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MATERNAL RECOGNITION OF PREGNANCY IN THE MARE III

13th – 16th November 2004
Barbados, West Indies

Editors: T. A. E. Stout and J. F. Wade

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EDITOR'S FOREWORD

A precisely regulated and intricate exchange of biochemical messages between a conceptus and its dam is critical to the establishment, maintenance and normal development of pregnancy in eutherian mammals. One of the most striking examples of embryo-maternal dialogue is 'maternal recognition of pregnancy' (MRP), the process by which the developing conceptus prevents luteolysis and, in so doing, prolongs the lifespan of the maternal primary corpus luteum (CL), the source of the progesterone that is essential to its continued survival and development. Given the essential nature of MRP, and the knowledge that a large proportion of detected pregnancy losses in mares occurs during or soon after the period of pregnancy recognition, it is surprising that the equine conceptus signal(s) responsible for MRP has yet to be identified. It is even more surprising given that, in the other large domestic animal species, not only are the primary anti-luteolytic signals described and characterised, but a wealth of information has been built up on the signalling pathways by which they operate and on 'back-up' luteostatic or anti-luteolytic messengers that further reduce the likelihood of inappropriate loss of the primary CL. There is also a growing body of literature on uterine proteins that are expressed and secreted in response to conceptus signals, and that appear to play important roles in trophectoderm-endometrium interaction during conceptus attachment, implantation and placentation. In short, it is clear that our understanding of conceptus-maternal signalling during early pregnancy in the mare currently lags behind that in other species, but that improving our understanding of this topic should be of interest to the horse breeding industry because it may lead to strategies to reduce the incidence of early pregnancy loss.

This workshop on Maternal Recognition of Pregnancy in the Mare was the third in a series sponsored by the Havemeyer Foundation. As with previous meetings in the series (1997 and 1999), the workshop benefited greatly from the input of scientists interested primarily in species other than the horse. It is sincerely hoped that the vivid descriptions of the biological pathways responsible for MRP in other species, and of the molecular biological techniques available for their investigation, will provide useful pointers on how to finally unravel aspects of the enigma of early equine pregnancy.

We are once again very grateful to the Havemeyer Foundation and, in particular to its President Mr Gene Pranzo, for their generous support of this workshop, and to Jan Wade and her colleagues at R&W Communications for arranging the venue, the programme and for performing the onerous organisational and administrative duties. It was a great shame that neither Mr Pranzo nor Jan were able to join us for the workshop, but Louise Holder was a more than able and very popular representative for both R&W and the Havemeyer Foundation.

On a more poignant note, the location of this meeting was chosen primarily to honour the memory of the late Francesca Stewart. The last Maternal Recognition of Pregnancy Workshop was also held in Barbados, and it proved to be the last scientific meeting that Francesca was able to attend; soon afterwards, the extent of her illness became evident. Francesca visibly enjoyed that last meeting both socially and scientifically; the subject area was one close to her heart. It seemed fitting, therefore, to return to Barbados for this follow-up meeting, a feeling that was reinforced by the number of references to Francesca and her work during the course of the workshop; she is missed by many, as both a scientist and a friend.

Tom Stout

HAVEMEYER SCIENTIFIC WORKSHOPS

1981 **First International Workshop on Lymphocyte Alloantigens of the Horse**
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Organiser: Dr D. F. Antczak

1982 **Second International Workshop on Lymphocyte Alloantigens of the Horse**
October - Cornell University, Ithaca, New York, USA
Organiser: Dr D. F. Antczak

1983 **Third International Workshop on Lymphocyte Alloantigens of the Horse**
April - New Bolton Center, University of Pennsylvania, USA
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1984 **First International Symposium on Equine Embryo Transfer**
October - Cornell University, Ithaca, New York, USA
Organisers: Drs D. F. Antczak and W. R. Allen

1985 **Fourth International Workshop on Lymphocyte Alloantigens of the Horse**
October - University of Kentucky, USA
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1986 **Workshop on *Corynebacterium equi* Pneumonia of Foals**
July - University of Guelph, Canada
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1987 **Fifth International Workshop on Lymphocyte Alloantigens of the Horse**
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1989 **Second International Symposium on Equine Embryo Transfer**
February - Banff, Alberta, Canada
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1990 **International Workshop on Equine Sarcoids**
April - Interlaken, Switzerland
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1992 **Workshop on Equine Neonatal Medicine**
January - Naples, Florida
Organisers: Drs D. F. Antczak and P. D. Rossdale

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1995

Equine Perinatology
July - Cambridge, England
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Second International Equine Leucocyte Antigen Workshop
July - Lake Tahoe, California, USA
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First International Workshop on Equine Gene Mapping
October - Lexington, Kentucky, USA
Organisers: Drs D. F. Antczak and E. Bailey

Erection and Ejaculation in the Human Male and Stallion: A Comparative Study
October - Mount Joy, Pennsylvania, USA
Organiser: Dr S. M. McDonnell

Bone Remodelling Workshop
October - Corcord, Massachusetts, USA
Organiser: Dr H. Seeherman

1997

Second International Workshop on Equine Gene Mapping
October - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Maternal Recognition of Pregnancy in the Mare
January - Dominican Republic
Organisers: Drs W. R. Allen and T. A. E. Stout

Uterine Clearance
March - Gainesville, Florida, USA
Organiser: Dr M. M. LeBlanc

Trophoblast Differentiation
September - Edinburgh, Scotland
Organisers: Drs D. F. Antczak and F. Stewart

1998

Third International Genome Workshop
January - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Third International Workshop on Perinatology: Genesis and Post Natal Consequences of Abnormal Intrauterine Developments: Comparative Aspects
February - Sydney, Australia
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Horse Genomics and the Genetic Factors Affecting Race Horse Performance
March - Banbury Center, Cold Spring Harbor, New York, USA
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Allergic Diseases of the Horse
April - Lipica, Slovenia
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Equine Placentitis Workshop
October - Lexington, Kentucky, USA
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Septicemia II Workshop
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Organiser: Dr M. R. Paradis

1999

Equine Genome Project
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June - Uppsala, Sweden
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Fourth International Meeting of OIE and WHO Experts on Control of Equine Influenza
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2000

Equine Genome Project
January - San Diego, California, USA
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5th International Symposium on Equine Embryo Transfer
July - Saari, Finland
Organiser: Dr T. Katila

2001

USDA International Plant & Animal Genome Conference
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Equine Immunology in 2001
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From Elephants to Aids
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International Equine Gene Mapping
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Second Meeting of the European Gamete Group (EEGG)
September - Loosdrecht, The Netherlands
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2002

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Comparative Neonatology/Perinatology
January - Palm Springs, California
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Stallion Behavior IV
June - Reykjavik, Iceland
Organisers: S. McDonell and D. Miller

Rhodococcus Equi II
July - Pullman, Washington
Organiser: J. Prescott

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August - Dublin, Ireland
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September - Boston, USA
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2003

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Embryonic and Fetal Nutrition
May - Ravello, Italy
Organiser: S. Wilsher

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Organiser: D. F. Antczak

Fifth International Gene Mapping Workshop
August - Kreuger Park, South Africa
Organiser: E. Baily and E. Vandyke

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September - Stratford-upon-Avon, UK
Organisers: P. Dixon and E. Robinson

Transporting Gametes and Embryos
October - Brewster, Massachusetts
Organiser: E. Squires

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October - Pardubice, Czech Republic
Organiser: J. and Z. Müller

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October - Lexington, USA
Organiser: F. Bain and J. Taub-Dargatz

2004

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January - San Diego, California

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August - Rio de Janiero, Brazil
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SESSION 1:

Luteolysis in the mare

Chairman:

K. J. Betteridge

LUTEAL AND ENDOMETRIAL INTERDEPENDENCE FOR EARLY EMBRYONIC DEVELOPMENT IN THE MARE

W. R. Allen, S. Wilsher, M. Kölling and A-C. Lefranc

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Nowadays in equine stud veterinary practice worldwide, twin conceptuses are reduced routinely to an ongoing singleton pregnancy by rupturing ('pinching') one conceptus via manual pressure exerted through the rectal wall, usually 14–18 days after ovulation (Morris and Allen 2002).

To investigate possible non-pharmacological methods to suppress unwanted oestrous behaviour in Sporthorse mares, a singleton conceptus was 'pinched' at 16–22 days after ovulation in 11 pregnant Pony mares. They all then passed into prolonged dioestrus ('pseudopregnancy') for a mean (±sd) of 82 ± 13 days in 10 of them (range 64–109 days), and for 228 days in the 11th mare when luteolysis was induced with a prostaglandin analogue. Oestrous behaviour was not expressed, uterine tone remained high, the cervix remained closed and peripheral serum progestagen concentrations were >2 ng/ml throughout the prolonged dioestrus in all the mares (Lefranc and Allen 2004).

Subsequently, a further 8 Thoroughbred mares were similarly induced to pass into prolonged dioestrus by 'pinching' a singleton conceptus at 14–18 days after ovulation, following which a good quality Day 7 blastocyst, recovered freshly from a healthy donor mare, was transferred non-surgically (Wilsher and Allen 2004) to the uterus of each mare in prolonged dioestrus. These transfers took place between 20 and 52 'equivalent days of gestation' in the recipient mares and despite the persistence of high serum progestagen concentrations after the transfer, none became pregnant.

Superfetation, the unintended establishment of a second pregnancy in an already pregnant female, has been verified in women (Soudre *et al.* 1992; Tuppen *et al.* 1999) and a cow (Hall 1987). Further, an unsubstantiated report in a 19th century farmer's journal described a mare, mated to a horse stallion in one oestrous period and a Jack donkey in the next; which produced twin foals, one a horse and one a mule. Due to the negative results obtained in the previous experiment when transferring embryos to recipient mares in prolonged dioestrus, an attempt was made to create asynchronous superfetation in the mare by transferring a second embryo to the uterus of 10 recipient mares that had been inseminated previously and were known to be pregnant on the basis of ultrasonographic recognition of a

TABLE 1: Attempts to induce superfetation in pregnant mares by asynchronous embryo transfer

Age of first embryo at time of transfer (days)	Age of second transferred embryo (days)	Degree of asynchrony (days)	Pregnancy outcome 7 days after ET	
			Existing embryo	Added embryo
P18	7	+11	1*	0
P15	7	+8	1	0
P14	7	+7	1	1†
P14	7	+7	1	0
P12	7	+5	0	0
P12	7	+5	1	0
P11	7	+4	1	0
P11	7	+4	0	0
P11	8	+3	1	1
P11	8	+3	1	1

P = pregnant; ET = embryo transfer; * this became an anembryonic trophoblast vesicle; † a 2 mm vesicle was visible for only 3 days

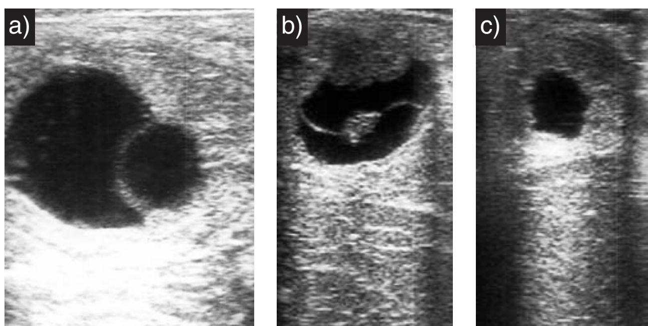

Fig 1: Ultrasound images of asynchronous conceptuses in 2 mares to which a second Day 8 embryo was transferred to the uterus containing an established Day 11 conceptus: a) the Day 17 and Day 14 conceptuses abutted to one another in the same uterine horn of one mare; b) a normal Day 32 conceptus in one uterine horn; and c) a severely retarded Day 29 conceptus in the other uterine horn of the second mare.

conceptus in the uterine lumen. The results are summarised in Table 1.

None of the 8 pregnant recipient mares that received a Day 7 blastocyst which was ≥ 4 days asynchronous with the age of the existing incipient conceptus, gestated twins, even though 5 (71%) continued to carry the first embryo normally after the transfer and a sixth mare remained 'pregnant', but with an anembryonic trophoblast vesicle. On the other hand, both mares that received an embryo which was only 3 days asynchronous with the incipient pregnancy, began to gestate twin conceptuses. In one mare the 2 differently sized conceptuses abutted against each other in one uterine horn (Fig 1a) and the smaller, transferred conceptus grew no further, failed to exhibit a visible embryo and disappeared completely between Days 25 and 30 of gestation, in the same manner and during the same period of gestation as one of naturally occurring unicornuate twin conceptuses is often observed to do (Ginther 1985); and presumably as a result of the histotrophe-imbibing choriovitelline portion of the conceptus membranes being abutted against its co-twin rather than the endometrium (Allen 2001). The other superfetation pregnancy was more interesting in that, despite the conceptuses being situated bi-cornually, the second transferred embryo remained very much smaller, and rather static, compared to the normally developing incipient conceptus between Days 14 and 31 after ovulation (Figs 1b and c). Intrauterine videoendoscopy of both conceptuses on Day 31, followed by their recovery using non-surgical uterine lavage, revealed the incipient conceptus to be of normal size and appearance for the stage, whereas the smaller transferred conceptus, although still clearly viable, measured only 2 cm in diameter and was more similar in size to a Day 12–14 conceptus than its nominal Day 28 stage (Fig 1c). Despite this dramatic reduction in size, the 'midget' conceptus nonetheless showed clear evidence of an allantois containing a typical tracework of primitive blood vessels developing within the chorion which appeared to be associated with a vestigial embryo-like structure.

These essentially negative results indicate that *in vivo* development of the equine embryo between Days 8 and 30 after ovulation requires obligatory, and seemingly stage-dependent, products of the progesterone dominated endometrium in order to proceed normally. Whether or not an existing embryo can actively suppress the development of a second 'passenger' embryo remains to be determined but, in any event, it must be concluded that prolonged or persistent dioestrus, rather than pseudopregnancy, would be more appropriate terminology to describe a non-pregnant mare that exhibits failure of cyclical luteolysis for whatever reason.

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CERVICAL DILATATION – A METHOD FOR STUDYING EMBRYO-MATERNAL INTERACTION IN MARES

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INTRODUCTION

The equine conceptus interacts very early – before classical maternal recognition – with the maternal organism. For example, it stimulates its own descent from the oviduct to the uterus by secretion of prostaglandin E_2 (PGE) from Day 5 after ovulation. Prostaglandins, secreted from the trophoblast, are also thought to be responsible for conceptus mobility in the uterus (reviewed by Allen 2001). Therefore, it was hypothesised that the equine conceptus might cause further changes in the maternal organism ensuring the establishment of pregnancy before maternal recognition takes place. To investigate pathways responsible, a method for interacting with the embryo-maternal system was developed: cervical dilatation (Handler *et al.* 2003b).

In cycling mares, cervical dilatation stimulates secretion of oxytocin, decreases plasma progesterone concentrations and shortens the length of dioestrus and the oestrous cycle by almost 2 days. However, cervical dilatation did not provoke the release of PGF2α (Handler *et al.* 2003b). These results led us to investigate the effects of cervical dilatation in pregnant mares, with special attention to the role of oxytocin (Handler 2001): 2 studies were performed in inseminated mares. In Experiment 1, cervical dilatation was combined with injection with the α-sympathomimetic agent xylazine, which inhibits the secretion of oxytocin by the posterior pituitary gland (Handler *et al.* 2002, 2003a). In Experiment 2, the effects of oxytocin, carbetocin (a long acting, synthetic analogue of oxytocin) and cervical dilatation on the pregnancy rate, embryonic development and the secretion of progesterone were compared.

MATERIALS AND METHODS

Cervical dilatation

Cervical dilatation was performed by a technique described earlier (Handler *et al.* 2003b). Briefly, a balloon-catheter was inserted into the cervical lumen via a tulip-formed insertion aid and the balloon inflated up to 4.5 cm in diameter on Day 7 after ovulation. The catheter remained in place for 10 min.

Experiment 1

Seven mares (4 Standardbreds, 2 Warmbloods, one Morgan) were inseminated with raw semen from a pony stallion of proven fertility, just prior to ovulation during 2 successive oestrous cycles. On Day 7 after ovulation, xylazine (0.4 mg/kg bwt; Rompun™) was administered iv to all mares shortly before treatment (control, dilatation) started. The mares were examined daily by ultrasonography during oestrous for detection of ovulation, and from Day 6 to 15 (ovulation = Day 0) to monitor ovarian function and embryonic development.

Experiment 2

Eight Haflinger mares were mated once or twice with a Haflinger stallion prior to ovulation during 4 successive oestrous cycles. Each mare had to undergo 4 treatments, which were performed on Day 7 after ovulation: 1) control; 2) oxytocin (10 IU/mare; Synpitan™); 3) carbetocin (280 µg/mare; Depotocin™); and 4) cervical dilatation. The mares were examined daily by ultrasonography during oestrous for detection of ovulation, and from Day 6 to 13 to monitor ovarian function and embryonic development.

