Effects of in vitro production on horse embryos morphology, cytoskeletal characteristics and blastocyst capsule formation

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ABSTRACT

Attempts to produce horse embryos in vitro (IVP) have resulted in low blastocyst rates. In addition, a characteristic and vital feature of blastulation in equids, namely capsule formation, does not occur in vitro. The aim of this study was to evaluate the impact of IVP on horse embryo development and blastocyst capsule formation. IVP embryos were produced by intracytoplasmic sperm injection of in vitro matured oocytes, followed by either culture in synthetic oviductal fluid (SOF) or temporary transfer to the oviduct of a progesterone-treated ewe. As controls, in vivo embryos were flushed from the uterus of mares on days 6-9 after ovulation. Embryo morphological quality and the degree of cytoplasmic fragmentation were investigated by light microscopy, and then by multiphoton scanning confocal microscopy. During the latter, microfilament distribution was visualized by means of AlexaFluo-Phalloidin staining, while the rate of apoptosis was examined using the TUNEL assay for DNA fragmentation combined with DAPI staining to evaluate nuclear morphology and total cell number. To examine the influence of culture on blastocyst capsule formation, embryos were stained with a monoclonal antibody (OC-1) specific for capsular glycoproteins. The blastocyst rate was significantly higher after transfer of 2 to 4 cell embryos to a sheep’s oviduct (16%) than after culture in SOF medium (6.3%). Compared to similarly aged in vivo embryos, Day 7 IVP embryos were small with low cell numbers, and compact with a small, or no, blastocoele and an indistinct inner cell mass. In addition, IVP embryos had high percentages of apoptotic cells (10% versus 0.3% for in vivo embryos) and irregular microfilament distribution, which highlighted the heterogeneity of cell size and shape. Finally, although IVP embryos secreted capsular glycoproteins, the latter failed to coalesce and form a confluent capsule enveloping the embryo, but instead adhered to and permeated into the zona pellucida or remained in patches on the trophocoele surface. This confirmed that the initial layer of capsule is made up of OC-1 reactive glycoproteins, and suggests that the missing element for capsule formation in vitro is a suitable microenvironment for crosslinking and coalescence of mucin-like glycoproteins.

INTRODUCTION

A variety of assisted reproductive techniques, including conventional in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), oocyte transfer (OT), and nuclear transfer, have been performed or attempted with equine gametes (Palmer et al., 1991; Carnevale and Ginther, 1995; Squires et al., 1996; Galli et al., 2002; Li et al., 2002). However, the relatively low efficiency of the in vitro culture
stages has limited their commercial application in this species. In particular, the current techniques for in vitro oocyte maturation, conventional IVF and culture of zygotes to the blastocyst stage are inadequate. For example, while only two foals have been produced via conventional IVF (Palmer et al., 1991; Bézard et al., 1992), the adoption of intracytoplasmic sperm injection (ICSI: Squires et al., 1996; Meitjes et al., 1996) has, by circumventing the need for sperm to penetrate the zona pellucida (ZP), resulted in the birth of several foals (Squires et al., 1996; Cochran et al., 1998; McKinnon et al., 2000; Li et al., 2001; Galli et al., 2001a,b). Still, the majority of ICSI pregnancies have resulted from transferring immediate post-cleavage zygotes to the oviduct of recipient mares (Squires et al., 1996; Cochran et al., 1998; McKinnon et al., 2000) while only a few have been produced via in vitro culture to the blastocyst stage and transfer to the uterus (Li et al., 2001; Galli et al., 2001a). In support of the apparent negative effect of culture, McKinnon et al. (2000) reported that even incubation for 26-28h between ICSI and oviductal transfer resulted in a lower pregnancy rate than transfer of presumptive zygotes 4-8 h after ICSI. Thus, if a viable complete in vitro horse embryo production (IVP) system is desired, and on grounds of cost and recipient welfare it is, one critical step is to devise a culture system that supports embryo development to a stage suitable for uterine, rather than oviductal, transfer. To date, two approaches to improving horse embryo culture have been explored. The first involved coculture of presumptive zygotes with somatic cells or their products (oviduct epithelial cells, Battut et al., 1991; cumulus cells, Li et al., 2001; equine trophoblast monolayer conditioned medium, Choi et al., 2001) and the second involved culturing zygotes in defined cell-free culture media such as synthetic oviductal fluid (SOF; Azuma et al., 1995; Galli and Lazzari, 2001), Dulbecco’s modified Eagles medium (DMEM; Li et al., 2001) or Human Tubal Fluid (HTF; Dell’Aquilla et al., 2001). In all of the above systems, however, only a low percentage of zygotes developed to the blastocyst stage (average 15%). Indeed, the fact that temporary transfer of ICSI-derived horse zygotes to the oviduct of progesterone treated ewes remains the most successful system for producing horse blastocysts ex vivo (rates approach 50%; Galli et al., 2002) emphasizes the inadequacy of current in vitro culture systems.

In vitro-produced mammalian embryos tend to exhibit characteristic differences to their in vivo counterparts, including lower cell numbers, an altered inner cell mass: trophoderm ratio, irregularly sized blastomeres and an increased incidence of cytoplasmic fragmentation, all of which are related to reduced developmental competence (pig – Kikuchi et al., 1999; cow - Kruip and den Daas, 1997). Programmed cell death (apoptosis) is a feature observed in both in vivo and in vitro produced mammalian blastocysts (e.g. mouse - Brison and Schultz, 1997; man -
Features of horse IVP embryo’s

Hardy, 1999; cattle - Byrne et al., 1999) and has been proposed as a means of eliminating cells that are damaged, nonfunctional, abnormal or misplaced (Hardy, 1999; Meier et al., 2000). It has been further suggested that apoptosis is a major contributor to embryonic arrest in sub-optimal culture conditions, such as an excess embryo: medium ratio (Brison and Schultz, 1997), heat shock (Paula-Lopes and Hansen, 2002), excess oxygen free radical concentrations (Van Soom et al., 2002) or following exposure of embryos to a high concentration of spermatozoa during IVF (Juriscova et al., 1995). Since IVP embryos exhibit relatively high levels of apoptosis (with varying degrees of cytoplasmic fragmentation) and a high incidence of developmental arrest during culture, parameters such as cell number and apoptosis rate may be valuable indicators of the health and developmental capacity of pre-implantation embryos.

Studies in the hamster (Barnett et al., 1997), rat (Matsumoto et al., 1998) and pig (Wang et al., 1999) have indicated that the actin filament organization of IVP embryos differs from that of in vivo embryos. Of course, the actin cytoskeleton plays an important role in the migration of cytoplasmic organelles and the nucleus, and in cell cleavage during mitosis (Alberts et al., 1994). Given that actin filament dynamics can be affected by environmental conditions such as temperature, pH, and ion concentrations, it would not be surprising if culture conditions affected embryo cleavage or compromised further development. Previous studies have indicated the likely roles of actin filaments during horse oocyte maturation, fertilization and early embryo development (Tremoleda et al., 2001, 2002), and disruption of the microfilament cytoskeleton has been proposed as a reason for the poor viability of frozen-thawed embryos (Huhtinen et al., 2001). However, it is not known if the actin cytoskeleton of IVP horse blastocysts differs significantly from that of in vivo embryos.

