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EQUINE EMBRYO TRANSFER

4th – 6th August 2004
Rio de Janeiro, Brazil

Editors: M. Alvarenga and J. F. Wade

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Picture: 'Splittin' Image' by Sheri Greves Neilson

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EDITOR'S FOREWORD

Since the last EET meeting in Finland we have observed important improvements in equine embryo transfer. The announcement of the first cloned horse is a very important development for the equine industry. Also, the commercial use of equine embryo transfer has had a remarkable increase in South and North America. Data from IETS showed that around 20,000 equine embryo transfers were performed in 2003. Unfortunately, very little progress has been made with regard to '*in vitro*' production of embryos, and we still cannot produce horse embryos consistently by the standard IVF technique.

The symposium receives ongoing funding from the Dorothy Havemeyer Foundation and, for the second time, was organised as an ICAR satellite meeting. Delegates attending both meetings had a wonderful opportunity to see how different and beautiful Brazil was after visiting Rio de Janeiro and Porto Seguro. Brazil has the third largest horse population in the world and was one of the first countries to use embryo transfer in horses on a large commercial scale. We were very proud to host the EET Symposium and thank Mr Gene Pranzo, President of the Havemeyer Foundation, for his support of the meeting. We are also grateful to BET Laboratories, Bioniche Animal Health and Sao Paulo State University-UNESP for additional support.

Small meetings like this, among scientists and practitioners from different cultures and with different experiences, are all too rare and they certainly provide fruitful discussions and conclusions.

We dedicated the meeting to the memory of João Junqueira Fleury a special friend who was the first veterinarian to perform equine embryo transfer in Brazil in 1986. He joined this international 'EET family' in Banff, Canada and was responsible for making equine embryo transfer a commercial viable technique in Brazil.

Finally, thanks to Fernanda for organising the scientific programme. She was the maestro and the authors the players of this orchestra; we enjoyed listening to you!

Marco Alvarenga
Chairman
Organising Committee

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1984 **First International Symposium on Equine Embryo Transfer**
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1999

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July - Pullman, Washington
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August - Dublin, Ireland
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October - Pardubice, Czech Republic
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October - Lexington, USA
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SESSION 1:

Early embryonic development and pregnancy loss

Chairman: W. R. Allen

TROPHOBLASTIC DISCS, YOLK-SAC TUBERCLES AND YOLK-SAC CORDS IN THE EQUINE CONCEPTUS DURING THE THIRD AND FOURTH WEEKS OF PREGNANCY

K. J. Betteridge, R. O. Waelchli, A. K. Smith* and M. A. Hayes†

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Introduction

Trophoblastic discs on the outside surface of the bilaminar yolk-sac wall of an equine conceptus of 21 days gestation, and apposing yolk-sac tubercles on the inside wall, were described by Ewart (1915) but have received scant attention since. During other studies (Raeside *et al.* 2004) we observed, in association with the discs and tubercles, cords of translucent material projecting into the yolk-sac cavity on Day 20, near the time of loss of the capsule (Fig 1). We are examining them, and their connection to the tubercles and discs, more closely using histology and scanning and transmission electron microscopy (SEM, TEM). This preliminary report describes the findings on 7 conceptuses collected transcervically between Days 18 and 26 of pregnancy (ovulation = Day 0).

Materials and methods

Management of the mares and methods of transcervical conceptus recovery have been described elsewhere (Raeside *et al.* 2004). After examination and photography as fresh specimens, conceptuses were fixed, whole, in 2% glutaraldehyde in Sorensen's buffer, pH 6.8, within 70 min of collection for a period of at least 24 h, then transferred to the same buffer, free of fixative, for several days. Under a dissecting microscope, areas of bilaminar omphalopleure containing discs and cords were excised from the fixed specimens and prepared for histology, SEM and TEM. For histology, strips were manipulated in 2% agar before paraffin embedding to ensure that transverse sections of the wall were cut. For SEM and TEM, specimens were post fixed in 1% OsO_4, then processed routinely.

Results

Preliminary observations indicate that the numbers and distribution of trophoblastic discs vary from conceptus to conceptus but that they were macroscopically obvious on all the specimens. Histologically, the disc-tubercle-cord complex varied considerably. The earliest sign of formation of the complex appeared to be an accumulation of amorphous material, staining like the basement membrane, between the trophectoderm and endoderm without hypertrophy of either. Thereafter, however, the discs generally comprise modified, highly active trophectodermal cells overlying pooled PAS-positive material that is continuous with thick, basement-membrane-like material between the trophectodermal and endodermal cells, which are also modified beneath

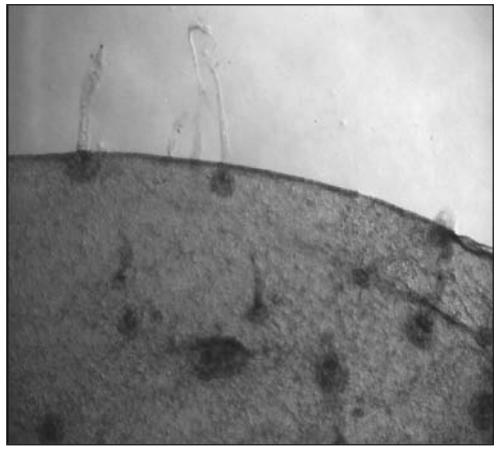

Fig 1: Translucent 'yolk-sac cords' attached to yolk-sac tubercles on the inner surface of the bilaminar omphalopleure and projecting into the cavity of the yolk-sac of an equine conceptus recovered 22 days after ovulation (fresh specimen, unfixed).

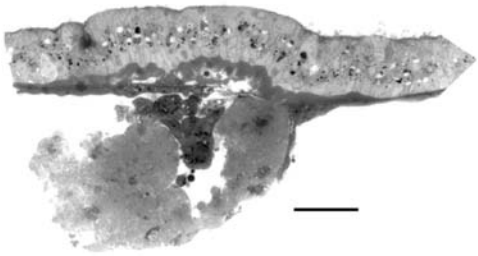

Fig 2: Light microscope section of a disc-tubercle-cord complex of a conceptus on Day 25±1. There is hypertrophy of both trophectoderm and endoderm and a thick basement membrane is visible between the two cell layers. An amorphous, acellular mass (the base of a yolk-sac cord) is attached to the enlarged endodermal cells. Osmophilic bodies (black) are prominent in the hypertrophied ectoderm (Photomontage, osmium stained; scale bar = 50 µm).

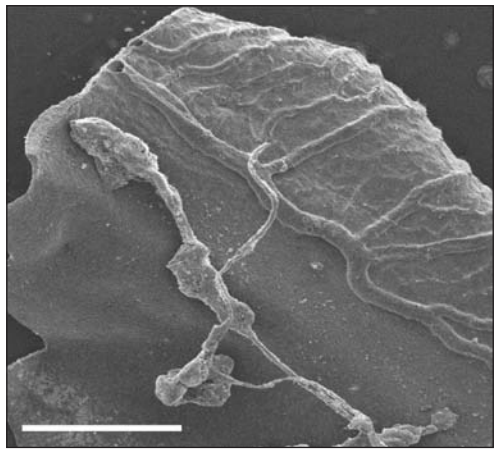

Fig 3: Scanning electron micrograph of the inside surface border between the vascularised trilaminar omphalopleure (top right) and the avascular bilaminar omphalopleure (bottom left). Scale bar = 1 mm.

the discs (Fig 2). The cords, made up of PAS-positive material apparently originating from the tubercles, became more extensive as the conceptus developed during the period examined. They are generally acellular and scanning electron microscopy shows them to be made up of bundles of smaller cords, often connecting one yolk-sac tubercle with another (Fig 3). The SEM appearance of the cords also suggests that they disperse gradually into the cavity of the yolk-sac.

Discussion

Ewart's (1915) histological observations have been confirmed and extended by our observations. The nature and function of the thick basement-membrane-like material is of special interest. Ewart considered it to be made up of material pinocytosed by tall columnar trophectodermal cells surrounding the discs for nutrition of the embryo. Amoroso (1952) drew attention to its resemblance to Reichert's membrane, which is a feature of rodent conceptuses (Boyd and Hamilton 1952) but is uncommon in other mammals. Amoroso concluded that 'This membrane may be of considerable importance as an element of the [equine] yolk-sac placenta; it should be more completely studied'.

Considering that the discs, tubercles and cords appear at a critical stage of pregnancy, they are being characterised further by immuno-histochemical and chemical analyses.

Acknowledgements

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INTRASPECIES EMBRYO TRANSFER IN DONKEYS

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INTRODUCTION

The Pantesca donkey, a Sicilian breed that has its roots in the island of Pantelleria, has been considered extinct since 1980. Morphological selection and genetic analyses performed on over 200 potential Pantesca crossbreed found 9 subjects (3 males and 6 females) with an estimated proportion of purity between 80 and 95% (IDFT, 1999). The breeding from these 9 donkeys resulted in a population of 35 animals.

Embryo transfer (ET) has been employed previously as a tool in the conservation of endangered equid species such as Przewalski's Horses (*Equus przewalskii*) (Summers *et al.* 1987) but data concerning ET in the donkey are still scarce and not encouraging (Allen *et al.* 1985; Allen *et al.* 1993; Vendramini *et al.* 1997). Allen showed that it was possible to recover embryos from donkeys by non-surgical flushing of the uterus and reported a 55.5% pregnancy rate at 40 days after their transfer in recipient mares by midline laparotomy (Allen *et al.* 1985). However, about 70% of these conceptuses were aborted within Day 100 of pregnancy, and half of the remaining fetuses died in the peri-partum period (Allen, 1993). Allen's works suggest strongly that mares are not suitable recipients for donkey embryos. Vendramini *et al.* (1997) transferred donkey embryos to donkeys, both surgically and non-surgically, but their rate of success was much lower than can be expected after intraspecies embryo transfer in other species, such horses or cattle. The aim of this study was to attempt to recover embryos from Pantesca jennies and obtain pregnancies after non-surgical transfer in jennies of a different breed.

MATERIALS AND METHODS

From March to December 2003, 4 Pantesca jennies (3–4 years old), and 8 Ragusana jennies (4–11 years old) were used as embryo donors and recipients, respectively. Two Pantesca jack stallions known to be fertile were used to mate the donors. The jennies were maintained in paddocks, the jacks in boxes; all animals were fed with a balanced ration of hay and commercial horse feed.

Oestrous was induced using 3 mg, im, of the $PgF_{2\alpha}$ analogue alfaprostol (Gabbrostim®, Vetem, Agrigento, Italy).

Ovarian activity of both donors and recipients was evaluated daily during oestrous by transrectal ultrasound (Toshiba JustVision 200 equipped with a 5–8 Mhz semi-convex transducer model PVF-738E).

From the detection of both a growing follicle ≥30 mm in diameter and oestrus behaviour, donors were mated naturally or artificially inseminated with fresh semen of one of the 2 jack stallions every second day until ovulation. Following each donor's ovulation, the recipient in oestrous with the largest follicle was treated with 2000 iu, iv, hCG (Vetecor®, Bio 98, Bologna, Italy) to induce ovulation.

Seven to 8 days after ovulation, donors were flushed 3 times for embryo recovery using a total of 2 litres of washing media kept at 37ºC.

The flushings were performed using either DPBS (ZE067, IMV, L'Aigle Cedeaux, France) and a one-way tubing system without filter (26 cycles; Lagneaux *et al.* 1988) or Ringer lactate and a self-built 'Y' tubing system using an EZ Way® filter (PETS, Canton, Tx, USA) (17 cycles). For both techniques, a 'AB Technology Equine

TABLE 1: Pregnancy rates after transcervical donkey to donkey embryo transfer

	Pregnancies at 14 days (%)		Pregnancies at 50 days (%)	
	PBS	Ringer	PBS	Ringer
Recipients at day 3-4	2/5 (40%)	0/6 (0%)	1/5 (20%)	0/6 (0%)
Recipients at day 5-8	0/10 (0%)	3/6 (50%)	0/10 (0%)	3/6 (50 %)
Total	2/15 (13.3%)	3/12 (25%)	1/15 (6.6%)	3/12 (25%)

TABLE 2: Number of cycles, ovulations, recovered embryos and pregnancies after transcervical donkey to donkey embryo transfer for each donor

Donors	Cycles	Ovulations	Embryos recovered	Pregnancies (50 days)
1	10	12	5	0
2	12	16	11	3
3	10	14	7[a]	0
4	11	12	6	1
Total	43	54	29	4

[a]2 embryos were lost during manipulations

Lavage Catether 32 Fr.'® (Pullman, WA, USA) was used. Before each flushing, for each donor's ovulation 20 ml of the medium used was separated and held at 37ºC to wash the embryos eventually recovered.

Each recovered embryo was evaluated for morphology (McKinnon and Squires 1989), washed 10 times, placed in a French straw and transferred transcervically in the recipient using a guarded French gun (Lagneaux *et al.* 1988). During evaluation, some of the embryos were measured.

Pregnancy diagnosis was performed by ultrasound 14, 16 and, if positive, 50 days after donor ovulation. Differences in pregnancy rates of recipients between the 2 media used were compared using the Fisher's Exact Test, and considered significant when $P<0.05$.

Results

Out of 43 cycles, 29 embryos (67.4%) from good to excellent quality were recovered. Mean diameter of 8 day old donkey embryos was 720±217.3 µm (n=8). Embryo recovery rate ranged from 1/4 (first cycle) to 4/4 (10th cycle). Overall pregnancy rates of recipients at 14 and 50 days were 5/27 (18.5%) and 4/27 (14.8%), respectively. Two embryos were not transferred as they were lost during the manipulations. When all recipients were considered, the flushing medium used had no effect on pregnancy rates ($P>0.05$ (Table 1). However, if only recipients on Days 5–8 were considered, Ringer lactate resulted in higher pregnancy rates than PBS (3/6 and 0/10, respectively, $P<0.05$). Embryo recovery rate and recipient pregnancy rate for each donor are shown in Table 2.

The first Pantesca foal from ET was born in June 2004, when the other 3 pregnancies were still ongoing.

Discussion and conclusion

The embryo recovery rate achieved was within the range normally reported for mares, and close to that described previously in donkeys: 53.3% (Allen *et al.* 1985) and 63.6% (Vendramini *et al.* 1997).

In particular, the 4 jennies studied yielded an embryo recovery rate from 50 to 92% each. Repeated uterine flushings (up to 12) for embryo recovery had no negative effects on the successive fertility of the donors. On the other hand, this study confirms that, to date, the limit of ET in donkeys is the very low pregnancy rate of recipients. The pregnancy rate obtained at 50 days (14.8%) was lower both than after the transfer of horse embryos to horses (50–75%, Squires *et al.* 1999) and than after the surgical transfer of donkey embryos to mares (67%, Allen *et al.* 1985), but similar to the pregnancy rate described previously after surgical or non-surgical transfer of donkey embryos to donkeys (5.5%, Vendramini *et al.* 1997). The reasons for these poor results are yet to be understood and, as we simply transferred to the donkey the ET techniques routinely employed for horses, it is possible than more factors had an effect

on the outcome. The optimal day for donkey embryo collection and the synchrony between donor and recipient ovulations could be different from those used in this study and commonly used in the mare. Donkey embryos may have different requirements in respect to flushing and washing media employed, and this study seems to indicate that Ringer lactate could be a better choice than PBS. Finally, although catheterisation of the cervix was more difficult than in mares, the transfer technique applied did not result in any apparent trauma or clinical sign of early luteolysis in the recipients. The results reported by Vendramini *et al.* (1997), where no differences were found in recipient pregnancy rates after surgical or non-surgical donkey to donkey embryo transfer, seems to support the hypothesis that cervical manipulation was not responsible for the low pregnancy rates observed in this study.