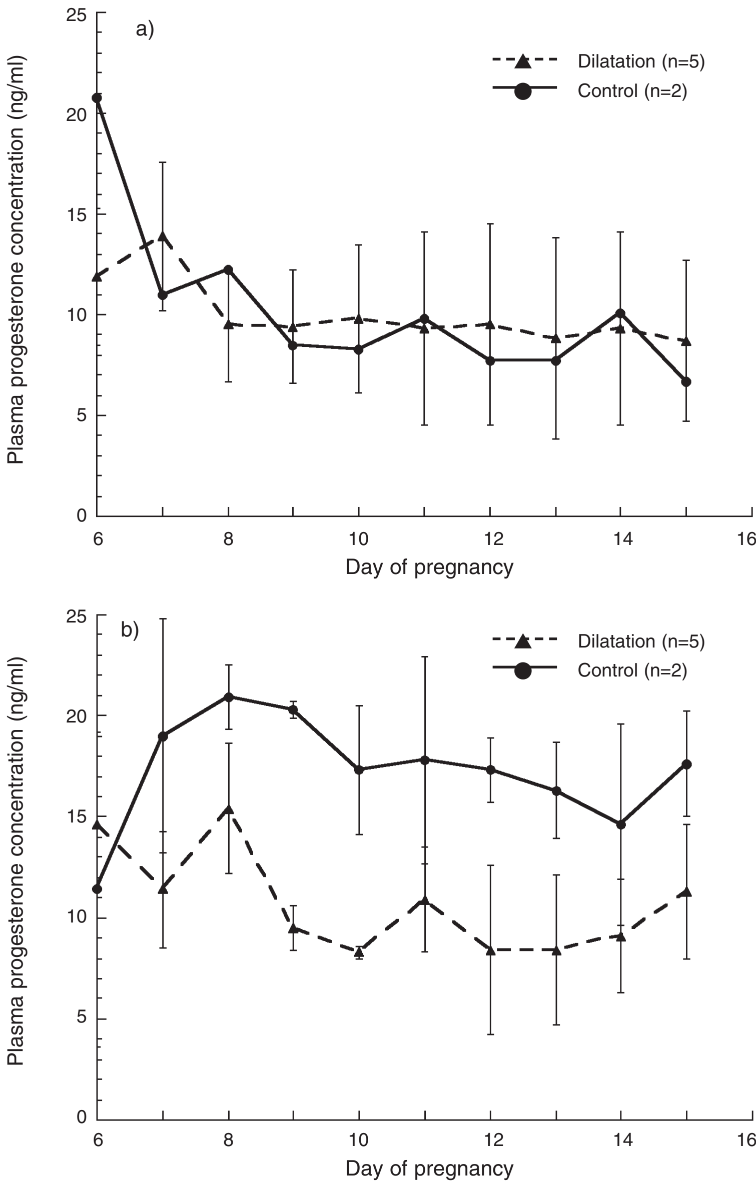

Fig 1: Daily plasma progesterone concentrations in mares carrying singleton (a) and twin (b) pregnancies in control and dilatation groups.

Statistical evaluations (descriptive statistics, Wilcoxon signed rank test) and calculations (embryonic cross-sectional area) were carried out using a software programme (Stat View 4.2; Abacus concepts, California, USA).

Results

Experiment 1

In inseminated mares, cervical dilatation did not affect pregnancy rates: all mares subjected to dilatation (7/7) became pregnant compared to 5 of 7 in the control group. Furthermore, embryonic growth rate from Day 12 to 15 after ovulation was significantly greater in the dilatation group than in controls (4.5 ± 0.4 vs. 3.9 ± 0.4 mm/day). During the whole observation period, there were no incidences of early embryonic death. Secretion of progesterone revealed variable patterns among treatments. In mares carrying singleton pregnancies, plasma concentrations of progesterone were similar in the control group (n=2) and the

dilatation group (n=5), whereas concentrations were higher in controls (n=3) than in the dilatation group (n=2) in mares carrying twins (Fig 1). Plasma concentrations of oxytocin remained at basal levels during both treatments.

Experiment 2

In inseminated mares, neither cervical dilatation nor treatments with an oxytocin preparation affected pregnancy rates. All mares (8/8) became pregnant in control, oxytocin and carbetocin groups and almost all mares (7/8) in the dilatation group. Evaluation of embryonic growth rate revealed no significant differences between treatments. Expansion rates between Days 10 and 13 after ovulation varied from 3.3 to 3.6 mm/day. Mean plasma concentrations of progesterone reached mean levels between 15 and 18 ng/ml at Day 7 after ovulation and remained almost at that level throughout the observation period in all treatment groups.

Discussion

Cervical dilatation at Day 7 after ovulation had no detrimental effects on the establishment and maintenance of early pregnancy in mares. Similarly, cervical dilatation and a single administration of oxytocin did not alter the pregnancy rate or the rate of expansion of conceptuses. Therefore, manipulation of the cervix itself is unlikely to contribute to varying success rates due to transcervical transfer of equine embryos. Other factors, eg oestrous cycle stage of recipient mares, irritation of the endometrium and bacterial contamination causing inflammation, seem to be of more influence on the success of embryo transfer in mares. Surprisingly, cervical dilatation even increased embryonic growth, if the mares were treated with xylazine before dilatation. We administered xylazine to block the release of oxytocin, which is stimulated by cervical dilatation (Handler *et al.* 2003b). However, we could not clarify, whether the suppression of oxytocin secretion, or possible direct effects of xylazine caused increased embryonic growth. Oxytocin itself did not change embryonic growth patterns, which makes it more likely, that the α-mimetic actions of xylazine may have contributed to this phenomenon.

Secretion patterns of progesterone yielded inconsistent results. Overall, in Treatment 1 secretion of progesterone seemed to be reduced in mares after dilatation, when compared to controls (Handler *et al.* 2002). But, differences among treatments were not significant and mares within the control group showed varying concentrations. Depending on whether mares carried singleton or twin pregnancies, progesterone patterns differed between treatments. Only mares carrying twins showed a decrease in progesterone secretion after cervical dilatation. However, the low number of mares used does not allow definite conclusions to be drawn from these observations. A single administration of oxytocin and cervical dilatation, respectively, without xylazine did not cause any long term changes in luteal function (Experiment 2).

In conclusion, the results of these studies suggest that the sympathetic autonomic nervous system might play a role in embryo-maternal interaction. However, the contribution of the α-mimetic action of xylazine to the effects of cervical dilatation on embryonic growth needs further investigation. Knowledge of the mechanisms responsible may allow the development of medications for supporting early embryonic growth and survival, which might improve reproductive performance in both embryo transfer recipient mares and subfertile mares.

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THE USE OF MECLOFENAMIC ACID TO EXTEND DONOR-RECIPIENT ASYNCHRONY IN EQUINE EMBRYO TRANSFER

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INTRODUCTION

Synchrony between embryonic development and the uterine environment is essential for the establishment of pregnancy. Asynchrony between embryo and uterus may preclude the former from conveying its maternal recognition of pregnancy signal to suppress the cyclical luteolytic response in the latter. Furthermore, the uterine environment changes markedly under the influence of progesterone so that the embryo when exposed to 'out-of-phase' growth factors in asynchronous uterine secretions may be retarded or accelerated in its development or it may die (Barnes 2000). Thus, uterine quiescence induced by luteostasis is not the only requirement for the successful establishment of a pregnancy. Rather, the ability of the embryo and uterus to achieve meaningful stage-dependent dialogue is equally important.

Embryo transfer studies in large domestic animal species have shown that the window of opportunity for achieving pregnancy is not limited to strict donor-recipient synchrony. Equids permit a wider degree of asynchrony than cattle and other ruminants and it is generally accepted that pregnancy rates after embryo transfer in the mare are maximal when the recipient has ovulated one or 2 days after the donor (Douglas 1982; Squires *et al.* 1982). An ability to manipulate and widen this window of acceptable asynchrony would be useful in commercial application of embryo transfer and as a tool to investigate early embryo-uterine communications. However, previous attempts to extend the window of 'permissible synchrony' in the mare have proved unsuccessful. For example, recipient mares that ovulated 2 or 3 days before the donor mare failed to maintain pregnancy even though treated with progestagens (Pool *et al.* 1987). The administration of the prostaglandin synthetase inhibitors, such as flunuxin meglumine or phenylbutazone, to mares also failed to prevent luteolysis and extend the luteal phase of the cycle (Daels and Hughes 1993). In the camel, however, Skidmore and Billah (2005) showed recently that administration of the prostaglandin synthetase inhibitor, meclofenamic acid, will prolong the lifespan of the corpus luteum and thereby allow asynchronous recipients to be used successfully for embryo transfer. In the present study the authors also investigated the effect of treating recipient mares with meclofenamic acid prior to asynchronous embryo transfer.

MATERIALS AND METHODS

The uteri of artificially inseminated donor mares were flushed on Day 7 or 8 after ovulation (Day 0) and the embryos recovered were transferred non-surgically, as described by Wilsher and Allen (2004), to either meclofenamic acid-treated or untreated control mares that had ovulated 2 or 3 days before the donor mare (n=16, in each group). Treated recipient mares received 1g/day of meclofenamic acid (Arquel, Parke Davis and Co.) orally beginning on Day 9 after ovulation and continuing until Day 7 after embryo transfer. Another group (n=16) of untreated mares that had ovulated 0 to -2 days with respect to the donor also received an embryo.

RESULTS AND DISCUSSION

All 16 (100%) of the control mares that ovulated 0 to –2 days with respect to the donor mare were pregnant at 16 days. The meclofenamic acid treated recipient mares, that ovulated +2 or +3

days with respect to the donor, showed higher pregnancy rates at Day 16 (13/16; 81%) compared to the untreated control mares with the same degree of asynchrony (7/16; 44%).

Cyclic luteolysis was not prevented in the mares treated with meclofenamic acid that failed to become pregnant. Thus, if meclofenamic acid does permit widening of the window of synchrony, as it appears to, it must act in a manner other than by suppression of luteolysis. The non-steroidal anti-inflammatory fenamates, of which meclofenamic acid is a member, have been shown to inhibit the 5-lipoxygenase pathway of the arachidonic acid cascade in addition to inhibiting the synthesis of cyclo-oxygenase (Civelli *et al.* 1991). Fenamates also prevent already formed prostaglandins from binding to PGE_2 receptors (Rees *et al.* 1988), they modulate the action of a large range of ion channels and they block gap junction intercellular communications (Harks *et al.* 2001). How such pharmacological effects may assist an equine embryo to overcome the dangers of an asynchronous uterine environment remains unclear.

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SESSION 2:

Steroid hormones and pregnancy maintenance

Chairman:

W. R. Allen

INFLUENCE OF THE EMBRYO ON EXTENDING LUTEAL FUNCTION IN THE PIG

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NATURE OF THE SIGNAL FOR MATERNAL RECOGNITION OF PREGNANCY IN THE PIG

Kidder *et al.* (1955) first reported, after attempting to manipulate the timing of oestrus, that oestrogen treatment could lengthen the inter-oestrous interval in the pig if the treatment occurred on Day 11 of the oestrous cycle. Gardner *et al.* (1963) followed up with an experiment specifically to test the effect of exogenous oestrogen on the maintenance of corpora lutea in the gilt, and demonstrated that it was dose-dependent. As described by Perry (1973), beginning on Day 10.5 to 11 of gestation the pig embryo secretes vast quantities of oestradiol-17ß (µg/day) which serves as the signal for maternal recognition of pregnancy. The production of oestradiol-17ß by the embryo results in a dramatic alteration in the secretions of the endometrium (Giesert *et al.* 1982a). Treatment of normally cycling gilts with oestradiol valerate on Day 11 and 12 leads to alterations in the composition of uterine luminal fluid similar to that induced by the presence of an embryo (Giesert *et al.* 1982b). Furthermore, there are effectively 2 periods of pregnancy recognition. The first signal for maternal recognition of pregnancy occurs on Day 11 to 12 and will extend luteal life-span for approximately one week, accounting for a large proportion of irregular returns with inter-oestrous intervals of approximately 28 days, which the authors referred to as short pseudopregnancy (Pusateri *et al.* 1996a). A second signal, also in the form of embryonic oestradiol-17ß must occur between Days 16 and 19 and will extend luteal lifespan for the duration of gestation; when resulting from exogenous treatment with oestradiol-17ß the authors termed this long pseudopregnancy. This second signal is sufficient to maintain corpora lutea for the duration of gestation, and long psuedopregnancy can last beyond the duration of gestation (Pusateri *et al.* 1996b).

MECHANISM OF OESTROGEN ACTION

Embryonic oestrogen does not affect the corpus luteum directly, but instead alters the profile of prostaglandins secreted by the endometrium. When corpora lutea are treated directly with oestradiol-17ß they are only maintained if enough oestradiol-17ß is given to maintain all of the corpora lutea. The endocrine secretion of prostaglandin $F_{2\alpha}$ (PGF), the luteolysin, is purported to be reduced in the pregnant as compared to non-pregnant pig. This assessment is based primarily on the lack of an increase in the concentration of PGF in uterine venous blood. However, the lack of an increase in PGF in uterine venous blood is coincident with a 4-fold increase in uterine blood flow coincident with the secretion of embryonic oestradiol-17ß (Ford 1982). In fact, the concentration of the major metabolite of PGF increases markedly in the peripheral circulation (Schille *et al* 1979). In conjunction with either the presence of an embryo or treatment with oestrogen there is an increase in the ratio of prostaglandin E_2 (PGE) to PGF approaching 4:1 (Christenson *et al.* 1994). This shift in the ratio of prostaglandins can be detected in uterine venous blood. In the unilaterally pregnant gilt there is only an increase in uterine blood flow to the gravid horn (Ford and Christenson 1979). When prostaglandins are then compared between the gravid and non-gravid horn in a unilaterally pregnant pig there is no difference in the uterine venous concentration of PGF

between the horns, whereas there is a dramatic increase in PGE in the uterine venous blood draining the gravid horn when compared to the non-gravid horn. Furthermore, treatment of individual corpora lutea with intraluteal implants containing a 4:1 ratio of PGE to PGF will extend luteal lifespan on the same ovary that implants with either 2:1 or 1:1 ratios of PGE to PGF will not.

IMPACT OF UTERINE-EMBRYO ASYNCHRONY

A window exists for the initiation of maternal recognition of pregnancy. Transfer of embryos to a uterus that is one day more or less advanced than the embryos results in apparently normal pregnancy rates. Asynchrony between the embryo and the uterus is not generally observed as a lack of maternal recognition of pregnancy, probably because there is significant variation in the degree of development among embryos within a litter. In fact, it is thought that variation amongst litter mate embryos contributes more towards early embryonic loss in the pig than any other component of development. Data from the prolific Meishan pig have led to the suggestion that the amount of oestrogen produced by the embryo will influence the magnitude of the uterine glandular secretory response to the presence of embryos. Meishan embryos secrete markedly less oestradiol-17ß than embryos of western breeds and the secretion of protein and prostaglandins into the uterine lumen at the time of maternal recognition of pregnancy is also reduced. The Meishan embryo is also smaller than embryos of western breeds and develops a smaller placenta. We reasoned that if the amount of oestradiol-17ß produced by the embryo determined the amount of uterine secretion of histotroph, then treating pregnant Meishan gilts with exogenous oestradiol-17ß should increase the secretion of histotroph thereby stimulating embryonic growth and ultimately increasing placental size. Indeed, treatment of pregnant Meishan gilts with exogenous oestradiol-17ß at the time of maternal recognition of pregnancy resulted in a 40% increase in placental size at term. It was also reasoned that if there is a window of opportunity for the embryonic signal for maternal recognition of pregnancy that the transfer of embryos to a more advanced uterine environment should provide for an increased secretion of histotroph in response to embryonic secretion of oestradiol-17ß. Embryos were transferred to recipient gilts that were in oestrus at the same time as the donors or 24 h before or after. This resulted in embryos being gestated in a uterus that was either more or less advanced than the embryos it contained. There were no differences in the conceptuses from females that received embryos of the same developmental stage or a more advanced stage than the uterus they were transferred to. However, when embryos are transferred to a uterus that is more advanced than the embryo, the uterus has presumably been exposed to luteal progesterone for a longer period and is potentially able to secrete a greater amount of histotroph once the signal for maternal recognition of pregnancy is received. Consequently, it was observed that conceptuses that had been transferred to a more advanced uterine environment had slightly larger placentae that were markedly more efficient. As a result the fetuses attached to these placentae were dramatically larger and heavier than those resulting from the transfer of embryos to a uterine environment that was either synchronous with or less developed than the embryos (Wilson *et al.* 2001).

CONCLUSIONS

In conclusion, the signal for maternal recognition of pregnancy in the pig is oestradiol-17ß, produced by the embryo from on Day 11–12 of gestation. The mechanism whereby this embryonic oestradiol-17ß extends luteal lifespan is not direct, but appears to involve an alteration in the secretion of prostaglandins from the uterine endometrium. This alteration appears specifically to involve an increase in the ratio of PGE to PGF, and may or may not also include a dramatic increase in uterine blood flow to effectively dilute all secretory products of the endometrium. Furthermore, shifting the synchrony between the conceptus and the uterine environment has the potential to dramatically alter the secretion of histotroph stimulated by embryonic oestradiol-17ß, which can then influence the growth and function of the placenta later in gestation.

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METABOLISM OF NEUTRAL STEROIDS BY THE EQUINE CONCEPTUS IN EARLY PREGNANCY

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INTRODUCTION

Establishment of pregnancy and support of the early conceptus in mammalian species depend in large measure on the steroid hormones, oestradiol (E2) and progesterone (P4). The source of these hormones may vary from maternal origin to synthesis by the conceptus itself, depending on the species (Heap *et al.* 1979). Early equine conceptuses show high production of oestrogens which are present in yolk-sac fluid along with large quantities of neutral steroids, such as P4 (Raeside *et al.* 2001). These steroids can serve as precursors for oestrogens but might also have other roles at this stage; P4 affecting the endometrial glands, for example. A concept which has gained strength in recent years is that local metabolism of a steroid hormone, to form other products within target tissues, has significance for the overall action of the hormone (Zhu and Conney 1998; Belanger *et al.* 2002). Therefore, we have studied the local metabolism of P4, pregnenolone (P5) and androgens, beyond their role as precursors for oestrogen formation, in the early stages of pregnancy in the mare.

MATERIALS AND METHODS

We examined metabolism of P5, P4, androstenedione (A4) and testosterone (T4) in trophoblast tissue from conceptuses collected by transcervical lavage (Sirois and Betteridge 1988) on Days 12, 15 and 18 (bi- and trilaminar) of pregnancy (n=3/day). In addition, we measured concentrations of these steroids in yolk-sac fluid by radioimmunoassays (RIA) for 64 conceptuses (Days 11–25). Trophoblast tissues were divided into 2–4 equal portions, washed with saline and dispensed into small flasks containing 2.5 ml TCM 199 to which [^{3}H]-labelled steroids (1×10^6 cpm) were added for incubation for 2h in a shaking waterbath at 37°C under 5% CO_2 in air.