An unusual and vital feature of early embryonic development in the horse is the formation of an acellular blastocyst capsule beneath the zona pellucida (ZP) on days 6-7 after ovulation, soon after the blastocyst enters the uterus (Flood et al., 1982). After the loss of the ZP, the capsule remains to envelop the conceptus throughout the second and third weeks of pregnancy (Betteridge, 1989). Although the precise functions of the capsule are not known (see Betteridge, 1989 for a review), it is thought to provide vital mechanical protection during the period when the conceptus is mobile and propelled throughout the uterine lumen by myometrial contractions (Ginther, 1985), where the resulting intrauterine migration is essential for the conceptus to distribute its maternal recognition of pregnancy signal to, and inhibit 

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primarily, or exclusively, a function of trophoblast cells (at least after day 11 of gestation: Oriol et al., 1993a; Albinh et al., 2003). However, the presence of large quantities of the endometrial lipocalin, P19 (Stewart et al., 1995), associated to the capsule argues that there may also be a maternal uterine contribution to capsule formation, as does the finding that equine embryos that blastulate in vitro are not able to produce a visible capsule (McKinnon et al., 1989; Hinrichs et al., 1990). On the other hand, the suggestion that the ZP is an essential requirement for capsule formation (Skidmore et al., 1989) has been disproven by the demonstration that zona-free bisected blastocysts develop an apparently normal capsule after transfer to the uterus of recipient mares (McKinnon et al., 1989). In any case, it has yet to be established whether the initial capsule is composed of the same trophoblast-produced molecules that predominate at later stages, and are recognized by the antibody OC-1 (Oriol et al., 1993a). Neither has the influence of in vitro culture on capsule glycoprotein production and coalescence been examined.

The aim of this study was to document the impact of in vitro production on the morphological and ultrastructural characteristics of horse embryos. IVP embryos were produced by ICSI followed by one of two different culture systems; the first was based on synthetic oviduct fluid (SOF: Galli et al., 2001b) and the second involved temporary transfer to the oviduct of a progesterone-treated ewe. As controls, in vivo produced embryos were flushed from the uterus of mares on days 6-9 after ovulation. Embryo morphology and degree of cytoplasmic fragmentation were analyzed by light microscopy, while multiphoton scanning confocal microscopy was used to evaluate total cell numbers, nuclear morphology (after DAPI counterstaining) and rates of cell apoptosis, where the latter was taken as the incidence of nuclear fragmentation or of DNA fragmentation as indicated by the deoxynucleotidyl dUTP terminal transferase (TUNEL) assay. To examine the influence of culture on cytoskeletal morphology and on capsule formation, day 6-9 in vivo and day 7 IVP late morulae, early blastocysts and IVP embryos cultured for a further 3 days, with or without horse adult fibroblast cells, were analysed with respect to microfilament distribution and capsular glycoprotein expression.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Milan, Italy) and plastic-ware from Nunc (Roskilde, Denmark).
In vitro production of Day 7 embryos

Collection and Culture of Cumulus Oocyte Complexes

Ovaries from slaughtered mares were transported to the laboratory in PBS at 25°C. Within 4 h of slaughter, cumulus oocyte complexes (COCs) were recovered by scraping the inside of follicles between 0.5 and 3.0 cm in diameter with a Jacobson curette. The scrapings were washed into Petri dishes using TCM199 supplemented with 25 mM HEPES, 1 mg/ml BSA and 10 µg/ml heparin. COCs were classified as either compact (Comp) or expanded (Exp) depending on cumulus and granulosa cell morphology, as described by Hinrichs et al. (1997). Thus, compact COCs were those surrounded by a dense cellular mass with a smooth cumulus hillock and homogenous colouration, whereas expanded COCs were those with cells protruding from the surface of the cumulus hillock and matrix visible between the cumulus cells. COCs were matured by culture for 22-24 h in TCM199 supplemented with 10% fetal calf serum (FCS), 1 µl/ml ITS (insulin, transferin, sodium selenite), 1mM sodium pyruvate, 100 ng/ml Long-IGF1, 50 ng/ml Long-EGF, and 0.1 IU/ml each of FSH and LH (Pergovet, Serono, Italy), in 4-well plates at 38.5°C in an atmosphere of 5% CO₂ in air (Galli et al., 2001b, Lazzari et al., 2002).

Preparation of oocytes and sperm for ICSI

After culture, oocytes were separated of their cumulus cells by incubation in 25 µg/ml hyaluronidase in Hepes-buffered SOF medium (H-SOF; Galli et al., 2001b) and then 2.5 mg/ml trypsin in PBS for 2 min, before aspirating them through a fine pipette. Oocytes with an intact cell membrane were returned to maturation medium for 2-4 h, after which those with an extruded first polar body were selected for sperm injection. Intracytoplasmic sperm injection (ICSI) was performed with frozen/thawed ejaculated sperm from a stallion of proven fertility. One hour before injection, the semen was thawed and the spermatozoa were rinsed free of cryoprotectant by centrifuging them at 750g through a discontinuous Percoll density gradient (45%: 90%) for 40 min at room temperature. The viable spermatozoa recovered from the bottom of the tube were washed in Ca²⁺ free TALP (Parrish et al., 1988) and repelleted by centrifugation at 400g for 10 min. This second sperm pellet was suspended at a concentration of 4 million sperm/ml in Hepes-buffered SOF medium supplemented with 6mg/ml fatty-acid-free (FAF) BSA, modified Eagle’s medium (MEM) amino acids, 1 µg/ml heparin, 20 µM penicillamine, 1 µM epinephrine, and 10 µM hypouthaurine (SOF IVF medium: Lazzari et al., 2002).
Just before ICSI, the sperm suspension was diluted 1:1 (v/v) with a 12% solution of PVP in SOF-IVF medium.

**ICSI**

Sperm injection was performed as described by Kimura and Yanagimachi (1995) using a Piezo micropipette-driving unit (Prima Tech, Japan) fixed on a micromanipulator (Narishige, Japan) and mounted on an inverted microscope equipped with a 37°C heated stage. A pipette with inner and outer diameters of 50 and 150 µm, respectively, was used to hold oocytes, and a pipette with a tip inner diameter of approximately 5 µm was used for sperm injection. A motile sperm was aspirated into the injection pipette and immobilized by applying two or three piezo-pulses to the tail-midpiece junction. The oocyte was held on the holding pipette by suction, with the polar body orientated to the 6 or 12 o’clock position, and the injection pipette was advanced through the ZP at the 3 o’clock position using the piezo-drilling motion. The core of ZP so excised was expelled into the holding medium and, finally, the injection pipette was advanced through the oolema using one piezo-pulse and the sperm was released into the ooplasm.

