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Influence of the uterine environment on the development of *in vitro*-produced equine embryos

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Abstract

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 recipient mares that had ovulated recently 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. Intrauterine transfer of cleavage-stage IVP equine embryos yielded neither ultrasonographically detectable pregnancies nor day 7 blastocysts, indicating that the uterus is not a suitable environment for pre-compact morula stage horse embryos. By contrast, exposure to uterocalin during IVP improved capsule formation, although it did not measurably affect the development or expression of a panel of genes known to differ between *in vivo* and *in vitro* embryos. Further studies are required to evaluate whether uterocalin serves purely as a carrier protein or more directly promotes improved capsule development.
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Introduction

Early embryonic development in the horse is characterised by a number of peculiarities (Betteridge 2007). First the equine embryo does not exit the oviduct via the prominent uterotubal papilla until as late as 144–156 h after ovulation (Freeman *et al*. 1991). Moreover, unfertilised eggs are not capable of stimulating passage through the ampullary–isthmic junction and are instead retained in the oviduct. The stimulus to this selective oviductal transport is the stage-specific production of prostaglandin E_2 by days 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 2007). Although the precise functions of the capsule are not clear, it has been proposed to function as a 'mailbox' that collects endometrial components such as signalling molecules and nutrients and transports them to the embryo (Herrler & Beier 2000), and to maintain the spherical shape of the mobile embryo and physically protect the while it migrates around the uterus to signal its presence to its dam and prevent luteolysis (Allen & Stewart 2001, Stout & Allen 2001). In addition, structural changes in the capsule are thought to be instrumental to the processes of fixation and orientation of the conceptus within the mare's 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 & Stewart 2001); moreover, during this prolonged pre-implantation period, the embryo is entirely dependent on endometrial secretions

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(histotrophe) for its nutrition. An undoubtedly important component of this histotrophe is the 19 kDa progesterone-dependent protein, uterocalin, which 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) suggest that there may be a functional correlation between uterocalin production and capsule persistence (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 (Crossett *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 *in vitro*, and therefore in the absence of the maternal tract, they differ markedly from their *in vivo* counterparts in terms of the kinetics of development, incidence of apoptotic cells, inner cell mass morphology and gene expression patterns (Tremoleda *et al*. 2003, Pomar *et al*. 2005, Smits *et al*. 2011). One of the more striking irregularities of *in vitro*-produced (IVP) horse embryos is the failure of normal capsule formation (Tremoleda *et al*. 2003); even though capsular mucin-like glycoproteins are produced *in vitro*, they fail to coalesce into the distinct continuous capsule observed around *in vivo* equine embryos from the early blastocyst stage. The reason(s) for the failure of capsular glycoprotein coalescence *in vitro* are not known, but may involve simple dispersion of the glycoproteins into the culture medium, thereby 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) (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 either to the complete uterine environment or to the endometrial protein, uterocalin, which is known to contribute substantially to the capsule of day 10–18 blastocysts. A recent study demonstrated that temporary transfer of IVP day 7 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 would enhance capsule formation and improve equine blastocyst development rates and quality, compared with culture *in vitro*. Earlier studies involving intrauterine transfer of small numbers of *in vivo*-derived horse embryos were not very successful (Allen & Rowson 1975, Ball *et al*. 1989, Weber *et al*. 1993), but 'premature intrauterine transfer' with large numbers of day 2–3 IVP horse embryos has not been described previously. In this latter respect, while it is common practice to culture embryos to the blastocyst stage before intrauterine transfer in most domestic species, in human medicine, premature intrauterine transfer of day 2 and 3 IVP 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. Currently, blastocyst rates of 25–35% can be obtained after ICSI and *in vitro* culture of cleaved horse embryos (Hinrichs 2010). However, only few laboratories are able to obtain such good results. If transfer of cleavage-stage IVP embryos to the uterus of the mare was successful, it would considerably simplify IVP, even if it did not 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 the 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* vs IVP horse embryos.

Results

Experiment 1: intrauterine transfer of cleavage-stage IVP embryos

A total of 99 cleaved (2–8 cell stage) embryos were transferred to the uterus of 12 synchronised (days 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 three of the six mares and no mare yielded more than a single embryo (overall recovery rate 6%). Moreover, none of the three recovered embryos had developed to the blastocyst stage; instead all three were clearly degenerate.