Steroids in the media were recovered by solid-phase extraction (Waters C_{18} Sep-Pak columns) as described previously. Unconjugated and conjugated steroids were eluted with 5 ml diethyl ether and 5 ml methanol in succession. The ether eluate was partitioned (NaOH/toluene) to give neutral and phenolic fractions. Conjugated material was solvolysed to yield a 'sulphate' fraction recovered as free steroids from a second Sep-Pak column. Profiles of steroid metabolites were generated using a gradient of acetonitrile-water on a Waters C18 column and HPLC system at a flow-rate of 0.7 ml/min with an online radiodetector and the absorbance monitored at 254 nm (Raeside *et al.* 2004).

RESULTS

Concentrations of steroids in yolk-sac fluid (mean values ± SE, determined by RIA) increased markedly with age /size, and ranged for Days 11–13 (n=23), Days 14–16 (n=23), Days 17–19 (n=12) and Days 20–25 (n=6) as follows: P5, 3.49 ± 0.74, 1.18 ± 0.19, 2.00 ± 0.33 and 8.05 ± 1.89 ng/ml, respectively. Values for P4 were 2.38 ± 0.20, 1.84 ± 0.30, 6.10 ± 1.40 and 7.58 ± 1.09; for A4, 28.78 ± 6.32, 47.90 ± 9.78, 108.90 ± 16.04, and 151.94 ± 45.73; and for T4, 2.21 ± 0.51, 1.46 ± 0.41, 9.36 ± 4.76 and 24.50 ± 12.91 ng/ml, respectively.

HPLC studies (Fig 1) with P5 and P4 showed that the radioactivity was largely as neutral steroids, with much remaining as substrate. The major metabolite appeared to be 17α-OH P4 and

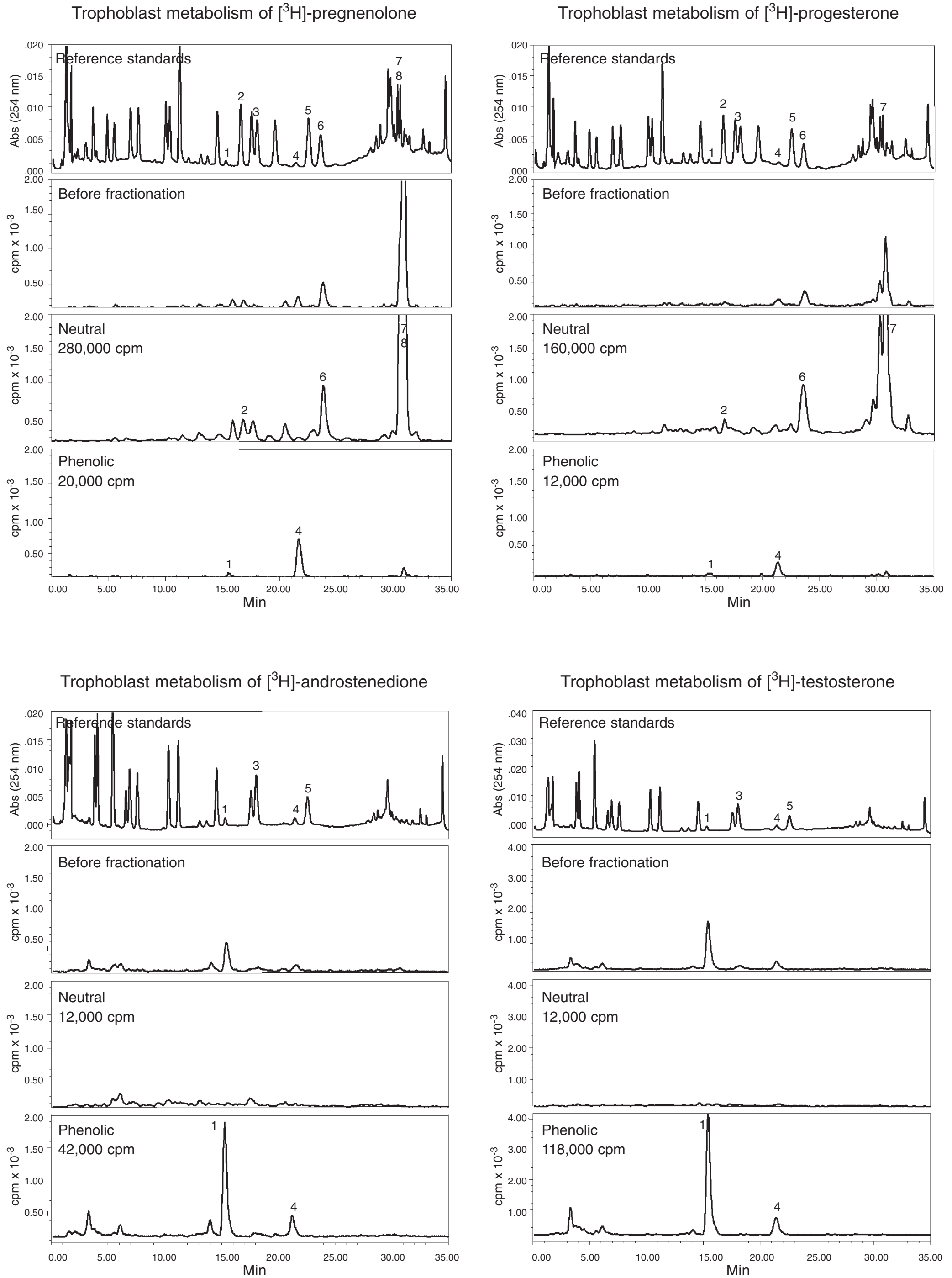

Fig 1: HPLC profiles of metabolites, in neutral and phenolic fractions, from incubation of trophoblast tissues with [3^H]-labelled steroids on Day 15 of pregnancy. Numbered reference standards are (1) E2; (2) 17α, 20ß-dihydro-P4; (3) T4; (4) E1; (5) A4; (6) 17α-OH-P4; (7) P4; (8) P5.

the presence of 17α, 20ß-OH P4 was also noted, along with other lesser peaks. Oestrone (E1) was present in small amounts in the phenolic fractions, with only traces of oestradiol-17ß (E2). With the androgens, aromatisation was almost complete; E2 was the principal product with lesser amounts of E1 and some more polar oestrogen metabolites (6-OH/6-oxo). Only trace quantities of neutral steroids were seen, and no substrate remained after incubations with A4 or T4.

DISCUSSION

Firstly, 2 points should be made in viewing the data on steroid concentrations in yolk-sac fluid against HPLC profiles: 1) RIAs may show cross-reactivity with other steroids present; and 2) length of incubation (2 h) may limit the extent of substrate utilisation. However, it is clear from the HPLC profiles that E1 and E2 can be formed from both C_{21} and C_{19} steroid precursors; the short incubations favouring the C_{19} substrates. Some preliminary evidence in earlier studies with equine conceptuses (Goff *et al.* 1993), and cell preparations from trophoblast tissues (Marsan *et al.* 1987), suggested the formation of E2 from longer incubation (8 h) with P5 and A4. They also pointed to 17α-OH P4 as the major metabolite, as was noted in our studies. The presence of both 17α,20ß- and 17α,20α-dihydroxy-P4, and other lesser peaks in the neutral fraction from P5 and P4, raises questions about their role as inactivation products from P4, or possibly as active agents in their own right. A similar view may be taken with regard to the polar metabolites of E2 and E1 seen in the profiles from A4 and T4 metabolism. In conclusion, it might be claimed that local metabolism of the neutral steroids present in yolk-sac fluid has significance for oestrogen production; and it may make an additional contribution to the establishment and maintenance of the early stages of pregnancy in the mare by the formation of other steroid products. Moreover, the localisation of an androgen receptor (AR), and the induction by E2 of AR mRNA abundance in the pig endometrium, underscores the need for understanding the interactions of androgens and oestrogens as they may relate to early embryo loss in the mare (Kowalski *et al.* 2004).

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EARLY EQUINE CONCEPTUSES EXPRESS mRNA FOR STEROID RECEPTORS

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INTRODUCTION

The dogma has long been that the stimulatory effects of progesterone and oestrogens on conceptus development are indirect, and mediated primarily via the endometrium. In addition, steroid hormones are classically thought to bind to intracellular receptors and to exert their effects via a relatively slow genomic pathway that involves the modulation of gene expression. However, recent studies have suggested that the reproductive steroid hormones may also have direct effects on the developing conceptus and, furthermore, that they may induce rapid, non-genomic responses by way of membrane-associated receptors and intracellular second messengers. In this respect, oocytes and embryos of various mammalian species have been shown to express mRNA for classical steroid hormone receptors (Hou and Gorski 1993; Ying *et al.* 2000) and, in several vertebrate species, a novel membrane-associated progesterone receptor (mPR) has been characterised and postulated to mediate the non-genomic effects (Meyer *et al.* 1996; Zhu *et al.* 2003a). For example, fish oocytes express mRNA for mPR, and progesterone appears to exert its rapid non-genomic effects via this receptor (Zhu *et al.* 2003b). In the horse, the expression of steroid receptors by conceptuses does not appear to have been examined. The aim of the current study was to use the reverse transcriptase polymerase chain reaction (RT-PCR) to determine whether equine conceptuses express mRNA for mPR, or for the classical intracellular progesterone (PR) and oestrogen (ERα and Erß) receptors. If mRNA expression was detected, quantitative RT-PCR (Q-PCR) would be used to examine whether the rate of expression changed during early intrauterine development.

MATERIALS AND METHODS

Collection of conceptuses

Conceptuses were recovered from 13 Warmblood mares inseminated at numerous oestrous cycles. Early in oestrus, mares were teased thrice-weekly using a vigorous stallion, and their reproductive tracts were examined by transrectal palpation and ultrasonography. Once the dominant ovarian follicle(s) exceeded 35 mm in diameter, the mare was inseminated with approximately 300 x 10^6 morphologically normal, progessively motile sperm from one of 2 fertile stallions. A total of 31 conceptuses were collected by uterine lavage 7 (n=10), 10 (n=10), 14 (n=10) or 16 (n=1) days after ovulation was detected during daily transrectal ultrasonographic examination of the ovaries. The embryos were collected by non-surgical uterine lavage using either Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 % (v:v) fetal calf serum (Day 7 conceptuses) or Ringer's solution (Day 10–16 conceptuses). After recovery, the conceptuses were washed in fresh flushing solution (without fetal calf serum), and the conceptus membranes were isolated, snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

PCR primer design

Primer pairs for mPR, PR, ERα, ERß, and an additional 2 'reference' genes (ribosomal RNA (rRNA) and ß-actin) were designed using the equine DNA sequence or, if this was not available, sequences of target gene DNA conserved well across other mammalian species. Preferably, each primer of a primer pair was located on a separate

gene exon. All primer pairs were validated using an equine cDNA panel before being applied to the embryonic samples.

RNA extraction and cDNA synthesis

Isolation of total RNA and on-column DNAse digestion were performed using the RNeasy Mini Kit and the RNAse-free DNAse Set (Qiagen, Valencia, USA), as per the manufacturer's instructions. Thereafter, cDNA was synthesised by reverse transcription using random primers; minus RT blanks were prepared using the same conditions in the absence of reverse transcriptase. The success of RNA extraction and cDNA production was tested using the polymerase chain reaction (PCR) with primers for ß-actin; samples that proved negative, or that were positive but accompanied by a positive minus RT blank, were excluded from further analysis.

Experiment 1: Qualitative PCR

cDNA samples for Day 7, 10, 14 (all stages, n=2) and Day 16 (n=1) conceptuses were analysed by PCR using primers for mPR, PR, ERα and ERß. The thermal cycling profile was as follows; initial denaturation and activation of the polymerase at 94°C for 15 min, followed by 40 cycles of 15 s at 94°C, 30 s at 55°C and 45 s at 72°C. Final extension was performed at 72°C for 10 min. After completion of the reaction, 10 µl of the product was resolved on a 1% agarose gel containing 0.4 µg/ml ethidium bromide to visualise any PCR products. A 100 basepair (bp) ladder was included as a reference for fragment size, and a standard sequencing procedure was used to verify the identity of the PCR products.

Experiment 2: Q-PCR

Q-PCR was performed on cDNA from Day 7, 10 and 14 conceptuses (n=8 at each stage). This involved real time PCR using primers specific for mPR and the 2 reference genes (rRNA and ß-actin), where quantification was based on the amount of the high affinity, double-stranded DNA-binding dye, SYBR green, intercalated. Standard curves were generated by using serial 10-fold dilutions of known amounts of PCR product, and then plotting the log of the starting amount of product versus the threshold cycle for detection. The amplification efficiency for each standard curve was considered acceptable if it exceeded 90% over a large dynamic range (6-8 orders of magnitude). For each sample, the starting quantity of cDNA for mPR was calculated, and expressed as the ratio of the quantity of a reference gene (rRNA or ß-actin). Melting curves were used to confirm the purity of the amplified products.

RESULTS

Experiment 1

mRNA for mPR and PR was expressed by conceptuses at all developmental stages examined (Day 7–16). By contrast, ERß was expressed by Day 10–16, but not Day 7, conceptuses and ERα mRNA was not expressed by horse conceptuses at any of the stages of development examined.

Experiment 2

Q-PCR demonstrated that the relative expression of mRNA for mPR (mPR/reference gene) by Day 10 and 14 conceptuses was significantly higher than that by Day 7 conceptuses ($p<0.05$).

DISCUSSION

This study describes the detection of mRNA for an equine mPR, and demonstrates that early equine conceptuses express mRNA for mPR, PR and ERß. These results suggest that progestagens and oestrogens may directly affect embryonic development in the horse. However, the exact significance of these steroid receptors and the nature of any direct effects of the reproductive steroids on conceptus development have yet to be examined. Nevertheless, the tissue specific rate of mPR expression increased during Days 7–14, a period during which the conceptus, *in vivo,* is mobile, undergoes rapid expansion and significant cell differentiation, and must physiologically signal its presence to its dam. It is therefore possible that maternal progesterone stimulates the conceptus to synthesise factors that play a role in pregnancy recognition, and ensure continued supply of luteal progesterone. At this stage, however, the possible direct effects of reproductive steroids on conceptus development are speculative.

Future studies will use QPCR analysis to examine whether the relative expression of mRNA

for PR and ERß also changes during conceptus development. These studies will investigate mPR, PR and ERß expression before, during and after the phases of conceptus mobility and maternal pregnancy recognition. Additional studies will examine whether mRNA expression is indeed followed by receptor synthesis, and in which cells the receptor is located. If receptor protein is detected, more complex experiments will be required to determine the effects of hormone-receptor binding, and whether these actions are mediated via non-genomic and/or genomic pathways.

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SESSION 3:

Oxytocin-prostaglandin interactions

Chairman:

T. A. E. Stout

ROLES FOR OXYTOCIN IN LUTEOLYSIS AND PREGNANCY RECOGNITION IN PIGS

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INTRODUCTION

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the uterine luteolysin in all domestic ungulates and is secreted from the uterine endometrium in a pulsatile manner to induce luteolysis in late dioestrus. In pigs, $PGF_{2\alpha}$ is released in an endocrine direction into the uterine-ovarian vein, from which it reaches the ovary to lyse the corpora lutea (Bazer and Thatcher 1977). Oxytocin (OT) is the major known stimulus for induction of luteolytic pulsatile secretion of $PGF_{2\alpha}$ in ungulates (Mirando *et al.* 1996). During luteolysis in pigs, OT is secreted primarily from the neurohypophysis (Kotwica *et al.* 1990) and the uterine endometrium (Trout *et al.* 1995; Boulton *et al.* 1996). Very little, if any, OT is secreted from the corpora lutea of pigs, in contrast to what occurs in sheep.

ROLE OF OXYTOCIN IN LUTEOLYSIS

Systemic administration of OT promotes endocrine secretion of $PGF_{2\alpha}$ in pigs. Endometrial response to OT develops during late dioestrus, before luteolysis is initiated and progesterone declines (Carnahan *et al.* 1996). Additionally, the inter-oestrous interval is decreased by 1–2 days in swine when exogenous OT is administered systemically (Prince *et al.* 1995; Sample *et al.* 2000). While the magnitude of the OT-induced decrease in inter-oestrous interval may seem small, it is essentially identical to that promoted by exogenous $PGF_{2\alpha}$ and, therefore, is a highly-consistent physiological effect. Also, luteolysis is induced by exogenous OT only when the uterus is present (Prince *et al.* 1995), consistent with an action upon the uterine endometrium to promote endocrine secretion of the luteolysin (Carnahan *et al.* 1996).

During development of the response to OT, the abundance of endometrial transcripts for OT receptors increases markedly during Days 10–16 post oestrus in cyclic gilts, while the number of endometrial receptors for OT increases only modestly (Ludwig *et al.* 1998). This indicates that increased transcription of the OT receptor gene does not yield corresponding increases in expression of functional OT receptors. The marked increase in response to OT in the absence of parallel increases in OT receptor binding activity (Ludwig *et al.* 1998) also indicates that development of the response to OT during dioestrus in swine is controlled at cellular sites subsequent to the OT receptor, possibly at the level of receptor coupling to the phosphoinositide signalling pathway.

AUTOCRINE ACTION OF OXYTOCIN

In vitro, the response to OT is greatest for stromal cells, intermediate in glandular epithelial cells and absent in luminal surface epithelial cells (Braileanu *et al.* 1999; Hu *et al.* 2001). This pattern is opposite to that in ruminants in which the response is almost exclusively in the epithelium (Asselin *et al.* 1997). Because expression of OT and its receptor occurs primarily in the luminal epithelium (Boulton *et al.* 1995, 1996), it was hypothesised that the apparent lack of response to exogenous OT in cultured luminal epithelial cells is partly due to an autocrine action of OT upon the epithelium. In support of this, treatment of endometrial explants *in vitro* with an OT antagonist suppresses basal secretion of

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$PGF_{2\alpha}$ in the absence of exogenous OT (Hu *et al.* 2001). Similarly, treatment of cultured luminal epithelial cells with an OT antagonist also suppresses basal $PGF_{2\alpha}$ secretion, whereas treatment of luminal epithelial cells with OT antiserum, to immunoneutralise endogenously-secreted OT and suppress basal $PGF_{2\alpha}$ release, promotes acquisition of a response to subsequent treatment with exogenous OT (Hu *et al.* 2001). Moreover, these effects are not apparent in stromal cells that are already responsive to OT (Hu *et al.* 2001). These results indicate that chronic exposure to an autocrine action of OT stimulates basal release of $PGF_{2\alpha}$ from luminal epithelial cells, thereby obscuring the response to exogenous OT.