**Culture of injected oocytes in vitro**

After sperm injection (Day 0), oocytes were cultured in groups of 20 in 20µl droplets of SOF medium supplemented with MEM amino acids and 16 mg/ml FAF BSA (SOF-BSA-AA; Lazzari et al., 2002), under mineral oil at 38.5°C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. On Day 2 of incubation, the cleavage rate was determined and morphologically normal two and four-cell embryos were selected for further culture. The selected embryos were incubated in one of two culture systems, namely: 1) *In vitro* culture (IVC) in 20 µl droplets of SOF-BSA-AA which was partially replaced (by adding 20 µl fresh medium and then removing 20 µl of the mix) with SOF-BSA-AA on Day 3 and with TCM199-BSA on Day 6; 2) Sheep-oviduct culture after surgical transfer of embryos embedded in agar chips to the ligated oviduct of a ewe implanted with an intravaginal progesterone-releasing device (Eazi-BREED CIDR; InterAg, Hamilton, New Zealand) on the day of transfer, as previously described by Willadsen (1979). For the IVC-group, both expanded and compact COCs were used to produce embryos, whereas for the Sheep-oviduct group only compact COCs were used. After 5 further days in culture, embryos in the IVC-group were assessed, and those lacking cellular compaction and displaying irregular embryonic cell sizes were removed from culture, fixed and stained to examine their developmental status. Sheep oviduct embryos were harvested 5 days after transfer into the oviduct, and the rate and quality of blastocysts from both systems was scored according to the criteria outlined in the Manual of the International Embryo Transfer Society (1998).
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In vitro production of Day 10 embryos

A proportion of the morphologically normal Day 7 blastocysts produced via the sheep oviduct system were maintained for a further 3 days in one of two different culture systems: 1) A semi-defined culture system consisting of 20 µl droplets of a 1:1 (v:v) solution of DMEM (Gibco BRL, Paisley, Scotland, UK) and TCM199, supplemented with 5% FCS and 5% Serum Replacement (Knockout™ SR; Gibco BRL) and incubated under mineral oil. The medium was partially replaced on Day 8 of culture. 2) In a cell co-culture system consisting of adult horse skin fibroblast cell monolayers (SFC) and 300 µl DMEM: TCM199 supplemented with 5% FCS and 5% SR, in 4-well plates. The SFCs were prepared from a small piece of subdermal tissue harvested from the chest of a 5-yr-old Haflinger mare, under local anesthesia. The tissue was sliced finely and digested for 30 min at 38°C in 0.5% trypsin-EDTA in PBS (v:v). The digested fragments were then washed twice in PBS by centrifugation at 700g for 10 min, and the resulting pellet of cells was resuspended in DMEM supplemented with 10% FCS (v:v) and cultured at 38.5°C in 5% CO₂, 5% O₂, and 90% N₂. Cells used for co-culture had been through 1-5 passages.

Collection of in vivo produced horse embryos

Sixteen early horse conceptuses were recovered from 5-10 year old Dutch Warmblood mares by non-surgical uterine lavage with modified Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 1% FCS, as described by Imel et al. (1981). The conceptuses were recovered on days 6, 7 or 9 after ovulation detected by daily ultrasonographic examination of the ovaries and were thus 6.5, 7.5 and 9.5 ± 0.5 days old at the time of collection.

Embryo Evaluation

In vitro and in vivo horse embryos of similar developmental stages were compared as follows: In Experiment 1, Day 7 in vivo embryos and day 7 IVP embryos from both the IVC and sheep-oviduct groups were analyzed for total cell number, apoptotic rate and microfilament distribution. In Experiment 2, Day 10 IVP embryos from both the semi-defined and SFC-monolayer culture systems were evaluated for total cell number, microfilament organization and blastocyst capsule formation. Four Day 7 embryos derived from SOF (3) or sheep-oviduct culture (1) and a range of day 6-9 in vivo embryos were also analysed in this way. In all cases,
embryos were measured and assessed morphologically immediately after collection or harvest and before further fixation and labelling.

**Morphological assessment**
Morulae and blastocysts produced *in vitro* (Day 7 and 10) or *in vivo* (Day 6, 7 and 9) were measured using a stereomicroscope fitted with an eyepiece micrometer.

**Table 1:** System used to grade the quality of equine embryos produced *in vitro**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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<tbody>
<tr>
<td>Grade 1</td>
<td><strong>Excellent or good:</strong> Symmetrical and spherical embryo mass with cells of uniform size, colour and density. Embryo mass has clear edges without indentation. No evidence of cellular fragmentation or loose cytoplasmic granules in the perivitelline space.</td>
</tr>
<tr>
<td>Grade 2</td>
<td><strong>Fair:</strong> Moderate irregularities in the overall shape of the embryo, for example a minor degree of cytoplasmic fragmentation evident as slightly convolution of the edges of the embryo and small cytoplasmic granules in the perivitelline space.</td>
</tr>
<tr>
<td>Grade 3</td>
<td><strong>Poor:</strong> Major irregularities in the shape of the embryonic mass or in the size, colour or density of the constituent cells. High degree of cytoplasmic fragmentation with extrusion or degeneration of cells of variable size reflected by highly lobulated, irregular edges of the embryonic mass and a large perivitelline space.</td>
</tr>
<tr>
<td>Grade 4</td>
<td><strong>Degenerate or dead:</strong> High degree of cytoplasmic fragmentation. Embryonic cells of irregular size and colour. Absence of cellular compaction and failure to form a clear embryonic mass. This group includes embryos with irregularly sized cells of a low number (&lt;4) inconsistent with the expected stage of development.</td>
</tr>
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</table>

Fig 1: Photomicrographs to illustrate the quality scoring of day 7 IVP horse embryos (Grades 1-4: good to degenerate). Grading was based on criteria laid down in the manual of the International Embryo Transfer Society and included degree of compactness, size and appearance of the perivitelline space, colour, presence of extruded cells, and the degree of cell granularity and cytoplasmic fragmentation.

A and B: GRADE 1
B and C: GRADE 2
D: GRADE 2-3
E: GRADE 3
F: GRADE 4
H: A group of grade 1-2 embryos cultured in SOF medium.
I: A grade 2 in vivo early blastocyst for comparative purposes.

Scale: In A-G bar = 45 µm, in H bar = 90 µm and in In vivo bar = 75 µm
Chapter 6

For Day 10 IVP embryos, separate measurements were made of the part of the embryo that remained enclosed within the ZP and the part that had herniated through the hole made during ICSI. In addition, each embryo was graded morphologically on a scale of 1-4, where 1 was good quality and 4 was indicative of degeneration. In this respect, *in vivo* embryos were graded using the system described by McKinnon and Squires (1988), while an adapted scale based on criteria published in the Manual of the International Embryo Transfer Society was developed for assessing IVP embryos (Table 1; Fig.1). This latter scale included parameters such as degree of compactness, size and appearance of the perivitelline space, colour, presence of extruded cells and the degree of cell granulation and cytoplasmic fragmentation (Hardy, 1999). After morphological assessment, embryos were fixed in 4% paraformaldehyde for 24 h and then stored at 4°C in PBS prior to staining.