In conclusion, these results suggest that more investigations are needed in this species before embryo transfer can be considered a reliable tool to preserve endangered donkey breeds.

ACKNOWLEDGEMENTS

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DOES COMPROMISED LUTEAL FUNCTION CONTRIBUTE TO FAILURE TO ESTABLISH PREGNANCY AFTER NON-SURGICAL EMBRYO TRANSFER?

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INTRODUCTION

Commercial equine embryo transfer (ET) programmes demonstrate that non-surgical ET can be extremely successful (>80% pregnancy rate: Losinno *et al.* 2000), if the operator is experienced and recipient mares are selected carefully. For less experienced practitioners, non-surgical ET is usually less successful than surgical transfer. The most common explanation given for this difference is luteal insufficiency resulting from $PGF_{2\alpha}$ release induced either by cervical and uterine trauma or from contamination introduced into the uterus during cervical transfer. Handler *et al.* (2003) reported that dilating the cervix on Day 5 or 7 after ovulation resulted in oxytocin release and a fall in circulating progesterone concentrations. However, they found no evidence of $PGF_{2\alpha}$ release, and a subsequent study failed to demonstrate any detrimental effect of cervical dilation on conceptus development in inseminated mares (Handler *et al.* 2002).

Many aspects of recipient quality undoubtedly affect the success of ET; poor uterine and cervical tone at transfer are associated with lower pregnancy and higher pregnancy loss rates (Carnevale *et al.* 2000). The maternal steroid-hormone environment may also affect conceptus development and vice versa. Maternal recognition of pregnancy is the clearest demonstration that a conceptus can dramatically influence maternal hormone concentrations while, in cattle, higher maternal blood progesterone and lower circulating oestradiol concentrations have been reported to, respectively, enhance conceptus growth and facilitate maternal recognition of pregnancy (Mann and Lamming 2001). In horses, it has been reported that exogenous progesterone administration enhances conceptus growth (Weithenauer *et al.* 1987) and that the growing conceptus stimulates increased maternal progesterone secretion from early in dioestrus (Sevinga *et al.* 1999). The current experiment aimed to examine the relationship between the success of non-surgical ET and corpus luteum (CL) activity, and to determine whether ultrasonographic measurement of CL size is a reliable indicator of function.

MATERIALS AND METHODS

Fifty Dutch Warmblood mares maintained at grass were synchronised repeatedly in groups of donors and recipients. Ovaries of mares in oestrus were examined daily. Once the dominant follicle reached 35 mm, donor mares were inseminated on alternate days until ovulation. Seven days after ovulation, embryos were recovered from the donors by non-surgical uterine lavage with Dulbecco's PBS supplemented with 0.5% fetal calf serum. After microscopic evaluation, embryos were transferred non-surgically using a sheathed embryo transfer pipette to the most suitable recipient available. A recipient was considered ideal if she had ovulated between one day before and 2 days after the donor.

Luteal function was measured in terms of peripheral plasma progesterone concentrations and the maximum ultrasonographic cross-sectional surface area of the CL. For the former, jugular vein plasma samples were collected daily during oestrus and until Day 3 after ovulation, and thereafter on alternate days until Day 19; progesterone concentrations were measured by radioimmunoassay. CL surface area (cm2) was measured on the day of embryo transfer and on Days 11, 15 and 19 after ovulation.

RESULTS

Fifty-two embryos were recovered and transferred to recipient mares that ovulated between 2 days before and 4 days after their respective donor; 29 (56%) of these recipients became pregnant. A higher pregnancy rate was achieved in 'properly' synchronised (27/42; 64%) than less well-synchronised (2/10; 20%) mares; these figures were not altered if synchronisation was based on the rise in plasma progesterone concentrations rather than ultrasonographic detection of ovulation. Only 2 non-pregnant mares showed evidence of premature luteolysis after ET, but progesterone profiles revealed that a further 2 were not actually in oestrus at the time of recorded ovulation; the remainder had a mean dioestrous length of 15.0 ± 0.4 days. One mare lost her pregnancy between Days 15 and 19 after ovulation, this was associated with a fall in progesterone concentrations to <1 ng/ml during Days 15–17.

Peak plasma progesterone concentrations varied greatly between mares, but there were no significant differences between pregnant and non-pregnant mares in the rate of the progesterone rise or in the mean plasma progesterone concentrations, until Day 13 after ovulation when non-pregnant mares began to undergo luteolysis. Mean CL surface area did not differ between pregnant and non-pregnant mares until cyclical luteolysis. Progesterone concentration (mean ± sem: 8.9 ± 1.4 vs 8.2 ± 1.3 ng/ml for pregnant and non-pregnant mares, respectively) and CL size (11.7 ± 0.7 vs 10.2 ± 0.6 for pregnant and non-pregnant mares) on the day of ET tended to be higher in mares that became pregnant, but the differences were not statistically significant. CL surface area was generally a good indicator of systemic progesterone concentration, but tended to over-estimate CL function at the time of ET and during luteolysis, when the decrease in CL size was slower than that in plasma progesterone concentrations. Indeed, the CL was ultrasonographically visible for at least 3 days after progesterone concentrations had reached baseline values.

Conclusions

Transfer-induced luteal compromise was not a significant cause of failed ET since luteal function did not differ between pregnant and non-pregnant mares until the onset of cyclical luteolysis. More often, failure to establish pregnancy was associated with inadequate donor-recipient synchrony. Nevertheless, the possibility that the overall maternal hormonal environment affects the likelihood of pregnancy, and that some deficiencies are reflected by sub-optimal uterine tone, cannot be ruled out. There was no indication that the presence of a viable conceptus affected maternal circulating progesterone concentrations, other than by preventing the onset of cyclical luteolysis. Moreover, even though circulating progesterone concentrations differed greatly between mares, higher or faster rising maternal progesterone concentrations did not improve the likelihood of pregnancy after ET. Finally, while CL surface area was a good estimator of the peripheral progesterone concentration in mares, it tended to over-estimate this parameter during luteolysis and would, therefore, be an unreliable indicator of luteal function in the face of impending pregnancy loss.

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NON-SPECIFIC IMMUNOMODULATION (NSI) CAN RECTIFY AN IMBALANCED UTERINE/OVARIAN MILIEU IN MARES SUSCEPTIBLE TO ENDOMETRITIS

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INTRODUCTION

Conception depends on a delicate balance between endocrine and immune mechanisms (Lee et al. 2000). Numerous studies have been performed to identify the mechanisms responsible for bi-directional communication between neuroendocrine and immune systems (Weigent *et al.* 1995). These relationships were observed in the human (Barañao 1997; Diaz Flores *et al.* 2001), bovine (Ohtani *et al.* 2004), and rat ovary (Barañao 1997) and endometrium (Simon *et al.* 1994)

Endometritis is a physiological response to mating or artificial insemination (AI). Normal, resistant mares (RM) can reduce this inflammation before the embryo arrives in the uterus, but some mares fail to do so and are considered susceptible (SM). SM accumulate neutrophils, plasma proteins, inflammatory mediators and nitric oxide resulting in a non-compatible environment for embryo development (Troedsson 1999; Alghamdi *et al.* 2002).

Bacillus of Calmete and Guerin and other mycobacterias behave as immunomodulators and it has been demonstrated that endometrial cells are susceptible to their anti-proliferative effects (Diaz Flores *et al.* 2001).

Mycobacterium phlei cell-wall extract (MCWE) administered during AI to RM and SM produced a significant decrease in neutrophils number in SM during dioestrus, to similar values in RM (Clayton *et al.* 2001). As neutrophils are hallmarks of acute inflammation, this may indicate a beneficial effect of treatment.

Cytokines are intercellular signalling proteins released by both immune and non-immune cells; in the uterus, they are produced by leucocytes and endometrial cells (Barañao 1997). Interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF-α), known as pro-inflammatory cytokines (PICs), modulate the acute phase response that involves potent systemic and local effects. In people, reproductive processes including ovulation, implantation, cervical ripening and dilation at term are inflammatory in nature, and have been connected with the activity of these cytokines (Barañao 1997). IL1β, IL6 and TNFα are reported to be over-expressed in SM with endometritis, in basal levels (BL) and after AI at oestrus and dioestrus (Fumuso *et al.* 2002).

Objectives

a) To determine if expression of endometrial PICs is related to estradiol and progesterone concentrations
b) To determine the effect of NSI on these parameters.

MATERIALS AND METHODS

Two groups of crossbred mares, 8 RM (control group) and 8 SM were used in this study. Criteria for susceptibility were based on reproductive records, a uterine clearance assay with *Strep. zooepidemicus* and the ability to become pregnant. Two endometrial biopsies and plasma samples were taken from each mare during 3 consecutive cycles as follows during oestrus: 1st cycle, BL: when follicles reached ≥35 mm; 2nd cycle, AI in oestrus 24 h post AI with dead semen; 3rd cycle, AI&NSI: 24 h after treatment with 1,500 µg of MCWE (Equimune IV®) and AI with dead semen and during dioestrus 7±1 days post ovulation. mRNA transcription levels (mRNAT) for IL1β, IL6 and TNFα were determined by Real Time

PCR. Briefly, total RNA was isolated, treated with DNAase-I and cDNA was synthesised. Relative quantitation of mRNAT was done using the comparative threshold method. Plasma oestradiol (E) and progesterone (P) levels were determined by a RIA, DPC, commercial test.

Data were statistically analysed by Wilcoxon's test for paired comparisons inside each group and by Mann-Whitney U. for unpaired comparisons between RM and the SM.

Results

First cycle BL: during oestrus, SM had significantly higher levels of mRNAT for the 3 PICs than the RM ($P<0.001$). Dioestrus mRNAT levels for IL1β, TNFα and E concentrations were higher in SM than in RM ($P<0.03$). Comparing oestrus and dioestrus, no differences were detected in E concentrations for SM ($P>0.05$), but E concentrations were lower in RM during dioestrus ($P<0.05$).

Second cycle AI: 24 h after AI, both RM and SM exhibited high PICs with no differences between groups ($P>0.05$), and normal E and P concentrations. During dioestrus only RM had reduced IL6, TNFα and E ($P<0.05$). An increased, normal P concentration was observed as compared to oestrus values. SM did not have a significant reduction either in PICs levels or E concentration ($P>0.05$) during dioestrus and P concentrations were not different to those exhibited in RM

Third cycle AI&NSI: in dioestrus, the levels of mRNAT for IL1β and TNFα in SM decreased to levels comparable to those RM. Significantly higher E concentrations were detected at oestrus compared to dioestrus ($P<0.05$) in both groups.

Discussion

SM had higher levels of PICs in the first cycle at BL and after AI than the RM. In addition SM had higher E concentrations during dioestrus in the first 2 cycles. However, when the NSI was administered (3rd cycle) the PICs and E levels decreased and became comparable to those in RM. Therefore, it can be assumed that imbalanced uterine/ovarian endocrine, paracrine, and/or autocrine milieu found in the SM was normalised by the use of MCWE. Although not yet studied in equine cells, the most likely mechanism of action of mycobacterium cell wall extract is the binding to TLR-4 and TLR-2 (Underhill *et al.* 1999) changing signals to produce PICs.

IL1 and TNFα were related to the local release of steroids and PGE_2. They were described as playing a key role in a local intermediatory/amplifying system in the bovine pre-ovulatory follicle (Acosta *et al.* 1998). During structural luteolysis, TNF-α may interact with $PGF_{2\alpha}$ to cause the drop in progesterone release and accelerate the process of luteolysis in the ovine (Ohtani *et al.* 2004).

PICs have effects on synthesis of prostaglandins (Erkinheimo *et al.* 2000) and also estradiol production by means of a steroid catalysing enzyme mediated action in the tissue (Honma *et al.* 2002). Whether the estradiol decrease detected after NSI treatment is directly related to the drop in cytokines, or whether it is an indirect consequence, cannot be established in this study and will be the subject of future experiments.

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IMMUNOHISTOCHEMICAL CHARACTERISATION OF EQUINE ENDOMETRIAL MALDIFFERENTIATION WITH SPECIAL EMPHASIS ON UTERINE SECRETORY PROTEINS

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INTRODUCTION

An important condition of equine fertility is a uniform and synchronous functional morphology of all endometrial structures (Aupperle *et al.* 2000).

Disorders are well known as possible causes of infertility in human gynaecopathology (Dallenbach-Hellweg 1987). In contrast, there is little information about comparable phenomena and their prognostic assessment in mares.

Equine endometrial functional disturbances were first described by Schoon *et al.* (2000). Endometrial maldifferentiation can be identified clearly even in H-E stained sections and is classified generally into 2 major features: irregular and unequal differentiation (Schoon *et al.* 2000).

Disorders may lead to permanent or temporary infertility, possibly resulting from an altered uterine microenvironment, for example a disturbed uterine secretory protein pattern, as described in endometrosis (Hoffmann *et al.* 2003).

The aim of this study was the immunohistochemical characterisation of endometrial maldifferentiation with special emphasis on uterine secretory protein patterns. Therefore endometrial biopsies of mares with endometrial maldifferentiation (i) due to ovarian disorders, (ii) caused by long term progestin application and (iii) of unknown aetiology were investigated.

MATERIALS AND METHODS

Endometrial biopsies (n=124) of 48 mares with endometrial maldifferentiation due to granulosa cell tumours (GCT) (n=15), progestin long term application (PLTA) (n=4) and of unknown aetiology (n=29) were examined.

The tumourous ovaries (n=15) were excised and endometrial biopsies (n=33) were collected pre- (n=15) and post surgically (n=18) to determine the therapeutic success of a unilateral ovariectomy. Serum hormone values (Table 1) were examined routinely in 14 patients before and after ovariectomy.

For investigating the influence of PLTA on endometrial reactions 4 adult pre-cycling mares were exposed to 2 different regimes of a Regumate®, long term application (Table 2). During the treatment periods and intervals as well as one month before and 3 months after the therapy endometrial biopsies (n=62) were collected. Clinical, gynaecological and endocrinological examinations were carried out weekly.

To characterise spontaneously occurring endometrial maldifferentiation (SOEM), biopsies of 29 mares (Table 3) were selected from routine specimens submitted for diagnostic purposes. Clinically, all mares with SOEM showed fertility problems during the physiological breeding season, barrenness for at least one year but mostly for several years without further clinical signs.

After routine fixation and preparation all biopsies were investigated by means of light microscopy (H-E) and special immunohistochemical methods (Ki-67 antigen, oestrogen-, progesterone receptors, vimentin, desmin, laminin). The uterine secretion pattern (uteroglobin, uteroferrin, uterocalin, calbindin)

TABLE 1: Serum hormone levels in mares (age: 4–14 years) with GCT

	Oestradiol (pg/ml)	Progesterone (ng/ml)	Testosterone (ng/ml)
Prior to ovariectomy	<1-33.2	<0.01-0.68	0.01-0.69
After ovariectomy	4-25	<0.09-3.4	<0.01-0.02

TABLE 2: Time schedule of Regumate®, long term application

Mares	Regumate®, application* (days)		
Age (5–14 years)	Treatment period	Interval	Treatment period
n=2	45	22	12
n=2	32	16	36

* Daily oral administration of 0.044 mg/kg BW altrenogest

TABLE 3: Characterisation of the mares (n=29) with SOEM

Years of age	n	Time of barrenness	n	Number of foalings	n
4-9	6	not barren	2	maiden	4
10-14	8	≤1 year	7	1 foal	6
15-19	8	2 years	7	2–3 foals	7
20-26	5	>2 years	8	5–8 foals	3
unknown	2	unknown	5	10–12 foals	3
				unknown	6

was examined immunohistochemically on selected specimens (n=51).