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%

Table 1 Influence of uterocalin on capsule formation and development of *in vitro*-produced equine blastocysts (*Equus caballus*).

	Control	Uterocalin	*P* value
Blastocyst percentage (%)	12.6 (±2.5)	13.3 (±3.5)	0.84
Mean cell count D9.5	551 (±47)	579 (±40)	0.66
Mean diameter (μm)	270 (±12)	247 (±15)	0.25
OC-1 specific fluorescence	2123 (±117)	2745 (±208)	0.03

Addition of uterocalin during IVC did not affect blastocyst development rate, embryo cell number or diameter. Total immunofluorescence of embryonic capsular glycoproteins was significantly increased by exposure to uterocalin. Mean values and their respective S.E.M. are shown.

of recovered oocytes reached the metaphase II (MII) stage and 76% of sperm-injected oocytes had cleaved 48 h after ICSI. In the control group, 25 of the 198 cleaved embryos developed to the blastocyst stage (12.6%); in the group cultured with uterocalin, 22 of the 165 cleaved embryos (13.3%) reached the blastocyst stage (Fig. 1A and B). Mean cell counts and embryo diameters (±S.E.M.) were 579 (±40) and 247 (±15) μm respectively for the blastocysts cultured with uterocalin ($n=11$) and 551 (±47) and 270 (±12) μm respectively for the control group ($n=11$) ($P>0.05$).

By contrast, total fluorescence after immunofluorescent labelling 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; $n=8$) ($P=0.03$; Table 1). Penetration of capsular glycoproteins into the transzonal channels was observed in both groups and was more obvious in smaller blastocysts (Fig. 1C and D). In the larger blastocysts, the capsular material appeared to form a more or less continuous layer (Fig. 1E and 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 (Fig. 2). Interestingly, in the uterocalin group, an apparently continuous area of capsule associated with the trophectoderm was observed in an area where the zona had detached locally around one blastocyst and in a zona-free area of another blastocyst (Fig. 3); similar findings were not observed in control embryos.

The five genes analysed by quantitative real-time PCR (RT-qPCR), *BEX2*, *FABP3*, *HSP90AA1*, *MOBKL3* and *ODC*, were chosen as markers for developmental quality because a previous study indicated downregulation of these genes in IVP compared with *in vivo*-derived equine blastocysts (Smits *et al.* 2011). It was hypothesised 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 ($n=11$) (Fig. 4). In this experiment, RT-qPCR efficiencies of ≥99% and correlation coefficients of ≥0.992 were obtained, indicating that the results were reliable.

Discussion

The ability to efficiently produce equine embryos *in vitro* is of interest for both research and the clinical treatment of (equine) infertility. Currently, the acceptable rates of production of viable embryos can be obtained using ICSI 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 overall efficiency (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