**Cell Number, Apoptosis index and Microfilament Distribution (Experiment 1)**

Apoptosis was detected using the TUNEL-labeling technique for DNA fragmentation. First, fixed embryos were incubated twice for 15 min in PBS containing 150 mM glycine and 1 mg/ml of polyvinylacohol (PVA), to reduce free aldehydes and block non-specific reactions. Next, they were permeabilized by immersion for 15 min at 4 °C in 0.1% (v:v) Triton X-100 in PBS. The permeabilized embryos were then washed twice in PBS containing 1 mg/ml of PVA (PBS-PVA; pH 7.4) before being incubated in 20 µl drops of fluorescein-conjugated dUTP and TdT (TUNEL reagents; Boehringer Manheim, Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 37 °C in a dark, moist chamber. Following TUNEL labeling, embryos were washed three times in 0.5% (v:v) Triton X-100 in PBS containing 1 mg/ml PVA (PBS-TX100-PVA). To enable microfilament detection, embryos were then incubated for 1 h with 15 IU/ml AlexaFluor™ 568 Phalloidin (Molecular Probes Europe BV, Leiden, The Netherlands; A-12380) in PBS-TX100-PVA. Finally, to enable DNA visualization, the embryos were washed twice in PBS-PVA and incubated with 0.1 µg/ml of 4,6-diamino-2-phenyl-indole (DAPI) in PBS for 10 min.

**Cell Number, Microfilament Distribution and Capsule immunolocalization (Experiment 2)**

Fixed embryos were washed and permeabilized in 0.1% Triton X-100, as described above. Capsular glycoproteins were labeled using a monoclonal antibody raised against day 13.5-15.5 equine capsule in mouse: OC-1 (Oriol et al., 1993a). For the labeling, embryos were first incubated for 45 min in a blocking solution (0.1M glycine, 1% v:v goat serum, 0.01% Triton X-100 and 0.5% w:v BSA) and then exposed to a 1:100 dilution of mAb OC-1 in PBS-TX100-PVA, for 1 h at 37 °C.
Next, the embryos were incubated with a 1:300 dilution of a goat anti-mouse IgG coupled to Alexa Fluor™ 488 (Molecular Probes; A-11029) in PBS-TX100-PVA, and then washed twice in PBS-PVA before incubation with AlexaFluor™ 568 Phalloidin (15 IU/ml in PBS-TX100-PVA) to label the microfilaments. Finally, the embryos were counterstained with DAPI (0.1 µg/ml in PBS) to label the nuclei. As controls, a few embryos were incubated with labeled goat anti-mouse secondary antibody without pre-exposure to OC-1. In addition, Day 6, 7 and 9 in vivo-produced embryos on which the capsule was clearly identifiable by light microscopy were stained with mAb OC-1 as positive controls, while in vitro and in vivo matured horse oocytes were labeled to control for cross-reactivity of OC-1 staining with the ZP.

Fluorescence and Multiphoton Laser Scanning Microscopy (MPLSM)
Stained embryos were mounted on glass slides with an antifade (Vectashield, Vector Lab. Burlingame, CA, USA). To avoid excessive pressure being exerted on the embryos, coverslips were supported at each corner by thick droplets of a Vaseline-wax mixture. Embryos were examined first using a Multiphoton Excitation Microscopy (MPEM) system combined with a Confocal Laser Scanning Microscope (CLSM; Bio-Rad Radiance 2100 MP) mounted on a Nikon TE300 inverted microscope. Imaging was performed using a 488-Argon-ion laser and a 543-HeliumNeon laser to simultaneously excite fluorescein and Alexa Fluor™ 568 (Experiment 1) or Alexa Fluor™ 568 and Alexa Fluor™ 488 (Experiment 2), respectively. DAPI staining was imaged by using a 100 fs pulsed 780 nm excitation laser source (a mode-locked Titanium:Saphire laser: Tsunami, Spectra Physics, Mountain View, CA, USA). To avoid cross talk of the images in the photomultiplier channels, specimens were analysed using a sequential scanning mode. Images were recorded digitally and processed using Adobe® Photoshop 5.5 software (Adobe Systems Inc., Mountain View, CA, USA). After MPLSM, mounted embryos were flattened, by pressing on the coverslip, to enable easier counting of total nucleus numbers using a conventional immunofluorescence microscope equipped with an eyepiece counting grid.

The method for calculating apoptosis rates was adapted from Spanos et al. (2000). Stained nuclei were categorized as follows: 1) Compact DAPI – interphase nuclei with a distinct round outline, uniform DAPI-staining and no TUNEL labelling (Fig. 2A). 2) Mitotic DAPI - nuclei in prophase, metaphase or anaphase with no TUNEL labelling. 3) Compact TUNEL - nuclei with strong DAPI and uniform TUNEL labelling. 4) Fragmented nuclei with TUNEL labelling. 5) Fragmented nuclei without TUNEL labelling. For each embryo, all nuclei were counted and
categorized in one of these 5 groups. The cells deemed to be apoptotic were all those with TUNEL-labelled nuclei plus those with fragmented nuclei but no TUNEL labelling. The following indices were then calculated:

a) apoptotic index = apoptotic nuclei / total nuclei x 100.
b) fragmented nucleus index = fragmented nuclei / total nuclei x 100
c) mitotic index = nuclei in mitosis / total nuclei x 100.

Statistical Analysis
All culture experiments included 5-7 replicates and statistical analyses were performed using GraphPad Prism® (San Diego, CA, USA). Cleavage and in vitro embryo development rates were compared using Fishers’ exact contingency test. The effect of culture conditions on total cell numbers and on the apoptotic, fragmentation and mitotic indices was examined using either unpaired Student’s tests or a one–way ANOVA followed by pair-wise multiple comparisons (Bonferroni t-test), after testing for normality (Kolmogorov-Smirnov test) and equivalence of variances (Levene Median test with Barletts test correction). Differences were considered statistically significant if $P<0.05$.

RESULTS

In vitro embryo development

A total of 666 oocytes displaying a first polar body after 24-26 h IVM were subjected to ICSI. Of these, 349 and 317 were derived from COCs categorized as respectively compact or expanded at the onset of IVM. There was, however, no effect of cumulus morphology at the onset of IVM on the cleavage rate after ICSI (61.3% versus 63.4%, Comp and Exp-COCs, respectively: Table 2). On the other hand, there was a significant effect of culture system on embryo development rate since zygotes from compact COCs incubated in the sheep-oviduct system yielding a significantly higher blastocyst rate (16% of injected and 23.7% of cleaved oocytes) than SOF-IVC with either compact (6.3% and 12.6%) or expanded (9.4% and 15%) COCs (Table 2).

Morphology and cellular characteristics of Day 7 Embryos (Experiment 1)

Of the 69 Day 7 embryos produced by ICSI and either culture in SOF ($n = 41$) or temporary transfer to the sheep oviduct ($n = 28$), 47 were analyzed with respect to cell number, apoptosis and microfilament organization. These were compared with 10 in vivo Day 7 embryos.
Table 2: Cleavage and development of horse embryos produced by ICSI of in vitro matured oocytes which had a compact or expanded cumulus at the onset of IVM, and subsequent culture for 7 days in SOF medium or the oviduct of a progesterone treated ewe.

