVP blastocysts. Blastocysts cultured in the presence of uterocalin showed more intense fluorescence following labelling with a capsule specific antibody (OC-1) than control embryos. Furthermore, where the zona pellucida was locally absent around blastocysts cultured in the presence of uterocalin, a distinct and confluent capsule was found associated with the trophectoderm (Figure 5); such zona-independent areas of continuous capsule were not seen on control embryos, and have not been described previously for IVP equine embryos. Indeed, when Tremoleda et al. (2003) separated the zona pellucida from a day 10 in vitro produced embryo, they found the capsular material to be stuck to the zona instead of forming a separate layer between zona and trophectoderm. This
apparent effect on capsular glycoprotein coalescence may reflect an additional function of uterocalin and, together with previous studies illustrating that uterocalin makes a considerable contribution to the total mass of the capsule, it suggests that uterocalin may be a maternally-derived structural component of the capsule rather than just a transiently associated transport molecule. Previous studies, have demonstrated that OC-1 reactive capsular glycoproteins are secreted by trophectoderm cells (Albihn et al., 2003), while the failure of normal capsule formation in vitro suggests the need for an additional maternal component (Tremoleda et al., 2003). In this respect, the 19kDa endometrial protein uterocalin was first isolated as one of the dominant proteins detected by SDS-PAGE of equine embryonic capsules (Stewart et al., 1995). Subsequent studies confirmed the presence of uterocalin in the capsule in a temporal pattern that suggested a functional correlation between the two (Crossett et al., 1996; Herrler & Beier, 2000; Quinn et al., 2007). Subsequently, and as a result of its molecular structure, uterocalin has been proposed to function primarily as a carrier of biologically important lipids and a source of essential amino acids for the developing conceptus (Suire et al., 2001, Kennedy, 2004), where the positive charge of uterocalin (Crossett et al., 1996) is thought to facilitate its binding to the negatively charged sialic acid residues of the capsule (Oriol et al., 1993). Since uterocalin has also been found in trophoblast cells (Crossett et al., 1996; Ellenberger et al., 2008), some of the molecule clearly passes through the capsule and presumably fulfils a role as a carrier protein. In addition, it seems likely that uterocalin contributes to the structure of the capsule, and plays a role in the initial aggregation and cross-linking of trophectoderm-produced OC-1 reactive glycoproteins. In summary, uterocalin appears to be instrumental in initial capsular glycoprotein coalescence, contributes significantly to the substance and structure of the capsule and plays an important role in transporting essential nutrients and or signalling molecules to the developing conceptus during the initial intra-uterine period.

The dynamics of embryonic covering formation and loss and, in particular, the addition of tubal and uterine secreted materials during development has been described in several species (for Review see Denker, 2000). In this respect, the equine embryonic capsule has been proposed to be most analogous to the neozena of the rabbit (Betteridge, 1989; Herrler & Beier, 2000). For example, while trophoectodermal secretions are important for neozena formation, maternal
components also appear to be critical (Fisher et al., 1991; Denker, 2000). Indeed, *in vitro* culture of rabbit embryos is associated with the deposition of granular material on the inside of the mucoprotein layer, but failure of normal neozona formation, aberrant herniation of embryonic cells through the zona, and incomplete or failed dissolution of the zona pellucida (Fisher et al., 1991). These features are reminiscent of the aberrations of capsule formation and hatching observed in IVP equine embryos (Tremoleda et al., 2003; Stout et al., 2005). However, in both species, these deviations are not necessarily irreversible and most can be corrected by exposure to uterine components. In this respect, addition of uterine flushings to rabbit embryo culture medium, or intra-uterine transfer of *in vitro* cultured embryos, has been shown to allow reactivation and completion of zona dissolution, although uterine flushings alone did not induce neozona formation *in vitro* (Fisher et al., 1991). In the horse, intra-uterine transfer of *in vitro* produced blastocysts results in successful pregnancies (Galli et al., 2007; Hinrichs et al., 2007; Smits et al., 2010b) with an apparently normal capsule (Choi et al., 2009), illustrating that the degree of initial aberration in capsule formation during IVP is not so severe as to preclude normal establishment of pregnancy, at least as long as exposure to the uterine environment is sufficiently early to remedy the aberrations; by contrast, total removal of the blastocyst capsule from day 6.5 embryos has been reported to be incompatible with embryonic survival after transfer (Stout et al., 2005); clearly the disruption to capsule formation suffered during IVP is not equivalent to removal.

In the current study, some aspects of aberrant equine embryonic capsule formation *in vitro* appeared to be ameliorated by the presence of uterocalin. However, it is not known how the uterocalin had this effect, and neither was uterocalin alone sufficient to completely normalize capsule formation; e.g. the ‘hatched’ areas of trophoderm still exhibited dispersed patches of OC-1 reactive glycoproteins that did not coalesce into a confluent layer (*Figure 4*). In addition, capsular material still penetrated into the transzonal channels as previously reported for IVP embryos (Tremoleda et al., 2003) but not seen in *in vivo* embryos; the transzonal penetration of capsular glycoproteins in this study was particularly evident for smaller embryos (*Figure 3*). While it is possible that there was insufficient uterocalin provided in the current study to completely normalize capsule production, it is more likely that this partial correction
reflects the continued absence of other components of the complex intra-uterine environment that are required for capsule formation.

Surprisingly, no clear influence of uterocalin on the development of equine IVP blastocysts was observed, as illustrated by equal blastocyst rates, embryo diameters and cell counts between control and uterocalin groups (Table 1). Moreover, exposure to uterocalin did not alter expression of 5 genes (BEX2, FABP3, HSP90AA1, MOBKL3 and ODC: Figure 6), previously found to be down-regulated in IVP embryos as compared to in vivo derived embryos (Smits et al., 2010a). A similar failure of endometrial proteins to influence early equine embryonic development was reported by Bøgh et al. (2002) who exposed day 8 in vivo derived embryos to a p19 (i.e. uterocalin) homologue during a 3h incubation, and found no obvious influence on subsequent embryonic growth and metabolism. The absence of effects on embryonic growth, metabolism (Bøgh et al., 2002), gene expression or quality (this study) might be an artefact due to measurement of factors not influenced by uterocalin. However, it is also possible that a crucial ligand(s) necessary for uterocalin to influence gene expression or metabolism was absent in the in vitro culture medium; this would seem logical if uterocalin is primarily a carrier protein, as suggested by its ability to bind several essential small lipids (Suire et al., 2001), rather than a stimulator of embryonic growth or development per se.

6.6 CONCLUSION

Several unusual features of early embryonic development in the horse illustrate the importance of embryo-maternal interaction. To optimize equine IVP it may well be necessary to further clarify, and develop methods to mimic, particular aspects of this interaction. In the current study, an essential role of the oviductal environment was illustrated by the failure of premature intrauterine transfer to yield any viable embryos. In addition, the need for the uterine environment of slightly older embryos was illustrated by the positive effect on capsule development of exposing IVP embryos to the maternal endometrial protein, uterocalin. However, since capsule formation in the presence of uterocalin was not completely normal, while embryo development and gene expression still differed markedly from in vivo embryos, it
is clear that many aspects of the maternal environment necessary to support optimal early embryonic development still need to be identified.