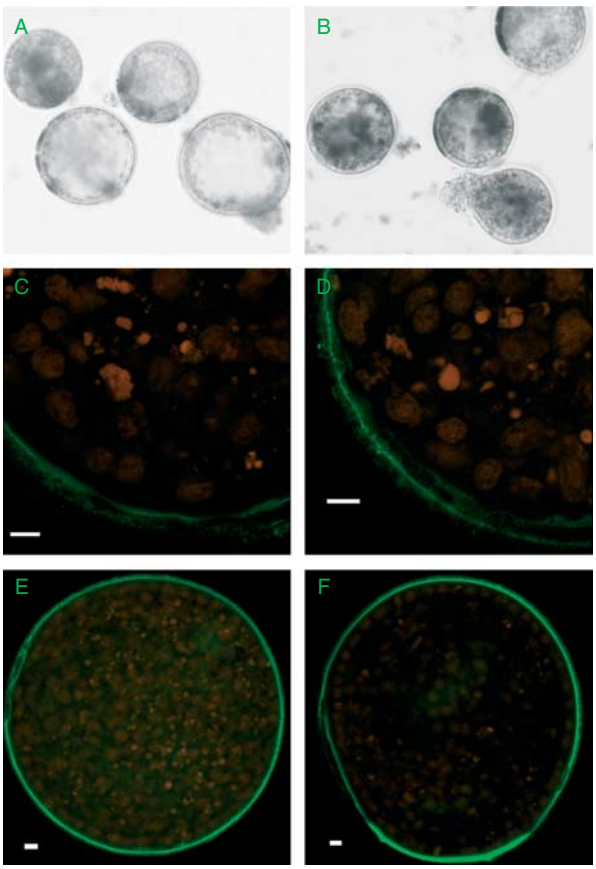

Figure 1 Day 9.5 blastocysts produced *in vitro* and cultured in the absence (A, C and E) or presence (B, D and F) of uterocalin (*Equus caballus*). (A and B) Equine blastocysts before fixation. Small blastocysts display a thin capsular line with penetration of capsular material into the transzonal channels 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) groups, but still show penetration of the glycoproteins into the thinned zona. White scale bar=10 μm.

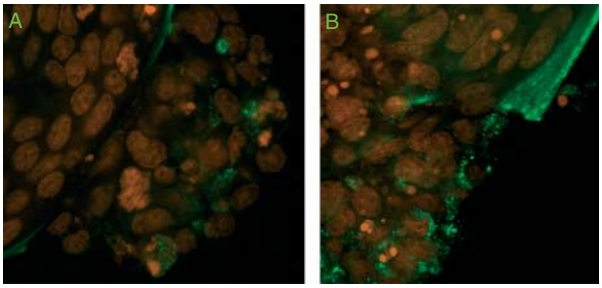

Figure 2 Hatching blastocyst produced *in vitro* (*Equus caballus*). Immunofluorescent staining with fluorescein-conjugated OC-1 illustrates capsular glycoproteins in the hatched part of an IVP blastocyst of the control group (A) and the uterocalin group (B). No confluent capsule is apparent.

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, intrauterine transfer of cleavage-stage IVP 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, and allowed loss of the embryos via the cervix. However, pregnancy after transfer of day 10 embryos to recipient mares on day 1 or 3 after ovulation has been described by Wilsher *et al.* (2010) and, although none of the embryos subsequently developed into normal pregnancies (i.e. with an embryo proper), it does illustrate that the uterus should be mechanically capable of retaining embryos introduced soon after ovulation. Wilsher *et al.*'s (2010) study also illustrates the possibilities and limits of embryo-uterine synchrony. Optimal synchronisation between donor and recipient mares yields the best results; it is, however, not known at which stage after ovulation the uterus of a recipient mare is best suited to nourishing embryonic stages that normally develop in the oviduct, especially when development may have been delayed by production *in vitro*. In addition, the large numbers of transferred embryos could have had a negative effect. *In vivo*, there are rarely more than two to three embryos in a mare's uterus. The choice to transfer an average of more than eight embryos per mare was based on recipient availability. Even if development of some embryos might have been impeded, recovery of others by flushing on day 7 would have been expected. Another 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 resulted in pregnancy and the birth of a live foal (Smits *et al.* 2010); 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 early cleavage-stage embryos. A similar failure to establish pregnancy following premature intrauterine transfer was reported for two mouse embryos of 292 days (Goto *et al.* 1993), and in previous small studies that described the intrauterine transfer of early *in vivo* derived horse embryos. For example, Weber *et al.* (1993) achieved no pregnancies following intrauterine transfer of two horse embryos of 7 days and, while Allen & Rowson (1975) did describe a single pregnancy after transfer of three equine embryos of 5 days, 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 compared with 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 the anatomical differences. In the mare, there is a distinct uterotubal papilla which presumably helps to maintain the marked differences in

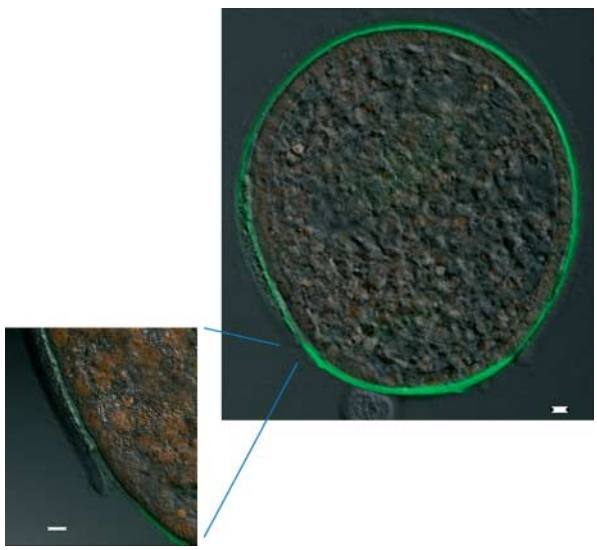

Figure 3 Confluent capsule adjacent to the trophectoderm (*Equus caballus*). This day 9.5 horse blastocyst was cultured in the presence of uterocalin; capsule formation was assessed by immunofluorescent OC-1 staining. An area of interrupted zona pellucida reveals a confluent piece of capsule adjacent to the trophectodermal surface. White scale bar = 10 μm.