Signalling pathway for oxytocin

At the cellular level, OT binds to its endometrial receptor to activate phospholipase C and initiate phosphoinositide signalling (Ludwig *et al.* 1998), releasing the intracellular messengers, inositol 1,4,5-trisphosphate, diacylglycerol and inositol 1,4,5-trisphosphate-sensitive calcium (Mirando *et al.* 1996; Braileanu *et al.* 1999). In turn, these intracellular signals activate calcium-dependent protein kinase C to stimulate downstream pathways that make arachidonic acid available for conversion to $PGF_{2\alpha}$. G-protein coupling of the OT receptor to phospholipase C appears to be a site for cellular regulation of $PGF_{2\alpha}$ release from porcine endometrium (Ludwig *et al.* 1998).

Pregnancy recognition

Maternal recognition of pregnancy in pigs occurs at Days 10–12 post oestrus. Endocrine secretion of $PGF_{2\alpha}$ must be attenuated to prevent luteolysis and provide the continued progesterone secretion needed to maintain pregnancy (Bazer and Thatcher 1977). During this time, $PGF_{2\alpha}$ secretion from the uterus is not decreased (Ludwig *et al.* 1998) but its release is re-oriented away from a luteolytic endocrine direction and toward the uterine lumen (in an exocrine direction) due to the paracrine effects of conceptus-secreted oestrogen (Bazer and Thatcher 1977). Although effects of increased blood flow to the uterus in early pregnancy could dilute $PGF_{2\alpha}$ exiting the uterus (Ford *et al.* 1995) or promote its retrograde transfer from uterine vein to uterine artery (Krzymowski and Stefanczyk-Krzymowska 2002), thereby contributing to reduced endocrine $PGF_{2\alpha}$ secretion, several studies utilising perifused endometrium clearly indicate that $PGF_{2\alpha}$ is re-oriented to an exocrine direction during pregnancy recognition in pigs (Gross *et al.* 1988, 1989, 1990).

Role of oxytocin in pregnancy recognition

During pregnancy recognition in swine, OT is not luteolytic and may be antiluteolytic (Sample *et al.* 2004). In contrast to the decrease in inter-oestrous interval caused by systemic administration of OT to cyclic gilts (Prince *et al.* 1995; Sample *et al.* 2000), exogenous OT does not prevent the extension of inter-oestrous interval promoted by oestradiol (Sample *et al.* 2000). In fact, OT does not promote luteolysis even when a very weak antiluteolytic oestradiol signal is provided (ie the lowest dose of oestradiol that promotes the shortest detectable extension of inter-oestrous interval). Presumably, the lack of response to OT in oestrogen-treated cyclic gilts results from a reduced endometrial $PGF_{2\alpha}$ secretory response, as development of the response to OT at Days 12–14 post oestrus in cyclic gilts is markedly attenuated during pregnancy recognition (Carnahan *et al.* 1996). Therefore, OT does not stimulate endocrine secretion of $PGF_{2\alpha}$ from the uterus to the same extent as occurs during luteolysis (Carnahan *et al.* 1996). Thus, OT-induced endocrine $PGF_{2\alpha}$ secretion is reduced during early pregnancy and OT is unable to promote luteolysis.

The reduced response to OT is not mediated through reduced endometrial expression of OT receptors because abundance of OT receptor transcripts increases in early pregnancy, whereas OT receptor binding activity is relatively unchanged (Ludwig *et al.* 1998). Conversely, the reduced response to OT in early pregnancy is mediated at cellular sites subsequent to the OT receptor, one of which includes uncoupling of OT receptor binding from activation of phospholipase C (Ludwig *et al.* 1998). Interestingly, progesterone can rapidly inhibit the response of pig endometrial stromal cells to OT through a non-genomic pathway. Thus, the antiluteolytic pregnancy recognition signal (ie oestrogen) also may abrogate OT-induced endocrine $PGF_{2\alpha}$ secretion by extending the progesterone block to development of endometrial response to OT.

Decreased endocrine $PGF_{2\alpha}$ secretion during pregnancy recognition in swine involves reduced endocrine $PGF_{2\alpha}$ release in response to OT (Carnahan *et al.* 1996). However, exocrine $PGF_{2\alpha}$ release into the uterine lumen increases during pregnancy recognition (Bazer and Thatcher 1977;

Carnahan *et al.* 1999). Oxytocin promotes exocrine secretion of $PGF_{2\alpha}$ to a greater extent in early pregnancy than an equivalent stage of dioestrus, regardless of whether OT is applied to the luminal surface of the endometrium (Gross *et al.* 1988) or on the vascular side (Carnahan *et al.* 1999).

Antiluteolytic action of oxytocin

In pigs, endometrial OT secretion into the uterine lumen increases up to 50-fold during pregnancy recognition (Trout *et al.* 1995), coincident with onset of conceptus oestrogen secretion and re-orientation of endometrial $PGF_{2\alpha}$ release (Bazer and Thatcher 1977). When OT is infused into the uterine lumen to mimic the high uterine OT content of early pregnancy, inter-oestrous interval is not decreased (Sample *et al.* 2000). In fact, intrauterine OT administration on Days 10–16 post oestrous decreases endocrine $PGF_{2\alpha}$ secretion on Days 14–16 and delays luteolysis (Sample *et al.* 2000). Thus, the large quantities of OT released into the uterine lumen during pregnancy recognition (Trout *et al.* 1995) may contribute to luteal maintenance by decreasing endocrine $PGF_{2\alpha}$ secretion and increasing its exocrine release into the uterine lumen. This is consistent with an autocrine action of OT to promote $PGF_{2\alpha}$ release from the luminal epithelium (Hu *et al.* 2001) and supports a role for endometrially-derived OT in re-orientation of $PGF_{2\alpha}$ release during maternal recognition of pregnancy, in contrast to its luteolytic role during the oestrous cycle.

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EFFECT OF CO-INCUBATION OF DAY-14 EQUINE CONCEPTUS AND ENDOMETRIAL TISSUES ON OXYTOCIN-INDUCED SECRETION OF ENDOMETRIAL PROSTAGLANDIN $F_{2\alpha}$

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INTRODUCTION

In non-pregnant mares, oxytocin (OT)-induced secretion of endometrial prostaglandin $F_{2\alpha}$ (PGF) causes luteolysis on Days 14 to 15 post ovulation. During pregnancy, the equine conceptus initiates maternal recognition of pregnancy by blocking OT-induced PGF secretion. The conceptus-mediated signal(s) of maternal recognition of pregnancy in the mare has not been identified. Studies have shown co-incubation of equine conceptus and endometrial tissues *in vitro* significantly decreased basal PGF secretion (Berglund *et al.* 1982; Sharp *et al.* 1984; Weithenauer *et al.* 1987; Watson and Sertich 1989; Sissener *et al.* 1996; Ababneh *et al.* 2000); however, the ability of conceptus tissue to suppress OT-induced PGF secretion *in vitro* has not been reported. The objective of this study was to determine if co-incubation of equine conceptus and endometrial tissues would suppress OT-induced endometrial PGF secretion.

MATERIALS AND METHODS

Three preliminary studies were done to determine the effect of pre-incubation time, OT dose, and biopsy order on uterine endometrial tissue responsiveness to OT *in vitro*. The first 2 studies were performed concurrently by collecting endometrial tissues from 5 non-pregnant mares on Day 14 (ovulation = Day 0). Endometrial tissue from each mare was rinsed with medium (TCM-199, 10% calf serum, 100 U penicillin/ml and 100 µg streptomycin/ml), and then dissected into sub-samples each weighing 20–80 mg. Samples from each mare were pre-incubated for 3 or 6 h, after which 6 sub-samples per mare were removed and weighed; 3 sub-samples were incubated in 1 ml fresh medium (without calf serum) to measure basal PGF secretion and 3 sub-samples were incubated in 1 ml fresh medium containing 1 µM OT to measure OT-induced PGF secretion (OT challenge). The OT dose response was performed by incubating 3 sub-samples per mare in medium with 0, 10 nM, 100 nM, 1µM, and 10 µM OT concentrations. These incubations were performed for 1 h, after which medium was collected and stored frozen until further analysis. The third preliminary study was performed to determine if repetitive collection of individual biopsy samples would affect each individual tissue sample's oxytocin responsiveness. Six biopsies were obtained sequentially from 11 non-pregnant mares on Day 14. Tissues were rinsed in medium and each biopsy was divided into 6 sub-samples and incubated. An OT challenge (described above) was performed after a 6-h pre-incubation period for each mare's individual biopsy samples, one through 6.

Two co-incubation studies were performed with endometrial and conceptus tissues. In Experiment 1, endometrial tissues were obtained from 6 non-pregnant mares and conceptuses were obtained from 6 pregnant mares on Day 14. Endometrial tissue from each mare was rinsed with medium and then dissected into 48 sub-samples. One-half of the sub-samples from each mare were incubated in medium (Control), while the remaining sub-samples were incubated in medium containing a single conceptus (Treated). Every 6 h during a 24 h period, 6 sub-samples were removed from Control and Treated dishes and weighed; 3 sub-samples were incubated in 1 ml fresh medium to measure basal PGF secretion and 3 sub-samples were incubated in 1 ml medium containing 1µM OT to measure OT-

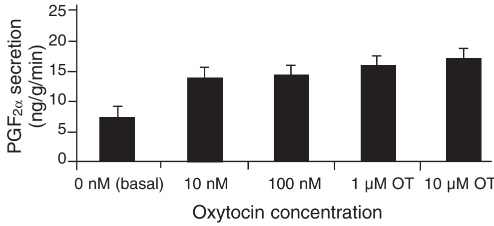

Fig 1: Effect of oxytocin concentration on endometrial secretion of $PGF_{2\alpha}$ in vitro. *Endometrium was collected from non-pregnant mares (n=5) on Day 14 post ovulation. All levels of oxytocin increased (P<0.05) $PGF_{2\alpha}$ secretion above basal (0 nM).*

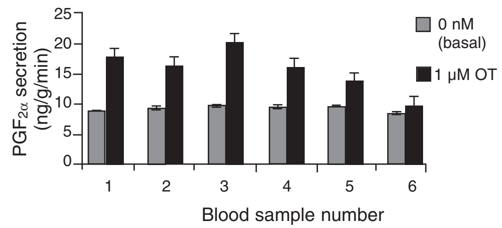

Fig 2: Effect of biopsy sample number on basal and oxytocin-induced endometrial $PGF_{2\alpha}$ secretion in vitro. *Six individual biopsy samples were collected sequentially from non-pregnant mares (n=11) on Day 14 post ovulation and then incubated in plain medium or medium with 1 μM oxytocin (OT). There was a main effect of oxytocin (P<0.001), but not of biopsy sample number or an interaction.*

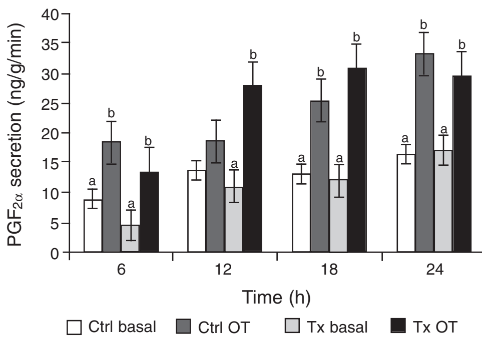

Fig 3: Co-incubation Experiment 1. Endometrial tissues were obtained from non-pregnant mares (n=6) and conceptuses were obtained from pregnant mares (n=6) on Day 14 post ovulation. Endometrial tissue samples were incubated in medium (Control), and in medium containing a single conceptus (Treated). Every 6 h sub-samples were removed from Control and Treated dishes and incubated for 1 h in fresh medium to measure basal $PGF_{2\alpha}$ secretion or medium containing 1 μM oxytocin (OT) to measure OT-induced $PGF_{2\alpha}$ secretion (OT challenge). a,b Bars without common superscripts (within treatment and time period) indicate an OT effect P<0.05 (basal vs. OT).

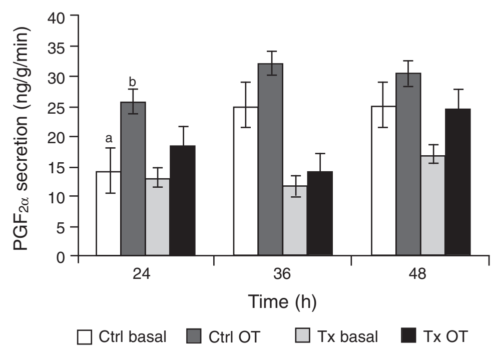

Fig 4: Co-incubation Experiment 2. Endometrial tissues were obtained from non-pregnant mares (n=5) and conceptuses were obtained from pregnant mares (n=5) on Day 14 post ovulation. Endometrial tissue samples were incubated in medium (Control), and in medium containing a single conceptus (Treated). After 24, 36, and 48 h of co-incubation sub-samples were removed from Control and Treated dishes and incubated for 30 min in fresh medium to measure basal $PGF_{2\alpha}$ secretion or medium containing 1 μM oxytocin (OT) to measure OT-induced $PGF_{2\alpha}$ secretion (OT challenge). The presence of a conceptus reduced (P<0.05) the overall production of PGF across time in the Treated group. a,b Bars without common superscripts (within treatment and time period) indicate an OT effect P<0.05 (basal vs. OT).

induced PGF secretion (OT challenge). Experiment 2 used similar procedures (n=5 non-pregnant and pregnant mares), except OT challenges were performed after 24, 36, and 48 h of co-incubation. Oxytocin challenges were performed for 1 h in Experiment 1 and 30 min in Experiment 2. For all studies, medium was analysed for PGF concentration by a validated radioimmunoassay. PGF secretion was expressed as ng/g tissue/min.

Results

Oxytocin increased (P<0.05) PGF secretion above basal levels after both the 3- and 6-h pre-incubation periods (data not shown); however, the response was more robust at 6 h. A 6-h pre-incubation period was used for the subsequent preliminary experiments. All doses of OT increased (P<0.05) PGF secretion above basal levels (Fig 1). Repetitive collection of biopsy

samples did not affect the ability of the tissue to secrete PGF, as there was a main effect of OT (P<0.001) and no effect of sample number or interaction (Fig 2).

In co-incubation Experiment 1, OT increased (P<0.05) PGF secretion above basal levels in both Control (endometrium) and Treated (endometrium with conceptus) groups at all time periods except Control at hour 12 (Fig 3). In Experiment 2, at hour 24, OT increased PGF secretion in the Control group, but not in the Treated (Fig 4). The presence of a conceptus reduced (P<0.05) the overall production of PGF across time in the Treated group (Fig 4).

Discussion

Three preliminary studies conducted to establish an *in vitro* system for assessing OT-induced endometrial PGF secretion demonstrated that: 1) OT stimulated increased PGF secretion from endometrial tissues after both a 3- and 6-h pre-incubation period, with a more robust PGF response to OT at hour 6; 2) all doses of OT tested stimulated increased PGF secretion above basal levels; and 3) repetitive collection of biopsy samples did not affect the endometrial tissue's ability to secrete PGF in response to OT treatment *in vitro*.

In co-incubation Experiment 1, OT increased PGF secretion above basal levels in the Treated group at all time points tested between 6 and 24 h, indicating that the conceptus did not inhibit OT-induced PGF secretion during that period. In co-incubation Experiment 2, the conceptus suppressed OT-induced PGF secretion in the Treated group at hour 24; however, the results at hours 36 and 48 were inconclusive, since OT failed to increase PGF secretion in both Control and Treated groups at those time points. The results of this study were consistent with previous studies, in that a generalised suppressive effect of the conceptus on PGF secretion from the endometrium was observed. Additional studies are needed to further develop this *in vitro* co-incubation model in order to assess fully the effect of the conceptus on OT-induced endometrial PGF secretion.

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EXPRESSION OF ENZYMES INVOLVED IN THE SYNTHESIS OF PROSTAGLANDINS IN EARLY EQUINE EMBRYOS

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INTRODUCTION

In contrast to ruminants and pigs, the equine conceptus does not elongate before maternal recognition of pregnancy and the embryonic signal which prevents prostaglandin $F_{2\alpha}$ synthesis is transferred to a sufficient amount of the endometrium by tremendous mobility of the embryo (Ginther 1983, 1985). The mechanisms leading to mobility of the equine conceptus seem to be of both maternal and embryonic origin. Recent studies suggest that prostaglandins secreted from the conceptus stimulate local myometrial contractions resulting in embryonic movement (Stout and Allen 2001, 2002). However, not much is known about the expression of enzymes involved in prostaglandin synthesis during early embryonic development in this species. Therefore, it was the aim of the present study to investigate the expression of specific genes involved in the synthesis of prostaglandins in early equine embryos.

MATERIALS AND METHODS

Oestrous mares were inseminated at 48 h intervals with fresh semen from a fertile stallion. Mares were checked for ovulation at 12 h intervals. Embryos were flushed from the uterus using routine methods and phosphate buffered saline (PBS). Embryos were scored for quality using a stereo microscope, washed in PBS and frozen at -80°C. After homogenisation of single embryos using a glass homogeniser, total RNA was extracted by TriReagent (Sigma). Quantification of total RNA was performed by spectrophotometry. Equal amounts of material were used for reverse transcriptase PCR (RT-PCR). The reaction was performed using the one-step RT-PCR kit (Qiagen) with enzyme specific primers designed from sequences present in the database. Expression of phospholipase A_2 (PLA), cyclo-oxygenase (Cox)-1 and -2, the membane-bound and cytosolic forms of prostaglandin E synthase (PGEs) and prostaglandin F synthase (PGFS) were determined in embryos of 8, 10, 12 and 14 days of age.