REFERENCES


GENERAL DISCUSSION
7.1 PRODUCTION OF EQUINE BLASTOCYSTS IN VIVO AND IN VITRO

This research focused on the equine blastocyst, a clinically relevant developmental phase because it is the physiological stage associated with presence in the uterus. Compared to oviductal stages, blastocysts can be obtained easily by flushing of the uterine cavity and can also be transferred by means of a non-invasive transcervical technique into the uterus. Equine embryonic development in vitro is retarded compared to in vivo development (Pomar et al., 2005). In this study, the in vivo derived blastocysts were recovered 7 days after ovulation whereas the in vitro produced blastocysts were harvested 9-9.5 days after ICSI in order to obtain comparable developmental stages rather than embryos of similar chronological age. However, considerable individual variation in the kinetics of development in vitro was observed, as illustrated in Figure 1.

Figure 1 Equine IVP blastocysts 9.5 days after ICSI (magnification 60x). Even though the 4 blastocysts have exactly the same age, there are differences in grade of expansion and hatching. The lower two blastocysts are more expanded than the upper two. The right blastocyst starts hatching.
Historically, research on equine blastocysts has been hampered by the difficulty of obtaining sufficient samples. Recovery of large numbers of *in vivo* embryos is hindered by the fact that the horse is a seasonal breeder with little or no ovarian activity during the winter months and, moreover, the mare is mono-ovulatory and does not react favourably to superovulatory treatments. In our studies, embryo recovery rates ranged around 50-60% (CHAPTER 4,5), which is in agreement with published figures (Squires et al., 2003).

Neither is IVP of equine blastocysts widespread nor efficient. Maturation (50%) and cleavage rates (70-80%) in our study were comparable to results published elsewhere (Galli et al., 2007). However, our blastocyst production rate was low and variable and improved from less than 2% in initial experiments up to more than 20% in later experiments. Even though media were constant throughout the period, there are some possible explanations for this improvement.

First of all, the original maturation time of 24h (Galli et al., 2007) was increased to 28h, which has been reported to improve cleavage rates for compact COCs (Choi et al., 2004). In this regard, it must be noted that the expanded COCs, for which a shorter maturation time might be preferential, matured to a high degree (up to 70%), which is in agreement with Galli et al. (2007) and Hinrichs and Schmidt (2000). However, the development of oocytes from expanded COCs up to the blastocyst stage was generally very poor when compared to that of compact COCs. This is in contrast to the results of Galli et al. (2007), who found a similar developmental competence for oocytes from both compact and expanded COCs. It should however be noted that these authors collected the oocytes by scraping the follicle wall, while in our laboratory aspiration of the follicles was performed. The latter is associated with the collection of a significant proportion of oocytes surrounded by only a few layers of cumulus cells, and they were classified as compact COCs, leaving the expanded COCs as a minority of the collected COCs (CHAPTER 1, *Table 1*). This difference in classification might have influenced the results observed.

A second factor that might have had a positive influence on the blastocyst production rate was the introduction of the piezo drill in December 2008. The introduction of the piezo drill for ICSI in the horse has been associated with better cleavage rates and more consistent results (Choi et
Possible explanations for the positive effect of piezo-assisted over conventional ICSI include the higher probability of oolemma breakage, reduced damage to the oocyte because mechanical suction of the oolemma is replaced by a single pulse and better permeabilization of the sperm membrane, which facilitates oocyte activation (Yanagida et al., 1998, Choi et al., 2002). Considering the possible influence of the injection technique on subsequent embryonic development, the laser was introduced as an alternative for the piezo drill. The pilot study presented here, determined the laser as a valuable option for ICSI in the horse.

A final consideration is the acquisition of experience over the years, coinciding with faster manipulation of the oocytes and reduced damage during subsequent IVP procedures.

The low blastocyst rate could be considered suboptimal. It could be criticized that IVP blastocysts, cultured in suboptimal conditions, will have more aberrations in gene expression. However, the focus of the research was on the identification of genes that were more highly expressed in the golden standard, which were in vivo derived equine blastocysts. Moreover, several stains (Figure 2) and the successful transfer of two IVP blastocysts leading to the birth of a foal (CHAPTER 3.2) proved the developmental competence and vitality of the produced blastocysts.

![Figure 2 Equine IVP blastocysts (day 9) (magnification 400x).](image)

*Fluorescent staining with Hoechst 33342 (A) and propidium iodide (B) illustrate the vital nuclei of horse blastocysts produced in vitro.*

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Despite the relatively low blastocyst rate, the foal rate is comparable to the results of an established laboratory in this field (Barbacini et al., 2009). The Italian group obtained 103 blastocysts out of 1553 collected oocytes. Considering the 3 ongoing pregnancies delivered a foal, 17 foals were produced out of 84 transferred blastocysts (20%). In summary, an average of 0.013 foals per collected oocyte can be calculated. In our study, one foal resulted from 52 collected oocytes (0.019). This is a good result, even though it only concerns a single experiment.

7.2 EMBRYO-MATERNAL INTERACTION

The early post-fertilization stages of embryonic development in the horse are supported by very specific micro-environments, offered by the oviduct and the uterus. Several aspects of this embryo-maternal interaction have been highlighted during the thesis and are summarized in Table 1.

Table 1 Embryo-maternal interaction in the horse. Several unusual aspects, both maternal and embryonic, contribute to specific interactions between the oviduct and the early embryo on the one hand and between the uterus and the later embryo on the other hand.

<table>
<thead>
<tr>
<th>Maternal environment</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oviduct – Cleavage/morula</td>
<td>Separated from the uterus by a distinct uterotubal papilla</td>
</tr>
<tr>
<td></td>
<td>Specific transport of embryos, while oocytes are retained</td>
</tr>
<tr>
<td>Uterus - Blastocyst</td>
<td>Nourishing histotrophe</td>
</tr>
<tr>
<td></td>
<td>Progesterone dependent proteins, including uterocalin</td>
</tr>
<tr>
<td></td>
<td>Mobile, signaling its presence to prevent luteolysis</td>
</tr>
</tbody>
</table>
Absence of the physiological maternal environment results in major or minor effects on the embryos. The oviductal environment supporting the early cleavage stage equine embryo is distinct to uterine effects, as illustrated by the failure of development after premature intra-uterine transfer of cleavage stage embryos (CHAPTER 6). On the other hand, the uterine environment is essential to attainment of specific features of a normal blastocyst. Blastocysts produced in vitro differ from their in vivo counterparts with regard to the kinetics of development, morphology and gene expression (Tremoleda et al., 2003; Pomar et al., 2005; CHAPTER 5). The addition of a specific component of the in vivo environment, for example uterocalin, to the in vitro culture medium appeared to partially restore one aspect, namely capsule formation, although other morphological and developmental features and expression of specific genes remained unaffected (CHAPTER 6). The absence of effects on embryonic growth, gene expression or quality might be an artefact due to measurement of factors not influenced by uterocalin. However, it is also possible that a crucial ligand(s) necessary for uterocalin to influence gene expression or metabolism was absent in the in vitro culture medium. More research is needed to better define the pieces of this complex embryo-maternal interaction.