<table>
<thead>
<tr>
<th>Culture</th>
<th>No. oocytes</th>
<th>No. cleaved</th>
<th>Cleavage rate</th>
<th>No Day 7 embryos</th>
<th>Embryo/oocytes (%)</th>
<th>Embryo/cleaved (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOF-IVC (Comp-COCs)</td>
<td>349</td>
<td>214</td>
<td>61.3 %</td>
<td>11</td>
<td>6.3 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.6 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sheep oviduct (Comp-COCs)</td>
<td></td>
<td></td>
<td></td>
<td>28</td>
<td>16 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.72 ± 5.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOF-IVC (Exp-COCs)</td>
<td>317</td>
<td>201</td>
<td>63.4 %</td>
<td>30</td>
<td>9.4 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 ± 1.5&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Within a column, values with a different superscript differed significantly (<sup>a</sup> <i>P</i> < 0.01; <sup>b</sup> <i>P</i> < 0.05).  * The embryo rate was adjusted to account for the number of cleaved embryos actually transferred into sheep oviducts (n=118).

Embryo diameter and total cell number

Day 7 <i>in vivo</i> embryos were significantly bigger than IVP embryos (mean ± sem embryo diameters: 374 ± 64.1, 139.9 ± 1.6 and 138.1 ± 2.5 mm for <i>in vivo</i>, SOF and sheep oviduct embryos, respectively: Table 3). Not surprisingly, <i>in vivo</i> embryos also contained significantly more cells (1736 ± 568: range 176-5720) than SOF (116 ± 15: range 20-363) or sheep oviduct (86 ± 9: range 31-141) produced embryos. With regard to the IVP embryos, COC morphology at the onset of culture did not affect eventual blastocyst diameter but did significantly influence day 7 cell number which was significantly higher for exp-COCs (133 ± 16) than for comp-COCs subsequently cultured via either the SOF or sheep oviduct systems (83 ± 31 and 86 ± 9, respectively: Table 3).

Apoptosis and nuclear morphology

Apoptosis occurred at a very low rate in <i>in vivo</i> embryos, with only 4 of the 10 embryos containing apoptotic cells and only 0.5% of all nuclei being classified as apoptotic. By contrast, all IVP embryos contained apoptotic nuclei and apoptosis rates were much higher. For this reason, the apoptotic, mitotic and fragmentation
indices were compared only between the different groups of IVP embryos. In this respect, the proportion of apoptotic cells was significantly higher in embryos produced from Comp-COCs and SOF (21.5 ± 6.4%; Fig. 2) than Exp-COCs and SOF (9.6 ± 1.3%) or Comp-COCs and sheep oviduct transfer (6.5 ± 1.5%).

**Table 3:** Diameter and total cell number of Day 7 horse embryos produced *in vivo* or *ex vivo* following ICSI of oocytes derived from compact and expanded COCs and cultured in SOF (IVC), or from compact COCs and temporary transfer to the oviduct of a sheep.

<table>
<thead>
<tr>
<th>Embryo production Method</th>
<th>No.</th>
<th>Diameter (µm)</th>
<th>Number of cells (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOF-IVC (Comp-COCs)</td>
<td>11</td>
<td>140.3 ± 3.2a</td>
<td>82.91 ± 30.7c</td>
</tr>
<tr>
<td>SOF-IVC (Exp-COCs)</td>
<td>21</td>
<td>139.7 ± 1.7a</td>
<td>132.9 ± 15.9d</td>
</tr>
<tr>
<td>Sheep oviduct (Comp-COCs)</td>
<td>15</td>
<td>138.1 ± 2.5a</td>
<td>85.8 ± 9.3c</td>
</tr>
<tr>
<td><em>In vivo</em></td>
<td>10</td>
<td>374 ± 64.1b</td>
<td>1736 ± 567.9e</td>
</tr>
</tbody>
</table>

Within a column, values that differ significantly have different superscripts (*a,b P< 0.001; c,d,e P<0.05*).

A similar pattern was seen for the incidence of fragmented nuclei (13.3 ± 3.5% versus 4.9 ± 0.9% and 4.5 ± 1% for Comp-COCs-SOF, Exp-COCs-SOF and Comp-COCs-sheep oviduct, respectively), but there was no apparent effect of COC morphology or culture system on the mitotic rate. More detailed analysis of the different classes of apoptosis (condensed-TUNEL positive, fragmented-TUNEL positive or fragmented-TUNEL negative) demonstrated that the bulk of the between-IVP group difference was in the proportion of fragmented-TUNEL positive cells; indeed, the proportion of TUNEL negative apoptotic cells did not differ between groups (Fig. 3). When apoptosis rates were compared between IVP embryos of different quality, it was found that embryos considered transferable (grades I and II: 76% of all IVP embryos) had lower apoptosis rates (9% of cells)
than embryos not considered fit for transfer (grades III and IV: 22% apoptotic cells).

![Bar graph showing the effect of cumulus morphology at the onset of IVM (compact or expanded) and subsequent culture system (SOF medium or sheep oviduct) on the mean (+ SEM) percentages of cells showing evidence of apoptosis, nuclear fragmentation or cell division (mitosis) in Day 7 IVP horse embryos. Embryos derived from Comp-COCs and cultured in SOF had a significantly higher proportion of apoptotic and fragmented nuclei. Within a group, different superscripts denote values that differ significantly (P<0.05).](image)

**Fig. 2.** The effect of cumulus morphology at the onset of IVM (compact or expanded) and subsequent culture system (SOF medium or sheep oviduct) on the mean (+ SEM) percentages of cells showing evidence of apoptosis, nuclear fragmentation or cell division (mitosis) in Day 7 IVP horse embryos. Embryos derived from Comp-COCs and cultured in SOF had a significantly higher proportion of apoptotic and fragmented nuclei. Within a group, different superscripts denote values that differ significantly (P<0.05).

**Microfilament organization**
Representative MPLSM photomicrographs of Day 7 *in vivo* and *in vitro* embryos are shown in Fig. 4. IVP embryos were not only smaller and had fewer cells than their *in vivo* counterparts, they were more compact such that most were categorized as morulae (Fig. 4A, B) or early blastocysts (Fig. 4C, D), whereas *in vivo* day 7 embryos were mostly expanded blastocysts (Fig 4H, I). The distinction between inner cell mass (ICM) and trophoectoderm (TE) was thus more difficult to discern in day 7 IVP embryos, which did not have the clear nucleus-dense domain seen in *in vivo* embryos. Apoptotic cells were scattered and not concentrated in particular areas, but were seen in higher numbers in poor quality IVP embryos (Fig 4E, F).
Fig. 3. The effect of cumulus morphology at the onset of IVM (compact or expanded) and culture system (SOF or sheep oviduct) on the rate of different categories of apoptosis in Day 7 horse embryos (mean ± SEM). Day 7 embryos derived from Comp-COCs and cultured in SOF had a significantly higher proportion of fragmented TUNEL-positive nuclei. (ab P< 0.05).

Microfilaments (MF) were primarily located along the inside of the cell plasma membranes, adjacent to the cell borders. In in vivo blastocysts, MF labeling of the contiguous cell borders was intense and homogeneous throughout the whole embryo. By contrast, in IVP embryos MF labeling was generally less intense and tended to be more marked around the periphery of the embryo (Fig. 4A, D) and weaker within, with some areas almost lacking labeling (Fig. 4G). On some occasions, the actin labeling in IVP embryos appeared to have conglomerated at the junctions of several cells (Fig. 4E). The blastocele cavity of IVP blastocysts tended to be small and irregular with its borders poorly stained for MF (Fig. 4C), especially when compared to the large, well delineated cavity of in vivo blastocysts (Fig. 4H).
Fig 4: Confocal laser scanning photomicrographs to demonstrate the features of, and differences between, morphology and microfilament distribution in *in vivo* and IVP horse embryos. In all cases, nuclei are coloured blue, microfilaments red and TUNEL labelled nuclei green (these nuclei contain DNA breaks and are considered to be apoptotic).