Results

In all mares with GCT an irregular differentiation was obvious. Two weeks post operatively the functional endometrial morphology was almost normal in two thirds of the patients.

In the PLTA study, the mares' endometria developed a secretory irregularity during progestin administration. Three months after final treatment the endometrial morphology had returned to normal in only 2 of the 4 mares, the other 2 still show an irregular differentiation.

The mares with SOEM revealed unequal as well as irregular endometrial differentiation.

Immunohistochemically, maldifferentiated epithelia revealed atypical expression patterns of secretory proteins.

All mares with GCT showed a disturbed protein secretion, independent of quality and degree of the maldifferentiation. There was no correlation to the variability of the serum hormone levels prior to ovariectomy (Table 1). The expression of uterocalin, uteroglobin and uteroferrin occured unequally. Despite the histomorphological endometrial normalisation within 2 weeks after ovariectomy, the secretion pattern of these proteins normalised after several months.

A multifocal expression of calbindin was recognisable in the majority of affected endometria. Only 3 mares revealed an atypical overexpression of this protein in nearly all glands, which was not detectable after surgery.

Endometria of mares with PLTA showed, at the beginning of the treatment, a physiological secretion pattern of all proteins investigated.

During the treatment periods, an unequal expression of uteroglobin and uteroferrin between and within the glands was obvious. Calbindin and uterocalin were atypically overexpressed in almost every gland towards the end of the treatment.

Three months after therapy, 2 of the 4 mares still showed endometrial irregularity with abnormal uterine secretion patterns. In 2 mares the endometrial morphology had returned to normal.

All endometria of mares with SOEM also revealed an atypical expression of secretory proteins.

In irregular differentiated endometria the secretion patterns corresponded to the findings in mares with GCT and with PLTA.

In cases of unequal differentiation the investigated proteins were expressed as follows: a uniform protein secretion pattern was obvious in areas differentiated in accordance with the stage of the ovarian cycle. Cycle asynchronous reaction patterns were detectable within foci of glands deviating from the normal morphology.

Furthermore, in all cases presented here, the endometrial maldifferentiation was also characterised by abnormal expression patterns of steroid hormone receptors, atypical stromal expression of desmin and a discontinuous glandular basal lamina.

Discussion

Functional disturbances have so far played a minor role in the diagnosis and the research of equine fertility problems.

The results of this study show, that the reason for an endometrial maldifferentiation remains unclear, if endocrine active tumourous ovarian disorders and a hormonal treatment can be aetiologically excluded.

The condition is neither related to age or parity of the mare (Table 3) nor to the annual season. Therefore, more detailed case control studies including investigations of the endocrine status are necessary for the understanding and prognostic assessment of this lesion.

Physiologically, during the oestrus cycle, the secretory proteins reveal typical reaction patterns (Hoffmann *et al.* 2003).

The disturbed secretory protein patterns detected in all maldifferentiated endometria of this study possibly lead to an altered uterine microenvironment, eventually resulting in an insufficient supply of the conceptus. This might be an important reason for the clinically reported fertility problems in these mares.

The reversibility depends on the aetiology, however, as long as the condition persists, a poor breeding prognosis results. Although maldifferentiation is not included in the categorisation system of Kenney and Doig (1986) it always has to be considered (Schoon and Schoon 2003) when reading equine endometrial biopsies. Since endometrial maldifferentiation cannot be detected by conventional clinical examinations, morphological investigations of endometrial biopsies should be indispensable especially for embryo recipient mares. However, a prognostic assessment in cases of SOEM remains impossible at this moment.

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FACTORS AFFECTING POST PARTUM REPRODUCTIVE PERFORMANCE IN THOROUGHBRED MARES

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INTRODUCTION

The equine is very unusual specie because a mare has a fertile oestrus within 2 weeks after parturition. Breeding at the first post partum oestrus can cause lower conception rates and higher embryo mortality rates. Many factors such as the parturition-first-breeding interval (Loy 1982), stallion fertility (Kenney *et al.* 1971) and age of the mare (Vanderwall *et al.* 1989; Carnevale and Ginther 1994) have been suggested as possible causes for the lower fertility observed when mares are bred at the foal heat (FH).

This study aimed to evaluate the effects of breeding either at the first or second post partum cycles, the age of the mare, the month of parturition and the interval from parturition to first breeding on the reproductive performance of Thoroughbred mares.

MATERIALS AND METHODS

In 424 cycles, the mares were mated at the FH while in 474 cycles mares were mated at the second post partum heat (SH). Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) was used in 57 cycles at the 20th day post partum in order to anticipate the second heat.

The mares were teased daily starting on the 5th day post partum and at the first sign of oestrus, the ovaries were scanned with a 5 MHz ultrasound until a 35 mm follicle was detected. The mares were then mated every other day by stallions with proven fertility until ovulation. Pregnancy diagnosis was done by ultrasonography on the 14th day after ovulation and repeated weekly up to the 45th day.

Mares were divided in 2 classes according to age and according to parturition-first-breeding interval (PBI). Dependent variables were per cycle pregnancy rate (PR) and embryo mortality (EM). PBI, age, month of parturition and breeding either at the FH or SH were considered categorical variables. Regression analysis and Chi-square were tests used for statistical analysis.

RESULTS AND DISCUSSION

Stevenson (1945), Bain (1957) and Tolksdorff *et al. (*1976) reported no significant difference in PR between the first and the second heat after foaling. We found a significantly higher PR for the SH than for the FH. The overall PR was 58.5%, and it was not affected by years analysed, month of parturition, month of first breeding and age of the mares, ($P>0.05$). Mares bred at the FH had a lower ($P<0.01$) PR (52.6%) than mares bred at the SH (63.7%). This higher PR (11 percent points) for the SH, are consistent with reports with by other authors, who observed a variation of 10 to 20 percent points (Caslick 1937; Chieffi *et al.* 1962; Loy 1982; Bell and Bristol 1986; Woods *et al.* 1987; Lowis and Hyland 1991).

The lower pregnancy rate in mares bred at the FH is not due to uterine involution time (Gigax *et al.* 1979), uterine contamination (Glatzel and Belz 1995), time of ovulation after foaling – if before or after the 10th day – (Loy 1982; McKinnon *et al.* 1988), the age of the mares (Vanderwall *et al.* 1989) or embryo mortality (Loy 1982; Bell and Bristol 1986; Woods *et al.* 1987; Vanderwall *et al.* 1989).

First matings were performed on 295 (69.5%) mares before the 10th day post partum and in 129 (30.4%) mares, after the 10th day. Loy (1982) and McKinnon *et al.* (1988) reported higher conception rate if first service occurred after the 10th day, when mares are bred at the FH. They suggested that higher fertility could be due to a

longer period for endometrium repair after foaling. In this study, the average foaling to ovulation interval for 424 mares bred at the FH was 10 days, which is in agreement with Loy (1982), who observed an average of 10.2 days. In this study, the difference observed in PR for mares bred before or after the 10th day, (50.9% and 56.6%, respectively) was not significant (P>0.3). The reason for these results could be attributed to the age distribution of the mares among groups. The mares that ovulated before the 10th day post partum were predominantly young (less than 10-years-old). This unbalanced age distribution and interval from parturition to ovulation interval might be the reason for the absence of significance in pregnancy rate.

Loy *et al.* (1975) and Loy (1982) using progesterone to postpone the first post partum ovulation obtained a higher fertility rate, suggesting that this procedure would allow more time for the endometrium repair before the embryo arrives from the oviduct, but in the present study, the PR did not differ (P>0.3), among mares bred at the FH (52.5% n=423) or at the $PGF_2\alpha$ anticipated SH (58.7% n=75). However, the overall PR for the SH, regardless of the use of $PGF_2\alpha$, was higher than the PR of the FH. These results suggest that time required for complete uterine involution should not be shortened by luteolytic products.

The PR for old (57.09%) or young mares (59.05%) was not different (P>0.5), although many papers (Vanderwall *et al.* 1989; Rocha 1994) indicated a significant decrease in PR as mares aged. Results observed in this study were similar to those reported by Loy (1982) where PR for older mares were lower than young mares (50% and 58.9% respectively).

In most of the papers reviewed older mares were considered as being more than 15 years of age (Carnevale and Ginther 1994) or more than 17 (Brinsko *et al.* 1994). In our study there were very few mares older than 15 (n=19), so the threshold of 10 years was used to group mares as old (n=268), or young (n=630), using the same criteria described by Rocha (1994) and Woods *et al.* (1987)). If in this work there were more mares older than 15, the results might be different, probably with magnified differences. Nowadays there is a trend to culling mares older than 15 due to the higher probability of reproductive problems found in this age group.

The lower reproductive performance reported for older mares is explained in this study by the higher EM rate observed in older mares (22.2%) than for younger mares (5.9%) when bred at the FH. Embryo mortality rate reported in the literature ranged from 8–18% (Woods *et al.* 1986; Ball 1988; Brinsko *et al.* 1994). When mares were not grouped according to age, EM rate was not different in mares were bred at the FH (10.4%) or at the SH (8%).

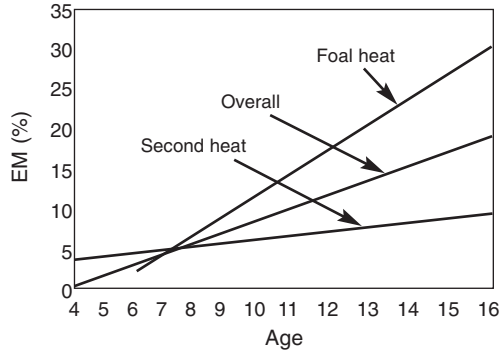

Fig. 1: Regression analysis of the embryo mortality rate as a function of the age of the mares.

Ball *et al.* (1986) reported no difference in conception rate between old and young mares 2 days after ovulation, however at 14 days after ovulation, there was a lower conception rate for older mares, suggesting a very early and higher EM rate in this group. The lower fertility of old mares has been attributed to abnormalities in the oviductal epithelium (Ball *et al.* 1986) determining an unfavourable environment for the early cleavage phases of embryo (Brinsko *et al.* 1994) or defective oocytes produced by older mares due to a longer follicular phases associated with slower rate of follicular growth (Carnevale and Ginther 1994).

When a regression analysis was performed estimating EM as a function of the age of the mares a significant trend of increased EM rate occured as the mares aged (Fig 1).

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THE CURRENT STATUS OF EQUINE CLONING

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INTRODUCTION

The ability to clone mammals using somatic cell nuclear transfer has been recognised as a scientific milestone, because it demonstrated that a fully differentiated somatic cell can be genetically 'reprogrammed' back to the undifferentiated state of a one-cell zygote, which can then initiate and undergo complete embryonic/fetal development resulting in the birth of an animal genetically identical to the original cell donor. Potential uses of equine cloning include: 1) preservation of genetics from individual animals that would otherwise not be able to reproduce such as geldings; 2) preservation of genetic material of endangered and/or exotic species such as Przewalski's horse; 3) because of the companion animal role that horses fill for some individuals, it is likely that some horse owners will have individual animals cloned for emotional fulfilment.

The year 2003 was notable for equine cloning. We reported the birth of 3 mule foals cloned from a fetal fibroblast cell line (Woods et al. 2003) and Galli et al. (2003) reported the birth of a horse foal cloned from an adult fibroblast cell line. We have also produced 7 cloned horse pregnancies using adult cumulus cells; however, all 7 pregnancies underwent spontaneous pregnancy loss prior to Day 80 of gestation (Vanderwall et al. 2004). Despite the recent success of equine cloning, like other species, the efficiency of equine cloning is low; less than 3% of cloned embryos produced with our current cloning system result in offspring. Based on experience with other species, the primary problem contributing to the inefficiency of cloning is 'cloned offspring syndrome', which is characterised by a high incidence of embryonic, fetal and/or placental developmental abnormalities that result in extremely high rates of embryonic loss, abortion and stillbirths throughout gestation; and compromised neonatal health after birth. Cloned offspring syndrome has been especially problematic in cattle and sheep where problems are manifested throughout gestation and the neonatal period, whereas in goats and swine, problems have generally been limited to early gestation. The embryonic/fetal and/or placental developmental abnormalities that result in abortion, stillbirth or compromised neonatal health are thought to reflect incomplete or abnormal genetic reprogramming of the donor nucleus, specifically related to critically important imprinted genes. Our experience with the live birth of 3 cloned mules has provided an opportunity to develop initial information on the characteristics of pregnancy, parturition and neonatal health of equine clones, which clearly has relevance to the clinical application of this emerging technology.

MATERIALS AND METHODS

Three light-horse mares ages 8 (#267), 10 (#285) and 12 years (#233) of unknown prior reproductive history that were pregnant with cloned mule embryos were monitored throughout gestation. The cloned mule embryos were produced from a fetal fibroblast cell line via nuclear transfer procedures (Woods *et al.* 2003). Immediately after completion of the *in vitro* nuclear transfer procedures, 4 one-cell cloned embryos were transferred to each mare by placing them surgically into the oviduct ipsilateral to ovulation, which had occurred during the preceding 24 h. An initial pregnancy examination was performed via transrectal ultrasonography from Days 13–16 of gestation (Day 0 = day of

transfer). Subsequent examinations were performed at 7- to 10-day intervals until Day 60, and then approximately every 2–4 weeks throughout gestation. Parameters evaluated at each examination during early gestation (<60 days) were size and location of the embryonic vesicles, presence of an embryo within the vesicle, and presence of an embryonic heartbeat. The primary parameter evaluated ultrasonographically and/or manually at each examination during mid-gestation (60–270 days) was fetal movement. Parameters evaluated during late gestation (>270 days) were fetal movement and transrectal ultrasonographic assessment of the combined thickness of the uterus and placenta (CTUP).

Results and discussion

Of the 12 cloned mule embryos transferred to these mares, 4 developed into ultrasonographically detectable conceptuses. Twins that subsequently became fixed in opposite uterine horns were detected in mare #233. The growth profile of each cloned conceptus was compared with the growth profile of non-cloned horse conceptuses that had normal development from Days 11–40; at each examination, all 4 cloned conceptuses were within the normal size range for non-cloned conceptuses (data not shown). An embryo was identified within the vesicle of all 4 cloned conceptuses between Days 21 and 29; however, a heartbeat was not detected in one of the twin conceptuses in mare #233. The conceptus without a heartbeat was spontaneously eliminated between Days 29 and 36. The 3 remaining conceptuses continued to have normal development and remained viable throughout gestation. During the 10th and 11th months of gestation, CTUP measurements of the cloned pregnancies were compared with CTUP measurements obtained from commercial broodmares with non-cloned horse pregnancies (Troedsson *et al.* 1997). At each examination at which it was measured, the CTUPs of the cloned pregnancies were within the reference range for non-cloned pregnancies.