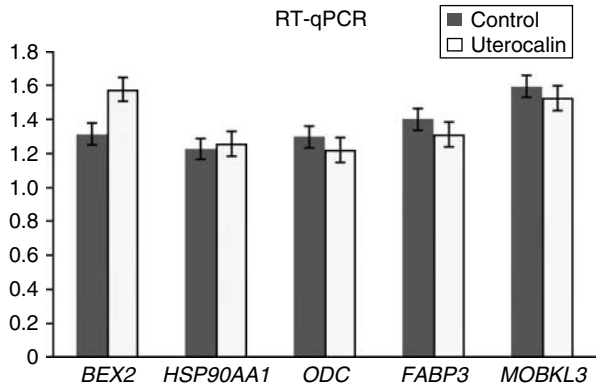

Figure 4 Expression of *BEX2*, *FABP3*, *HSP90AA1*, *MOBKL3* and *ODC* genes in blastocysts cultured with or without uterocalin, as determined by RT-qPCR (*Equus caballus*). The expression of these five genes was found to be downregulated in IVP equine blastocysts compared with *in vivo*-derived equine blastocysts. However, this figure shows that the addition of uterocalin, an abundant protein in uterine secretions during early pregnancy, to the *in vitro* culture medium from days 6 to 9 did not influence the relative expression of these genes. The error bars represent the S.E.M.

fluid composition observed in different parts of the oviduct and uterus in both horses and other domestic and laboratory species; this is in contrast to the lack of an anatomically distinct uterotubal separation in women due to 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 (Fig. 3); 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 IVP embryo, they found the capsular material to be stuck to the zona instead of having formed 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 contributes considerably 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). Indeed, the 19 kDa endometrial protein, uterocalin, was first isolated by SDS-PAGE as one of the dominant proteins released by equine embryonic capsules (Stewart *et al*. 1995). Subsequent studies confirmed the presence of uterocalin in the capsule in a temporal pattern, which suggested a functional correlation between the two (Crossett *et al*. 1996, Herrler & Beier 2000, Quinn *et al*. 2007). As a result of its molecular structure, uterocalin has since 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. This study suggests that uterocalin also contributes to the structure of the capsule and may play 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 to the structure of the capsule and plays an important role in transporting essential nutrients and/or signalling molecules to the early intrauterine conceptus.

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 neozona of the rabbit (Betteridge 1989, Herrler & Beier 2000). For example, while trophectodermal secretions are important for neozona formation, maternal components also appear to be critical (Fischer *et al*. 1991, Denker 2000). Indeed, *in vitro* culture of rabbit embryos is not only associated with the deposition of granular material on the inside of the mucoprotein layer, but also with failure of normal neozona formation, aberrant herniation of embryonic cells through the zona, and incomplete or failed dissolution of the zona pellucida (Fischer *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 intrauterine 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* (Fischer *et al.* 1991). In the horse, intrauterine transfer of IVP blastocysts results in successful pregnancies (Galli *et al.* 2007, Hinrichs *et al.* 2007, Smits *et al.* 2010) with an apparently normal capsule (Choi *et al.* 2009), illustrating that the degree of initial disturbance to capsule formation during IVP is not so severe as to preclude normal 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 is 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 this study, some aspects of aberrant equine embryonic capsule formation *in vitro* appeared to be ameliorated by uterocalin. However, it is not known how uterocalin had this effect, and neither was uterocalin alone sufficient to completely normalise capsule formation; e.g. the 'hatched' areas of trophectoderm still exhibited dispersed patches of OC-1-reactive glycoproteins that did not become confluent (Fig. 2). In addition, capsular material still penetrated into the transzonal channels (Fig. 1) as previously reported for IVP embryos (Tremoleda *et al.* 2003), but not seen around *in vivo* embryos. While it is possible that there was insufficient uterocalin provided to completely normalise capsule production, it is more likely that this partial correction reflects the continued absence of other components of the complex intrauterine 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 formation rates, embryo diameters and cell counts between control and uterocalin groups (Table 1). Moreover, exposure to uterocalin did not alter the expression of five genes (*BEX2*, *FABP3*, *HSP90AA1*, *MOBKL3* and *ODC*; Fig. 4) previously found to be downregulated in IVP embryos compared with *in vivo*-derived embryos (Smits *et al.* 2011). 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 3-h 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.