### 7.3 Gene Expression

#### 7.3.1 Gene expression in mammalian embryos

Several reciprocal interactions exist between embryonic development, embryonic gene expression and embryo culture conditions (Figure 3). The embryonic environment affects embryonic development, which is in turn reflected by the genes expressed by the embryo. On the other hand, environmental conditions can induce changes in gene expression, which can in their turn interfere with embryonic developmental competence. Altered embryo quality can then induce secondary changes in gene expression.
Figure 3 Reciprocal interactions between embryonic development, gene expression and environmental circumstances.

Marked morphological and developmental differences between in vivo derived and IVP embryos have been shown in several species, and they have been associated to culture environment induced changes in mRNA abundance (Corcoran et al., 2006). This differential gene expression is evident at several levels. Firstly, IVP embryos exhibit a different gene expression pattern when compared to their in vivo derived counterparts, as shown in various mammals, including cattle (Mohan et al., 2004; Corcoran et al., 2006; Goossens et al., 2007), pigs (Magnani and Cabot, 2008) and mice (Fernández-González et al., 2009). Furthermore the gene expression level is influenced by IVF per se (Giritharan et al., 2007), by the type of embryo culture medium (Rinaudo and Schultz, 2004) and by embryo density (Hoelker et al., 2009). Therefore the expression of specific developmentally important genes has been used to evaluate the effects of different culture media and of the addition of particular components to the embryo culture medium (McElroy et al., 2008; Chin et al., 2009). For example, the presence of FCS during IVC has been associated with the upregulation of the pro-apoptotic Bax-gene in bovine (Rizos et al., 2003) and porcine (Cui et al., 2004) embryos. Moreover FCS in murine IVC medium modifies the expression of genes related to epigenetic mechanisms (Fernández-González et al., 2009). Since early epigenetic modifications affect the later phenotype, aberrant expression of the related genes might provide the link between suboptimal embryonic culture conditions and long-term phenotype perturbation (Fernández-González et al., 2009). Epigenetic changes due to suboptimal culture conditions have also been associated with the ‘large offspring syndrome’ in sheep (Young et al., 2001). This illustrates the importance of gene expression analysis as a tool to understand the link between the early embryonic environment
and the long-term phenotype on the one hand, and to evaluate IVC conditions in order to optimize the IVP of embryos on the other.

### 7.3.2 Gene expression in equine embryos

As gene expression reflects embryo quality and the effects of embryo culture conditions, we aimed to identify genes that are expressed at a higher level in golden standard *in vivo* derived equine blastocysts than in IVP equine blastocysts, with the ultimate purpose of inducing similar expression patterns *in vitro* by optimization of embryo culture conditions. The most suitable technique at that moment to attain this purpose was SSH. The SSH method as developed and described in detail by Diatchenko et al. (1996) uses cDNA of a tester (*in vivo* blastocysts) and a driver (*in vitro* blastocysts) and combines several hybridizations and amplifications resulting in suppression of non-target DNA amplification and the selective amplification of the tester specific sequences (Addendum 2). Suppression subtractive hybridization has proven extremely useful for studying early embryonic development in other mammals including cattle (Mohan et al., 2004; Goossens et al., 2007), mice (Wen and Guang-xiu, 2007) and monkeys (Sun et al., 2004) because of a number of particular advantages (Bui et al., 2005; Pfeffer et al., 2007).

1. No prior knowledge about the genes of interest is required. At the start of this project in 2007, little was known about gene expression in equine embryos and the equine genome was poorly annotated. Therefore SSH was preferred over micro-array, which requires specific probes.

2. The genetic material can be pre-amplified without biasing the outcome of the SSH. This is very valuable considering the difficulty of obtaining blastocysts in the horse and the very low amount of RNA in each embryo.

3. It enriches lowly abundant tester-specific transcripts, while common sequences are blocked, leading to the identification of tester-specific genes of interest in very small amounts of initial cDNA.

Even though initial identification of the amplified target sequences via BLAST analysis against the NCBI database (www.ncbi.nlm.nih.gov) was hindered by the poor annotation of the horse
genome and frequent changes through updates, final identification of 62 genes with a higher expression in *in vivo* derived equine blastocysts was achieved. Functional analysis revealed particular involvement of these genes in protein synthesis and energy metabolism. This is in agreement with reports in other species about impaired mitochondrial function (Mtango et al., 2008) and transcription/translation (Corcoran et al., 2006) *in vitro*, when compared to the situation *in vivo*.

As for other large scale screening techniques such as microarray, SSH is at risk of false positive results. Therefore, differentially expressed genes require confirmation by means of a very sensitive and specific method, as offered by RT-qPCR (Addendum 3). RT-qPCR permits accurate quantification of the difference in expression between *in vivo* derived and IVP equine blastocysts, but in order to ensure reliability of the results, the RT-qPCR assays must be carefully conducted. Most importantly, accurate normalization is obligatory for correct interpretation (Vandesompele et al., 2002; Bustin and Nolan, 2004; Bustin et al., 2005). Since no RT-qPCR normalisation studies had been reported for horse embryos, a preliminary trial was conducted to optimize the RT-qPCR standard operating procedure for equine embryos (Smits et al., 2008) and reference genes for comparing *in vivo* and *in vitro* produced equine blastocysts were evaluated (CHAPTER 4). Similarities in reference gene stability between the 3 different types of equine blastocysts and differences to reference genes reported for other species supported respectively the reliability and the need for the experiment. Meanwhile, RT-qPCR has been used in equine embryos to examine expression of progesterone and oestrogen receptors (Rambags et al., 2008), POU5F1 (Choi et al., 2009) and HSPA1A (Mortensen et al., 2010). However, in these studies, only one reference gene was used, namely ACTB (Rambags et al., 2008; Choi et al., 2009) or 18S rRNA (Mortensen et al., 2010). Even though ACTB appeared stable in our study, the use of a single reference gene can lead to erroneous normalization (Dheda et al., 2005).

Of the six genes selected from the SSH, five (ODC, HSP90AA1, BEX2, MOBKL3 and FABP3) were confirmed to be expressed more highly in the *in vivo* derived horse blastocysts by means of RT-qPCR (83%). This is a good result, as it is lower than the reported incidence of false positive
sequences in SSH, being of the order of 30% (Mohan et al., 2004) and 25% (Goossens et al., 2007).

Even though statistical significance was shown, biological relevance remains hard to interpret. Although several transcriptomic analyses have been performed in different species, data on the function of many identified genes are much scarcer. To obtain this functional information, several methods can be applied, including:

1. Knocking out specific genes (Guan et al., 2010). Through knocking out a specific gene and subsequent observation of the phenotype, the function of this gene can be deduced. For practical and ethical reasons this method is mainly restricted to mice.

2. RNA interference (Schellander et al., 2007; Perrimon et al., 2010). Posttranscriptional gene silencing includes the introduction of siRNA, dsRNA or shRNA, leading to degradation of the target mRNA. This technique has proven valuable in functional genomics not only in murine, but also in bovine and porcine oocytes and embryos in vivo and in vitro.