IVP embryos had relatively few cells and were relatively compact, such that most were categorized as morulae (Fig. 4A, B) or early blastocysts (Fig. 4C, D). Day 7 *in vivo* embryos were mostly expanded blastocysts (Fig 4H, I) with a clear distinction between trophectoderm (TE) and inner cell mass (ICM). Apoptotic cells tended to be scattered but more numerous in poor quality IVP embryos (Fig 4E, F: f denotes a fragmented nucleus). Microfilaments (MF) lined the inside of the cell plasma membranes and were intense and sharp in *in vivo* embryos. In IVP embryos, MF labeling was
generally less intense but tended to be stronger around the periphery of the embryo (Fig. 4A, D) and weaker to almost absent within (Fig. 4G). On some occasions, the actin filaments were clumped at the junction of several cells (Fig 4E). The blastocoele cavity of IVP blastocysts tended to be small and much less clearly delineated by a MF border (Fig. 4C) than that of in vivo blastocysts (Fig. 4H).

Scale: In A-G bar = 30 µm, in H bar = 40 µm and in I bar = 120 µm.

Cp = compact DAPI-labeled nucleus; f and c = fragmented and compact TUNEL-labeled nuclei, respectively

Capsule glycoprotein expression (Experiment 2)

In total, twenty day 10 IVP embryos were stained with mAb OC-1 (Table 4) to examine capsule formation. These embryos had been produced by 5 day culture in sheep oviducts followed by 3 days either in a cell-free system (n=10) or with a monolayer of adult horse fibroblast cells (n=10). All day 10 IVP embryos were classified as partially hatched, since part of the embryo had herniated via the hole made during ICSI. In these embryos, a distinct layer of OC-1 positive ‘capsule’ was visible lining the inside of the ZP (Fig. 5A, B) and extending into the transzonal channels of the latter (Fig. 5E). When the ZP was separated from its contained embryo by micromanipulation, the capsular material remained stuck to the ZP and not to the trophectoderm (Fig 5F) demonstrating that it was more intimately associated to the former. In the area of embryo herniated from the ZP, capsular glycoprotein was present on the apical surface of the trophectoderm cells (Fig. 5A, B) as scattered small patches that were not assembled into a confluent layer (Fig. 5 I, H). Day 7 IVP embryos (3 from SOF and 1 from sheep-oviduct cultures) showed only weak OC-1 labeling in scattered patches on the apical surface of trophectoderm cells, again without assembly into a confluent capsule and, in these cases, with little infiltration into the substance of the ZP (Fig 5. C, D). By contrast, in two day 6 in vivo embryos examined (categorized as late morulae) a clear thick confluent capsule was sandwiched between the relatively thick ZP and the trophectoderm surface and there was no infiltration into the transzonal channels of the ZP (Fig. 5J). Older in vivo hatched and expanded blastocysts, had a complete capsule apposed tightly to the trophectodermal surface and these capsules displayed the classical bilaminar appearance (Fig. 5K) described previously by Oriol et al. (1993).

Because OC-1 showed such strong affinity for the ZP of IVP embryos, a number of in vitro matured COCs (n=4) were also stained with OC-1, to ensure that there was no cross-reaction with ZP after in vitro culture. In these cases, there was no OC-1 staining of any part of the ZP (Fig. 5L), demonstrating clearly that staining of the ZP in IVP embryos was a function of embryonic secretion of OC-1 reactive glycoproteins.
Features of horse IVP embryo’s
Fig 5: Confocal laser scanning micrographs to show the distribution of OC-1 reactive molecules (capsular glycoproteins) in IVM oocytes, and IVP and in vivo embryos. In all cases, nuclei are labeled blue, microfilaments red and capsular glycoproteins green.

A. Capsule material lining the inside of and permeating through the ZP of a day 10 IVP embryo. OC-1 staining is also seen (arrow) on the outer surface of the trophectoderm cells that have herniated out of the ZP via the hole made during ICSI.

B. Day 10 IVP embryo with strong OC-1 staining on the herniated ZP-free trophectoderm cells. Cell density in the ZP enclosed portion of the embryo is much higher than in the ‘unrestricted’ herniated area.

C. Capsular material on the trophectodermal surface of a day 7 IVP embryo derived from sheep-oviduct culture.

D. Accumulation of capsule material beneath the ZP of a day 7 IVP embryo derived from in vitro culture.

E. Capsular material permeating through the transzonal channels of a day 10 IVP embryo.

F. Separation of the ZP from the trophectoderm by micromanipulation demonstrated that the ‘pseudocapsule’ was adhered to the ZP rather than the trophectoderm.

G. Day 10 IVP embryo with a dense actin ‘neck’ between the zona-contained and herniated parts of the embryo.

H. Herniated part of a Day 10 IVP embryo with considerable quantities of capsular material on the surface but without coalescence into a confluent layer.

I. A day 10 IVP embryo that, during culture with a fibroblast monolayer, attached to the surface of the well. It is presumed that failure of OC-1 reactive glycoproteins to coalesce prevented them from exerting the anti-adhesive properties that have been ascribed to the capsule.

J. Day 6.5 in vivo early blastocyst with a distinct well-defined capsule and a very clear microfilament cytoskeleton delineating individual cells.

K. High magnification image of the capsule and trophectoderm of a day 7 in vivo blastocyst to show the bilaminar appearance of the capsule after staining with mAb OC-1.

L. Cumulus oocyte complex stained after IVM to demonstrate that mAb OC-1 did not cross react with zona pellucida.

Scale: In A-C, I and K bar = 50 µm and in D-G, J and L bar = 10 µm
### TABLE 4. Immunolocalization of capsular material on early *in vivo* and IVP horse embryos.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Stage</th>
<th>No.</th>
<th>Size (µm)</th>
<th>No. of nuclei</th>
<th>Site of mAb OC-1 staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 6</td>
<td>2</td>
<td>200; 290</td>
<td>364; 979</td>
<td>Thin capsule sandwiched between ZP and trophectoderm</td>
</tr>
<tr>
<td>In Vivo</td>
<td>Day 7</td>
<td>1</td>
<td>730</td>
<td>3939</td>
<td>Distinct capsule enveloping the conceptus</td>
</tr>
<tr>
<td></td>
<td>Day 8</td>
<td>1</td>
<td>940</td>
<td>10,400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 9</td>
<td>1</td>
<td>1640</td>
<td>44,500</td>
<td></td>
</tr>
<tr>
<td>In Vitro</td>
<td>Day 10</td>
<td>10</td>
<td><em>165 ± 9.0 / 136.5 ± 5.0</em></td>
<td>531 ± 45.8b</td>
<td>Accumulation on the inside of the ZP and infiltration into the transzonal channels. Scattered labelling of trophectoderm cells of the “herniated” areas</td>
</tr>
<tr>
<td></td>
<td>Day 10</td>
<td>10</td>
<td><em>148 ± 2.9 / 122 ± 29.1</em></td>
<td>438.6 ± 61.1b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>3</td>
<td>137 ± 1.2</td>
<td>112 ± 61.7</td>
<td>Small patches of labelling on the surface of trophectoderm cells</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>1</td>
<td>140</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>COCs</td>
<td>IVM Comp-COCs</td>
<td>2</td>
<td>130 ± 4.5</td>
<td>Metaphase II stage</td>
<td>No labelling</td>
</tr>
<tr>
<td></td>
<td>IVM Exp-COCs</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*mAb OC-1: monoclonal antibody raised against the capsule of day 13-15 horse conceptuses (Oriol et al., 1993)*

* Diameters of the embryonic portions within/herniated from the ZP.