Conclusion

Several unusual features of early embryonic development in the horse illustrate the importance of embryo–maternal interaction. To optimise equine IVP, it may well be necessary to further clarify, and develop methods to mimic, particular aspects of this interaction. In this study, an essential role of the oviductal environment was illustrated by the failure of premature intrauterine transfer to yield viable embryos. In addition, the need for contact with the uterine environment of slightly older embryos was illustrated by the positive effect on capsule development of exposure 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.

Materials and Methods

Experiment 1: intrauterine 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.* (2010). Briefly, slaughterhouse oocytes were matured *in vitro* for 24 h in a DMEM/F12-based medium in an atmosphere containing 5% CO_2 (Galli *et al.* 2007). MII oocytes were fertilised by conventional ICSI and cultured *in vitro* in DMEM/F12 with 10% FCS at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2. On day 2–3 after ICSI, the cleaved embryos were transferred by means of a transcervical pipette (IMV Technologies France, L'Aigle, 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 synchronised mares (average of 8.25 embryos per mare). The transfers were performed by an experienced veterinarian, who obtained average pregnancy rates of 85% on day 14 and 79% on day 45 when using grades 1 and 2 day 7 horse blastocysts. On the day of transfer, the recipient mare was injected intravenously with 1.1 mg/kg of the nonsteroidal anti-inflammatory agent, flunixin meglumine (Emdofluxin, Emdoka Bvda, Hoogstraten, Belgium); in addition, daily *per os* treatment with 0.044 mg/kg of the synthetic progestogen, altrenogest (Regumate, Intervet, Boxmeer, 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 the other half were subjected to embryo recovery by transcervical uterine lavage,

as described by McKinnon & Squires (2009), on day 7 after ICSI. Briefly, flushing was performed with 6 l of lactated Ringer's solution introduced and recovered using a Bivona catheter (Minitüb, Tiefenbach, Germany); the recovered fluid was passed through an EZ filter (Bioniche Pharma, Galway, Ireland). Any embryos recovered were stained with Hoechst 33342 (Invitrogen) to assess cell viability and number.

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 Prof. M W 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 (BioRad) 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 intrauterine insemination as described by Velazquez *et al.* (2010), while subsequent uterine lavage resulted in the recovery of an embryo, thereby confirming that the mare had been 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 ∼4 mg/ml. Subsequent analysis of the uterine secretions of three other pregnant mares revealed an average concentration of 0.8 mg/ml. This physiological uterocalin concentration was mimicked in the experiment by 1 mg/ml recombinant uterocalin.

IVP 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 28 h and ICSI was performed by 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% FCS at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2. 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 Prof. K J Betteridge (University of Guelph, Canada). Day 9.5 blastocysts were fixed in 4% paraformaldehyde (P6118; Sigma–Aldrich) 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 min at room temperature, the blastocysts were washed three times in phosphate buffered saline (PBS) containing 1 mg/ml polyvinylpyrrolidone (PVP). Non-specific staining was blocked by incubation in 10% (v/v) goat serum (16210-064; Invitrogen) for 30 min 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 1.5 h 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; Invitrogen), 1/100 dilution) was performed for 1 h at 37 °C, followed by another washing step. Nuclei were then stained by incubation with 2% propidium iodide (Invitrogen) for 30 min at room temperature, after which the embryos were fixed in Dabco (Thermo Fisher Scientific, Geel, Belgium) on siliconised glass and enclosed under a coverslip 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 motorised Nikon TE2000-E inverted microscope (Nikon BeLux, Brussels, Belgium) and identical settings. Subsequent fluorescence measurements were performed by Nikon EC-V1 FreeViewer software. Since some blastocysts were slightly squeezed by the coverslip, intact capsules of six uterocalin blastocysts and eight control blastocysts in similar condition were evaluated. For each embryo, the total fluorescence of three areas in different, randomly selected spots of the capsule was measured and the mean of these three measurements was recorded (Fig. 5).