When the estimated date of parturition approached, the mares were monitored for clinical signs indicative of preparation for parturition, including udder development, relaxation of the sacrosciatic ligaments and vulva, and cervical relaxation or dilatation. For each of these parameters, all 3 mares had the typical temporal pattern of change prior to parturition. The 3 mares spontaneously initiated and completed parturition without assistance on Days 340 (#285) and 346 (#233 and #267). All 3 foals were active and healthy at birth, and all 3 mares spontaneously expelled the placenta within 90 min of parturition. Physical characteristics of each placenta were evaluated and compared with previously published data (Whitwell and Jeffcott 1975) for Thoroughbred horses; of note was the finding that the non-pregnant horn was longer than the pregnant horn in all 3 cloned placentas, whereas the pregnant horn is typically longer than the non-pregnant horn in equine placentas. The clinical importance, if any, of this finding was unknown. In addition, the placenta of mare #267 had gross and microscopic evidence of mild, focal microcotyledonary necrosis in 3 areas (4.0 x 5.0, 3.0 x 1.5, and 2.0 x 2.0 cm) that were associated with minimal inflammatory changes (lymphocytic placentitis), and yielded growth of moderate numbers of Aeromonas and Klebsiella organisms. There was no evidence that these areas of focal placentitis compromised fetal or neonatal health of this mare's foal. Although the inciting cause of the placentitis was unknown, it is unlikely that it was related to the cloning procedure.

After birth, all 3 foals were weighed, received routine post partum care and underwent a complete physical examination. All 3 foals stood unassisted within 30 min of birth. Between 24 and 36 h after birth, blood samples were collected from each foal for routine haematologic and serum biochemical analyses. All 3 foals had mild to moderate thrombocytopenia, which resolved spontaneously and was not associated with any clinical signs. The underlying cause of the thrombocytopenia was unknown, but may have been the result of absorption from colostrum of alloantibodies against foal platelets. Clinically, the foals remained healthy throughout the neonatal period, and continue to be healthy and vigorous at the time of this writing (18–21 months of age).

Three of the 4 cloned mule conceptuses that established ultrasonographically detectable pregnancies remained viable throughout gestation and had prototypical patterns of embryonic, fetal, and placental development; parturition; and neonatal health. The twin conceptus in mare #233 that was spontaneously lost between Days 29 and 36 was one of 18 cloned mule conceptuses in a larger study (Woods *et al.* 2003) that were spontaneously lost prior to Day 60; therefore,

cloned mule conceptuses had a high rate of early pregnancy loss (18/21 [86%]), which is similar to that seen with cloned conceptuses in other species. However, the normality of fetal and placental development, parturition, and neonatal health in the 3 mule conceptuses that remained viable after Day 60 was markedly different from that typically observed in clones of many other species, most notably cattle and sheep. The absence of mid- to late-gestation losses and neonatal health problems in these cloned mules bodes well for the clinical application of cloning in the equine species.

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SESSION 2:

Follicular development, oocyte maturation and embryo *in vitro* production

Chairman: E. Palmer

OVARIAN TISSUE REMODELLING AND PRE-OVULATORY FOLLICLE DEVELOPMENT

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INTRODUCTION

The slow and variable time course of follicular development in horses compared with other domestic species is due largely to the unusual anatomical architecture of the equine ovary, (Walt *et al.* 1979; Ginther 1992). The 'inside-out' nature of the equine ovary, and the fact that follicular rupture can occur only at the ovulation fossa, means that follicles must undergo considerably more growth and development in horses than other species. In most species, the peripheral location of primordial follicles places them close to the ovarian surface, and they have no need to migrate to a specific location for ovulation.

We speculate that the long oestrus period of mares reflects a greater amount of ovarian tissue remodelling before follicle rupture can take place. The extracellular matrix, (ECM) through which follicles must enlarge or expand to reach the surface germinal epithelium and ovulate, provides a specialised microenvironment composed of proteinaceous and non-proteinaceous molecules. These molecules provide the means by which cells interact during the remodelling process. Receptors on cell surfaces, called integrins bind to their respective ligands, including the family of enzymes known as matrix metalloproteinases, (MMPs). MMP-2 and MMP-9 are the predominant matrix metalloproteinases found in the equine ovary, (Song *et al.* 1999; Desvousges *et al.* 2000; 2001) and are stimulated by administration of hCG.

To maintain extracellular matrix integrity, the enzymatic action of MMPs must be held in check by some process. For that purpose, another class of proteins exists with inhibitory activity toward the MMPs. Such inhibitory proteins are called Tissue Inhibitors of Matrix MetalloProteinases (TIMPs). The most commonly studied TIMP, (TIMP-1) has been proposed to control the site and extent of follicular connective tissue remodelling associated with ovulation.

The rationale for these studies then was to study changes in MMP-2 and TIMP-1 in equine ovarian pre-ovulatory follicles to understand better the process of tissue remodelling required for ovulation.

MATERIALS AND METHODS

Experiment 1

To test the efficacy of transvaginal folliculocentesis for studying tissue remodelling, follicular fluid (FF; 500 µl) was collected at varying times from first detection of a 30 mm pre-ovulatory follicle from 20 cycling mares as follows: Group 1: 0 h (first detection), Group 2: 48 h, Group 3: 72 h and Group 4: 0, 24, 48 and 72 h. MMP-2, TIMP-1 and progesterone were quantified in the FF.

Results

Both MMP-2 and TIMP-1 increased in FF from time of first detection of a 30 mm follicle to 72 h later, while FF progesterone decreased. The method of acquiring follicular fluid sample (cross-sectionally with different mares or serially, with repeated sampling from the same mares) did not appear to alter the results or interpretation of them (see Table 1).

Experiment 2

In this experiment gonadotropins were reduced through negative feedback of progesterone (LH)

TABLE 1: P4, MMP-2 and TIMP-1 in follicular fluid collected at a single time point from different mares (cross-sectional; n=5 each time period) or repeatedly from the same mares (serial; n=5)

Endpoint	30 mm diam.	+24 h	+48 h	+72 h
P4 (ng/ml)				
Cross-sectional	972.6 ± 166.5		652.2 ± 166.5	330.3 ± 204.0
Serial	973.7 ± 69.2	682.6 ± 69.2	431.0 ± 98.0	312.3 ± 97.9
MMP-2 (density)				
Cross-sectional	0.91 ± 0.14		1.18 ± 0.15	2.2 ± 0.15
Serial	1.0 ± 0.14	1.0 ± 0.14	1.2 ± 0.19	2.2 ± 0.19
TIMP-1 (density)				
Cross-sectional	0.66 ± 0.03		.92 ± 0.03	1.1 ± 0.03
Serial	0.64 ± 0.03	0.77 ± 0.03	.96 ± 0.04	1.1 ± 0.04

TABLE 2: P4, MMP-2 and TIMP-1 in follicular fluid of vehicle, P4 (150 mg/day) and P4+E2 (150 + 1-mg/day, respectively) treated mares

	Control vehicle	P4	P4+E2
P4 (ng/ml)	7.32 ± 2.1	10.1 ± 2.1	21.00 ± 2.57
MMP-2 (Rel. Density)	0.67 ± 0.02	0.33 ± 0.02	0.24 ± 0.02
TIMP-1 (Rel. Density)	0.67 ± 0.11	0.51 ± 0.11	0.79 ± 0.20

TABLE 3: P4, MMP-2 and TIMP-1 production *in vitro* and effects of hCG and MMP-specific inhibitor

	Control media	hCG *in vitro*	MMP inhibitor
P4 (ng/ml)	14.1 ± 1.20	8.3 ± 1.10	16.1 ± 1.20
MMP-2 (Rel. Density)	0.39 ± 0.01	0.60 ± 0.02	0.25 ± 0.02
TIMP-1 (Rel. Density)	0.74 ± 0.03	0.62 ± 0.03	0.61 ± 0.02

and oestrogen + progesterone (LH + FSH) to study the effects on MMP-2 and TIMP-1. Eight mares in the luteal phase were administered prostaglandin F 2α (PGF) if they had a follicle >15 but <25 mm diameter. Control vehicle (n=3) or progesterone (150 mg/day; n=3) were begun on day of PGF. Oestradiol + progesterone (10 and 150 mg/day, respectively; n=2) administration was begun after PGF, but not until the time of follicle deviation. When follicles reached 30 mm, mares were ovariectomised, FF collected, and follicle wall tissue incubated for 24 h in MEM (37°C, $N:O_2:CO_2$, 45, 50 5%) and the media evaluated for MMP-2, and TIMP-1. Follicle wall tissue was exposed *in vitro* to hCG (0.01 iu/ml) or specific MMP-2 cyclic inhibitor; 10 µm/ml).

Results

P4 treatment did not reduce MMP-2 in FF at surgery, but did reduce (P<0.001) MMP-2 production in culture. P4 + E2 treatment significantly reduced MMP-2 in FF at surgery, and reduced *in vitro* MMP-2 production (P< 0.001) (Table 2 and 3). Addition of hCG to *in vitro* cultures increased MMP-2 production (P<0.001). TIMP-1 production by follicle wall cultures was unaffected by *in vivo* and *in vitro* treatments.

CONCLUSIONS

These data indicate that: 1) MMP-2 production appears to require gonadotropin, both for production and mRNA expression (data not shown), 2) hCG increased MMP-2 *in vitro* production by follicle wall, 3) TIMP-1, on the other hand, does not appear to require gonadotropin, as removal of gonadotropin resulted in either no change (*in vitro* production) or increased (mRNA; data not shown), suggesting that regulation of TIMP-1 may be less tightly linked to gonadotropin than MMP-2. The authors conclude that ovarian tissue remodelling for follicle development and ovulation depends on a

positive gonadotropin-MMP-2 interaction.

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PRELIMINARY RESULTS ON INTRAFOLLICULAR TRANSFER OF *IN VITRO*-MATURED OOCYTES

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INTRODUCTION

Culture conditions for *in vitro* (IVM) maturation of mammalian oocytes are still not well defined (Sutton 2003). Oocytes matured *in vitro* are compromised in their developmental competence compared with those matured *in vivo* (Bousquet *et al.* 1999; Lonergan 2003; Rodriguéz 2004).

Despite efforts to develop a system for oocyte IVM, little is known about the specfic requiremets of the Cumulus-oocytes complexes (COCs) on energy substrates, hormones, growth factors and other undefined factors (Sutton 2003). A range of IVM media is used as oocytes from different species respond differently.

Oocyte cytoplasmic and nuclear maturation, which are partially independent processes, are a culmination of a prolonged period of oocyte growth and development bathed in follicular fluid (FF) (Goudet 1997b; Driancourt 1998). Different proportions of FF were used to replace serum or other protein sources in IVM media for buffalo (Chauhan 1997), bovine (Romero-Arredondo 1996), porcine (Funahashi 1993) and equine COCs (Dell`Aquila 1997; Aguilar 1998; Schmid, 1998) with suitable results. Pre-ovulatory follicular fluid has been used 100% pure as maturation medium for equine oocytes and apparently provides the necessary factors to induce adequate *in vitro* maturation (Aguilar 2001; Bogh 2002). Intrafollicular transfer of immature oocytes has resulted in progression of meiosis as an alternative for *in vitro* maturation (Goudet 1997a) and in fertilisation and subsequent embryo development *in vivo* (Hinrichs and DiGiorgio 1991).

The aim of this study was to compare 2 different media to mature equine oocytes *in vitro* and to evaluate the ability of these oocytes to become embryos after being transferred into a pre-ovulatory follicle.

MATERIALS AND METHODS

Ovaries from a local abattoir were transported to the laboratory at 25°C within 1 h in PBS. Individual follicle dissection and scraping were used to recover COCs. Only compact COCs were included in this study. Groups of 10–16 compact COCs were matured in droplets of 125 µl under mineral oil for 36 h at 38°C in a humidified atmosphere with 5% CO_2. One hundred percent equine FF from pre-ovulatory follicles or BiTCM-199 containing FSH 5 µg/ml, LH 2 µg/ml, IGF-1 100 ng/ml, EGF 100 ng/ml, and 10% FCS were used as maturation media. *In vitro* maturation was initiated when a growing follicle of 34–38 mm was detected ultrasonographically in the recipient mares showing signs of oestrus. Human Chorionic Gonadotrophin (2,500 iu, iv) was used to induce ovulation when the recipient mare was detected with a follicle of 35–38 mm in diameter. The next day after hCG injection, the recipient mares were artificially inseminated with fresh extended semen receiving at least 2 x 10^9 progressively motile spermatozoa from a fertile stallion. After 36 h of *in vitro* maturation, the COCs were placed in Hepes TCM-199 containing 10% FCS, and loaded in 300 ml of holding medium in a flexible plastic IVF catheter (Veterinary Concepts Inc, USA) connected to a 1 ml syringe. Recipient mares were sedated with xylacin, butorphanol and morphine, prepared for standing abdominal puncture and 10 ml of lidocaine were administered SC and IM at the point of injection. An ultrasound-guided spinal needle (18G) inserted through the flank was used to transfer the COCs into the pre-ovulatory follicle while an operator was holding transrectally the

ovary against the abdominal wall. Ovulation was confirmed to occur within 6 h after the transfer. Uterine lavage was performed at Day 8 post ovulation for embryo recovery. A total of 6 recipient mares were used. Three transfers were performed for each medium containing 12, 10 and 10 COCs for TCM and 16, 13 and 10 COCs for FF. Chi square test was used to compare the percentage of embryos derived from *in vitro*-matured oocytes. The probability $P<0.05$ was considered of statistical significance.

Results and discussion

Overall, embryos were recovered in 3/6 lavages (50%). Subtracting the number of embryos that each recipient mare might have provided based on the number of ovulations; the embryo recovery rate was 0% (0/32) for TCM and 5.1% (2/39) for FF ($P=0.07$). In only one uterine lavage 2 embryos (3–1) were considered to derive from *in vitro*-matured oocyte. They both were expanded blastocyst Grade 1. The intrafollicular transfer was considered simple and safe to practice with no complications detected in the mares. More studies are needed to improve the consistency of the technique. Five unfertilised eggs (4 and 1) were recovered from 2 mares that received COCs matured in TCM. In conclusion, compact COCs matured *in vitro* for 36 h in 100% equine FF had the capacity of becoming transferable embryos at a rate of 5.1%.

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THE USE OF OOCYTE TRANSFER TO EVALUATE *IN VITRO* MATURATION OF EQUINE OOCYTES IN DIFFERENT CULTURE CONDITIONS

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INTRODUCTION

An efficient system to mature equine oocytes *in vitro* has not been developed yet. Since nuclear maturation only indicates the ability of oocytes to restart meiosis in culture, surgical transfer of *in vitro* matured oocytes to the oviduct of recipient mares can be used to determine oocyte competence to develop after fertilisation (Hinrichs *et al.* 1998a; Hinrichs *et al.* 1998b; Hinrichs *et al.* 1999; Carnevale *et al.* 2000). The present study aimed to evaluate the ability of equine oocytes matured in different media containing heterologous or homologous FSH, associated with equine growth hormone, to undergo nuclear maturation and development after oocyte transfer (OT).

MATERIALS AND METHODS

Equine oocytes were obtained after the slicing (Choi *et al.* 1993) of slaughterhouse ovaries transported at 20–25ºC over 4–5 h. Only oocytes with a spherical, non-fragmented cytoplasm and showing at least one layer of compact granulosa cells were used. Five replicates were performed using 30 oocytes per experimental group, matured during 36 h at 38.5°C in 5% CO2 in air with 100% humidity.