3. Systems biology (Romero et al., 2006). The central dogma describes the transcription of genes to mRNA and further translation to the protein, which actually exerts the biological function. Accordingly, combining genomics, transcriptomics, proteomics and metabolomics is essential to understand the role of specific genes in the associated biological system. This approach was followed for example to clarify the preterm parturition syndrome in human (Romero et al., 2006). Genomics was used to determine a genetic predisposition for spontaneous preterm labour. Then, mRNA changes in reproductive tissues, associated with preterm birth, were determined by transcriptomics. By means of proteomics, differential protein profiles in the amniotic fluid from patients with premature labour were established. Finally, metabolic profiling of amniotic fluid was used to evaluate the risk for preterm delivery. As a whole, the combination of all these techniques, called high-dimensional biology, can provide insight into a complex biological phenomenon.
The involvement in embryonic development of the six genes that were selected out of the 62 genes shown to be differentially expressed by SSH, has been documented in several other species as discussed extensively in CHAPTER 5. However, a lack of data on gene expression in horse embryos hinders comparative analysis in this field. A recent study describing the expression of heat shock protein 70 (HSP1A1) in equine \textit{in vivo} and \textit{in vitro} blastocysts (Mortensen et al., 2010) is interesting considering that this was one of the genes shown to be differentially expressed by SSH. Mortensen et al. (2010) found an increase of HSP1A1 mRNA relative to 18S rRNA in IVP equine blastocysts when compared to \textit{in vivo} derived grade 1 equine blastocysts, but this was primarily due to lower concentrations of 18S rRNA in the IVP blastocysts. This difference in analysis compared to the methods used in this thesis makes it difficult to compare and interpret the results.

The purpose of examining gene expression includes fundamental understanding of early development on the one hand and implementation of the results in order to improve IVP efficiency on the other. Regarding the latter, it needs to be considered that the expression of a gene can either reflect a direct influence of the embryo culture medium or it can be secondary through induction by a downstream effect (embryonic environment $\rightarrow$ deregulation of certain genes $\rightarrow$ altered embryo quality $\rightarrow$ deregulation of other genes) (\textit{Figure 3}). The expression of a particular gene does not necessarily mean that the addition of its substrate to the culture medium will benefit embryonic development. Okawara et al. (2009) detected the expression of glycerol kinase in bovine oocytes and embryos, indicating a physiological role of glycerol; however, the addition of glycerol to the maturation medium appeared to negatively influence subsequent maturation rates. On the other hand, changes in the culture medium are reflected by gene expression patterns. For example, during IVP in mice KSOM resulted in higher blastocyst rates when compared to Whitten’s medium and when, in a later microarray experiment, gene expression was compared to that of \textit{in vivo} derived embryos, culture in KSOM induced far less aberrant gene expression than the suboptimal Whitten’s medium (Gardner and Lane, 2005).
To improve understanding of both the meaning of the genes identified in CHAPTER 5 and the influence of uterocalin on IVP embryos, the expression of ODC, HSP90AA1, BEX2, MOBKL3 and FABP3 was evaluated in equine IVP blastocysts cultured in the presence or absence of uterocalin. It was hypothesized that the addition of a maternal component to the embryo culture medium could result in a more ‘in vivo-like’ gene expression pattern. However, uterocalin did not appear to affect the examined genes.

7.4 General conclusions

In a first part of the thesis, the procedure for in vitro embryo production in the horse was optimized.

1. IVP of equine blastocysts was successfully introduced to our laboratory. After an initial period of conventional ICSI, piezo drill assisted ICSI was introduced and subsequently compared with laser assisted ICSI. Both devices have specific advantages and disadvantages, but no major effect on subsequent embryonic development was observed (CHAPTER 3.1).

2. The viability of the IVP equine blastocysts was illustrated by successful embryo transfer, resulting in the birth of the first ICSI-foal in the Benelux (CHAPTER 3.2).

Based on the results presented in this thesis, the following conclusions can be drawn:

1. A method for reliable determination of gene expression in equine blastocysts was developed by the optimization of a standard operating procedure for RT-qPCR and through the determination of the most stable reference genes for normalization. The geometric mean of UBC, ACTB, RPL32 and GAPDH is recommended as a normalization factor for RT-qPCR in equine in vivo and in vitro derived blastocysts (CHAPTER 4).

2. Upregulation of genes in in vivo derived equine blastocysts, when compared to IVP equine blastocysts, was determined by SSH. A total of 62 upregulated genes was identified, with major involvement in protein synthesis and energy metabolism. The differential expression was confirmed by RT-qPCR for five out of six developmentally important genes, namely ODC, HSP90AA1, BEX2, MOBKL3 and FABP3 (CHAPTER 5).
3. The premature intra-uterine transfer of cleavage stage equine IVP embryos (day 2-3) did not result in successful embryo development (CHAPTER 6).

4. The addition of recombinant uterocalin to the embryo culture medium on day 6 improved capsule formation in equine IVP blastocysts, but did not influence embryonic development nor the expression of the examined genes (CHAPTER 6).

7.5 FUTURE PERSPECTIVES

The IVP of equine embryos has evolved greatly in recent years, creating the possibility to obtain foals through the combination of OPU, IVM, ICSI, IVC and ET for research (Jacobson et al., 2010) as well as for clinical (Colleoni et al., 2007) purposes. However, each of these steps needs optimization through further research in order to allow large scale application as it happens already in cattle (Blanco et al., 2009). Recent publications stress the limitations of superovulation and OPU techniques (Blanco et al., 2009; Jacobson et al., 2010), IVM (Galli et al., 2007), ICSI (Choi et al., 2002), IVC (Choi et al., 2004) and ET (Panzani et al., 2009). Taken into account a current blastocyst rate of 10% in one of the most experienced commercial labs (Barbacini et al., 2009), there is considerable room to improve both the quantity and the quality of transferable blastocysts by optimizing all these crucial steps.

A clear influence of the maternal environment on embryonic development was observed. An important role of the oviductal environment was also shown (CHAPTER 6), and further information on the mechanism involved or on the composition of equine oviductal fluid and the influence of oviduct epithelial cell secretions on early embryonic stages would be very interesting. Considering the blastocyst and its uterine environment, a link was made between the maternal protein uterocalin and the embryonic capsule. However, elucidating underlying mechanisms of this phenomenon, influence of the addition of ligands for uterocalin and the role of other components of the uterine histotrophe all require further research.

One way to evaluate embryos is to look at gene expression. Since genetic research in equine embryos is still in its infancy, plenty of opportunities exist to expand this field in order to achieve a level of information, which is comparable to that in other species. Growing
knowledge of the equine genome and advances in genetic technologies are opening new avenues for rapid generation of large amounts of data. Recent work on endometrial gene expression was performed with a home-made microarray (Klein et al., 2010). Microarray analysis has generated substantial information on murine (Fernández-González et al., 2009) and bovine (Vigneault et al., 2009) embryonic gene expression. Embryonic biopsy, combined with microarray and DNA fingerprinting can contribute to the identification of markers to predict embryonic developmental competence (Jones et al., 2008). Currently, microarray is in its turn being replaced by even more advanced techniques like next generation sequencing (deep sequencing), providing massive amounts of information in a short time (Hurd and Nelson, 2009). Combining knowledge from genomics and transcriptomics with proteomics and metabolomics is crucial in order to achieve functional insights (Romero et al., 2006; Werner et al., 2010) in the complex puzzle called ‘early embryonic development’.

REFERENCES


transcriptomics, proteomics, and metabolomics) to understand the preterm parturition syndrome. *BJOG* 113 (Suppl3) 118-135.