RT-qPCR

For both the uterocalin and the control groups, 11 blastocysts were selected on day 9. After washing in Dulbecco's PBS (DPBS),

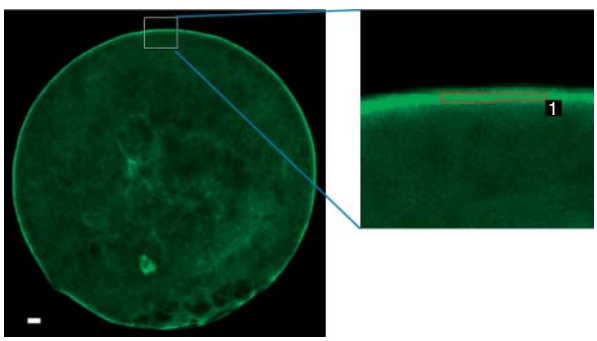

Figure 5 Measurement of capsule-specific fluorescence (*Equus caballus*). For each blastocyst, capsular fluorescence was measured in three areas of at least 10 µm^2 as represented in the figure. Random places on different aspects of the blastocyst that presented a continuous area of capsule-specific fluorescence were assessed. For each embryo, the mean of these three measurements was calculated.

individual blastocysts were transferred to cryotubes with 2 µl lysis buffer, frozen in liquid nitrogen for 3 min and stored at −80 °C. RNA extraction was performed by the RNeasy Micro Kit (Qiagen) and, after RT minus control, the RNA was converted into cDNA using the iScript cDNA synthesis Kit (BioRad). The expression of the five development 'marker' genes (*BEX2*, *FABP3*, *HSP90AA1*, *MOBKL3* and *ODC*) was quantified by RT-qPCR as described by Smits *et al.* (2011). Normalisation of data was performed by *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 χ^2 test (SPSS 16.0; SPSS, Inc., Chicago, IL, USA). Cell number and capsular fluorescence were compared between the groups by *t*-tests, while gene expression between the uterocalin and control groups was compared with a Mann–Whitney *U* test using GraphPad InStat 3 (Graphpad Software Inc, La Jolla, CA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

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References

Albihn A, Waelchli RO, Samper J, Oriol JG, Croy BA & Betteridge KJ 2003 Production of capsular material by equine trophoblast transplanted into immunodeficient mice. *Reproduction* **125** 855–863. (doi:10.1530/rep.0.1250855)

Allen WR & Rowson LEA 1975 Surgical and non-surgical egg transfer in horses. *Journal of Reproduction and Fertility Supplement* **23** 525–530.

Allen WR & Stewart F 2001 Equine placentation. *Reproduction, Fertility and Development* **13** 623–634 (Review). (doi:10.1071/RD01063)

Ball BA, Little TV, Weber JA & Woods GL 1989 Survival of day-4 embryos from young, normal mares and aged, subfertile mares after transfer to normal recipient mares. *Journal of Reproduction and Fertility* **85** 187–194. (doi:10.1530/jrf.0.0850187)

Betteridge KJ 1989 The structure and function of the equine capsule in relation to embryo manipulation and transfer. *Equine Veterinary Journal Supplement* **8** 92–100.

Betteridge KJ 2007 Equine embryology: an inventory of unanswered questions. *Theriogenology* **68S** S9–S21. (doi:10.1016/j.theriogenology.2007.04.037)

Blanco IDP, Devito LG, Ferreira HN, Araujo GHM, Fernandes CB, Alvarenga MA & Landim-Alvarenga FC 2009 Aspiration of equine oocytes from immature follicles after treatment with equine pituitary extract (EPE) alone or in combination with hCG. *Animal Reproduction Science* **114** 203–209. (doi:10.1016/j.anireprosci.2008.09.002)

Bøgh IB, Steeves T, Cartwright G, Gurevich V, Greve T & Hyland J 2002 Effects of a progesterone-induced protein with a molecular weight of 20 kDa (PIP-20) on carbohydrate metabolism and growth of day 8 equine embryos in culture. *Theriogenology* **58** 751–754. (doi:10.1016/S0093-691X(02)00894-4)

Bromer JG & Seli E 2008 Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. *Current Opinions in Obstetrics and Gynecology* **20** 234–241. (doi:10.1097/GCO.0b013e3282fe723d)

Choi YH, Harding HD, Hartman DL, Obermiller AD, Kurosaka S, McLaughlin KJ & Hinrichs K 2009 The uterine environment modulates trophectodermal POU5F1 levels in equine blastocysts. *Reproduction* **138** 589–599. (doi:10.1530/REP-08-0394)

Crossett B, Allen WR & Stewart F 1996 A 19 kDa protein secreted by the endometrium of the mare is a novel member of the lipocalin family. *Biochemical Journal* **320** 137–143.