Experiment 1: Different maturation media were tested

Group TCM 199 – oocytes were matured in TCM-199 medium containing 1 mM L-glutamine, 0.32 mM sodium pyruvate, 3.3 mM sodium lactate, 0.4 mM cystein, 0.1mM taurine, 0.4 mM glycine, 8 mg/ml BSA, 10ng/ml IGF-1, 50 ng/ml EGF, 500 ng/ml E_2 (all from Sigma, St. Louis, USA) and 5 µg/ml bFSH (Foltropin-V®, Vetepharm, Canada).

Group SOFaa – oocytes were matured in SOFaa medium (Embriocare BT-FIV, Cultilab, Campinas, Brazil), with the same factors as in group TCM-199.

Group HTF:BME – oocytes were matured in HTF (Irvine Scientific, California, USA): BME media (Gibco BRL, NY, USA) (1:1), with the same factors as in group TCM-199.

Experiment 2: The effect of addition of bovine or equine FSH and equine growth hormone was tested

Group bFSH - oocytes were matured in HTF:BME media (1:1), containing 1 mM L-glutamine, 0.32 mM sodium pyruvate, 3.3 mM sodium lactate, 0.4 mM cystein, 0.1 mM taurine, 0.4 mM glycine, 8 mg/ml BSA, 50 ng/ml EGF, 500 ng/ml E_2 (all from Sigma, St. Louis, USA) and 5 µg/ml bFSH (Foltropin-V®, Vetepharm, Canada).

Group eFSH - oocytes were matured in HTF: BME media (1:1), with the same factors as in group bFSH + 5 µg/ml eFSH (Bioniche, Canada).

Group eFSH + eGH (Equine Growth Hormone) – the oocytes were matured in HTF:BME media (1:1), with the same factors as in group bFSH + 5 µg/ml eFSH (Bioniche, Canada) + 100 ng/ml eGH (EquiGen™, BresaGen).

Nuclear maturation was evaluated by staining the oocytes with 10ml Hoechst 33342 (Sigma, St. Louis, H33342, USA). All data of nuclear maturation were analysed with a single factor Analysis of Variance (ANOVA).

For oocyte transfer six non-cycling recipient mares (2 for each group) were treated with Oestradiol Cipionate (ECP, Rhodia-Merieux, Paulínia, Brazil) for 3 days (2–5 ml/day). After

transfer of 12.8 oocytes on average, each recipient was inseminated 12 h pre- and post surgery with 2 x 10^9 live sperm cells from the same stallion. After sedation and local anesthesia, the ovary and oviduct was exposure through a flank incision, and the oocytes transferred to the infundibulum using a glass pipette (Carnevale 2004). After the surgery all recipients received 200 mg/day of progesterone (BET Laboratory, Rio de Janeiro, Brazil) until pregnancy detection 15 and 90 days after transfer.

Results and discussion

No statistical difference was observed between metaphase II rates in the present study when different culture systems were used (29.2; 23.8% and 13.8% for HTF:BME; SOFaa and TCM 199, respectively, P=0.95). These results were lower than many observed in the literature. This could be related to the absence of serum in the culture media used (Ali and Sirard 2002). Another detrimental factor was the long holding time of the oocytes firstly between the slaughterhouse and the arrival at the laboratory and, secondly between oocyte extraction from the follicle and the beginning of culture (Franz *et al.* 2002). Love *et al.* (2003) also reported the effect of storage of equine ovaries on the IVM rates. Maturation rates of the control group, where the oocytes were matured right after collection (57%), were significantly higher (P<0.001) when compared to the storage of ovaries at 22–25ºC for 15–18 h (14%).

Although many studies have been performed attempting to improve equine oocyte culture conditions, the results were still low. The addition of homologous gonadotrophin sources seems to increase maturation rates. Willis *et al.* (1991) working with bLH, eLH and with FSH, had better results of cumulus expansion using eLH and FSH. The addition of eLH or eGH in the culture media also significantly increased the percentage of MII (32% for the control versus 51% for eLH and 43% for eGH, P<0.05) (Marchal *et al.* 2003). In Experiment 2, eFSH and eGH were introduced in to the culture media as an attempt to improve the MII rates. However, the results obtained did not show significant differences between the media supplemented with bFSH, eFSH and eFSH + eGH (37.1%; 25.8% and 29.8%, respectively, P=0.545). This finding could be due to the lack of studies to determine a dose: effect relation of eFSH and eGH in the culture media.

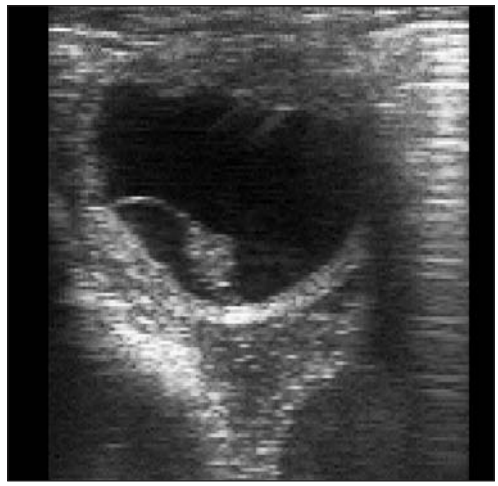

Fig 1: Ultrasonographic image of the embryonary vesicle 28 days after OT.

The pregnancy rates were 10% for the bFSH group and 6.7% for the eFSH + eGH group (Fig 1). These results were similar to those obtained by Scott *et al.* (2001), for oocytes collected during dioestrus and submitted to *in vitro* culture (7%) and for oocytes from slaughterhouses ovaries matured *in vitro* (10%). Preis *et al.* (2004) obtained an 18% pregnancy rate after OT of *in vitro* matured slaughterhouses oocytes shipped for 18–24 h at 22ºC. Landim-Alvarenga (1999), working with xenogenic fertilisation of equine oocytes matured *in vitro*, obtained 15% fertilisation rates in rabbit oviducts. Because oocytes collected from pre-ovulatory follicles resulted in 82% of pregnancy rates after OT (Scott *et al.* 2001) it can be concluded that *in vivo* matured oocytes have a high embryo development potential, comparing with those cultured *in vitro*. These data showed that *in vitro* maturation systems are still not adequate for nuclear and cytoplasmtic maturation of equine oocytes (Squires *et al.* 2003).

Based on these results we can conclude that the use of homologous FSH and GH did not improve nuclear maturation in equine oocytes cultured in HTF/BME media. Although OT resulted in pregnancy, the percentage of competent *in vitro* matured oocytes is still low.

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CENTROSOME CHANGES DURING MEIOSIS OF HORSE OOCYTES AND FIRST EMBRYONIC CELL CYCLE ORGANISATION FOLLOWING PARTHENOGENESIS, FERTILISATION AND NUCLEAR TRANSFER

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Introduction

Centrosomes are found at the poles of mitotic spindles, which are microtubule organising centres (MTOC) of mammalian cells during the cycle of somatic cell division (Kirschner and Mitchison 1986; Mazia 1987; Joshi *et al.* 1992; Karp 1999). There are 2 types of centrosome structure; i) centrioles surrounded by pericentriolar material (PCM) and, ii) acentriolar centrosomes. Some animal cells lack centrosomes entirely yet they are still capable of forming complex microtubular structures such as the meiotic spindle. Although centrioles are part of centrosomes, centrioles may not be essential for the meiotic spindles when oocytes were generated from germline cells and their microtubules are still organised by PCM which can be found in mammalian oogenesis (Szollosi *et al.* 1972; Maro *et al.* 1985; Karp 1999).

The patterns of centrosome inheritance during gametogenesis and fertilisation differ among different mammalian species and very little information relevant to this subject is available in horses. In this study, we investigated the behaviour of the centrosomes of horse oocytes during the meiotic cell cycle and their changes at the first cycle in embryos produced *in vitro* by parthenogenesis, fertilisation and nuclear transfer.

Materials and methods

Meiotic oocytes of GV, GVBD, MI and MII stages were prepared from cumulus-oocyte complexes (COCs) recovered from the ovaries of slaughtered mares and cultured *in vitro*. MII oocytes were subjected to parthenogenetic stimulation, ICSI or nuclear transfer using somatic cells as described previously by Li *et al.* (2001 and 2003). The treated oocytes, including denuded GV to MII stages, and oocytes treated by parthenogenesis, ICSI and nuclear transfer, were fixed in a glycerol-based microtubule-stabilising solution followed by 2.5% paraformaldehyde in PBS. Meiosis and first cell cycle organisation were visualised by indirect immuno-fluorescent staining using a mouse anti-α-tubulin antibody which stained the microtubules green and a rabbit anti-α-tubulin antibody which stained the centrosome red, followed by the application of a second antibody conjugated with respectively, FITC and Texas-red. The stained oocytes were mounted under a coverslip in anti-fade medium containing TOTO3 (staining chromatin blue), and examined by confocal microscopy. The appearance of the centrosome in somatic cells was also monitored as the experimental control.

Results and discussion

Meiosis in the germline halves the ploidy of the gametes to ensure that the ploidy of the organism is maintained in the next generation. Similarly, the number of centrosomes must also be reduced during gametogenesis or fertilisation to maintain the correct number of centrosomes in the zygotes (LeGuen and Crozet 1989). In most species of mammals the sperm contributes the centrioles, and the egg contributes the PCM, to the zygotic centrosomes, but there are some exceptions to the paternal inheritance of centrioles rule. For example, spermiogenesis in mice and parthenogenetic development in wasps (Messinger and Albertini 1991; Schatten 1994). On the other hand, MTOC are not found in metaphase II (MII)

oocytes of pigs, sheep and cows, compared to the situation in mouse MII oocytes where they have been found at the spindle poles and in the cytoplasm. Also, several PCM foci can be obtained at the acentriolar meiotic spindle poles, as well as in the cytoplasm (Lee *et al.* 2000).

Our results show that, during meiosis commencing from the GV stage, the oocyte does not show a centriolar structure following organisation of the microtubules but only a pericentriolar material-like structure of α-tubulin surrounding both poles of the MI and MII stage spindles. In parthenogenetically activated oocytes centrioles were also absent from both poles of the microtubular spindles following their further development after separation of the chromosomes. However, red stained α-tubulin did remain associated with the microtubules. On the other hand, 1–2 red-stained centrosome-like structures were observed in oocytes fertilised by ICSI, or in oocytes reconstructed by nuclear transfer, and these appeared to be associated with the introduced donor chromatin. These findings suggest that centrioles are not an essential component in the formation of the metaphase spindle during meiotic horse oogenesis, but they are introduced into the oocytes from the spermatozoon or the donor cell following ICSI or nuclear transfer.

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EMBRYO DEVELOPMENT OF EQUINE OOCYTES MATURED *IN VITRO* IN THE PRESENCE OF CYSTEAMINE AND FERTILISED BY ICSI

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INTRODUCTION

Oxidative stress, via the reactive oxygen species (ROS), is one of the main processes affecting proper *in vitro* mammalian embryo development. In most cells, the damaging effects of ROS can be attenuated by means of efficient antioxidant systems that scavenge the active oxygen species. Equine oocytes and embryos carry large amounts of lipid droplets in their cytoplasm. This lipid content not only gives them their characteristic dark cytoplasm appearance, but might also raise their susceptibility to oxidative stress.

Glutathione (GSH) is the major non-protein sulphydryl compound in mammalian cells and participates in the protection of the cell from oxidative damage. GSH is synthesised during oocyte development and maturation (Perreault *et al.* 1988) and accumulates to form a pool which will participate at different stages of post-fertilisation and embryo development. After fertilisation, GSH is involved in sperm decondensation, oocyte activation and in the transformation of the sperm head into the male pronucleus (Perreault *et al.* 1984; Calvin *et al.* 1986; Perreault 1990; Yoshida *et al.* 1992; Yosida *et al.* 1993). Moreover, it has been demonstrated the addition of cysteamine to *in vitro* maturation (IVM) medium of bovine (de Matos and Furnus 2000), bubaline (Gasparrini *et al.* 2003) and ovine (de Matos *et al.* 2002) oocytes induces GSH de novo synthesis improving further embryo development *in vitro*. Although recent progress has been achieved on the *in vitro* culture of equine embryos (Choi *et al.* 2004), the results obtained with this species are still low. Therefore, the aim of this study was to investigate the effect of cysteamine during IVM of equine oocytes and its effect on *in vitro* embryo development after fertilisation by Intracytoplasmic Sperm Injection (ICSI).

MATERIALS AND METHODS

In vitro maturation

Equine cumulus-oocyte complexes (COCs) were obtained from salughterhouse ovaries from mares of unknown reproductive history from spring 2003 to end of summer 2004 in Buenos Aires, Argentina (Latitude: 34° 20' South). Ovaries were transported to the laboratory at room temperature in sterile saline within 4–5 h of slaughter. All follicles of less than 3 cm in diameter were aspirated and flushed 5 times with PBS additioned with 10% FBS and 2 iu/ml heparin, using a syringe and a 18 G needle. Each aspirated follicle was opened with a scalpel blade and the walls scraped using a rounded end spatula. All oocytes recovered were matured *in vitro* in groups of 10 in 50 ml droplets of TCM 199 (Gibco, USA) with 5 mg/ml FSH (Bioniche, Canada), 2 mg/ml LH (Bioniche, Canada), 100 ng/ml IGF-I, 100 ng/ml EGF, 10% FBS (Gibco, South America) and 0, 50 or 100 mM cysteamine. After 30 h of IVM, COCs were incubated in a hyaluronidase-trypsin solution for 10 min and their cumulus cells were removed by vigorous pipetting through a bore glass pipette. All oocytes with a visible polar body and an intact cytoplasm were selected for injection.

Sperm preparation

Fresh semen was collected from a stallion using a Missouri Model artificial vagina. Routine semen evaluation parameters of concentration and total number of spermatozoa were measured using a hemocytometer. Samples were then diluted to a final concentration of 50 x 10^6 spermatozoa/ml in

Kenney's extender, stored at 5°C in an Equitainer and transported to the laboratory. One h before ICSI, 2 ml of the sperm suspension were centrifuged at 800g for 15 min and the pellet was transferred to the bottom of a conical tube which contained 2 ml of Fert-TALP medium at 38°C. Thirty minutes later, motile spermatozoa were selected by the swim-up procedure. One microlitre of the sperm fraction was added to a 4 ml droplet of 10% polyvinylpyrrolidone in HEPES buffered salt solution.

Intracytoplasmic sperm injection (ICSI)

The ICSI technique used was the same as described by Palermo *et al.* (Palermo *et al.* 1992). Briefly, sperm cells were immobilised by disruption of the plasma membrane with the injection pipette. Each oocyte was injected with one stallion sperm while it was held in place by the holding pipette. All injected oocytes were exposed to 30 μM Ca^{++} ionophore A23187 for 10 min to induce activation and cultured *in vitro* in DMEM:F10 (1:1) (Gibco, USA) with 10% FBS and 2.2 mM $CaCl_2$ at 38°C, 7% O_2 and 5% CO_2 for 48 h. At this time, they were observed under light microscopy in order to determine embryonic stage of development. Unless indicated, all reagents were purchased from Sigma, USA.