SUMMARY

In vitro produced equine embryos are valuable, not only to investigate aspects of early embryonic development, but also from a clinical point of view. The low success of conventional IVF contributed to the initial slow development and implementation of IVP in the horse. However, once ICSI was introduced almost 15 years ago progress became much more rapid. Despite this advance, the other steps of the IVP process (oocyte collection - IVM – ICSI – IVC) still pose specific problems, and need optimization to enhance the efficiency of the overall procedure.

The blastocyst stage embryo is particularly interesting, being on the one hand the endpoint of the IVP process and on the other hand the first embryonic stage, which is unique to the uterus, allowing the possibility toatraumatically collect in vivo blastocysts from donor mares as well as to transfer in vivo or in vitro produced blastocysts to recipient mares. Comparing IVP blastocysts with their counterparts derived in vivo reveals differences in the kinetics of development and in morphology, reflecting the influence of the unique equine maternal environment. CHAPTER 1 represents a general introduction.

The purpose of this thesis (CHAPTER 2) is to contribute to the optimization of the IVP of equine embryos by considering some technical aspects of the process (CHAPTER 3) and through fundamental understanding of differences between in vitro produced and in vivo derived equine blastocysts, with the emphasis on gene expression (CHAPTER 4,5) and on the influence of the maternal environment on early equine embryonic development (CHAPTER 6).

The method of choice for fertilizing equine oocytes is ICSI. After an initial period of conventional ICSI, the piezo drill was introduced and was associated with higher cleavage rates and more consistent results, even though no direct comparison with conventional ICSI had been performed. Since the piezo drill also has some disadvantages, like the toxicity of mercury, other advanced injection technologies should also be considered. Therefore in CHAPTER 3.1 piezo-assisted ICSI was compared with laser-assisted ICSI. No major influence on subsequent embryonic development was observed; a slightly higher cleavage rate was obtained after laser-
assisted ICSI, but blastocyst rates were not significantly affected. Further technical advantages of the laser included the absence of mercury and the ease of use, since it was less sensitive to exact 3-dimensional positioning than the piezo drill. On the other hand, laser-assisted ICSI requires the making of several adjacent holes in the zona pellucida, after which conventional ICSI needs to be applied anyway to penetrate the oolemma. This occurs less fluently than in piezo-assisted ICSI, in which both the zona and the oolemma are penetrated in one manipulation. However, the difference in duration of the procedure was not significant.

The IVP of equine embryos was introduced to the laboratory. The feasibility of the IVP protocol used in this thesis and the viability and developmental competence of the resulting IVP blastocysts was confirmed through the birth of the first ICSI foal in the Benelux, as described in CHAPTER 3.2.

The major part of this thesis was focusing on the differences between equine blastocysts produced in vitro and those derived in vivo. The evaluation of gene expression was selected as an approach to gain fundamental insight into these differences. The aim was to identify genes which were upregulated in golden standard in vivo derived blastocysts, when compared to in vitro produced embryos. Considering the limited knowledge on embryonic gene expression in the horse and the scarcity of embryonic RNA, SSH was at the time the method of choice for initial genetic analysis (CHAPTER 5). Using this technique, 62 genes were identified that were expressed at a higher level in in vivo derived equine blastocysts than in their in vitro produced counterparts. Functional analysis revealed substantial involvement of these genes in protein synthesis and energy metabolism. The possibility to generate false positive results by SSH required confirmation of these results using a very sensitive and specific technique, as offered by RT-qPCR. Six genes, resulting from the SSH and described in the literature as being involved in embryonic development, namely BEX2, FABP3, HSP90AA1, MOBKL3, MCM7 and ODC, were selected for RT-qPCR.

In order to obtain reliable results by RT-qPCR, careful assay and data analysis is crucial. An important aspect is correct normalization of the data. The method of choice to achieve this consists of the calculation of a normalization factor, from the geometric mean of the most
stably expressed reference genes in the tissue of interest. To this end, the expression of eight reference genes was evaluated in *in vivo* derived and in fresh and frozen-thawed *in vitro* produced equine blastocysts. This analysis indicated the geometric mean of ACTB, UBC, RPL32 and GAPDH as a reliable normalization factor for RT-qPCR data in experiments with equine *in vivo* and *in vitro* blastocysts (CHAPTER 4).

This normalisation factor was subsequently used for the analysis of the RT-qPCR data of the 6 developmentally important genes, generated from SSH. For 5 of these genes, namely BEX2, FABP3, HSP90AA1, MOBK3, and ODC, a higher level of expression in *in vivo* derived horse blastocysts, when compared to *in vitro* produced blastocysts, was confirmed by means of RT-qPCR (CHAPTER 5).

In CHAPTER 6 the influence of the maternal environment during embryonic development was examined. A first experiment examined the necessity of the oviduct for normal early embryonic development through the premature intra-uterine transfer of day 2-3 equine embryos produced *in vitro*. Flushing of the recipient mares on day 7 yielded no viable blastocysts and pregnancy diagnosis on day 14 revealed no pregnancies, suggesting that the uterus cannot replace the oviductal environment as a support for the development of early cleavage stage horse embryos.

In a second experiment the influence of the uterus was assessed, and more specifically the influence of an important protein component of endometrial secretions, uterocalin. Recombinant uterocalin was added to IVC medium on day 6 and subsequent *in vitro* produced blastocysts were evaluated on day 9-9.5. No differences in blastocyst production, blastocyst diameter or cell number were found between the embryos cultured in the presence or absence of uterocalin. Uterocalin did not affect the expression of the genes shown to be upregulated *in vivo* embryos (CHAPTER 5) either. However, the presence of uterocalin in the IVC medium appeared to stimulate embryonic capsule formation.

CHAPTER 7 represents a general discussion and the conclusions of this thesis:
In a first part of the thesis, the procedure for *in vitro* embryo production in the horse was optimized.

1. IVP of equine blastocysts was successfully introduced to our laboratory. After an initial period of conventional ICSI, piezo drill assisted ICSI was introduced and subsequently compared with laser assisted ICSI. Both devices have specific advantages and disadvantages, but no major effect on subsequent embryonic development was observed (CHAPTER 3.1).

2. The viability of the IVP equine blastocysts was proven by successful embryo transfer, resulting in the birth of the first ICSI-foal in the Benelux (CHAPTER 3.2).

Based on the results presented in the second part of this thesis, the following conclusions can be drawn:

1. A method for reliable determination of gene expression in equine blastocysts was developed by the optimization of a standard operating procedure for RT-qPCR and through the determination of the most stable reference genes for normalization. The geometric mean of UBC, ACTB, RPL32 and GAPDH is recommended as a normalization factor for RT-qPCR in equine *in vivo* and *in vitro* derived blastocysts (CHAPTER 4).

2. Upregulation of genes in *in vivo* derived equine blastocysts, when compared to IVP equine blastocysts, was determined by SSH. A total of 62 upregulated genes was identified, with major involvement in protein synthesis and energy metabolism. The differential expression was confirmed by RT-qPCR for five out of six developmentally important genes, namely ODC, HSP90AA1, BEX2, MOBKL3 and FABP3 (CHAPTER 5).

3. The premature intra-uterine transfer of cleavage stage equine IVP embryos (day 2-3) did not result in successful embryo development (CHAPTER 6).