Denker HW 2000 Structural dynamics and function of early embryonic coats. *Cells Tissues Organs* **166** 180–207. (doi:10.1159/000016732)

Ellenberger C, Wilsher S, Allen WR, Hoffmann C, Kölling M, Bazer FW, Klug J, Schoon D & Schoon H-A 2008 Immunolocalisation of the uterine secretory proteins uterocalin, uteroferrin and uteroglobin in the mare's uterus and placenta throughout pregnancy. *Theriogenology* **70** 746–757. (doi:10.1016/j.theriogenology.2008.04.050)

Fischer B, Mootz U, Denker HW, Lambertz M & Beier HM 1991 The dynamic structure of rabbit blastocyst coverings. *Anatomy and Embryology* **183** 17–27.

Flood PF, Betteridge KJ & Diocee MS 1982 Transmission electron microscopy of horse embryos 3–16 days after ovulation. *Journal of Reproduction and Fertility Supplement* **32** 319–327.

Freeman DA, Weber JA, Geary RT & Woods GL 1991 Time of embryo transport through the mare oviduct. *Theriogenology* **36** 823–830. (doi:10.1016/0093-691X(91)90348-H)

Galli C, Colleoni S, Duchi R, Lagutina I & Lazzari G 2007 Developmental competence of equine oocytes and embryos obtained by *in vitro* procedures ranging from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Animal Reproduction Science* **98** 39–55. (doi:10.1016/j.anireprosci.2006.10.011)

Goto Y, Noda Y, Masahide S, Kishi J, Nonogaki T & Mori T 1993 The fate of embryos transferred into the uterus. *Journal of Assisted Reproduction and Genetics* **10** 197–201. (doi:10.1007/BF01239221)

Herrler A & Beier HM 2000 Early embryonic coats: morphology, function, practical applications. *Cells Tissues Organs* **166** 233–246. (doi:10.1159/000016736)

Hinrichs K 2010 *In vitro* production of equine embryos: state of the art. *Reproduction in Domestic Animals* **45** (Suppl 2) 3–8. (doi:10.1111/j.1439-0531.2010.01624.x)

Hinrichs K, Choi YH, Walckenaer BE, Varner DD & Hartman DL 2007 *In vitro*-produced equine embryos: production of foals after transfer, assessment by differential staining and effect of medium calcium concentrations during culture. *Theriogenology* **68** 521–529. (doi:10.1016/j.theriogenology.2007.04.046)

Hunter RHF 1998 Have the fallopian tubes a vital role in promoting fertility? *Acta Obstetricia et Gynecologica Scandinavica* **77** 475–486. (doi:10.1080/j.1600-0412.1998.770501.x)

Kennedy MW 2004 Uterocalin – provider of essential lipids and amino acids to the pre-placentation equine conceptus. *Havemeyer Foundation Monograph Series* **16** 53–56.

McKinnon AO & Squires EL 2009 Embryo transfer and related technologies. *Proceedings of the 11th Annual Resort Symposium of the American Association of Equine Practitioners, January 25–28, Gold Coast, Australia* 27–57.