Statistical analysis

Results were analysed by 2-way ANOVA. A probability of $P<0.05$ was considered significant.

RESULTS

A total of 331 follicles from 97 ovaries were processed obtaining 169 viable oocytes from which 89 extruded a polar body after 30 h of IVM.

TABLE 1: Embryo development after IVM in the presence of different concentrations of cysteamine

	Cysteamine concentration			
	0 μM	50 μM	100 μM	Total
n	53	55	61	169
MII (%)	24 (45)	31 (56)	34 (56)	89 (53)
Injected	19	22	20	61
Cleaved	2	6	4	12
2 cell	2	4	4	10
4-6 cell	0	2	0	2

The number of MII oocytes, embryos with 2 or 4-6 cells after 48 h of *in vitro* culture and the total number of cleaved embryos did not differ between oocytes matured *in vitro* in the presence of 0, 50 or 100 μM of cysteamine (Table 1) ($P<0.05$). Fertilisation rates were not determined since injected oocytes were not scored for pronuclear formation.

CONCLUSIONS

It has been demonstrated GSH is synthesised during the *in vitro* maturation of hamster (Perreaullt *et al.* 1988), mouse (Calvin *et al.* 1986), pig (Yoshida *et al.* 1993), buffalo (Gasparrini *et al.* 2003) and cattle (Miyamura *et al.* 1995) oocytes. To our knowledge, no studies have been conducted to determine if this also occurs during the IVM of equine oocytes. The addition of cysteamine to the *in vitro* maturation medium has been shown to improve developmental parameters of *in vitro* produced embryos of different species such as the bovine, ovine and bubaline and this improvement has been related to an increase of the intracellular GSH levels.

In the present study cleavage rates of equine oocytes matured *in vitro* without cysteamine were lower than those reported by other authors using conventional ICSI in the equine (Dell'Aquila *et al.* 1997; Guignot *et al.* 1998; Li *et al.* 2000), and these rates did not improve with the addition of 50 or 100 μM cysteamine. Other parameters, such as male pronuclear formation were not studied in this work, but could improve on *in vitro* fertilised equine oocytes, given GSH synthesis occurs *in vivo* and it can be induced *in vitro*. In order to determine if cysteamine or another low molecular weight thiol such as β-mercaptoethanol, are able to induce GSH synthesis in equine oocytes *in vitro*, further experiments should be designed to detect the intracellular increase of GSH in the presence of these compounds.

In conclusion, our work demonstrated that the addition of cysteamine to the *in vitro* maturation of equine oocytes did not modify embryo cleavage rates 48 h post ICSI. GSH concentration throughout equine *in vivo* oocyte maturation and early embryo development needs to be studied to determine if this compound can exert any improvement on the *in vitro* production of equine embryos.

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TRANSFER OF *IN VITRO*-PRODUCED EQUINE EMBRYOS

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INTRODUCTION

Little information is available on the production of equine embryos *in vitro*, because methods for repeatable *in vitro* fertilisation have been slow to develop. Recently, procedures for intracytoplasmic sperm injection (ICSI) have allowed high rates of fertilisation in the laboratory (Choi *et al.* 2002; Galli *et al.* 2002), and embryo culture methods are being developed which can support up to 35% blastocyst production (Hinrichs *et al.* 2005). Because these procedures are only now being developed, limited information is available on the ability of *in vitro*-produced equine embryos to establish normal pregnancy after transfer to the uteri of recipient mares.

Two laboratories have reported pregnancies from transfer of ICSI-derived, *in vitro*-cultured (IVC) equine embryos. Li *et al.* (2003) transferred 6 *in vitro*-produced (*in vitro* oocyte maturation (IVM)/ICSI/IVC) blastocysts and obtained 4 pregnancies (67% pregnancy rate), of which 2 resulted in birth of normal foals. Galli et al. (2002) and Lazzari et al. (2002) transferred 16 blastocysts (some previously frozen and thawed) produced by IVM/ICSI and cultured either *in vitro* or in the oviducts of sheep. Multiple embryos were transferred to each recipient mare. Overall, 8 of the 16 embryos (50%) resulted in formation of embryonic vesicles after transfer. Two IVC embryos were transferred and both formed vesicles; 6 of 14 oviduct-cultured embryos formed vesicles. Because of twinning after transfer of ≥2 embryos, some pregnancies were terminated intentionally; 2 foals were born (Galli, personal communication 2004). The aim of the present study was to explore the rate of normal fetal development after transfer of blastocysts produced by ICSI/IVC in our laboratory.

MATERIALS AND METHODS

Equine oocytes were recovered from slaughterhouse-derived ovaries and matured *in vitro* in M199 with Earles salts containing 10% FBS and 5 μU/ml FSH for 24–30 h in 5% CO_2 in air at 38.2°C. In addition, *in vivo*-matured equine oocytes were obtained by aspiration of the pre-ovulatory follicle through the flank in the standing mare, 24–30 h after administration of hCG or deslorelin (Hinrichs *et al.* 1998). Oocytes in metaphase II were subjected to ICSI with frozen-thawed sperm, using the Piezo drill (Choi *et al.* 2002, 2003), then cultured in 1 of 4 media: G media (1.2/2.2 or 1.3/2.3), DMEM/F-12 medium containing 10% fetal bovine serum, with or without co-culture with equine oviductal epithelial cells, or DMEM/F-12 medium with 5 mg/ml BSA. Embryos were cultured for 6–8 days before transcervical transfer to the uteri of recipient mares. Two embryos in the DMEM/F-12/serum group were transported for 5 h in gassed DMEM/F-12/serum medium in a portable incubator before transfer.

RESULTS

A total of 20 blastocysts were transferred and 7 pregnanciees were established. Of 2 blastocysts produced from *ex vivo*-aspirated oocytes (embryos cultured in DMEM/F-12/co-culture), one resulted in formation of an embryonic vesicle after transfer. Eighteen blastocysts produced from IVM oocytes were transferred. The rates of formation of embryonic vesicles were 0/5, 0/1, 1/3 and 5/9 for embryos cultured in G media, DMEM/F-12/BSA, DMEM/F-12/co-culture and DMEM/F-12/serum, respectively.

One of the pregnancies (DMEM/F12/serum group) was terminated intentionally to recover the

vesicle. Of the 6 remaining pregnancies, 3 vesicles formed trophoblast only and were lost around 30 days of gestation. This included the vesicle from the *ex vivo*-recovered oocyte, and 2 vesicles from IVM oocytes in the DMEM/F-12/serum group. Three pregnancies continued with apparently normal fetal development; these were all from IVM oocytes. Two pregnancies (DMEM/F-12/co-culture and DMEM/F-12/serum groups) were carried to term and resulted in the birth of live foals, both fillies. The remaining pregnancy (DMEM/F-12/serum) is currently ongoing at 120 days.

Discussion

These data suggest that the pregnancy rate after transcervical transfer of *in vitro*-produced blastocysts can be acceptable (7/14, 50% for embryos cultured in DMEM/F-12/serum +/- co-culture); however, 3 of 6 pregnancies followed past 20 days were lost due to failure of the embryo proper to develop. One of these failed pregnancies developed from an oocyte recovered *ex-vivo*, suggesting that this phenomenon is not due solely to inadequacies associated with *in vitro* oocyte maturation. In addition, we have noted failure of formation of the embryo proper in 2 vesicles resulting from transfer of ICSI blastocysts that had been cultured in the oviduct of a live mare. The embryos were transferred to the oviduct of the primary recipient immediately after ICSI; blastocysts were recovered from the uterus 8 days afterward and transferred transcervically to the uteri of secondary recipients (Hinrichs and Choi, unpublished data). The overall incidence of formation of trophoblast-only pregnancies associated with *in vitro*-manipulated embryos is unclear. However, this phenomenon has been reported in embryos recovered from the uterus *ex vivo* and cultured for as little as 24 h (Pruitt *et al.* 1991). Further work is needed to define factors affecting developmental competence of *in vitro*-produced equine embryos, to maximise the ability of these embryos to result in normal pregnancy.

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DETECTION OF MIXOPLOIDY IN EQUINE EMBRYOS PRODUCED *IN VIVO* OR *IN VITRO*

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INTRODUCTION

Chromosomal abnormalities have been proposed, but not proven, to be a significant cause of pregnancy failure in the mare. However, chromosomally abnormal cells are a fairly common finding in morphologically normal conceptuses of other species, in which they may affect the success of embryonic and fetal development. The majority of chromosomal anomalies probably arise spontaneously during meiotic divisions, fertilisation or early embryonic cell divisions. In cattle conceptuses, conventional karyotypic analysis of dividing cells has suggested that most spontaneous chromosomal aberrations are various types and degrees of mixoploidy, ie diploid-polyploid mosaics (Hare *et al.* 1980).

The development of DNA probes that can be used, via fluorescent in situ hybridisation (FISH), to label specific chromosomes in non-dividing (interphase) nuclei has enabled the analysis of chromosome copy number in the majority of embryonic cells without the need for preparatory cell culture and treatment to drive those cells into metaphase. Performing FISH with chromosome specific probes on interphase cells therefore represents a more reliable way of identifying embryos containing cells with abnormal ploidy. Indeed, FISH has been used recently to demonstrate that a large proportion of *in vitro* produced human (Bielanska *et al.* 2002) and bovine (Viuff *et al.* 1999) blastocysts are mixoploid. By contrast, the few reports on chromosomal anomalies in horse conceptuses have used conventional karyotypic analysis and, to date, have failed to demonstrate the occurrence of any such abnormalities (Blue 1981; Romagnano et al. 1987). This study aimed to validate FISH probes for the identification of specific equine chromosomes in interphase nuclei, and to use these probes to examine the incidence of abnormal ploidy in *in vivo* and *in vitro* produced (IVP) embryos.

MATERIALS AND METHODS

Embryo production and spread preparations

In vivo developed embryos were flushed from the uterus of mares on Day 6 or 7 after ovulation. *In vitro* produced (IVP) embryos were generated by *in vitro* maturation of oocytes from slaughtered mares, intracytoplasmic sperm injection and subsequent culture *in vitro* for 2 days and in the oviduct of a progesterone-treated ewe for 5 days. The IVP embryos were frozen for transport, and thawed immediately prior to preparing the cell nuclei spreads. Cell spreads were made by lysing an embryo with a 0.1% Tween/HCl solution and letting the cells disperse onto a glass microscope slide. After fixing the nuclei with a 3:1 (v:v) mixture of methanol:glacial acetic acid, the spread preparations were air dried and stored at –80°C.

Production and validation of FISH probes

FISH probes were prepared from bacterial artificial chromosomes (BACs) specific for equine chromosomes 2 and 4 (a gift from the horse BAC library, INRA, France). After culture of the bacteria and isolation of the BAC DNA, the probes were labelled with either biotin or digoxigenin (both from Roche Diagnostics, Penzberg, Germany) so that they could be distinguished using an epifluorescence microscope as red (streptavidin-CY3) or green (anti-digoxigenin-fluorescein) signals, respectively. The remaining nuclear DNA was counterstained blue using 4',6'-diamino-2-phenyl-indole (DAPI; Vector Laboratories, Burlingame, California, USA).

Analytical criteria

Fluorescent signals detected within a nucleus were considered to reflect true and separate chromosomes if they were of similar size and shape, and were more than the diameter of a single signal apart. A nucleus was considered to be diploid if either 2+2, 2+1 or 2+0 signals were detected, triploid if there were 3+3, 3+2, 3+1, or 3+0 signals and tetraploid if 4+4, 4+3, 4+2, 4+1, or 4+0 signals were recorded. Nuclei lacking signals (eg 0+0, 0+1, 1+1) were considered false negatives and, along with damaged or overlapping nuclei, they were classified as unscorable. Finally, only embryos in which >50% of a minimum of 30 nuclei could be scored were included in further analyses.

RESULTS

Cell numbers

In total, 44 embryos (22 *in vivo* and 22 IVP) were examined. However, 2 of the IVP embryos were excluded from further analysis because of an abnormally low cell number (<30 cells/embryo). The remaining *in vivo* and IVP embryos had means of 1,670 and 150 cells per embryo, respectively.

Chromosomal abnormalities

Over 30,000 cells were examined and, while the vast majority were diploid (2n), both triploid (3n) and tetraploid (4n) nuclei were also recorded. Of the 22 *in vivo* embryos, 18 were entirely diploid, 3 were mixoploids containing less than 30% polyploid cells and one embryo was a mixoploid (2n/3n) in which >60% of the nuclei were triploid. Of the 20 IVP embryos, 12 were entirely diploid, 7 contained less than 30% polyploid cells and one was completely triploid.

DISCUSSION

To the authors' knowledge, this study is the first to demonstrate that equine embryos do occasionally contain chromosomally abnormal cells. However, the majority of 'abnormal' embryos detected were mixoploids (2n/3n, 2n/4n, 2n/3n/4n) that contained a relatively small proportion (<30%) of polyploid nuclei, and it is unclear whether such degrees of mixoploidy would compromise embryo viability. However, it is very unlikely that either of the 2 embryos with more than 60% polyploid nuclei would have given rise to a live foal at term. As in the bovine (Viuff *et al.* 1999), *in vitro* production tended to increase the likelihood of an embryo containing chromosomally abnormal cells (40% versus 18% for *in vivo* embryos); this may contribute to the reduced viability of IVP embryos.

The current study only aimed to examine abnormalities of ploidy (extra sets of chromosomes), whereas in women autosomal trisomies are the most common chromosomal defect identified in spontaneous abortions (Eiben *et al.* 1990). It is therefore probable that the current study underestimated the true incidence of chromosomal aberrations in horse embryos. Future studies could usefully concentrate on the development of probes or chromsome paints for all 32 chromsome pairs and thereby examine other types of aneuploidy or anomaly. The distribution of abnormal cells between inner cell mass and trophectoderm, the effect of stage of development on the proportion of abnormal cells and the effects of maternal age and/or the manner of *in vitro* embryo production on the incidence of chromosomal abnormalities also deserve further investigation.

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SESSION 3:

Embryo cryopreservation

Chairman: F. Landim-Alvarenga

EMBRYO DEVELOPMENT RATES AFTER VITRIFICATION AND TRANSFER OF EQUINE EMBRYOS

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INTRODUCTION

Cryopreservation of equine embryos has been successful with small (≤300 µm) embryos. Cryopreservation of large embryos (>300 µm) has resulted in lower early pregnancy rates (Slade *et al.* 1985; Squires *et al.* 1989). Vitrification is an alternative to conventional methods of cryopreservation that requires less time and equipment. During vitrification, the embryo is rapidly exposed to high concentrations of cryoprotectants before being plunged into liquid nitrogen. Results of studies *in vitro* (Hochi *et al.* 1995; Chaves *et al.* 1997; Oberstein *et al.* 2001) have suggested that small equine embryos can be successfully vitrified. However, development *in vitro* may not be an accurate reflection of developmental competence *in vivo*.

Objectives of this study were to compare embryo development rates after vitrification, warming and transfer of: 1) small (≤300 µm) and large (>300 µm) embryos, 2) small embryos that were transferred after dilution of cryoprotectants in steps or after in-straw dilution and direct transfer, and 3) large embryos vitrified with different concentrations of ethylene glycol. Embryo collection rates were compared for Day 6 (small embryos) versus Days 7 to 8 (large embryos).