4. The addition of recombinant uterocalin to the embryo culture medium on day 6 improved capsule formation in equine IVP blastocysts, but did not influence embryonic development nor the expression of the examined genes (CHAPTER 6).
SAMENVATTING

*In vitro* geproduceerde paardenembryo’s zijn waardevol, niet alleen voor onderzoek met als doel fundamenteel inzicht te verwerven in de vroege embryonale ontwikkeling, maar ook vanuit een klinisch standpunt. Het beperkte succes van conventionele IVF heeft bijgedragen tot een initiële trage opgang van de IVP bij het paard. ICSI werd echter met succes geïntroduceerd en tijdens het laatste decennium werd een snelle vooruitgang geboekt. Ondanks deze progressie stellen de verschillende stappen van het IVP proces (eicelverzameling – IVM – ICSI - IVC) nog steeds meerdere specifieke problemen, waarbij optimalisatie vereist is om de efficiëntie van de techniek te verbeteren.

Het blastocyststadium is in het bijzonder interessant omdat het enerzijds het eindpunt van het IVP proces betekent en omdat het anderzijds het stadium is dat zich onder fysiologische omstandigheden in de baarmoeder bevindt, waardoor het mogelijk is om op atraumatische wijze zowel *in vivo* blastocysten te verzamelen van donormerries, als om *in vivo* of *in vitro* geproduceerde blastocysten over te planten in receptormerries. Wanneer IVP blastocysten met hun *in vivo* verzamelde tegenhangers worden vergeleken, zijn er verschillen in ontwikkeling en morfologie, die de invloed van de maternale omgeving weerspiegelen. Een algemene inleiding wordt gegeven in HOOFDSTUK 1.

Het doel van deze thesis (HOOFDSTUK 2) is om bij te dragen aan de optimalisatie van de IVP van paardenembryo’s door bepaalde technische aspecten van het proces te onderzoeken (HOOFDSTUK 3) en door de fundamentele verschillen tussen *in vivo* en *in vitro* geproduceerde paardenblastocysten trachten te begrijpen, met de nadruk op genexpressie (HOOFDSTUK 4,5) en op de invloed van de maternale omgeving op de vroege embryonale ontwikkeling bij het paard (HOOFDSTUK 6).

De voorkeurstechniek om paardeneicellen te bevruchten is ICSI. Na een initiële periode van conventionele ICSI werd de piëzo drill geïntroduceerd voor ICSI bij het paard, wat werd geassocieerd met hogere delingspercentages en meer herhaalbare resultaten. Een directe vergelijking met conventionele ICSI werd echter nooit gemaakt. Aangezien de piëzo drill ook
problemen stelt, zoals de toxiciteit van kwik, moeten andere vooruitstrevende technologieën ook overwogen worden. Daarom werd in HOOFDSTUK 3.1 piëzo-geassisteerde ICSI vergeleken met laser-geassisteerde ICSI. Er werden geen grote invloeden op de daaropvolgende embryonale ontwikkeling vastgesteld. Een iets hoger delingspercentage werd bekomen na laser-geassisteerde ICSI, maar de blastocystpercentages werden niet significant beïnvloed. Verdere technische voordelen van de laser behelsend de afwezigheid van kwik en het gebruiksgemak, waarbij de laser ook minder gevoelig is voor de 3-dimensionale positionering van de micromanipulatoren dan de piëzo. Aan de andere kant moeten er bij laser-geassisteerde ICSI verschillende gaten in de zona pellucida gemaakt worden, waarna alsnog conventionele ICSI toegepast dient te worden om de eicelmembraan te penetreren. Dit verloopt minder vlot dan bij piëzo-geassisteerde ICSI, waarbij zowel de zona als de eicelmembraan in één vloeienende manipulatie kunnen worden gepenetreerd. Er was echter geen significant verschil in tijd tussen beide technieken.

De IVP van paardenembryo’s werd in het laboratorium geïntroduceerd. De kwaliteit van het protocol voor IVP dat in deze thesis gebruikt werd, wordt bevestigd door de vitaliteit en het ontwikkelingspotentieel van de resulterende IVP blastocysten en door de geboorte van het eerste ICSI-veulen van de Benelux, die beschreven wordt in HOOFDSTUK 3.2.

In deze thesis lag de nadruk echter op de verschillen tussen in vitro versus in vivo geproduceerde paardenblastocysts. De evaluatie van hun genexpressie werd gekozen als benadering om fundamenteel inzicht te verwerven in deze verschillen. Het doel was om genen te identificeren die opgereguleerd worden bij in vivo blastocysten, die als gouden standaard gebruikt worden, in vergelijking met in vitro blastocysten. Rekening houdend met de beperkte kennis over embryonale genexpressie bij het paard en de schaarsheid aan embryonaal RNA, was SSH de voorkeurstechniek voor een initiële genetische analyse (HOOFDSTUK 5). Op deze manier werden 62 genen geïdentificeerd, die meer tot expressie kwamen bij in vivo geproduceerde paardenblastocysts dan bij hun in vitro geproduceerde tegenhangers. Functionele analyse onthulde een belangrijke betrokkenheid van deze genen in de eiwitsynthese en het energiemetabolisme. De mogelijkheid van het genereren van vals
positieve resultaten met SSH vereist echter een extra bevestiging door middel van een zeer gevoelige en specifieke techniek, namelijk RT-qPCR. Uit de SSH werden zes genen geselecteerd op basis van hun in de literatuur beschreven betrokkenheid bij de embryonale ontwikkeling, namelijk BEX2, FABP3, HSP90AA1, MOBKL3, MCM7 en ODC.

Om betrouwbare resultaten te verkrijgen met RT-qPCR is een zorgvuldige methode en data-analyse cruciaal. Hierbij is een correcte normalisatie van de data van belang. De voorkeursmethode om dit te bereiken bestaat uit de berekening van een normalisatiefactor, zijnde het geometrisch gemiddelde van de stabielste referentiegenen in het weefsel van interesse. Daarom werd de expressie van 8 referentiegenen geëvalueerd zowel in in vivo geproduceerde als in verse en ontdoode in vitro geproduceerde paardenblastocysten. Deze analyse bepaalde het geometrisch gemiddelde van ACTB, UBC, RPL32 en GAPDH als een betrouwbare normalisatiefactor voor RT-qPCR data in experimenten met zowel in vivo als in vitro paardenblastocysten (HOOFDSTUK 4).

De op deze manier bepaalde normalisatiefactor werd vervolgens gebruikt voor de analyse van de RT-qPCR data van de zes geselecteerde genen. Voor vijf van deze genen, namelijk BEX2, FABP3, HSP90AA1, MOBKL3, en ODC, werd de hogere expressie in in vivo geproduceerde paardenblastocysten in vergelijking met in vitro geproduceerde paardenblastocysten bevestigd door middel van RT-qPCR (HOOFDSTUK 5).

In HOOFDSTUK 6 werd de invloed van de maternale omgeving tijdens de embryonale ontwikkeling onderzocht. Een eerste experiment stelde de cruciale rol van de eileider in vraag door gedeelde embryo’s van 2 à 3 dagen oud vroegtijdig over te planten naar de baarmoeder. Er werden geen blastocysten gevonden na baarmoeserspoeling van de receptormerries op dag 7 en er werden ook geen drachten vastgesteld na echografie op dag 14 wat suggereert dat de baarmoeder de eileideromgeving niet kan vervangen om de ontwikkeling van vroege gedeelde embryonale stadia bij het paard te ondersteunen.