*a,b* Within a column, values which did not differ significantly have the same superscripts (*P*>0.001).
DISCUSSION

This study demonstrated that in vitro produced horse embryos differ markedly from their in vivo produced counterparts by having fewer cells, lower morphological quality scores and a higher incidence of apoptotic cells (Table 3). These observations are similar to those for IVP bovine embryos which are consistently of lower quality than embryos produced in vivo, both in terms of morphological quality (Fair et al., 2001) and with regard to the expression of developmentally important genes (Rizos et al., 2002). In cattle, these differences have been shown to affect embryo survival following cryopreservation adversely and to compromise embryonic development (Rizos et al., 2001). On the other hand, because only a few foals have so far been produced from IVP embryos (Galli et al., 2001a, 2002; Li et al., 2001), it is too early to determine if and to what degree in vitro production adversely affects embryo development in this species. It is, however, clear that despite the apparently poor quality and low cell numbers of IVP equine embryos, they are able to establish pregnancies following transfer to recipient mares (Galli et al., 2001a; Li et al., 2001). With regard to embryo cell number, Allen and Pashen (1984) similarly demonstrated that embryos produced by the mechanical separation of blastomeres from 2-to-8 cell horse embryos and insertion into evacuated pig zona pellucidae were able to establish pregnancies despite low cell numbers. Together, these observations suggest that low cell number can be rapidly compensated for after transfer of embryos into the uteri of recipient mares.

As in previous studies (Galli and Lazzari, 2001; Galli et al., 2002), embryo development was more efficient when zygotes were transferred temporarily to the oviduct of progesterone treated sheep (23% blastocysts) than when they were cultured solely in vitro (14%). However, the number of cells in day 7 IVP embryos derived from compact COCs and either cultured in SOF medium or transferred to a sheep’s oviduct did not differ. By contrast, cell numbers were higher in day 7 IVP embryos originating from expanded COCs and cultured in SOF medium. The effects of this relatively rapid increase in cell number in vitro, of embryos derived from expanded as compared to compact COCs, on later embryo development is however difficult to predict. At first glance, a higher cell number would appear to be a positive sign of embryo viability. On the other hand, in other species, relatively rapid development in vitro has proven detrimental to the resulting offspring (van Wagendonk-de Leeuw et al., 2000). That the rate of apoptosis (cells with DNA strand breaks and/or nuclear fragmentation) was significantly higher in SOF than sheep oviduct embryos (22 versus 7%), and much higher in both than in vivo produced embryos suggests a detrimental effect of culture on embryo development. Similarly increased rates of apoptosis have been observed in cattle (Byrne et al.,
1999) and pig (Kidson et al., 2002) embryos produced in vitro and it has been
further suggested that sub-optimal culture conditions may cause the proportion of
affected cells to increase to a level where further embryonic development is
critically compromised, particularly in embryos with low cell numbers (Brison and
Schultz, 1997; Jurisicova et al., 1998). On the other hand, apoptosis has also been
proposed to be a physiological process during mammalian preimplantation
embryogenesis, presumably acting as a quality control mechanism to eliminate
aberrant cells (Jurisicova et al., 1995; Meier et al., 2000). In either case, the
marked difference in the proportion of apoptotic cells between in vivo and IVP
horse embryos, suggests that culture adversely affected embryo development.

IVP embryos were produced at similar rates from oocytes that had a compact or an
expanded cumulus at the onset of IVM, although the overall percentage of injected
oocytes developing into blastocysts was low (mean=14%). Previous studies have
suggested a greater ability of oocytes with an expanded cumulus at recovery to
form a male pronucleus after ICSI (Dell’Aquila et al., 1997, 2001), although no
differences in nuclear maturation rates were observed. One possible explanation for
this finding was that COCs with an expanded cumulus had better cytoplasmic
maturation and were thereby better able to support embryo development. Although
the current study did not show any significant difference in cleavage rates between
Exp- and Comp-COC oocytes, embryos resulting from SOF culture after ICSI of
Exp-COC oocytes had more cells and a lower incidence of apoptosis than those
from Comp-COCs. Interestingly, the major difference in the proportion of
apoptotic cells was a higher level of cells with fragmented nuclei in Comp-COC
derived embryos. Apoptosis is considered to progress from condensation of
chromatin and cytoplasm to DNA fragmentation accompanied by indentation and
convolution of the nuclear membrane. TUNEL labels the DNA breaks in situ and
thus identifies cells fairly early in the apoptotic process (Gavrieli et al., 1992)
whereas fragmentation of the convoluted nucleus with blebbing and fragmentation
of the whole cell into membrane bounds apoptotic bodies occurs much later in the
apoptotic process (for review see Wyllie et al., 1980). The raised incidence of
nuclear fragmentation in Comp-COC derived embryos is therefore indicative of
more advanced apoptotic changes which could relate to a higher sensitivity to
culture conditions and/or presence of more serious internal defects in these
embryos (Jurisicova et al., 1995). However, the quality (based on apoptotic index)
of embryos derived from Comp-COCs was significantly improved when zygotes
were transferred to and cultured in sheep oviducts, thereby identifying sensitivity to
the culture conditions as the most likely critical factor. Overall, this data suggests
that current IVM protocols are still sub-optimal with respect to the acquisition of
developmental competence by equine oocytes, especially those less advanced oocytes with a compact cumulus at the time of recovery.

Staining of the microfilament cytoskeleton helped to identify differences in cellular organization between in vivo and in vitro embryos. In vivo day 7 embryos were expanded blastocysts in which the microfilaments delineated the cell borders and highlighted the division between a distinct ICM and the TE layer (Fig 4F). By contrast, IVP embryos were compact without, or with only a small, blastocoel cavity and patchy microfilament distribution. Actin microfilaments play an important role in cell cleavage during embryo development (Maro et al., 1986) and are essential for the distribution of mRNA and organelles such as mitochondria and the Golgi apparatus between daughter cells (Bassell et al., 1994; Volderrama et al., 1998). The polymerization and depolymerization of actin filaments are, however, dynamic processes susceptible to disruption by environmental conditions (temperature, pH, culture medium; Barnett et al., 1997). Moreover, microfilament disruption can adversely affect the structural integrity of cells, with serious consequences for their metabolic activity. Abnormal actin filament distribution has been suggested as a reason for abnormal embryo cleavage in IVP pig embryos (Wang et al., 1999) because embryo division can be similarly blocked by cytochalasin D, an inhibitor of microfilament polymerization. In general, cells store a large pool of nonfilamentous actin (G-actin; Wang et al., 2000) to maintain their ability to quickly reorganize filamentous actin in response to environmental changes or need. Whether the pool of G-actin is low or impaired in IVP horse embryos, thereby decreasing their potential to overcome suboptimal culture conditions, has yet to be investigated.