MATERIALS AND METHODS

Embryos were collected on Days 6 to 8 (Day 0=day of ovulation detection). For the collection of small (≤300 µm) embryos, embryo collection attempts were performed 8 days after the administration of human chorionic gonadotropin (hCG, 2,000 iu, iv) to donors, when ovulation was detected 2 d after hCG administration. Embryos were classified by developmental stage and diameter (small, ≤300 µm or large, >300 µm). Embryos, within the same size category, were randomly assigned to one of 4 groups. In Groups 1 and 2, small (EG/G-S, n=22) and large (EG/G-L, n=10) embryos were exposed to vitrification solutions in base medium of PBS (phosphate-buffered saline without calcium and magnesium and supplemented with 0.3 mM sodium pyruvate, 3.3 mM glucose and 20% FCS) in 3 steps: 1) 1.4 M glycerol for 5 min, 2) 1.4 M glycerol + 3.6 M ethylene glycol for 5 min, and 3) 3.4 M glycerol + 4.6 M ethylene glycol (EG/G) for <1 min. Embryos were loaded into the centre of a 0.25 ml polyvinyl chloride straw, separated by air bubbles from 2 columns containing 20 µl of final vitrification solution. Straw ends were loaded with 60 µl of 0.5 M galactose in PBS. In Group 3 (XEG/G-L, n=10), large embryos were exposed to a final vitrification solution that contained 6.6 M ethylene glycol + 1.4 M glycerol. Straws were loaded as described above. In Group 4 (DT-S, n=26), vitrification procedures were modified to allow direct transfer of embryos into the uteri of recipients. Embryos were vitrified in the same solutions as Groups 1 and 2; however, loading of straws was modified so that the embryo, within 30 µL of EG/G, was loaded into the centre of the straw and separated by 2 air bubbles from columns (90 µL) of 0.5 M galactose.

For warming, straws were held in air for 10 s before immersion into a waterbath at 20°C for 10 s. Embryos in Groups 1 through 3 were expelled into a Petri dish, and the solutions were stirred. Embryos were moved into a solution with 0.25 M galactose for 5 min before being transferred into PBS for <10 min until transfer into a recipient. For direct transfer (Group 4), straws were warmed, flicked 4 to 6 times, and held at room temperature for 4 to 5 min before loading into a transfer gun. Recipients ovulated 5 or

6 days before the day of transfer. Uteri of recipients were scanned with ultrasound at 5, 7 and 9 days after transfer for detection of embryonic vesicles. Fisher's exact test was used for statistical comparisons with significance at $P<0.05$.

Results

For mares inseminated before ovulation, number of embryos collected per ovulation were similar ($P>0.1$) on Day 6 (28/36, 78%, range of diameters of 140 to 250 µm) versus Days 7 and 8 (30/48, 63%, 150 to 1,150 µm). Mean diameters (µm) ± SEM for embryos were 186.1 ± 8.6 for EG/G-S, 181.7 ± 5.4 for DT-S, 575.0 ± 82.0 for EG/G-L, and 643.5 ± 83.3 for XEG/G-L. As detected with ultrasound, embryo development rates by Day 16 were similar ($P>0.1$) after vitrification and transfer of small embryos using step dilutions (EG/G-S, 10/22, 45%) or direct transfer (DT-S, 16/26, 62%). One recipient, pregnant with an embryo from the direct-transfer group, had fluid imaged within the uterine lumen and no embryonic vesicle at the final pregnancy examination. After the transfer of small embryos, the pregnancy rate per recipient was higher ($P<0.05$) for recipients with no transfer the previous cycle versus recipients that were pregnant (n=1) or not pregnant (n=7) during the previous cycle (21/30, 70% versus 2/8, 25%, respectively). Embryo development rates were lower for large versus small embryos, with no embryonic vesicles detected in uteri of recipients that received large embryos (EG/G-L and XEG/G-L).

Discussion

The study demonstrated that small embryos can be reliably collected for cryopreservation. The basic procedures used have recently been used for the cryopreservation of embryos from water buffalo (Gasparrini *et al.* 2001). In a preliminary study in our laboratory (Caracciolo di Brienza *et al.* 2004), 9 equine embryos were vitrified similarly to Groups 1 and 2 (EG/G) in the present study. None of 3 large embryos developed, while 4 of 6 small embryos developed by Day 16. Two of the pregnancies were allowed to continue until Day 38, at which time they appeared normal. In the present study, 5 pregnancies were allowed to continue to Day 35. No embryo losses were observed, and the pregnancies appeared normal upon ultrasound imaging.

In this study, vitrification of large embryos was unsuccessful. Pregnancies have been established for large embryos after conventional cryopreservation methods; however, large embryos do not survive the procedure as well as small embryos (Slade *et al.* 1985; Squires *et al.* 1989). The large equine embryo has unique characteristics that could affect cryopreservation, including a capsule and large blastocoele.

Direct transfer of vitrified embryos resulted in an easy and time efficient method to cryopreserve and transfer embryos. Contamination by liquid nitrogen and storage problems were minimised as straws were sealed at both ends.

Conclusions

Large embryos did not result in pregnancies after vitrification. Small embryos were successfully collected on Day 6, vitrified and transferred to result in pregnancies. Direct transfer was a simple and successful procedure with clinical applications.

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SLOW FREEZING VS OPEN PULLED STRAW (OPS) VITRIFICATION FOR EQUINE EMBRYO CRYOPRESERVATION

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INTRODUCTION

Slow freezing methods have been applied with reasonable success to small (<250 µm) equine embryos. Recently, an ultra rapid vitrification method has provided encouraging results in other species. This study aimed to compare the efficiency of 2 techniques for cryopreservation of equine embryos: slow freezing and OPS vitrification.

MATERIALS AND METHODS

Embryo recovery and handling

Embryos (n=38) were recovered by transcervical flushing of the uterus using 3 x 1L of modified PBS medium warmed at 37°C (MPBS; phosphate-buffered saline supplemented with penicillin/streptomycin and 4 g/l BSA) (IMV-l'Aigle France). Embryos were washed four times in fresh, sterile MPBS at 25°C. Embryos from each age group (Day 6.5 or 6.75) were randomly divided between slow freezing and OPS vitrification. Eleven embryos were assigned to each of the four groups.

Slow freezing and thawing procedure

Embryos frozen as reported by Lagneaux *et al.* (2000) were placed in 4 successive baths containing MPBS supplemented with 100 mM glutamine (Sigma) and increasing glycerol (Sigma) concentration (2.5, 5, 7.5 and 10% [v/v]) for 5 min each at room temperature. Embryos were then loaded into 0.25 ml plastic straws, placed in a programmable freezer. The straws were cooled from room temperature (22–25°C) to – 7°C at –3°C/min, seeded and held at –7°C for 9 min, then cooled to –30°C at -0.3°C/min and plunged into liquid nitrogen. Embryos were thawed by placing straws in a 37°C water bath for 30 sec. Cryoprotectant was removed by transferring the embryos in 5 successive baths containing MPBS and decreasing glycerol and sucrose (Sigma) concentrations: 7.5% (v/v) glycerol + 0.25 mM sucrose; 5% (v/v) glycerol + 0.1mM sucrose; 2.5% (v/v) glycerol + 0.1 mM sucrose; 0% glycerol + 0.1 mM sucrose and MPBS only for 5 min each at room temperature.

OPS vitrification and warming procedure

Embryo vitrification was performed using the OPS procedure of Berthelot *et al.* (2000). The basic medium for cryoprotectant dilution was TCM 199 Hepes (TCM, Sigma) supplemented with 20% (v/v) newborn calf serum (NBCS, Sigma). Dimethyl-sulphoxide (DMSO, Sigma) and ethylene glycol (EG, Sigma) were used as cryoprotectants. Embryos were rinsed in TCM for 2 x 1 min, then equilibrated in TCM + 7.5% (v/v) DMSO + 7.5% (v/v) EG for 3 min and in TCM + 18% (v/v) DMSO + 18% (v/v) EG + 0.4 M sucrose (Sigma) for 1 min. During the last step, embryos were drawn up in a 2 µl drop, loaded into the narrow end of an OPS straw (SZIGTA Co., Denmark) by capillary action and immediately plunged into liquid nitrogen. At warming, straws containing embryos were removed from the liquid nitrogen and held in air for 2 sec. The narrow end was immersed in TCM containing 0.13 M sucrose and the embryos moved out of the straw into the medium. After 1 min, embryos were transferred into another well with the same medium for 5 min, then 5 min in TCM containing 0.075 M sucrose and a further 5 min in TCM alone.

In vitro culture

To evaluate *in vitro* development, thawed embryos

were cultured for 3 h. *In vitro* culture was conducted in 500 µl of Synthetic Oviduct Fluid (SOF) at 38.8°C, in presence of 5%CO2, 5%O2 and 90% N2 at 100% humidity.

Assessment of embryo viability

Morphological and diameter evaluation: At collection, post thawing and after culture, embryos were measured using an eyepiece micrometer and graded based on morphology as described by McKinnon and Squires (1988).

DAPI-labelling

After culture, thawed embryos were washed 3 times in PBS+0.05% BSA and placed in PBS+0.05% BSA containing 1 µg/ml 4',6-diamidino-2-phenylindole DAPI (Sigma) for 15 min at 38°C. Embryos were then washed 3 times in PBS+0.05% BSA. The number of dead cells (DAPI positive, fluorescent cells) per embryo was determined using an inverted fluorescence microscope (Olympus, IMT-2) with appropriate filters.

Characterisation of the S-phase

The S-phase was characterised by immuno-cytochemical detection of 5'Bromo-2'deoxy-Uridine (BrdU; Sigma) incorporated into newly synthesised DNA strands. The BrdU was evidenced with a first antibody (monoclonal mouse immunoglobuline G of anti-BrdU, Sigma) and a second polyclonal antibody (goat immunoglobulin anti-IgG of mouse conjugated with fluorescein isothiocyanate, FITC; Sigma) according to the modified method of Moussa *et al.* (2004). Non-parametric Kruskal Wallis test was used to compare embryo diameter, morphological grades, percentage of dead cells, percentage of cells entering S phase and effect of embryo age between the 2 treatments (significance at P<0.05).

RESULTS

Morphology and diameter evaluation

No significant differences between 2 treatments for mean diameter and morphological grade assessed before and after 3h of culture (Table 1).

Percentage of dead cells and degenerated embryos

The percentage of dead cells per embryo was similar (42 ± 6 vs 46 ± 9) for slow freezing and OPS (Table 2). The percentage of dead cells was divided into 2 categories; embryos having <15% dead cells (viable embryos) and >40% dead cells (degenerated embryos). Mean percentage of dead cells for the 2 categories is summarised in Table 2. The percentage of degenerated embryos after 3h of culture was similar for slow freezing and OPS (67% [12/18] vs 50% [10/20]).

Percentage of cells entering S phase

The percentage of cells incorporated with BrdU into newly synthesised DNA strands did not differ significantly between the procedures (Table 2). The percentage of cells incorporating BrdU in viable embryos were 54 ± 5 and 52 ± 4 for slow freezing and OPS, respectively. However, the percentage of cells incorporating BrdU for degenerated embryos were significantly lower for slow freezing and OPS methods than for viable embryos (15 ± 2 vs 6 ± 3). Percentage of dead cells and percentage of cells entering S phase did not differ significantly between embryos aged 6.5 vs 6.75 days.

TABLE 1: Morphological grade and diameter of embryos (means ± SEM)

Treatment Groups	Morphological Grade			Diameter (µm)		
	Initial	Post-thaw	Post-3h culture	Initial	Post-thaw	Post-3h culture
Slow freezing (n=18)	1	2.4 ± 0.2[a]	2.2 ± 0.2[a]	183 ± 7[b]	175 ± 7[b]	175 ± 6 [b]
Vitrification (OPS) (n=20)	1	2.8 ± 0.2[a]	2.7 ± 0.3[a]	187 ± 6[b]	176 ± 5[b]	179 ± 6[b]

[a, b] Different superscripts within columns indicate significant difference (P < 0.05)

TABLE 2: Effect of cryopreservation on percentage of dead cells and percentage of cells entering in S phase (means ± SEM)

	Group 1 (slow freezing)			Group 2 (OPS) Vitrification			
	<15% dead cells (6/18)	>40% dead cells (12/18)	Total	<15% dead cells (9/20)	>40% dead cells (10/20)	15-40% dead cells (1/20)	Total
Diameter (µm)	160 ± 7	183 ± 9	175 ± 7	177 ± 9	175 ± 6	200	176 ± 5
Number of cells	244 ± 32	343 ± 39	310 ± 30	317 ± 45	312 ± 24	420	314 ± 24
% of dead cells	7 ± 2[a]	59 ± 3[b]	42 ± 6	3 ± 1[a]	81 ± 8[b]	19	46 ± 9
% of cells entering S phase	54 ± 5[c*]	15 ± 2[d]	27 ± 5	52 ± 4[c*]	6 ± 3[d]	40	26 ± 6

a, b, c, d Different superscripts within rows indicate significant difference (P< 0.05)
* One embryo from each treatment was lost most of cells during BrdU staining and these embryos not included in results because the number of BrdU no determined.

DISCUSSION

The results of the *in vitro* evaluation of embryonic viability suggest that there is no significant difference (P>0.05) between the 2 techniques. These observations are in agreement with Oberstein *et al.* (2001) who reported that there was no significant difference in embryo grade or post-thaw viability between 3 methods of cryopreservation of equine embryos (vitrification, cryoloop ultra rapid vitrification and slow freezing). Bruyas *et al.* (1999) reported that the vitrification did not efficient to cryopreserved horse embryos, this finding was concluded on a smaller number of vitrified embryos (n=6) and all vitrified embryos were degenerated without cellular or nuclear membranes. Hochi *et al.* (1995) determined viability following the vitrification of equine blastocysts less than 300 µm in diameter, 6/8 developed and re-expanded after thawing, while only 2/8 blastocysts of more than 300 µm re-expanded after thawing. In a recent study, Caracciolo *et al.* (2004) obtained four pregnancies on Day 16 after transfer of 6 vitrified small embryos but they reported no pregnancies after transfer of 3 vitrified expanded blastocysts.

The results presented in the current study demonstrate that the rates of DNA synthesis in embryonic cells of frozen-thawed embryos did not differ significantly between the two techniques. In a previous study, we have reported that the percentage of cells entering S phase in 6.5 day-old fresh equine embryos was 74% (Moussa *et al.* 2004). The percentage of cells incorporating BrdU for frozen-thawed equine embryos was lower than for fresh embryos. In conclusion, vitrification with OPS system is simple, rapid and may be as efficient as slow freezing for the cryopreservation of equine embryos. However, these results should be confirmed by the transfer of OPS vitrified embryos to recipients.

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CRYOPRESERVATION OF EQUINE EMBRYOS BY VITRIFICATION

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The aim of the present study was to evaluate the *in vitro* viability of equine embryos vitrified in 3 different solutions.

Twelve mares were used as embryo donors. Embryos were recovered on Day 6 or 6.5 post ovulation by transcervical uterine flushing with phosphate buffered saline (PBS) containing 1% fetal bovine serum. Embryos were washed 6 times PBS with 15% fetal bovine serum. Their total diameter, and the morphology were evaluated and the embryos were given a quality score and only Grade 1 or 2 morulae and early blastocysts were used for vitrification.