In een tweede experiment werd de invloed van de baarmoeder nagegaan en meer specifiek de invloed van een belangrijk eiwit in de baarmoedersecreties, uterocaline. Recombinant uterocaline werd toegevoegd aan het IVC medium op dag 6 en de in vitro geproduceerde
Samenvatting

blastocysten werden 9 tot 9,5 dagen na ICSI geëvalueerd. Geen verschillen in blastocystpercentage, diameter of celaantal werden gevonden tussen de embryo’s die werden gekweekt in de aan- of afwezigheid van uterocaline. Uterocaline had ook geen invloed op de expressie van de genen die opgereguleerd zijn in vivo (HOOFDSTUK 5). De aanwezigheid van uterocaline in het cultuurmedium bleek echter wel de embryonale kapselvorming te stimuleren.

HOOFDSTUK 7 bestaat uit een algemene discussie, gevolgd door de conclusies van deze thesis:

In een eerste deel van de thesis werd de procedure voor de in vitro productie van paardenembryo’s geoptimaliseerd.

1. De IVP van paardenembryo’s werd in ons laboratorium ingevoerd. Na een initiële periode van conventionele ICSI werd piezo-geassisteerde ICSI geïntroduceerd en vergeleken met laser-geassisteerde ICSI. Beide apparaten hadden specifieke voor- en nadelen, maar geen groot effect op de daaropvolgende embryonale ontwikkeling werd vastgesteld.

2. De leefbaarheid van de IVP paardenblastocysten werd bewezen door succesvolle embryotransplantatie die resulteerde in de geboorte van het eerste ICSI-veulen van de Benelux.

De conclusies op basis van het onderzoek in het tweede deel van deze thesis zijn:

3. Een methode voor betrouwbare bepaling van genexpressie bij paardenblastocysten werd op punt gesteld door middel van de optimalisatie van het protocol voor RT-qPCR en door de bepaling van de stabielste referentiegenen voor datanormalisatie. Het geometrisch gemiddelde van ACTB, UBC, RPL32 en GAPDH wordt aanbevolen als normalisatiefactor voor RT-qPCR van in vivo en in vitro geproduceerde paardenblastocysten.

4. Opregulatie van genen in in vivo geproduceerde paardenblastocysten, in vergelijking met in vitro geproduceerde paardenblastocysten, werd bepaald met SSH. In totaal werden 62 genen geïdentificeerd, die een belangrijke betrokkenheid bij eiwitsynthese
en energiemetabolisme vertonen. De differentiële expressie werd bevestigd door RT-qPCR voor vijf van de zes belangrijke ontwikkelingsgenen, namelijk BEX2, FABP3, HSP90AA1, MOBKL3 en ODC.

5. De voortijdige transplantatie naar de baarmoeder van IVP gedeelde paardenembryo’s ondersteunde de verdere embryonale ontwikkeling niet.

6. De toevoeging van recombinant uterocaline aan het embryocultuurmedium op dag 6 stimuleerde de kapselvorming bij IVP paardenblastocysten, maar had geen invloed op de embryonale ontwikkeling of op de expressie van de onderzochte genen.
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CURRICULUM VITAE


Op 1 oktober 2006 trad ze in dienst van de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde als doctoraatsbursaal (BOF). Daar verrichtte ze onderzoek naar de ontwikkeling en genexpressie van in vitro geproduceerde paardenembryo’s. Naast haar onderzoek was Katrien Smits ook werkzaam in de kliniek verloskunde, waar ze participeerde in de nacht- en weekenddiensten.

Katrien Smits is auteur of mede-auteur van verschillende publicaties in internationale en nationale wetenschappelijke tijdschriften en nam actief deel aan diverse internationale en nationale congressen.
BIBLIOGRAPHY

PUBLICATIONS


ORAL PRESENTATIONS AND POSTERS AT NATIONAL AND INTERNATIONAL CONFERENCES


ADDENDUM 1  PROTOCOL IN VITRO PRODUCTION EQUINE EMBRYOS

Based on Galli et al., 2007; Lagutina et al., 2005

MEDIA

- **Flush medium**
  - DPBS, 50 µg/ml BSA, 25 IU/ml heparin

- **Dissection medium**
  - HEPES buffered TCM199, 1 mg/ml bovine serum albumin (BSA), 1 mg/ml polyvinyl alcohol (PVA), 10 µg/ml heparin, 50 µg/ml gentamicin
  - pH: 7.45; 280 mosm

- **Maturation medium**
  - DMEM-F12, 10% (v:v) serum replacement, 50 ng/ml epidermal growth factor, 10 µg/ml pFSH, 2 µg/ml pLH, 0.1 µg/ml cystine, 50 ng/ml cysteamine, 0.5 µl/ml lactic acid, 0.1 µg/ml glutamine, 0.075 µg/ml ascorbic acid, 25 ng/ml PVA, 5 ng/ml myoinositol, 1 mM sodium pyruvate, 1 µl/ml insulin transferring sodium selenite, 50 µg/ml gentamicin
  - For DMEM-F12: pH:7.43; 286 mosm

- **SOF-IVF**
  - Synthetic oviductal fluid, no glucose, 1µg/ml heparin
  - pH: 7.45; 280 mosm

- **Ca++free TALP**
  - pH: 7.4; 280 mosm

- **H-SOF**
  - HEPES-buffered synthetic oviductal fluid
  - pH: 7.38; 275 mosm

- **Culture medium**
  - DMEM-F12, 10% fetal calf serum
For DMEM-F12: pH: 7.43; 286 mosm

OOCYTE COLLECTION

- Preparations before going to the slaughterhouse:
  - Put 3 l of sterile NaCl-solution in the heating bath (38°C)
  - Media
    - Prepare flush medium and keep at room temperature
    - Put dissection medium in the incubator without CO₂
    - Prepare maturation medium and put 500 µl of it in each well of a 4-well-plate. Put the 4-well plate in the incubator with 5% CO₂. The remaining medium is also placed in the incubator with unscrewed lid.

- Collection ovaries at slaughterhouse
- Transport: 1 hour, ambient temperature, isolated box
- Removal adherent tissues
- Ovaries rinsed twice in sterile NaCl-solution (38°C)
- Ovaries kept in clean sterile NaCl-solution (38°C) on heated surface (37°C) during further procedure
- Aspiration of follicle fluid into sterile recipient with 16-G needle connected to vacuum pump and flushing of the follicles with flush medium, using a 20 ml-syringe with 18-G needle
- Recipient with follicle fluid on heated surface (37°C) during entire procedure
- 10 ml of follicle fluid from the bottom of the recipient is pipetted into large Petri dish and evaluated under stereomicroscope
- Oocytes collected in dissection medium; only oocytes with at least 3 layers of cumulus cells
- Last 2 steps are repeated until no more oocytes are found
- Oocytes washed twice in dissection medium
MATURATION

- **Maturation medium**
- Wash oocytes in first 3 wells of maturation medium
- 20-50 oocytes / 500 µl medium
- 24 h-28h
- 5% CO₂
- 38.5 °C