Oriol JG 1994 The equine embryonic capsule: practical implications of recent research. *Equine Veterinary Journal* **26** 184–186. (doi:10.1111/j.2042-3306.1994.tb04366.x)

Oriol JG, Sharom FJ & Betteridge KJ 1993 Developmentally regulated changes in the glycoproteins of the equine embryonic capsule. *Journal of Reproduction and Fertility* **99** 653–664. (doi:10.1530/jrf.0.0990653)

Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Faterni H, Tarlatzis B & Devroey P 2008 Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. *Human Reproduction* **23** 91–99. (doi:10.1093/humrep/dem339)

Peyrot LM, Little TV, Lowe JE, Weber JA & Woods GL 1987 Autotransfer of day 4 embryos from oviduct to oviduct versus oviduct to uterus in the mare. *Theriogenology* **28** 699–708. (doi:10.1016/0093-691X(87)90287-1)

Pomar FJ, Teerds KJ, Kidson A, Colenbrander B, Tharasanit T, Aguilar B & Roelen BA 2005 Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. *Theriogenology* **63** 2254–2268. (doi:10.1016/j.theriogenology.2004.10.015)

Quinn BA, Hayes MA, Waelchli RO, Kennedy MW & Betteridge KJ 2007 Changes in major proteins in the embryonic capsule during immobilization (fixation) of the conceptus in the third week of pregnancy in the mare. *Reproduction* **134** 161–170. (doi:10.1530/REP-06-0241)

Smits K, Goossens K, Van Soom A, Govaere J, Hoogewijs M, Vanhaesebrouck E, Galli C, Colleoni S, Vandesompele J & Peelman L 2009 Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen–thawed *in vitro* blastocysts. *BMC Research Notes* **2** 246. (doi:10.1186/1756-0500-2-246)

Smits K, Govaere J, Hoogewijs M, De Schauwer C, Vanhaesebrouck E, Van Poucke M, Peelman LJ, Van den Berg M, Vullers T & Van Soom A 2010 Birth of the first ICSI foal in the Benelux. *Vlaams Diergeneeskundig Tijdschrift* **79** 134–138.

Smits K, Goossens K, Van Soom A, Govaere J, Hoogewijs M & Peelman L 2011 *In vivo* derived horse blastocysts show upregulation of developmentally important genes compared to *in vitro* produced horse blastocysts. *Reproduction, Fertility and Development* **23** 364–375. (doi:10.1071/RD10124)

Stewart F, Charleston B, Crossett B, Barker P & Allen WR 1995 A novel uterine protein that associates with the embryonic capsule. *Journal of Reproduction and Fertility* **105** 65–70. (doi:10.1530/jrf.0.1050065)

Stout TA & Allen WR 2001 Role of prostaglandins in intrauterine migration of the equine conceptus. *Reproduction* **121** 771–775. (doi:10.1530/rep.0.1210771)

Stout TA, Meadows S & Allen WR 2005 Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival *in vivo*. *Animal Reproduction Science* **87** 269–281. (doi:10.1016/j.anireprosci.2004.11.009)

Suire S, Stewart F, Beauchamp J & Kennedy MW 2001 Uterocalin, a lipocalin provisioning the preattachment equine conceptus: fatty acid and retinol binding properties, and structural characterization. *Biochemical Journal* **356** 369–376. (doi:10.1042/0264-6021:3560369)

Tremoleda JL, Stout TAE, Lagutina I, Lazzari G, Bevers MM, Colenbrander B & Galli C 2003 Effects of *in vitro* production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biology of Reproduction* **69** 1895–1906. (doi:10.1095/biolreprod.103.018515)

Velazquez MA, Parrilla I, Van Soom A, Verberckmoes S, Kues W & Niemann H 2010 Sampling techniques for oviductal and uterine luminal fluid in cattle. *Theriogenology* **73** 756–767. (doi:10.1016/j.theriogenology.2009.07.004)

Weber JA, Douglas AF, Vanderwall DK & Woods GL 1991 Prostaglandin E_2 hastens oviductal transport of equine embryos. *Biology of Reproduction* **45** 544–546. (doi:10.1095/biolreprod45.4.544)

Weber JA, Woods GL, Freeman DA & Vanderwall DK 1993 Oviductal and uterine influence on the development of day-2 equine embryos *in vivo* and *in vitro*. *Theriogenology* **40** 689–698. (doi:10.1016/0093-691X(93)90205-J)

Wilsher S, Clutton-Brock A & Allen WR 2010 Successful transfer of day 10 horse embryos: influence of donor–recipient asynchrony on embryo development. *Reproduction* **139** 575–585. (doi:10.1530/REP-09-0306)

Younis JS, Radin O, Izhaki I & Ben-Ami M 2009 Does first polar body morphology predict oocyte performance during ICSI treatment? *Journal of Assisted Reproduction and Genetics* **26** 561–567. (doi:10.1007/s10815-009-9368-9)

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