RESULTS AND DISCUSSION

A semiquantitative evaluation of the RT-PCR results for PLA, Cox-1, Cox-2, membrane-bound PGES (mPGES), cytosolic PGES (cPGES) and PGFS in embryos on Day 8, 10, 12 and 14 after ovulation is given in Table 1.

TABLE 1: Expression of enzymes involved in prostaglandin synthesis in equine embryos at different stages of development

Embryo	PLA_2	Cox-1	Cox-2	cPGES	mPGES	PGFS
Day 8	++	+	(+)	+	+	++
Day 10	++	+	+	++	+	++
Day 12	++	+	+	++	++	+
Day 14	++	+	+++	++	+	++

+ = weak; ++ = medium; +++ = strong expression

To compensate for the existence of a small trophoblastic surface of the conceptus, the embryonic signal for maternal recognition in the horse is transferred to a sufficient amount of the endometrium by mobility of the embryo within the uterine lumen (Ginther 1983, 1985). Between Days 10 and 16 after ovulation, the embryo moves constantly through the uterine cavity, traversing the full length of each uterine horn and the uterine body several times per day (Stout and Allen 2001). Suppression of mobility during that time results in luteolysis and subsequent embryonic loss (McDowell *et al.* 1988). Simulated embryonic vesicles of a size comparable to a Day 12 embryo were mobile within the uterus, but the rate of movement was significantly lower than that of viable conceptuses indicating that the embryo itself provides an active stimulus increasing mobility (Ginther 1985).

The hypothesis that prostaglandins might be involved in the process of maternal recognition of pregnancy in the horse was supported for the first time by the finding that early equine embryos seem to be capable of secreting prostaglandins of the E and F family *in vitro* (Watson and Sertich 1989). Secretion of prostaglandin E was shown to be the major stimulus of the morula and early blastocyst for passage from the oviduct to the uterine lumen (Weber *et al.* 1991). Recent investigations suggested that prostaglandin $F_{2\alpha}$ secreted by the conceptus stimulates local peristaltic contractions of the myometrium leading to mobility of the conceptus (Stout and Allen 2001, 2002). Determination of prostaglandins in fluid from the different embryonic cavities supported the hypothesis that the substance is produced by the embryo itself and not by the endometrium (Stout and Allen 2002). The present study demonstrates for the first time that in the early equine embryo, enzymes for the synthesis of prostaglandins of the E and F family are actually expressed. In all tissues investigated so far, prostaglandins are produced from membrane phospholipids by the action of phospholipase A_2 (PLA_2), cyclo-oxygenase (Cox) and specific prostaglandin synthases (Goff 2004). In the embryos investigated in this study, expression of all of these enzymes could be demonstrated. Activation of PLA_2 is known to play a key role in the initiation of prostaglandin synthesis by delivery of phospholipids from cell membranes. Expression of this enzyme was demonstrated in embryos of all stages. In most tissues investigated, Cox-1 is constitutively expressed and is considered to play a housekeeping role, while expression of Cox-2 is induced by a variety of factors (eg oxytocin; Goff 2004). In embryos from Day 12 and 14 but not Days 8 and 10 Cox-2 was expressed strongly, while Cox-1 was expressed at a constant low level at all times investigated. The increase in Cox-2 expression on Day 14 of pregnancy could coincide with a stimulus-dependent activation of the Cox-2 pathway around the time of maternal recognition of pregnancy. These findings support the idea of an involvement of prostaglandins in the antiluteolytic system of the equine embryo. However, the signal activating prostaglandin synthesis is still unknown.

Acknowledgements

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SESSION 4:

Regulation of conceptus secretory activity

Chairman:

M. A. Mirando

UTERINE-DERIVED FACTORS INFLUENCE INTERFERON-TAU EXPRESSION IN THE BOVINE PLACENTA

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INTRODUCTION

The first placental cell type to develop in the early embryo, the trophectoderm, comprises the outermost layer of the placenta and acts as a proliferative stem cell for the fetal portion of the placenta. Ruminant trophectoderm proliferates extensively prior to its attachment to the uterus. During this time it produces interferon-tau (IFN-τ), which promotes the continuation of pregnancy in the *Ruminantia* suborder of mammals (cattle, sheep, goats, deer, antelope, giraffe; Roberts *et al.* 2003). IFN-τ is first produced at the blastocyst stage in cattle (Day 7–10 of pregnancy), and its production increases profoundly from Day 14 to 21 of pregnancy. During the second and third weeks of pregnancy, IFN-τ interacts with the endometrial epithelium and prevents corpus luteum regression and return to oestrus by limiting the pulsatile release of prostaglandin $F_{2\alpha}$, a luteolytic agent, from the endometrium. IFN-τ also stimulates the endometrial synthesis and release of prostaglandin E_2, a putative luteotrophic factor and regulates the expression of several uterine-derived factors that potentially function to prepare the uterus for placental attachment, modify the uterine immune system, and regulate early conceptus development.

Substantial embryonic loss occurs in cattle during the period when IFN-τ must interact with the uterine endometrium so that a functional corpus luteum is maintained. Reduced rates of trophectoderm proliferation and diminished IFN-τ gene expression and protein synthesis are likely causes of failed pregnancy recognition in cattle and other ruminants. Glandular epithelium is essential for normal conceptus development in sheep. Ewes that fail to develop extensive uterine glandular epithelium, a model created by exposing neonatal ewes to progestins, exhibit defects in conceptus survival and elongation between Day 9 and 14 of pregnancy (Gray *et al.* 2001). Several uterine- and conceptus-derived factors have been implicated in regulating peri-implantation conceptus development. In addition, mitogenic proteins have been detected in uterine flushes from pregnant ewes (Bird *et al.* 1988; Ko *et al.* 1991). In summary, it is clear that uterine- and conceptus-derived factors modulate peri-implantation conceptus development in ruminants but the key factors controlling this process remain undefined.

UTERINE-DERIVED GROWTH FACTORS

Cytokines and growth factors present within the uterine secretory milieu that are known to regulate trophectoderm proliferation and IFN-τ production include insulin-like growth factor- (IGF-) I and II (Ko *et al.* 1991), granulocyte-macrophage colony stimulating factor (GM-CSF; Imakawa *et al.* 1993), and fibroblast growth factor-2 (FGF-2; A. Ealy *et al.,* unpublished observations). The effects of FGF-2 are of particular interest to our laboratory due to its potent angiogenic, mitogenic and gene regulatory activities. In vertebrates, the FGF family comprises at least 23 members that function as mitogenic, angiogenic, chemotactic, and differentiation factors (Ornitz and Itoh 2001). The primary protein formed from the FGF-2 gene is 16–18 kDa, but additional proteins that range from 20 to 24 kDa can be produced by premature initiation of translation. Like FGF-1, FGF-2 does not contain a secretory signal peptide sequence and is not released from cells through secretory granules derived from the endoplasmic reticulum and golgi apparatus. The mechanism of FGF-2

release remains unknown. The membrane-associated receptors that bind FGFs (FGFR) are derived from 4 distinct genes, 3 of which are alternatively spliced to generate various receptor isoforms (Ornitz and Itoh 2001). FGF-2 is known to interact primarily with FGFR1 (IIIb and IIIc spliced variant forms), FGFR2-IIIc, FGFR3-IIIc, and FGFR-4. Cytoplasmic domains of FGFR contain intrinsic tyrosine kinase activity that regulates numerous signalling pathways, including Ras-mediated systems and PKC.

There is substantial evidence that FGF-2 is involved with regulating conceptus development. Early bovine embryo development *in vitro* is increased by addition of FGF-2 and transforming growth factor-beta (TGFß; Larson *et al.* 1992). Immunoreactive FGF-2 is expressed by the uterus and has been localised to glandular and luminal epithelium, stroma and myometrium. Uterine FGF-2 expression is influenced by pregnancy in some species. In the rat, immunoreactive FGF-2 is detected in luminal epithelium and uterine luminal flushes at Day 4 to 5 of pregnancy, but not before (Carlone and Rider 1993). In the pig, intense FGF-2 immunoreactivity is observed within the luminal epithelium beginning at Day 10 of pregnancy whereas luminal localisation of FGF-2 is less pronounced in non-pregnant gilts (Gupta *et al.* 1997). Several FGFRs, including those that interact with FGF-2, are detectable in bovine and mouse embryos (Rappolee *et al.* 1998), in post implantation ovine placentae (Chen *et al.* 2000) and in various peri- and post implantation placental and embryonic tissues in mice (Rappolee *et al.* 1998).

FGF-2 AFFECTS TROPHECTODERM FUNCTION

The aim of the present work was to determine if FGF-2 is expressed in the bovine uterus and investigate whether FGF-2 modulates the rate of trophectoderm proliferation and the production of IFN-τ mRNA and protein in cattle.

Western blotting was performed to establish the existence of FGF-2 protein in the bovine uterine lumen. When using a polyclonal antiserum generated against bovine FGF-2, 2 prominent immunoreactive bands (~18 kDa and ~37kDa) were detected in uterine flushes from pregnant and non-pregnant cows. The same 2 immunoreactive proteins were detected with the use of polyclonal antibodies directed against human or mouse FGF-2 or with a monoclonal antibody directed against human FGF-2. The less intense ~18 kDa band is similar in size to FGF-2 proteins derived from other tissues (16–18 kDa). FGF-2 mRNA was readily detected in bovine endometrium using quantitative real-time RT-PCR and *in situ*-hybridisation. The relative abundance of FGF-2 mRNA did not differ between endometrial samples isolated from pregnant and non-pregnant cows at Day 17 post oestrus. FGF-2 mRNA was localised specifically to the luminal and glandular epithelium of the bovine endometrium. There was no discernable difference in the intensity of FGF-2 mRNA staining in the luminal and glandular epithelium between pregnant and non-pregnant cows.

A bovine trophectoderm cell line, termed the CT-1 cell (for cow trophectoderm), was used to examine if FGF-2 influences proliferation and IFN-τ production during early pregnancy in cattle. This cell line was generated by serial passage of trophectoderm derived from a single bovine blastocyst (Talbot *et al.* 2000). These cells grow as a monolayer in distinct colonies and retain characteristic features of trophectoderm cells (small, cuboidal shape; prominent nuclei; numerous secretory granules). Moreover, CT-1 cells secrete IFN-τ constitutively into culture medium.

To determine if FGF-2 supplementation increases the rate of IFN-τ protein production from CT-1 cells, boFGF-2 was supplemented at various concentrations to CT-1 cells. After 72 h, IFN-τ concentrations were measured by antiviral assays and normalised by CT-1 cell number at the end of the incubation period. Supplementation with boFGF-2 increased IFN-τ concentrations in medium at 72 h post treatment in a dose-dependent manner. Quantitative real-time RT-PCR was completed to determine if FGF-2 supplementation increases the concentration of IFN-τ mRNA in CT-1 cells. Supplementing CT-1 cells with boFGF-2 for 24 h increased the relative abundance of IFN-τ mRNA versus control (normalised with the abundance of 18S RNA). FGF-2 also was found to stimulate the rate of trophectoderm proliferation. Supplementing CT-1 cells with boFGF-2 increased the rate of [3H]-Thymidine incorporation into DNA when compared to non-treated controls.

In summary, FGF-2 is expressed by the endometrium and present within the uterine lumen

during early pregnancy in cattle. Moreover, FGF-2 acts as a mediator of trophectoderm proliferation and IFN-τ production in bovine trophectoderm cells *in vitro.* The impact of FGF-2 on the overall production of IFN-τ is substantial when one considers the combined influence that FGF-2 has on the extent of IFN-τ gene transcription and trophectoderm proliferation. In conclusion, the uterus plays an active role in regulating the establishment and maintenance of pregnancy in ruminants by secreting factors, such as FGF-2, that control trophectoderm proliferation and IFN-τ production. Suboptimal production of these factors is likely to compromise the establishment and maintenance of pregnancy in ruminants. It is anticipated that continued effort to identify and characterise uterine-derived factors that regulate IFN-τ production and conceptus development will provide opportunities for developing schemes to improve conceptus survival in cattle and other ruminants.

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GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) STIMULATES INTERFERON-TAU (IFNτ) SECRETION BY EARLY OVINE EMBRYOS: IMPLICATIONS FOR LATER DEVELOPMENT?

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INTRODUCTION

After its discovery and subsequent recognition as an interferon in the late 1980s, interferon-tau (IFNτ) has received much attention because it is the primary embryonic signal for maternal recognition of pregnancy in ruminants (Demmers *et al.* 2001; Spencer *et al.* 2004). It is therefore an ideal experimental paradigm for investigating signalling between the implanting embryo and maternal uterine tissues. For example, there is good evidence for interactions between secretion of IFNτ by the implanting embryo and GM-CSF by uterine tissues (Emond *et al.* 2004). However, it is also a property of blastocyst-stage embryos whether produced *in vivo* or *in vitro* (IV) that IFNτ is expressed by trophectoderm cells and secreted into culture media. The expression and secretion of IFNτ by embryos is influenced by environment (IV culture (IVC) conditions), sex of embryo and sire and dam factors (Kubisch *et al.* 2001). It is not clear whether IFNτ secretion by blastocyst-stage embryos is functionally related to current quality or subsequent ability to implant; indeed it has been suggested that IFNτ secretion may be a stress-related response (Kubisch *et al.* 2001). Although addition of GM-CSF to IVC media improves embryo yield and quality in a number of species (cattle; de Moraes and Hansen 1997), its ability to modify secretion of IFNτ by ruminant embryos has not been investigated. Therefore, the data presented here describe 2 studies investigating the effect of GM-CSF on IFNτ secretion *in vitro*; the results suggest relationships to *in vivo* mechanisms.

MATERIALS AND METHODS

Full experimental details have been submitted (Rooke *et al.* 2005). Brief, in both studies, presumptive ovine zygotes were produced from abattoir-derived oocytes by conventional IV maturation and fertilisation procedures. Zygotes were then cultured for 6 days in SOF-based media (changed at 48 h intervals) containing either 10% albumin w/v and amino acids (A) or 10% w/v adult ewe serum (S). Recombinant ovine GM-CSF was added to media, where stipulated, at 5 ng/ml. In the first study, using a 2 × 2 factorial arrangement, zygotes were cultured in groups of 10 in SOF plus either albumin or serum in the presence (P) or absence of GM-CSF (O) until Day 7 post insemination. On both Days 6 and 7, blastocysts were removed from group culture. They were then reallocated in a balanced manner to individual culture for a further 24 h in one of the 4 treatments. In the second study, zygotes were cultured in SOF plus albumin. GM-CSF was absent or present during Periods 1 (Days 1–3), 2 (Days 3–5) or 3 (Days 5–7) of IVC in 6 combinations: OOO, OOP, OPP, PPP, PPO and POO. Conditioned culture media samples were recovered after each 48 h period during group culture, or after individual culture. Integrity of samples from each individual drop was maintained and samples were assayed for immunoreactive IFNτ by ELISA. Data were analysed by Anova, after log transformation of IFNτ concentrations.

RESULTS

In Study 1, media recovered on Day 7 after 48 h culture of groups of embryos in the presence of either GM-CSF or serum contained greater IFNτ concentrations (both $P<0.001$; Fig 1) than controls. Serum increased IFNτ concentrations to a greater extent than GM-CSF. When blastocysts were cultured for 24 h in individual micro-drops

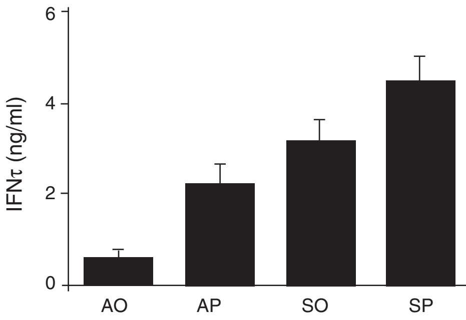

Fig 1: IFNτ concentrations (mean ± se) in conditioned media after 48 h group culture of embryos between Days 5 and 7.

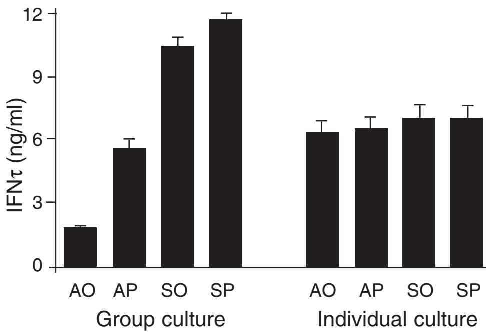

Fig 2: Effects of culture media (albumin v serum) and presence of GM-CSF (5 ng/ml) either during group culture or subsequent individual culture.

(Fig 2), it was the media-type in which they had been cultured previously, rather than the medium in which they were cultured individually, which influenced IFNτ secretion. Both serum and GM-CSF increased IFNτ concentrations (P<0.001). The effects of GM-CSF and serum were not additive (interaction P<0.001).

In the second study, effects of GM-CSF on IFNτ concentrations in conditioned media were assessed after each 48 h period of culture (Fig 3). IFNτ was detectable in some micro-drops on Day 3 with more micro-drops being positive for IFNτ (p<0.001) when GM-CSF was present (Fig 3a). Concentrations of IFNτ successively increased from Day 3 through Day 5 to Day 7. On both Days 5 (Fig 3b) and 7 (Fig 3c), it was inclusion of GM-CSF in culture between Days 1 and 3 of culture that resulted in significant increases (P<0.001) in IFNτ concentration. Inclusion of GM-CSF between Days 3 and 5 and between Days 5 and 7 had little or no effect on IFNτ concentrations.