The combination of monoclonal antibody OC-1 and confocal microscopy enabled three-dimensional imaging of capsule glycoprotein distribution in early embryos. In turn, this enabled us to demonstrate that the capsule assumes its classical bilaminar appearance around in vivo embryos from very soon after its initial formation (Fig. 5K); previously OC-1 expression and appearance of the capsule after OC-1 staining had not been reported for embryos recovered earlier than day 11 of gestation (Oriol et al., 1993a). Production of capsular glycoproteins by the trophoblast cells of IVP embryos was also demonstrated by OC-1 labeling of the apical surface of these cells, thereby confirming the hypotheses that the initial layer of capsule is formed from OC-1 reactive glycoproteins and that early trophoblast cells secrete capsular glycoproteins independent of a maternal (endometrial) input (Albihn et al., 2003). Intriguingly, in vitro the capsular glycoproteins failed to assemble into a normal and complete capsule enveloping the embryo, suggesting either that some aspect of the uterine environment is necessary for glycoprotein
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coalescence or that some aspect of the in vitro environment otherwise hindered capsule formation. In the former respect, because the glycoproteins of the capsule are mucin-like, Oriol et al. (1993a) postulated that they, like other mucins, may coalesce to form a gel-like mucus layer by hydration and cross-linking (Verdugo, 1991) after they have accumulated in sufficient quantities on the surface of the trophoderm. Oriol et al. (1993a) further suggested that failure of capsule formation in vitro might thus be due to dispersal of the glycoproteins into the culture medium, or unsuitability of the microenvironment for hydration and cross-linking. The current study suggests that the former is a minor problem since capsule glycoproteins were found on the surface of the trophoblast and lining and permeating through the ZP. Nevertheless, it cannot be discounted that a degree of dispersal prevented glycoprotein concentrations reaching a critical concentration needed to initiate coalescence. In this latter respect, it is also likely that the absolute production of capsular glycoproteins by IVP embryos was relatively low, due primarily to their low numbers of cells. There are, however, comparable reports of reduced secretion of other high molecular weight mucins in culture such that they fail to assemble into a mucus layer (Virmani et al., 1992). One interesting observation with regard to the proposed functions of the capsule, was that the herniated part (ZP-free) of one of the embryos cultured with an AFC monolayer adhered to the cell layer, despite the presence of capsular glycoproteins on the surface of the adhered cells (Fig 5I). The capsule has been proposed to confer anti-adhesion properties, by means of its high content of negatively charged sialic acid residues, on the mobile pre-day 17 conceptus (Oriol et al., 1993b; Chu et al., 1997). The adhesion of glycoprotein secreting trophoderm cells to the monolayer suggests that the anti-adhesive property is acquired only after coalescence into a complete layer.

In the present study, considerable quantities of capsule material were detected as an accumulation between the trophoblast and the ZP of IVP embryos (Fig. 5D). This was reminiscent of the flocculent material detected between the trophoblast and ZP of in vivo embryos by Flood et al.(1982) and Wilson et al.(1986) which they postulated, but could not prove, to be capsule precursor material. An striking feature of IVP embryos was the level to which the ZP was lined and infiltrated with capsular glycoproteins, something not seen in in vivo embryos. The possibility that mAbOC-1 was cross-reacting with the ZP per se was ruled out by the failure to label the ZP of either in vivo embryos or IVM oocytes (Fig. 5L). Instead it appears that during culture in vitro, capsule material adheres to the inside of the ZP and permeates through the cumulus cell-created transzonal channels, presumably as a consequence of the failure of the glycoproteins to coalesce on the trophodermal surface. By contrast, in vivo capsule formation does not require the presence of a
ZP since zona-free bisected blastocysts have been shown to form a capsule after transfer to the uterus of recipient mares (McKinnon et al., 1989). This again indicates that the greatest obstacle to capsule formation in vitro is probably that the microenvironment fails to replicate the conditions necessary for glycoprotein cross-linking. In day 10 IVP embryos, a ‘pseudo’-capsule formed lining the inside of the ZP (Fig. 5E) that was not seen around ZP-free areas of embryo (Fig. 5H). This capsule was tightly adhered to the ZP, as demonstrated during removal of the latter by micromanipulation (Fig. 5F); the capsule peeled away with the ZP.

In the current study, in vitro cultured embryos escaped from their ZP by herniating through a hole in the ZP (in this case the hole made during ICSI), as previously reported by Hinrichs et al. (1990) and Hochi et al. (1993). In other species, the exact mechanism of hatching from the ZP is also unclear, although it is thought that, in vivo, proteolytic enzymes released by the maternal endometrium are most likely to be responsible for ZP dissolution (O’Sullivan et al., 2002). The current observations demonstrate that loss of the horse ZP in vitro is very different to the apparently rapid loss in vivo (intermediate stages are rarely found), in which the ZP is thought to be attenuated and ruptured by a combination of a uterine zonalytic and blastocyst expansion (Betteridge et al., 1980; Flood et al., 1982). Although the uterus seems to critically influence the mode of zona loss from horse embryos, the existence of a uterine zonalytic has yet to be demonstrated in this species (NB there appears to be no uterine zonalytic in cattle; Betteridge et al., 1980). It is therefore tempting to speculate that the essential roles of the uterus during physiological zona loss and capsule formation may be linked. Finally, it has been proposed that the capsule is essential to the survival of horse embryos in vivo because embryos transferred after capsule removal either do not develop into pregnancies (Stout et al., 1997) or do so only after forming a new capsule (McKinnon et al., 1989). If IVP embryos do not form a normal capsule during culture then it is likely that transfer to the uterus of the recipient mare at a stage when capsule formation can still occur will be essential to the success of this procedure. Since the transfer of day 7 IVP late morulae or early blastocysts has resulted in pregnancy in mares, it must be assumed that subsequent capsule coalescence can and does occur.

In summary, the present study represents the first detailed description and comparison of the morphological, cytoskeletal and developmental characteristics of in vitro and in vivo produced horse embryos. Day 7 IVP embryos were smaller, had fewer cells and were more compact than in vivo embryos of similar age. In addition, Day 7 IVP embryos had a small or nonexistent blastocoele cavity, an indistinct inner cell mass and had still not properly (or normally) hatched after 10
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days of culture. IVP embryos also displayed high percentages of apoptotic cells (10% compared to 0.3% for in vivo embryos), a disturbed pattern of microfilament distribution and irregularities in cell size and shape. Finally, while IVP embryos remained viable, continued to develop for at least 10 days in vitro and secreted capsular glycoproteins, the latter failed to coalesce to form a confluent capsule, a structure that is a prominent and apparently vital feature of in vivo blastulation.

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