ICSI

**Preparation sperm**

- Put Percoll 90% and Ca²⁺free TALP at room temperature 1h before preparing sperm
- Thaw SOF-IVF at room temperature, vortex and leave in incubator at 38.5°C
- Preparation Percoll discontinuous density gradient (45% over 90%)
  - 2 ml of Percoll 90% in tube A
  - 1.5 ml of Percoll 90% mixed with 1.5 ml of Ca²⁺free TALP in tube B
  - 2 ml of 45% solution of tube B is gently pipetted on top of the 2 ml of 90% Percoll in tube A
- Thawing sperm 1h before ICSI in 26°C H₂O
- Sperm is gently put on top of Percoll gradient with 20-G needle on 1 ml syringe
- Centrifugation
  - 750 x g = 2041 t/min
  - 40 min
  - 26°C
- Removal supernatans
- Resuspension sperm pellet in 5 ml of Ca²⁺free TALP
- Centrifugation
  - 400 x g = 1490 t/min
  - 10 min
- 26°C
- Removal supernatans
- Resuspension sperm pellet in 0.3 ml of SOF-IVF
- Just before ICSI: little bit of sperm solution is pipetted on left bottom side of drop of 9% polyvinylpyrrolidone (PVP)

**Preparation oocytes**

- Decumulation
  - Plate with 2 droplets of 45 µl hyaluronidase (1µg/ml) and 10 droplets of H-SOF under mineral oil
  - In hyaluronidase (1µg/ml)
    - 3 min
    - Partial denudation with 170 µm pipette
  - In H-SOF
    - Denudation by gently pipetting with 130µm pipette
    - MII and MI are separated

- *Maturation medium*
  - MII oocytes and MI oocytes in separate droplets of 50 µl of maturation medium under mineral oil at 38.5°C and 5% CO₂ until ICSI

**Time scheme preparation ICSI**

- 4 h before ICSI: Thaw *culture medium* at RT, make a 4-well with 20µl droplets under mineral oil
  - Make a 4-well with 50 µl droplets of *maturation medium*
  - under mineral oil for denuded oocytes

- 2.5 h before ICSI: take out Percoll, Ca²⁺free TALP and SOF-IVF

- 1.5 h prepare plates
- 2 decumulation plates: plate with 2 droplets of 45 µl of hyaluronidase and 10 droplets of 45 µl of H-SOF under mineral oil
- 2 ICSI plates: plate with 13 droplets of 5 µl of H-SOF under mineral oil

1 h   Prepare sperm
50 min   1st centrifugation
10 min   Denudation oocytes
2nd centrifugation
Preparation microscope and micromanipulation

ICSI

Preparation plate
- 1 blue droplet of H-SOF is replaced by 9% PVP
- Orange droplets are numbered and +/- 10 oocytes are divided over numbered droplets (MII oocytes first)
- Sperm is pipetted on left bottom site of PVP-droplet
- The 3 steps are repeated until all mature oocytes are injected

Heated plate
Olympus inverted microscope
Narishige micromanipulation
Injection of 10 oocytes takes about half an hour
- Injected oocytes: *culture medium* at 38.5 °C and 5% CO₂ until all oocytes are injected

**CULTURE**

- *Culture medium*
- Prepare 4-well plate with 20 µl droplets under mineral oil
- Put in the CO₂ incubator for at least two hours to equilibrate
- 10-20 embryos/20µl-droplet
- 5% CO₂, 5% O₂, 90% N₂: Put the plates in a turtle, let the gas mix flow through the turtle during 2 minutes and then close the turtle thoroughly
- 38.5 °C
- Day 2,5:
  - Change ½ of medium by adding *culture medium*
  - Only cleaved embryos are kept
- Day 6: Change ½ of medium
- Day 8-9: Evaluation embryos

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ADDENDUM 2  SUPPRESSION SUBTRACTION HYBRIDIZATION (SSH)

This scheme represents the principle of suppression subtractive hybridization, based on Diatchenko et al., (1996). The tester cDNA is subdivided into two portions, and each is ligated with a different adaptor. The SSH involves two hybridizations. In the first one, an excess of driver cDNA is added to each tester sample. The samples are denatured and allowed to anneal, generating the type a, b, c and d molecules in each sample. In the second hybridization, the two samples from the first hybridization are mixed together without prior denaturation, again with an excess of driver cDNA. Only the remaining equalized and subtracted ss tester cDNAs can reassociate and form new type e hybrids. After the ends are filled in, the type e molecules have
two different primer annealing sites. Subsequently, all molecules are subjected to PCR to amplify the desired differentially expressed sequences. Type a and d molecules lack primer annealing sites and can not amplify during the PCR. Most type b molecules form a ‘pan-like’ structure. This suppression effect prevents amplification of the type b molecules. Type c molecules, having only one primer annealing site, amplify linearly. Only the type e molecules, representing the differentially expressed sequences, have two different adaptors and can amplify exponentially. Finally, a second PCR is performed to further reduce background and enrich the differentially expressed tester specific sequences.
**ADDENDUM 3 REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)**

![Diagram of PCR reaction steps: 1. Denaturation, 2. Primer annealing, 3. Elongation](image)

**Figure 1 qPCR.** This scheme represents the PCR reaction, using SYBR Green for quantification. The 3 PCR steps, denaturation, primer annealing and elongation, are repeated in a cyclical pattern. This results in exponential amplification of the target sequence. Binding of SYBR Green to the double stranded DNA results in fluorescence, which is measured during each cycle of the qPCR.
When working with RNA, the RNA strand is first transcribed into complementary DNA (cDNA), using the enzyme reverse transcriptase (RT). The cDNA can be amplified by means of the polymerase chain reaction (PCR) (Peake et al., 1989; Mullis, 1990). The PCR cycle consists of 3 different steps. Firstly, DNA denaturation is caused by heating to 95°C. The second step involves the annealing of primers, specifically developed for the region of interest. In third instance, the complementary strands are synthesized, using a thermostable DNA polymerase enzyme. Cyclical repeat of this process results in exponential amplification of the region between the two target specific primers.

Reverse transcription quantitative PCR (RT-qPCR) is based upon this PCR reaction (Morrison et al., 1998; Bustin, 2000; for review see Van Guilder et al., 2008). Not only can specific sequences be detected and amplified, qPCR also allows quantification of the genetic material in the initial samples. Quantification can be achieved through different methods. In this thesis, a DNA binding fluorescent dye, namely SYBR Green, was used (Figure 1). Unbound dye exhibits little fluorescence. Binding to double stranded DNA, makes the dye fluoresce. During qPCR, this fluorescence is measured during the elongation step of each cycle. The sample fluorescence is plotted against the qPCR cycle (Figure 2). For each sample, the quantification cycle (cq) value is determined. This is the point where the sample fluorescence exceeds the background fluorescence, as represented by the threshold line. A lower cq value means that the threshold fluorescence is achieved in few cycles, so that the original sample contained a large number of copies of the target sequence. Vice versa, a high cq value equals many cycles needed to reach the threshold, representing little material in the initial sample. A difference of 3.3 cq reflects a 10-fold difference in the original template.
Figure 2 qPCR amplification graph. Sample fluorescence is measured during each cycle and plotted against the qPCR cycle. Five samples were evaluated. The orange line represents the threshold line. Lower levels of fluorescence are considered as background. The quantification cycle ($c_q$) value represents the point where the sample fluorescence exceeds this threshold line, for example around 17 for the green sample.