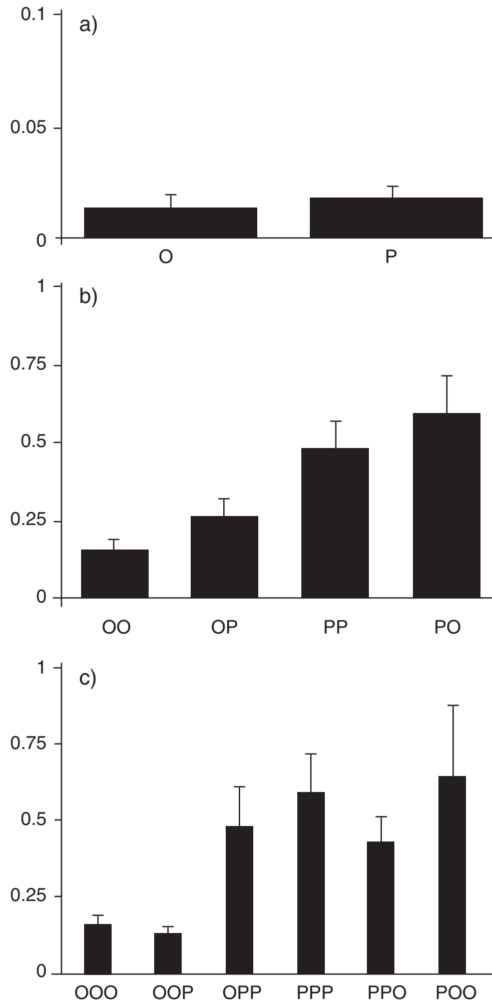

Fig 3: IFNτ concentrations in conditioned media (ng/ml) recovered after 48 h culture on Days 3 (a). 5 (b) and 8 (c) of in vitro *culture. Zygotes were exposed (P) or not exposed (O) to 5 ng/ml GM-CSF in different combinations of 48 h.*

Discussion

The 2 studies briefly reported here lead to 3 main conclusions. First, this is the first report that IFNτ secretion by blastocyst-stage embryos is increased by exposure to GM-CSF. Secondly, immuno-detectable IFNτ was found in conditioned media much earlier than the blastocyst stage reported previously as the onset of secretion. Finally, inclusion of GM-CSF in culture between Days 1 and 3 stimulated IFNτ secretion at later stages of culture.

GM-CSF has been implicated as a maternal component of the signalling pathways involved in maternal recognition of pregnancy in ruminants. It is expressed and synthesised by ruminant endometrial cells, its expression is stimulated by IFNτ and, in turn, IFNτ secretion by a bovine trophectoderm cell line is stimulated by GM-CSF. Indeed an amplification loop involving IFNτ and GM-CSF has been suggested to be active at implantation. The present studies suggest an earlier involvement of GM-CSF in IFNτ secretion by ruminant embryos. Consideration of the stage of embryo development at which GM-CSF temporally stimulated IFNτ secretion is suggestive of an event at or prior to the maternal-zygotic transition when major embryo gene activation occurs. If this is true, then the stimulation of IFNτ secretion by GM-CSF may depend on maternal mRNA and thus a response not mediated at the transcriptional level; this is in agreement with studies on bovine trophectoderm cells (Ocon *et al.* 2004). Therefore the ability of a bovine embryo to produce IFNτ may in part be dependent on oocyte-specific factors and therefore upon oocyte 'quality'.

An important question then is the biological relevance of stimulation of IFNτ secretion by GM-CSF between 24 and 72 h post insemination of oocytes *in vitro*. Semen has been postulated to have a priming effect on the female reproductive tract. In mice, insemination stimulates a transient immune response part of which is up-regulation of uterine epithelial expression of GM-CSF, with seminal plasma $TGFß_1$ being implicated (Tremellen *et al.* 1998). Recently, GM-CSF expression in pig endometrium 34 h after intra-uterine infusion of seminal plasma was shown to be 5-fold greater than in control pigs (O'Leary *et al.* 2004). This timing is similar to the timing of the GM-CSF effect observed in the current study *in vitro* in the ovine. There do not appear to be any parallel studies in ruminants on the effects of seminal plasma on female reproductive function. However, if findings in the pig and mouse can be extrapolated to ruminants, then results from the current study suggest that seminal plasma may have a priming effect on the recently fertilised ovum, albeit indirectly through stimulation of endothelial GM-CSF secretion. This may have developmental consequences for the ability of the embryo to signal its presence at implantation.

Acknowledgements

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PREGNANCY-ASSOCIATED GLYCOPROTEINS: A COMPLEX FAMILY OF TROPHOBLAST GENES EXPRESSED BY THE UNGULATE PLACENTA

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OVERVIEW

The pregnancy-associated glycoproteins (PAGs), found in ruminant ungulates, comprise a complex multi-member family of trophoblast-expressed proteins. In cattle alone, there are predicted to be dozens of expressed genes. So far, 21 distinct full-length cDNAs have been identified in cattle, 9 in sheep and 11 in the goat (Garbayo *et al.* 2000; Green *et al.* 2000). More recently, 10 additional PAG clones have been identified in white tailed deer (Vawter *et al.* 2004).

As the name suggests, the PAGs are glycoproteins with extensive glycosylation (both n- and o- linked). In cattle, they range in molecular weight from 55 to 90 kDa and much of their mass is due to glycosylation; the unglycosylated molecular mass of most mature PAGs is predicted to be 37 kDa. Besides glycosylation, these proteins undergo additional post-translational modifications such as phosphorylation.

EVOLUTIONARY RELATIONSHIPS

Based on their evolutionary relationships, PAGs can be classified into 2 distinct classes: A group of ‘ancient’ PAGs that arose ~87 million years ago (MYA) and more recently evolved ‘modern’ PAGs that arose subsequently at ~52 MYA (Hughes *et al.* 2000). The PAGs belong to the aspartic peptidase family of enzymes, a grouping that includes gastric enzymes such as pepsin and chymosin, lysosomal enzymes such as cathepsin D & E, and metabolic enzymes such as renin. Despite their relationship and structural similarity to aspartic peptidases, many of the modern PAGs are believed to be inactive as enzymes due to mutations in key residues comprising the catalytic center. The ancient PAGs, on the contrary, have retained an intact catalytic centre and are predicted to be functional peptidases; however, it is worth noting that none of the ancient PAGs has been shown to exhibit any proteolytic activity thus far. Nevertheless, both the modern and ancient PAGs have retained their capacity to bind peptides as demonstrated by their ability to bind pepstatin-A, a specific hexapeptide inhibitor of aspartic peptidases. This property of PAGs to bind pepstatin, although with different affinities, has been effectively harnessed to purify various subsets of native PAGs from placental extracts.

PAG EXPRESSION

In addition to the phylogenetic and structural variations, both the ancient and modern PAGs also exhibit differences in their expression patterns. Localisation of PAG mRNAs within the placentome by *in situ* hybridisation demonstrated that, while some PAGs are found uniformly throughout the trophoblast, the majority are expressed exclusively in the invasive trophoblast binucleate cells (BNCs) (Green *et al.* 2000). Interestingly, the localisation pattern corresponded to the ‘ancient’ and ‘modern’ groupings, with the ancient members being expressed in all trophoblasts and the modern PAGs being localised to BNCs. In fact, the ruminant ungulate’s unusual trophoblast BNCs and synepitheliochorial type of placentation are believed to have appeared around the time of their divergence from the swine lineage, which is relatively recent (50–55 MYA). This correlation in the estimate of when the modern PAGs first arose and the evolution of the synepitheliochorial placenta (from the epitheliochorial type in swine)

of the ruminant lineage is more than likely to be a coincidence.

The PAGs also exhibit differences in their temporal expression patterns. While some BNC-PAGs ('modern PAG'), such as bovine PAG-9, appear very early in pregnancy coinciding with the earliest appearance of BNCs (~ Day 19 in cattle), others are only seen later in pregnancy. Recent immuno-histochemical localisation studies with antibodies specific to ancient and BNC-PAGs have contributed to the expansion of our understanding of PAG distribution. Within the placentome, the BNC-PAGs, apart from localising to binucleate cells, are also seen in association with the maternal caruncular stroma adjacent to chorionic villi. Likewise, the ancient PAGs in the placentome are found in abundance lining the microvillar junction at the maternal-fetal interface, but are confined to the BNCs within the interplacentomal regions. Consequently, these new observations may necessitate a revision of the PAG nomenclature since it is apparent that the 'BNC-PAG' designation used for the past few years to describe the modern PAGs is not entirely accurate.

IDENTIFICATION OF PAG-LIKE GENES

Although the PAG family is quite large and complex, most of the family members are restricted to ruminant ungulates (*Ruminantia* suborder). Many attempts have been made to define the family in species outside the *Artiodactyla* order. These studies have led to the identification of a small group of PAG-like genes. These molecules resemble PAG family members more closely than any other aspartic peptidase, including pepsin A and cathepsin D, and are believed to represent the ancestral gene from which the PAG family arose. They display trophectoderm-specific localisation and fall outside the *Ruminantia* suborder. The PAG-like molecules have been identified in the horse (*Perisodactyla*), cat (*Carnivora*), rabbits (*Lagomorpha*), mice and rats (*Rodentia*) (Green *et al.* 1999; Kageyama *et al.* 2000; Chen *et al.* 2001). The first member in this group to be described was that from rabbit (Kageyama *et al.* 2000). It was found to be transiently expressed in the neonatal gastric mucosa, specifically within gastric chief cells, and was named 'pepsinogen F' or 'pepF'. Orthologous proteins in other species were also found to be localised to the neonatal stomach, but not the adult stomach, as well as to the placenta. Indeed, the expression of pepF in both of these organs distinguishes these genes from the PAG family. Another distinctive characteristic of the pepF group is that pepF possesses little, if any, glycosylation. Additionally, recombinant equine and mouse pepF do exhibit general proteolytic activity. The proteolytic activity of the pepF group suggests that they are functionally distinct from the other ruminant PAGs and unlike the diverse multigene PAG family of the ruminant ungulates, the PAG-like proteins (PepF) appear to be unigenic.

PAG FUNCTION

There are 2 major prevailing hypotheses regarding the functional role of the BNC-PAGs. According to one hypothesis, BNC-PAGs function as a signal from the conceptus to indirectly maintain progesterone production by the ovary. For instance, Del Vecchio *et al.* (1995) reported an increase in luteotrophic PGE_2 and progesterone production from primary bovine luteal cell cultures upon treatment with boPAG-1 (PSPB). Likewise, following treatment of ovine endometrial cells with boPAG-1, Weems *et al.* (2003) recorded an increase in the ratio of the luteotrophic prostaglandin, PGE_2, versus luteolytic $PGF_{2\alpha}$. Another hypothesis hints at an immunological role for these PAGs. According to this hypothesis, the PAGs are proposed to function as immunomodulatory agents by protecting the fetus from destruction by the maternal immune system. For example, Dosogne *et al.* (1999, 2000) reported a decrease in oxidative burst activity of polymorphonuclear leukocytes by boPAG-1 *in vitro*. Similarly, Hoeben *et al.* (1999) reported a reduction in proliferative activity of bovine haematopoietic cells by boPAG-1 *in vitro*. Among the 2 predominant hypotheses, the argument for BNC-PAGs as luteotrophic agents is perhaps the least convincing, as one may wonder about the need for so many BNC-PAGs as 'luteotrophic agents', when one or a few would suffice. Alternately, the aggregation of BNC-PAGs to the proximal uterine stroma would place them in a position to protect the fetal allograft by manipulating lymphocyte and PMN leukocyte migration and or activation. Indeed, the changes in prostaglandin profiles reported by Del Vecchio *et*

al. (1995) and Weems *et al.* (2003) may be reflective of an immunological role, as prostaglandins are known immunomodulatory agents. In contrast to the BNC-PAGs, the role of the proteolytically active ancient PAGs at the maternal fetal interface is still not clear and is under investigation.

Summary

The PAGs are a group of trophoblast-expressed genes with considerable diversity in number, organisation, activity and distribution. Given these characteristics, they appear to represent a class of proteins that have evolved to meet the specialised challenges that have arisen as a result of the unique synepitheliochorial placenta of ruminant ungulates. They seem to be constituents of a wide array of active molecules produced by the fetus, engaged in an active dialogue with the mother to manipulate her physiology.

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SESSION 5:

Conceptus protein expression

Chairman:

C. Aurich

MAJOR PROTEINS IN THE EMBRYONIC CAPSULE, AND IN YOLK-SAC AND UTERINE FLUIDS, DURING THE SECOND TO FOURTH WEEKS OF PREGNANCY IN THE MARE

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INTRODUCTION

Once in the uterus, the equine conceptus becomes enveloped by a polysaccharide-rich capsule that is modified and lost during the third week of gestation (Oriol *et al.* 1993; Betteridge 2000). At about Day 16, the conceptus loses its mobility in the uterine lumen and becomes 'fixed' at the site of future placentation (Ginther 1983; Enders and Liu 1991). From the time of fixation, the amount of sialic acid in the capsular polysaccharides decreases relative to amounts of galactose (Gal) and N-acetylgalactose (GalNAc) (Oriol *et al.* 1993). These changes in capsular polysaccharides might be passive consequences of degradation of the capsule. Alternatively, the loss of sialic acid might have some functional role in fixation, for example by exposing saccharides that interact with the endometrium or by modifying the permeability of the capsule to substances produced by the endometrium or the conceptus. L-selectins expressed by the endometrium initiate attachment of the human blastocyst (Fazleabas and Kim 2003; Genbacev *et al.* 2003) but, for the horse, endometrial proteins that bind specifically to the capsular polysaccharides have not been defined.

In these studies, the main objective was to characterise the changes in proteins that are preferentially associated with the capsule before, during and after fixation. To better identify the origin of capsular proteins, the authors also looked for similar proteins in the yolk-sac and in the uterine lumen.

MATERIALS AND METHODS

For the main part of this study, individual equine conceptuses (n=37) collected between Days 13 and 25 were processed and samples stored briefly at –70°C until analysed. Additional conceptuses (n=20) of similar age from previous breeding seasons were stored at –20°C and examined separately to help the authors recognise artifacts that increase with storage or processing. Conceptuses were recovered transcervically by flushing the uterus with 1 litre of phosphate-buffered saline and transferred into a petri dish for yolk-sac fluid collection by puncture and aspiration (Waelchli and Betteridge 1996) and separation of entire capsules. The capsules were then collected on a paper filter, washed with PBS and cut into portions for analysis. Lavage and yolk-sac fluids were centrifuged immediately to remove cells and particulate debris. Proteins in cell-free uterine flush and yolk-sac fluids were concentrated 50 or 10 times respectively by centrifugal ultrafiltration (5 kDa cutoff).

All samples were subjected to one-dimensional SDS-PAGE (15%) under reducing conditions. Proteins were eluted from the capsule by boiling in SDS-PAGE sample buffer under reducing conditions. Multiple electrophoretic runs surveyed the changes over the 13 to 25 day age sequence for capsule, yolk-sac fluid and uterine lavage samples. All time points were represented by at least 3 conceptuses. Major bands in the capsule that changed most obviously and reproducibly over the fixation interval (14–17 days) were first defined by reduced kDa and compared with bands of similar mobility in uterine lavage and yolk-sac samples.

RESULTS AND DISCUSSION

Proteins in capsule extracts were sparse at all time points. Two (p10 and p17) were most prominent

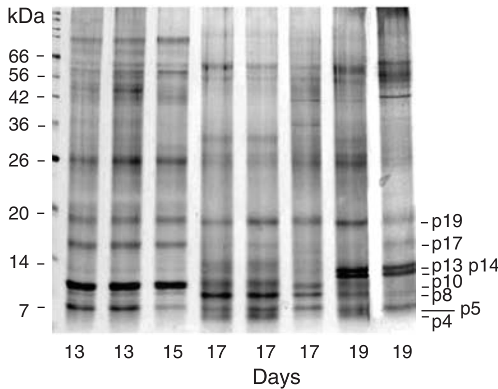

Fig 1: Silver stained SDS-PAGE gel (reducing) comparing proteins extracted from the capsule of equine conceptuses of various ages.

before Day 16, then decreased rapidly after Day 17 and became undetectable after Day 18 (Fig 1). The more abundant of these (p10) was a strong match for equine beta-2-microglobulin (B2M) by MALDI-TOF MS and N-terminal amino acid sequence (GI:231622) (Ellis and Martin 1993). B2M is expressed in the chorionic girdle of equine trophoblasts at 33 days as part of the MHC class 1 complex (Bacon *et al.* 2002), and in the human villous syncytiotrophoblast where it is associated with HFE iron uptake (Leitner *et al.* 2002). However, in the capsule of pre-fixation conceptuses in this study, the authors did not observe bands that corresponded to the MHC class I heavy chains.

The trypsin peptide fragment masses from p17 matched most closely with equine α lactalbumin A (GI:125991), a 14 kDa milk protein that is a member of the lysozyme family. N-terminal sequencing attempts were unsuccessful. In tissues other than the mammary gland, α lactalbumin A is a galactosyl transferase that transfers Gal to oligosaccharides (Kaminogawa *et al.* 1984).

A less prominent 19 kDa band in all capsule preparations was relatively constant through the period of interest (Fig 1). This corresponded with a 19 kDa band that was the most abundant in uterine lavage fluids (Table 1). MALDI-TOF MS analysis of a similar prominent band in a Day 13 endometrial membrane preparation provided a strong match with equine uterocalin (GI:1403310) (Crossett *et al.* 1996). While p19-uterocalin is a major protein in the uterine flush fluids through Days 14–25, it was observed as a major protein in the yolk-sac fluid only during the period of fixation (Days 14–16), after which it was no longer detectable (Table 1). This is consistent with its proposed role in transport from endometrium to embryo (Suire *et al.* 2001).

A band designated p4 was abundant in all uterine lavage samples in which p19-uterocalin was prominent (Table 1). A band of similar size was seen in capsule samples at Day 15–18. A p4 band also appeared in yolk-sac fluids along with p19-uterocalin during Days 15–16 (Fig 1).

A prominent band of ~8 kDa (p8) was consistently observed in fresh or archived samples

TABLE 1: Changes in major protein bands in electrophoretic samples of the capsule of the equine conceptus, compared with yolk-sac and uterine flush samples

Band kDa	Protein	Amounts in various samples		
		Capsule	Yolk-sac	Flush
p19	Uterocalin	2+ Days 13-25	2+ Days 13-14 4+ Days 15-16	3+ Days 13-25
p17	α-Lactalbumin ?	2+ Days 13-15	ND*	ND
p14	α-Haemoglobin	1+ Days 16-25	4+ Days 19-25	2+ Days 13-25
p13	ß-Haemoglobin	1+ Days 16-25	4+ Days 19-25	2+ Days 13-25
p10	ß2-Microglobulin	3+ Days 13-15 1+ Days 15-17	ND	ND
p8	Unknown	3+ Days 15-18 1+ Days 18-19	ND	ND
p5	Unknown	2+ Days 18-25	ND	ND
p4	Uteroglobin	1+ Days 15-18	2+ Days 15-16	3+ Days 13-25

*ND = not detected

of capsule collected at Days 17–18 (Fig 1). Minimal amounts of protein in the 8 kDa region were detectable in the capsule before Day 17. By Day 19, amounts of p8 in the capsule were reduced and subsequently undetectable. There was no obvious p8 counterpart in either yolk-sac or uterine lavage preparations at any time in the 13–25 day period. This indicates that p8 is preferentially associated with the capsule at the time of fixation. Another band (p5) was observed most prominently in the capsule after Day 18 (not shown).

After Day 17, a pair of bands of 13 and 14 kDa appeared in variable amounts in the capsule (Fig 1). These were identified by MALDI-TOF MS as the α and ß subunits of equine haemoglobin (GI: 230632, GI: 122614). Similar p13 and p14 bands were also observed in most uterine lavage fluids collected at various stages of gestation, and they became abundant in yolk-sac fluid after Day 20 (Table 1). It is possible that the altered capsule binds maternal or embryonic haemoglobin more readily, but the ubiquity of haemoglobins suggests that they are non-specific contaminants.

These studies indicate that the capsule of the equine conceptus undergoes a rapid change in protein composition at the time it becomes fixed within the uterus (Table 1). It will be important to determine how the capsule changes its ability to preferentially bind, produce or alter these proteins and how this transition is involved in normal and impaired fixation.

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LOW MOLECULAR WEIGHT PROTEINS SECRETED BY THE EARLY EQUINE CONCEPTUS

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INTRODUCTION

The horse is one of the few commercially important animal species in which the maternal recognition of pregnancy (MRP) signal, ie the conceptus factor(s) responsible for ensuring prolongation of the primary corpus luteum (CL), has yet to characterised. Of the candidate MRP signals examined, interferons are produced only in small amounts by equine conceptuses and neither oestrogens nor PGE_2, both of which are produced in considerable quantities during Days 10–18, have been definitively proven to extend dioestrus or effect the absolute reduction in endometrial $PGF_{2\alpha}$ secretion that underlies MRP in the mare.

One approach that has yielded progress with regard to the possible nature of the equine MRP signal, is to investigate the size of the conceptus product that inhibits $PGF_{2\alpha}$ secretion by equine endometrium incubated *in vitro*. In this respect, Weithenauer *et al.* (1987) concluded that the equine conceptus MRP factor must have a molecular weight of 1–6 Kda, based on the molecular weight cut-offs of dialysis tubing that either did or did not allow a contained conceptus to inhibit endometrial $PGF_{2\alpha}$ secretion. Ababneh *et al.* (2000) used size fractionation of conceptus products to similarly conclude that the MRP factor must have a molecular weight of 3–10 Kda. In a preliminary experiment, Stout, Stewart and Allen (unpublished data) used SDS-PAGE and n-terminal amino acid sequencing to identify 2 proteins isolated in the yolk-sac fluid of, and culture medium conditioned by, Day 10–18 equine conceptuses as ubiquitin (8.5 Kda) and the ß-chain of insulin (3.5 Kda). Insulin was particularly interesting because it appeared to be released in greater quantities by Day 10–18 than older conceptuses. The current study aimed to verify gene expression for ubiquitin and insulin by early equine conceptuses using RT-PCR. In addition, because circulating insulin has been reported to affect endogenous secretion of LH and progesterone, a second experiment was performed to examine the effects of systemic insulin administration on luteal lifespan in cycling mares.

MATERIALS AND METHODS

In Experiment 1, 31 conceptuses were recovered from Warmblood mares on Days 7–16 after ovulation by non-surgical uterine lavage using either Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 % (v:v) fetal calf serum (FCS: Day 7 conceptuses) or Ringer's solution (Day 10–16 conceptuses). After recovery, the conceptuses were washed in fresh flushing solution (without FCS), and the membranes were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Conceptus RNA was isolated, and endogenous DNAse digested simultaneously, using the RNeasy Mini Kit and the RNAse-free DNAse Set (Qiagen, Valencia, USA). Thereafter, cDNA was synthesised using random primers, and RT-PCR was performed using primers for ubiquitin and insulin based, respectively, on the equine sequence or regions of DNA conserved well across other mammalian species. In the case of ubiquitin, quantitive PCR was then used to determine whether the pattern of mRNA expression altered with gestational age (technique described by Rambags *et al.* 2005).

In Experiment 2, 6 Warmblood mares were treated daily on Days 7–17 after ovulation of 2 successive oestrous cycles with either insulin (0.01 iu Actrapid and 0.2 iu Caninsuline per kg bwt) or control saline. Mares were assigned randomly to

initial treatment group and switched to the other group during the subsequent cycle. Daily blood samples recovered throughout the treatment period and until the subsequent ovulation have yet to be assayed for concentrations of LH, progesterone and insulin. However, to get an immediate indication of cycle stage and luteal activity the tone and ultrasonographic appearance of the uterus were monitored *per rectum,* and the maximum cross-sectional surface area of the primary CL was recorded; the latter is a useful estimator of luteal function (Stout *et al.* 2002). In addition, from Day 12 after ovulation, the mares were teased on alternate days with a vigorous stallion to determine when they returned to behavioural oestrus.

Results

Experiment 1

Day 7–16 equine conceptuses expressed mRNA for ubiquitin. However, the relative rate of expression (calculated as the ratio of ubiquitin expression to that of a reference gene; ribosomal RNA) was very low (approximately 1,000 times lower than that of ribosomal RNA) and did not differ between conceptuses recovered on Days 7, 10 or 14 after ovulation (3.0 ± 0.7, 2.1 ± 0.3 and 2.5 ± 0.5; all $\times 10^{-3}$). Frustratingly, we have been unable to design PCR primers able to detect insulin mRNA in any equine tissue (including pancreas).

Experiment 2

Daily administration of insulin did not delay the return to behavioural oestrus (16.2 ± 0.4 versus 17 ± 0.9 days in treated and control mares, respectively) or the time of the subsequent ovulation (20.8 ± 1.3 vs 21.8 ± 1.9 days). Furthermore, daily insulin administration had no effect on maximum luteal cross-sectional surface area (see Fig 1).

Conclusions

The molecular size of insulin (6 Kda; ß-chain 3.5 KDa) and its apparent secretion in readily detectable quantities by Day 10–18, but not later stage, conceptuses earmarked it as a candidate equine MRP signal. While yolk-sac synthesis of insulin has been reported previously in the rat (from Day 14 of gestation: Muglia and Locker 1984), it does not appear to have been examined in many other species and it is unclear whether yolk-sac insulin is physiologically significant and, if so, what its functions are. On the other hand, recent studies have suggested that elevations in circulating insulin concentrations, usually as a result of nutritional state, may boost LH and progesterone secretion in various animal species including the horse (Sessions *et al.* 2004), although the veracity and anatomical level of any effects of insulin on luteal function in the mare are still to be confirmed. Disappointingly, the current study demonstrated that chronic insulin administration does not extend dioestrus in the mare and, since the intrauterine infusion of 875 mg insulin in 10 ml 0.9% saline on Days 10, 12 and 14 after ovulation also failed to prolong dioestrus in 4 cycling mares (Stout, Stewart and Allen, unpublished data), it seems unlikely that conceptus insulin is involved directly in MRP in the horse. Rather, if insulin synthesis by early equine conceptuses is confirmed, it may function to support fetal and placental development, as part of the IGF system.

Ubiquitin is an intracellular molecule used

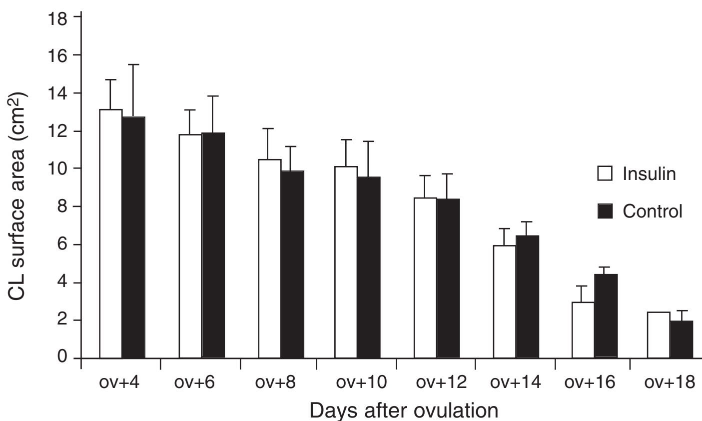

Fig 1: Mean (+ sem) corpus luteum cross-sectional surface area in mares treated daily with insulin or control saline during Days 7–17 after ovulation. CL surface area decreased over time, but did not differ between treatment groups.

primarily for covalently marking proteins prior to degradation by a large cytosolic protease (the 26S proteasome), in a process known as ubiquitination. However, it appears that ubiquitin can also act extracellularly to label cell surface proteins for subsequent internalisation and degradation in the lysosome system. Moreover, ubiquitin expression is upregulated during early pregnancy in both human and baboon endometrium (Bebington *et al.* 1999) where it is accompanied by the appearance of ubiquitin cross reactive protein, a head-to-tail ubiquitin-ubiqutin polyprotein that was first detected as an IFN-τ induced secretory product of pregnant cow endometrium (Austin *et al.* 1996). At present, it is believed that ubiquitin and ubiquitin cross-reactive protein secreted during early pregnancy help prepare the endometrium for conceptus adhesion. It will, therefore, be interesting to see whether a similar upregulation of ubiquitin-like molecules occurs in equine endometrium during early pregnancy, and it is postulated that equine conceptus-secreted ubiquitin may function to mark conceptus, capsule or uterine proteins for intracellular lysis.

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UTEROCALIN – PROVIDER OF ESSENTIAL LIPIDS AND AMINO ACIDS TO THE PRE-PLACENTATION EQUINE CONCEPTUS

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EQUINE UTEROCALIN

The equine conceptus does not form an effective placenta until around Day 40 of gestation. For about 22 days of this period it is enclosed in a glycoprotein capsule that presents a barrier between mother and embryo preventing the close tissue contact that would otherwise facilitate nutrient transfer. The conceptus's only source of nutrition, therefore, is secretions of the endometrial glands, the components of which must pass through the capsule and be absorbed by the trophoblast.

A notable protein component of endometrial gland secretions is uterocalin (also known as P19), which is a member of the lipocalin family of proteins, and has a molecular mass of ~19 kDa (Crossett *et al.* 1996; Suire *et al.* 2001). Uterocalin is only detectable in uterine secretions until about 23 days of pregnancy, and its production largely ceases around the time of escape from the capsule. The protein is detectable in the epithelial cells of the endometrium and its gland cells, and mRNA encoding it is only found in the endometrium and nowhere in the conceptus itself (Stewart *et al.* 1995; Crossett *et al.* 1998; Stewart *et al.* 2000). Uterocalin is detectable within the trophoblast cells of the embryo and the yolk-sac, so maternally-derived protein must be absorbed and internalised. Whether a proportion of this is subsequently released back to the mother is not known, but the likelihood is that most of it is withheld by the embryo.

HYDROPHOBIC LIGAND BINDING

Lipocalins have very similar tertiary structures and many transport sparingly soluble or chemically sensitive hydrophobic ligands (Flower 1996), although some bind quite different types of compound in order to play an anti-bacterial role (Goetz *et al.* 2002). We investigated ligand-binding

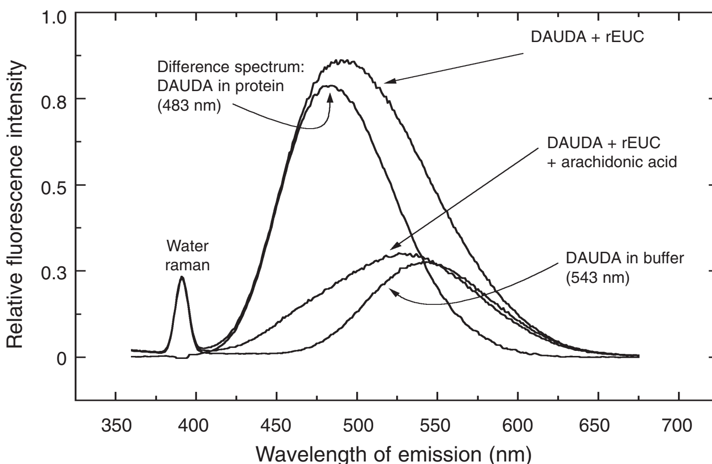

Fig 1: Equine uterocalin binds fatty acids. Fluorescence emission of a fluorophore-labelled fatty acid (11-(dansylamino)undecanoic acid; DAUDA) alone in buffer or after the addition of recombinant equine uterocalin (rEUC). The wavelength of peak emission by DAUDA when located in rEUC's binding site is as indicated by the difference spectrum. Also shown is the reversal of changes in DAUDA emission by competition with a polyunsaturated essential fatty acid (arachidonic acid) added to the rEUC:DAUDA complex. For full details see Suire et al. *(2001).*

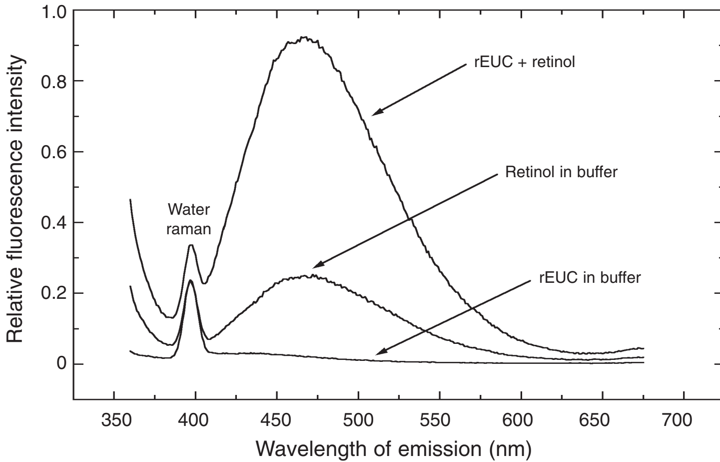

Fig 2: Equine uterocalin binds retinol. Fluorescence emission spectra of retinol in buffer, or when added to a solution containing recombinant equine uterocalin (rEUC). Although not shown, the addition of oleic acid to the mixture resulted in a reversal of the enhancement of retinol fluorescence emission. For full details see Suire et al. *(2001).*

properties of uterocalin, using recombinant protein produced in bacteria, and fluorescence-based lipid binding assays. These exploit the intrinsic fluorescence emission of natural lipids (such as retinol), or use lipids to which fluorophores are covalently attached. The methods rely on a change in the fluorescence emission of the ligand upon entry into a protein binding site, usually manifested as an increase in the intensity of fluorescence emission, and often accompanied by a shift in the wavelength of peak emission. Recombinant equine uterocalin (rEUC) was found to bind dansylated fatty acids (Fig 1), and the natural fatty acid *cis*-parinaric acid, and retinol (Fig 2; Suire *et al.* 2001). Dansylated fatty acids are artificial compounds, but can be competively displaced from a protein's binding site by a natural compound. Such experiments showed that EUC binds a range of saturated, monounsaturated and polyunsaturated fatty acids (Suire *et al.* 2001; Table 1). The former two may be crucial to the embryo's energy metabolism and membrane construction, and the latter may be required for nervous system lipids and as precursors of signalling molecules, such as prostaglandins, that are important in mammalian embryogenesis (Dutta-Roy 2000). Retinol and its derivatives are involved in a plethora of biological processes, including gene regulation and morphogenesis during development (usually as isoforms of retinoic acid) (Morris-Kay and Sokolova 1996), in addition to vision (as retinal).

EUC's prime function may therefore be as a carrier protein for essential lipids that are sparingly soluble, oxidation sensitive, or both. We also have preliminary information, however, that it binds progesterone (which could have implications for hormone communication bewteen mother and conceptus), but also an antibiotic, Rifampin. The latter finding could mean that this and other drugs might thereby concentrate adversely in the fetus, or be excluded.

ESSENTIAL AMINO PROVIDER

Another major requirement for a developing embryo is essential amino acids, the only source being the mother. The amino acid composition of

TABLE 1: Ligand binding specificity of recombinant equine uterocalin

Binding	No detectable binding
11-(dansylamino)undecanoic acid (DAUDA)*	cholesterol
dansyl-D,L-α-amino-octanoic acid (DACA)*	noradrenaline /arterenol
cis-parinaric acid**	dehydroergosterol*
oleic acid	
arachidonic acid	
docosahexaenoic acid	
cis-eicosapentaenoic acid	
γ-linolenic	
linoleic	
Vitamin A / retinol**	
progesterone?	
Rifampin?	

*Synthetic, fluorophore-conjugated lipids.
**Natural, fluorescent lipids.

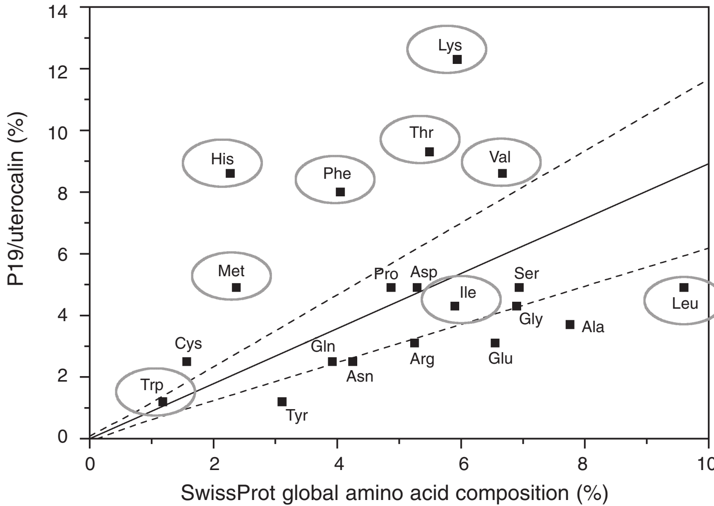

Fig 3: Equine uterocalin is enriched in essential amino acids. The graph shows the relationship between the percent amino acid composition of equine uterocalin and the percent amino acid composition average of all the proteins entered into the SwissProt database. The fitted lines are for guidance only, and are a trendline fitted to go through the origin and with arbitrary confidence intervals. The amino acids in ellipses are essential amino acids for mammals.

uterocalin itself shows an unusual bias such that it is peculiarly rich in most of the essential amino acids for mammals - histidine, lysine, phenylalanine, methionine and valine in particular (Fig 3). This is not apparent for any other known protein present in uterine secretions, or for any other lipocalin produced by equines. Uterocalin has an approximately average content of tryptophan and isoleucine, and the leucine content is slightly below average, but not notably so.

So, uterocalin appears to be a source of essential amino acids for protein synthesis, in addition to having the properties of a carrier of essential lipids for growth, metabolism and morphogenesis. The high content of histidines in uerocalin may also indicate that it binds metal ions, and our molecular modelling studies indicate a potential cluster of histidine side chains on the external surface of the protein, similar to that found in another metal-binding lipocalin.

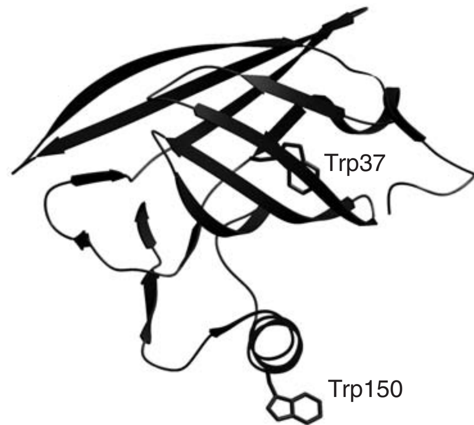

Fig 4: Unusual external exposure of tryptophan 150 in equine uterocalin. Molecular model of equine uterocalin positioned to show the predicted orientation of Trp150, pointing directly out from the major helix and into solvent. Such an orientation is unusual in proteins unless they are involved in interactions with other proteins or membranes. EUC has one other tryptophan, Trp-37, which is buried within the structure of the protein and is conserved in virtually all lipocalins. For full details see Suire et al. *(2001).*

Interaction with the trophoblast surface – direct or receptor bound?

An unusual feature of the molecular model of uterocalin is the presence and orientation of the side chain of a tryptophan (Trp150) on the external face of the single large helix (Fig 4). Tryptophans are moderately hydrophobic and are usually embedded within the structure of a protein, and sometimes in the wall of hydrophobic ligand binding sites. A tryptophan or similar aromatic amino acid in this position is highly unusual, if not unique, amongst lipocalins. Proteins possessing externally directed tryptophans are usually involved either in interaction with other proteins, or with membranes (reviewed in Kennedy and Beauchamp 2000; Suire *et al.* 2001). So, uterocalin might be designed to interact directly with the surface membrane of the trophoblast cells, or with a receptor embedded within it. Such interactions with the trophoblast would potentially provide a

mechanism for the rapid uptake of the protein, and may also avoid re-absorption by the maternal epithelium so long as the latter does not possess the appropriate receptor, or is of a composition such that uterocalin fails to interact significantly.

Concluding remarks – two-way traffic?

It seems clear that equine uterocalin's molecular and biochemical characteristics suit it for transport of essential nutrients, and possibly also hormones and other growth factors, to the embryo. But, is the traffic entirely one-way? Is it possible that some proportion of the protein is re-exported by the conceptus in order to carry poorly soluble hormones back to the mother to maintain the pregnant state and promote the production of transferable nutrients? Or, might diffusion of signal molecules (eg steroid hormones, eicosanoids) from conceptus to endometrium be facilitated by relay from one uterocalin molecule to another, through the matrix of the capsule (with which uterocalin associates).

It is conceivable that the efficiency of uterocalin's loading with ligands, the quality of that cargo, and its transfer to the embryo, may all be crucial to the quality of the offspring. Further investigation is clearly now required to identify uterocalin's true biological ligands rather than merely those that can bind *in vitro*. It must also be borne in mind that the protein might be loaded with different lipids, or varying proportions of lipid types, as gestation proceeds. Ultimately, it might be profitable to investigate the gene encoding uterocalin, and those of any receptors it may have. There might, for example, be polyporphisms in the protein-encoding regions, and/or hormone-sensitive promoter regions. And a final speculative thought is whether or not male and female conceptuses induce the production of different quantities of this crucial nutritional protein by the mother.

Acknowledgements

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SESSION 6:

The capsule

Chairman:
M. W. Kennedy

EQUINE EMBRYO ENCAPSULATION: EPHEMERAL, ESSENTIAL AND ENIGMATIC

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INTRODUCTION

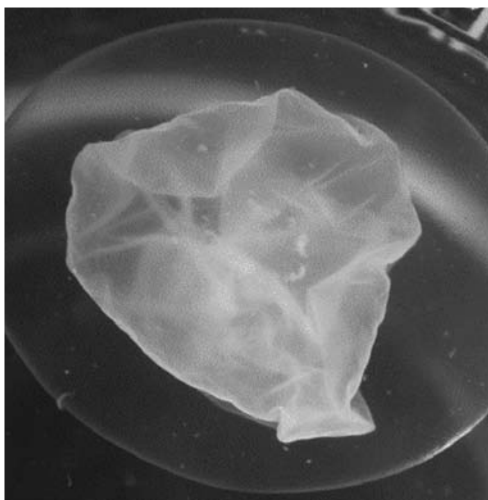
Acellular blastocyst coverings replace the zona pellucida before implantation or attachment of the conceptus to the endometrium in several mammalian species (Betteridge 1989; Denker 2000). One such covering is the embryonic capsule that envelops the horse conceptus throughout the second and third weeks of pregnancy. This presentation reviews briefly what we know about how the capsule is formed, modified during its brief existence, and lost at the beginning of the fourth week. Further, since the capsule can be assumed to play an important role in the dialogue between the mare and her embryo at a time critical to the maintenance of pregnancy, discussion is focused on how the capsule may affect the dialogue, and how this might be studied experimentally.

THE EPHEMERAL NATURE OF THE CAPSULE

Formation of the capsule normally begins on the inside of the zona pellucida soon after the embryo enters the uterus and forms a blastocyst (references in Betteridge 1989; Oriol *et al.* 1993a,b; Albihn *et al.* 2003). The zona pellucida is not essential to the process but the uterus probably is; to date, capsule production by embryos cultured *in vitro* has not been observed (Choi *et al.* 2004). Structurally, the capsule consists of mucin-like glycoproteins (Oriol *et al.* 1993a,b) but it also contains many other proteins involved in the transport of materials into (and presumably out of) the developing conceptus (Crosset *et al.* 1998; Suire *et al.* 2001; Quinn *et al.* 2005). The trophoblast has been shown to produce the mucin-like glycoproteins (Albihn *et al.* 2003). An important developmentally-regulated modification of the capsule's structure is the loss of sialic acid at about the time of conceptus fixation (Day 16); the consequent loss of negative charge may contribute to the cessation of the conceptus' mobility at that time (Oriol *et al.* 1993b). The physical nature of the capsule also changes markedly at about the time of fixation (Fig 1). The dry weight of the capsule increases until about day 18, then declines (Oriol *et al.* 1993b) until the capsule is lysed by mechanisms which remain uncertain but may involve proteases (Denker *et al.* 1987). Part of the weight increase is due to the capsule's content of non-structural proteins. The composition of these also differs markedly before and after fixation, probably reflecting important metabolic changes on both sides of the capsule (Quinn *et al.* 2005). Histological studies of conceptuses fixed *in situ* indicate that the capsule disintegrates gradually between Days 20 and 22 of pregnancy, with remnants remaining between the trophoblast and endometrium until at least Day 36 (Enders and Liu 1991).

FUNCTIONS OF THE CAPSULE

The capsule is presumed to be essential to the continuance of pregnancy because, experimentally, half-embryos, denuded of their zona pellucida before transfer, need to form a capsule to survive (McKinnon *et al.* 1989). Its structure suggests that it plays both a protective role, and participates in communication at the conceptus' interface with the endometrium (Flood *et al.* 1982; Oriol *et al.* 1993b). The change in charge and physical structure that coincides with fixation at about Day 16 is consistent with the proposal that the capsule's



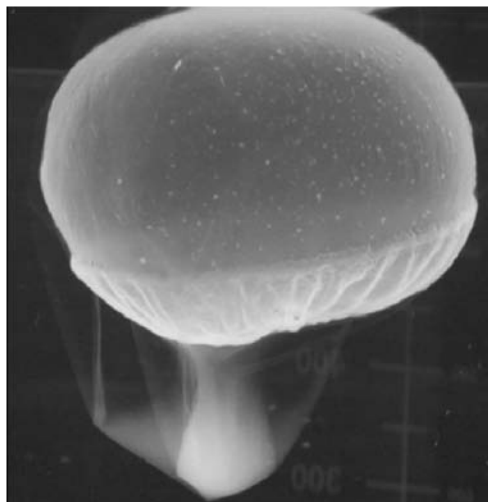


Fig 1: Before conceptus fixation, the embryonic capsule is resilient and maintains its shape even after collapse of the trophoblastic vesicle due to trauma at collection, as in the conceptus on the left, measuring 24 x 27 mm by ultrasonography 15.5 ± 1.5 days after ovulation. After conceptus fixation, the capsule becomes much more flaccid, as in the conceptus on the right, 24 mm in diameter, recovered 18.5 ± 1.5 days after ovulation.

anti-adhesive properties help its intrauterine mobility before that time, and that its subsequent 'stickiness' plays a role in the fixation and orientation processes. Adhesion of one conceptus to the other in cases of unilateral twin pregnancy can also involve the capsule (unpublished observations). Non-physiological, but practically important functions of the capsule from its first appearance include its impedance of embryo micromanipulation and, possibly, penetration of cryoprotective agents (Hochi 2003).

Some remaining enigmas of capsule structure and function

Experimental studies of capsular function *in vitro* are hampered by our inability to culture horse embryos satisfactorily. However, *in vivo,* after prostaglandin-induced luteolysis, the conceptus, including the capsule, can continue to develop (Kastelic *et al.* 1987; Hinrichs and Watson 1988). In such circumstances, the capsule does not lose sialic acid at Day 16 and is more susceptible than normal to proteolysis (Chu *et al.* 1997). These changes are concomitant with the disturbances of fixation and orientation that follow progesterone withdrawal (Kastelic *et al.* 1987). Similar models of pregnancy failure may be useful means of unravelling how the capsule functions. Analysis of capsular structure and contained proteins following clinical conceptus rupture for suppression of oestrus (Lefranc and Allen 2004) could also be instructive, as could analogous comparisons of the capsule of the surviving and ruptured conceptus soon after clinical twin reduction. The permeability of the capsule to various substances before and after fixation also deserves intensive study.

Acknowledgements

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FORMATION OF THE EQUINE BLASTOCYST CAPSULE

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INTRODUCTION

An enigmatic feature of early horse embryo development is the formation of an acellular glycoprotein 'capsule' between the trophectoderm and zona pellucida (ZP) on Days 6–7 after ovulation, soon after the embryo enters the uterus. The ZP is then shed to leave the expanding blastocyst surrounded by a tight fitting capsule that increases in dry weight until approximately Day 18 of gestation and, thereafter, is attenuated steadily until it disappears by around Day 23 (Oriol *et al.* 1993). The capsule appears to be essential to conceptus survival *in vivo* (Stout *et al.* 2005), probably because it protects the conceptus from mechanical damage when it is propelled throughout the uterine lumen. Because of its position at the maternal-conceptus interface, the capsule has also been proposed to play roles in maternal-conceptus communication (Herrler *et al.* 2000), conceptus nutrient uptake (Crossett *et al.* 1998) and protection of the conceptus against maternal immunological recognition (Betteridge 1989). Growth of the capsule appears to be primarily a function of trophoblast-secreted glycoproteins (Oriol *et al.* 1993), but a uterine contribution has been proposed to explain why embryos that blastulate *in vitro* fail to produce a visible capsule (see Betteridge 1989). The current study aimed to determine whether initial capsule formation is from the same trophectodermal glycoproteins that predominate during later growth, and to investigate why the capsule fails to form *in vitro.*

MATERIALS AND METHODS

Horse embryos were produced *in vitro* (IVP) by injecting a spermatozoan into the cytoplasm of oocytes matured *in vitro,* and incubating the injected oocytes for 2 days in modified synthetic oviductal fluid (SOF). At the end of the initial incubation, cleaved zygotes were selected for additional culture for 5 days in one of 2 systems; 1) 20 µl droplets of modified SOF, under oil at 38.5°C in an atmosphere containing 5% CO_2; or 2) the ligated oviduct of progesterone-supplemented ewes. At the end of the 5 day culture, the embryos were harvested and either fixed (Day 7 IVP embryos) or cultured for a further 3 days in a 1:1 mixture of DMEM and M199 supplemented with 5% FCS and 5% serum replacement (Day 10 IVP embryos). *In vivo* developed embryos were flushed from the uterus of inseminated mares 6–9 days after ovulation.

After recovery, embryos were fixed for 24 h in 4% paraformaldehyde and then stored in PBS at 4°C. Before staining, the embryos were permeabilised with 0.1% Triton X-100. Capsular glycoproteins were then labelled using a monoclonal antibody raised in mice against Day 13.5–15.5 equine capsule (OC-1: Oriol *et al.* 1993) and a goat anti-mouse second antibody coupled to the fluorochrome Alexa Fluor™ 488 (Molecular Probes). The embryos were concurrently stained with 4,6-diamino-2-phenyl-indole (DAPI) to visualise cell nuclei, and either AlexaFluor™ 568 Phalloidin (Molecular Probes) or wheat-germ agglutinin-Alexofluor™ 594 (WGA) to label, respectively, the actin microfilaments or lectin-binding elements of the cell plasma membranes and embryonic coverings. Finally, the pattern of OC-1 expression was examined using a multiphoton laser-scanning microscope.

RESULTS

There were no discernible differences in development or capsular glycoprotein expression between IVP embryos produced in SOF medium or in a sheep's oviduct. Compared to equivalently aged *in vivo* developed embryos, Day 7 IVP embryos were less well developed, had fewer cells and less distinct demarkation of the blastocoele cavity, the inner cell mass and the actin

cytoskeleton lining the cell membranes (see Tremoleda 2003). Day 6 *in vivo* embryos had a confluent capsule sandwiched between the ZP and trophectoderm, with no infiltration of capsular glycoproteins into the ZP. Older *in vivo* embryos had hatched completely from their ZP and were surrounded by a capsule with a bilaminar staining pattern, as described previously by Oriol *et al.* (1993). By contrast, Day 7 IVP embryos showed only scattered patches of OC-1 staining over the surface of the trophectoderm cells, together with loose aggregations of capsule within the perivitteline space. Day 10 IVP embryos had partially hatched and, where the trophectoderm had herniated from the ZP, capsular glycoproteins were again scattered in patches over the surface of the trophectoderm. Within the ZP of Day 10 IVP embryos, a relatively thick layer of capsule was evident which infiltrated extensively into the substance of the ZP. Removal of the ZP by micromanipulation demonstrated that this capsular lining was firmly adhered to the ZP. WGA labelled the cell membranes, the ZP and the capsule. In a Day 6 *in vivo* embryo around which the capsule was not yet visible using light microscopy, a complete WGA positive layer was observed between the embryo and the ZP. When a Day 6.5 *in vivo* embryo was co-stained with WGA and OC-1, an inner layer of the capsule was apparent that stained with WGA but not with OC-1.

Conclusions

This study demonstrated that the initial layer of capsule formed between the trophectoderm and ZP of a Day 6 horse embryo contains abundant OC-1 reactive glycoproteins; previously, conceptus OC-1 expression had not been examined before Day 11 after ovulation (Oriol *et al.* 1993). As the trophectoderm cells of IVP embryos also expressed OC-1, it appears that the initial layer of capsule is composed predominantly of OC-1 reactive glycoproteins secreted by trophectoderm cells, together with WGA positive/OC-1 negative glycoproteins of unknown origin. *In vitro,* the trophectoderm-derived OC-1 glycoproteins fail to coalesce into a confluent layer, presumably either because they fail to reach a critical concentration or because some vital aspect of the uterine environment is missing. In the former respect, the retarded development of Day 7 IVP embryos and the presence of the ICSI-derived hole in the ZP could have hindered glycoprotein accumulation; however, the latter is unlikely to be significant given that an intact ZP is not an absolute requirement for capsule formation *in vivo* (McKinnon *et al.* 1989). More probably, the critical role of the uterus in capsule formation is to provide a microenvironment that supports crosslinking and hydration of the mucin-like glycoproteins.

In the absence of the uterine micro-environment, capsular glycoproteins adhered to, and infiltrated into, the ZP; a feature not seen in *in vivo* embryos. This infiltration of the ZP with capsular glycoproteins may help to explain the aberrant mechanism of ZP loss *in vitro,* ie herniation through a small hole in the ZP. Infiltration with capsular glycoproteins would dramatically alter the response of the ZP to both stretching and enzymatic digestion. As a result of the current findings, it is suggested that IVP horse embryos should be transferred into the uterus of recipient mares by no later than the late morula to early blastocyst stage, to ensure that survival is not compromised by aberrant capsule formation.

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