Eighteen embryos were distributed in 3 groups according to the vitrification solution. Embryos of Group 1 were vitrified with 40% ethylene glycol (EG) in PBS, while embryos of Groups 2 and 3 with 40% EG, 18% ficoll, 0.3M sucrose in PBS (Hochi *et al.* 1994a) or 0.3M trehalose in PBS, respectively. After morphological evaluation and diameter measurement, all embryos were exposed to 20% EG in PBS (v/v) solution for 10 min. Afterwards, they were transferred to a vitrification solution and loaded individually into a 0.25 ml straws. The straws were sealed, cooled (1 min.) in liquid nitrogen vapour and immersed in liquid nitrogen. The straws were warmed at 20°C for 20 s and the embryos were expelled out into a 0.5M sucrose in PBS solution. After 10 min, the embryos were transferred to PBS solution (10 min). The diameters of the embryos were measured again and morphological (Grade 1–5) and embryonic viability were evaluated. To assess the percentage of viable cells embryos were stained with Propidium iodide and Hoechst 33258.

Embryos of Groups 2 and 3 showed the highest morphological quality and percentage of viable cells (19.2% and 26.7%, respectively).

INTRODUCTION

Transfer of fresh embryos has been carried out succesfully in the equine since 1974 (Oguri and Tsutsumi 1974). However, the possibility of transporting embryos and the difficulties in transfering fresh embryos has stimulated research in the cryopreservation of equine embryos (Griffin *et al.* 1981). The first pregnancy from a cryopreserved equine embryo was reported by Griffin *et al.* (1981). The first birth of a foal from a frozen embryo was reported by Yamamoto *et al.* (1982).

The controlled freezing was laborious and time consuming. Consequently many people in several countries have tried to simplify this procedure and to investigate factors affecting sucess of embryo freezing as well as new cryopreservation methods (Massip *et al.* 1989).

Rall and Fahy (1985), reported the first successful cryopreservation of mouse embryos by vitrification. Embryos are suspended in a highly concentrated solution which can be cooled by liquid nitrogen without crystallising. The cryopreservation of embryos by vitrification is much quicker than controlled freezing and does not require computerised equipment (Rall 1987; Massip *et al.* 1989). The vitrification approach eliminates the need for controlled slow freezing and concomitant extracellular ice by producing the necessary dehydration prior to cooling. In fact, the rate of cooling for vitrification is relatively unimportant as long as it is rapid enough to prevent crystallisation (Rall 1987). However, the high concentrations of cryoprotectants can be toxic to the embryo. Kasai *et al.* (1990) reported a simple method using a less toxic vitrification solution. The vitrification of equine embryo is

using this solution resulted in 2 pregnancies from 5 tranfers (Hochi *et al.* 1994a).

The aim of the present work was to evaluate the *in vitro* viability of equine embryos vitrified in 3 different solutions.

Materials and methods

Embryos

Twelve Brazilian jumping mares (3–10 years of age) were used as embryo donors. During oestrus, mares were palpated every 8 h to detect ovulation. Embryos were recovered on Day 6 or 6.5 post ovulation (day of ovulation = Day 0) by transcervical uterine flushing with phosphate buffered saline (PBS) containing 1% fetal bovine serum. Embryos were washed 6 times PBS with 15% fetal bovine serum. Their total diameter, including *zona pellucida* was measured using an ocular micrometer. The morphology was evaluated and the embryos were given a quality score using the classification discribed by McKinnon and Squires (1988). Embryo diameters ranged from 170 µm to 235 µm and only Grade 1 or 2 morulae and early blastocysts were used for vitrification.

Vitrification and thawing

Eighteen embryos were distributed in 3 groups according to the vitrification solution. Embryos of Group 1 were vitrified with 40% ethylene glycol (EG) in PBS, while embryos of Groups 2 and 3 with 40% EG, 18% ficoll, 0.3M sucrose in PBS (Hochi *et al.* 1994a) or 0.3M trehalose in PBS, respectively. After morphological evaluation and diameter measurement, all embryos were exposed to 20% EG in PBS (v/v) solution for 10 min. Afterwards, they were transferred to a vitrification solution and loaded individually into a 0.25 ml straws. The straws were sealed, cooled (1 min) in liquid nitrogen vapour and immersed in liquid nitrogen. The straws were warmed at 20°C for 20 s and the embryos were expelled out into a 0.5M sucrose in PBS solution. After 10 min, the embryos were transferred to PBS solution (10 min). The diameters of the embryos were measured again and morphological (Grade 1 to 5) and embryonic viability were evaluated. To assess the percentage of viable cells embryos were stained with Propidium iodide and Hoechst 33258.

Treatment differences were analysed by non-parametric tests Kruskal-Wallis and Mann-Whitney ($P \leq 0.05$).

Results and discussion

The recovery rates of Day 6.5 equine embryos in this study were 57.8% (26/45). This rate is lower than the 82% reported by Hochi *et al.* (1995). However, they were superiour to the 46% and 50% reported by Poitras *et al.* (1994) and Ferreira *et al.* (1997). Lack of fertilisation, or the failure of some embryos to enter the uterus by Day 6 or early embryonic death before Day 6 may due to reduced recovery rates (Ball *et al.* 1986).

In this study, after thawing, the proportion of embryos with *zona pellucida* damage was 23%. Zona damage is thought to be associated with solidification of the embryo suspension medium during cooling (Whittingham and Adams 1976) and its subsequent liquefaction during warming (Leibo 1984). Contrasting results related to pregnancy rate, associated with the presence of damage in the *zona pellucida* of equine embryos were observed. Although Squires *et al.* (1989) did not find a correlation between *zona pellucida* damage and pregnancy rates, Poitras *et al.* (1994) and Ferreira *et al.* (1997) reported that damage to the *zona pellucida* decreases embryo viability.

TABLE 1: Variation of pre- and post vitrification embryo diameter and grade and percentage of viable embyro cells post vitrification between the groups

Group	Variation of pre and post vitrification		Post vitrification viable cells (%)
	Diameter	Grade	
1	28.4 ± 8.1[a]	3.4 ± 0.5[a]	0.8 ± 1.1[a]
2	5.7 ± 11.6[b]	1.3 ± 0.5[b]	26.7 ± 26.6[b]
3	18.0 ± 9.3[ab]	1.3 ± 1.0[b]	19.2 ± 8.01[b]

Different superscripts in the same column differ ($P \leq 0{,}05$). Group 1: 40% ethylene glycol/PBS, Group 2: 40% ethylene glycol + 18% ficoll + 0.3M sucrose, Group 3: 40% ethylene glycol + 18% ficoll + 0.3M trehalose

The incidence of damage to the *zona pellucida* of the thawed embryos in this study (23%) was similar to the figure reported by Hochi *et al.* (1995) (13%–25%) for equine embryos vitrified in the presence of ethylene glycol, ficoll and sucrose. In this study the percentage of viable cells and embryo morphological grade after vitrification were highly correlated r= -0.625 (P= 0.007). Thus morphological grade was an acceptable indicator of viability.

A reduction in embryo quality and in the percentage of viable cells were observed, that was significantilly higher in Group 1 (Table 1). Embryos of Groups 2 and 3 showed the highest morphological quality and percentage of viable cells (19.2% and 26.7%, respectively).The higher percentage of viable cells observed in Groups 2 and 3 could be associated to the addition of ficoll and sucrose or trehalose, respectively in the solution of vitrification. However, there were no differences between percentage of viable cells and embryo grades post thawing when we compared the use of sucrose or trehalose in vitrification solution.

Conclusion

The addition of non-permeating cryoprotectants (macromolecules and sugars) to vitrification solutions containing ethylene glycol improved the morphology and the percentage of viable cells afther thawing.

The type of sugar (sucrose and trehalose) did not interfere in the grade and the percentage of viable cells after thawing.

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SESSION 4:

Insemination and fertilisation

Chairman: B. Colenbrander

CAPACITATION AND THE ACROSOME REACTION IN STALLION SPERM

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INTRODUCTION

During fertilisation, the oocyte must fuse with just one of the billions of sperm deposited in the mare's uterus at mating, so that development of a diploid zygote can proceed. To aid selection of a single competent sperm, sperm transport in the female reproductive tract and sperm-oocyte interaction are very precisely regulated processes. Moreover, because many of the changes essential in preparing a sperm to bind to and penetrate the *zona pellucida* (ZP) occur at the level of the outer plasma membrane, this membrane is precisely organised and very dynamic. With regard to the changes involved in the acquisition of fertilising capacity, it is noteworthy that, after leaving the testis, sperm are transcriptionally inactive and therefore incapable of synthesising new membrane components. Instead, the modifications that sperm undergo as they mature and become motile during transit through the epididymis, and those related to capacitation and the acrosome reaction (AR) within the female tract, are driven primarily by the surrounding genital fluids. Of course, studying capacitation and the AR *in vivo* is impractical because the salient events occur within the uterus and oviduct. And, while both processes can be mimicked *in vitro*, care is needed because the plasma membrane of capacitated sperm is fragile and artefacts can be induced by even minor changes in incubation conditions. Ideally, therefore, investigation of sperm activation should be performed in conditions as close to physiological as possible. This paper will review the techniques available for monitoring the changes that occur in the sperm plasma membrane in preparation for fertilisation (see also Gadella *et al.* 2001).

Sperm-oocyte interaction

When ejaculated, stallion sperm are mature and motile but must still undergo a series of sequential modifications before they are able to fertilise an oocyte. First, the sperm undergoes a battery of changes, known collectively as capacitation, that increase the affinity of its plasma membrane for the ZP; these changes include the removal of stabilising molecules originating from the epididymis and exposure to stimulatory factors in uterine and oviductal fluids. In turn, sperm-ZP binding initiates the AR, an exocytotic event characterised by the release of hydrolytic enzymes that help the sperm penetrate the ZP and enter the perivitelline space. Finally, the sperm will fuse with the oolemma via a specialised area of membrane at its equatorial segment.

Sperm plasma membrane topology

The mature sperm is a highly specialised cell divided into 3 major regions; a flagellum for propulsion, a midpiece containing mitochondria to power that motility, and a head that is critical to oocyte interaction and contains the DNA and an acrosomal vesicle loaded with zonalytic enzymes. The sperm head's plasma membrane is specialised for its role in oocyte interaction and its composition differs markedly from that of somatic cells. In particular, the sperm plasma membrane is rich in cholesterol and its glycolipid fraction is composed almost exclusively of seminolipid, an unusual glycolipid found only in sperm and Schwaan cells. As in somatic cells, the membrane phospholipids are distributed asymmetrically across the bilayer; this asymmetric distribution is maintained enzymatically and appears to be vital to maintaining the plasma membrane in a stable, non-fusogenic state. On the outside of the plasma

membrane is a carbohydrate-rich glycocalyx that interacts with both the membrane and the surrounding fluids.

Monitoring capacitation

Chlortetracycline (CTC) staining was long the method of choice for investigating sperm activation because it allowed differentiation between non-activated, capacitated and acrosome-reacted sperm. However, CTC staining can only be performed on fixed cells and, because it is not amenable to flow-cytometric analysis, is laborious. Furthermore, the molecular basis of the capacitation-related changes detected by CTC staining is unclear, except that it is Ca^{2+} dependent and probably occurs very late in capactitation.

In vitro, capacitation can be induced by bicarbonate; bicarbonate may also be instrumental *in vivo* since it is scarce in seminal plasma (<4 mM) but abundant in oviductal fluid (>20mM). One dramatic effect of bicarbonate stimulation is the activation of a plasma membrane 'scramblase' that breaks down the asymmetrical distribution of phospholipids and thereby stimulates an increase in fusogenicity and zona-binding affinity. This membrane 'scrambling' can be detected using the fluorescent dye merocyanin 540, which intercalates only into disordered membrane bilayers. An increase in merocyanin fluorescence is therefore a sensitive and early indicator of capacitation, and can be monitored in living sperm using flow cytometry or confocal microscopy with a life chamber.

Depletion of plasma membrane cholesterol also appears to be a critical, possibly rate limiting, step in the tyrosine phosphorylation initiated second messenger systems underlying capacitation and the AR; capacitation in stallion sperm is presumably slow therefore because of the relatively high membrane cholesterol content. The changes in membrane cholesterol distribution can be followed using the fluorescent probe, filipin. Capacitation involves a spatial reduction in cholesterol distribution from over the whole sperm head to only over the acrosomal cap, followed, in the presence of a cholesterol acceptor such as albumin, by the loss of approximately 45% of that cholesterol. Interestingly, stallion seminal plasma contains cholesterol rich vesicles (prostasomes) that block cholesterol efflux and may help delay capacitation until the appropriate time.

Sperm plasma membrane progesterone receptors

Capacitation also involves changes in the glycocalyx coating the sperm head; among these changes is the exposure of progesterone binding sites. Although definitive identification of the binding sites is pending, they are thought to be specific membrane receptors that allow a rapid (non-transcriptional) response to progesterone and play a critical part in the AR. Indeed *in vitro*, progesterone can induce the AR in capacitated sperm, even in the absence of ZP, by inducing a rapid rise in intracellular Ca^{2+}; *in vivo*, progesterone is present in huge quantities in follicular fluid, and progesterone impregnated in the ZP has been proposed to contribute to the physiological AR. Sperm plasma membrane progesterone receptor exposure can also be monitored flow-cytometrically, using a fluorescent progesterone conjugate such as progesterone-BSA-FITC.

Detecting the AR

Although CTC staining allows the discrimination of acrosome intact versus non-intact sperm, it is now more common to monitor acrosome integrity in live sperm using lectins with specific binding characteristics; eg FITC-PNA, a peanut agglutinin-fluoroscein conjugate that binds specifically to the outer acrosomal membrane. Combination with a supra-vital stain, such as propidium iodide, allows the AR in live sperm to be detected as an increase in FITC-PNA fluorescence, since the lectin can only access the outer acrosomal membrane once the latter has begun to fuse with the plasma membrane during the AR.

Applications to clinical practice

In practice, capacitation and the AR are of interest because they influence sperm longevity and fertilising capacity. For example, capacitation results in a more reactive sperm plasma membrane and, therefore, reduced sperm longevity; storing semen in liquid form would, therefore, benefit from keeping the sperm in a reversible 'decapacitated' state, as occurs in the epididymis. Because the AR is a one-off event, prematurely acrosome-reacted sperm are no longer able to fertilise. Not surprisingly then, the percentage of acrosome-intact sperm correlates well with the fertility of frozen stallion semen, because freeze-

thawing commonly induces acrosome damage. For fresh semen, where membrane damage would not be expected, the ability of sperm to undergo the important processes of capacitation, progesterone receptor exposure and the AR in response to bicarbonate and progesterone, are more useful indicators of fertility (Colenbrander *et al.* 2003). Similarly, preliminary data suggests that an important reason for the poor fertility of stallion epididymal sperm is the inability of sperm bearing a cytoplasmic droplet to capacitate, while the principal problem during conventional *in vitro* fertilisation appears to be failure of sperm to complete the AR after binding to the ZP. It is anticipated that the use of fluorescent markers to track sperm activation in 'physiological' conditions (flow-cytometry, confocal microscopy with a life chamber) will improve our understanding of what goes wrong in these various situations and help in the development of approaches to bridge the deficits.

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CASEIN, NATIVE PHOSPHOCASEINATE AND CASEINOGLYCOPEPTIDE ENHANCE BINDING OF EQUINE SPERM TO BOVINE ZONAE PELLUCIDAE

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INTRODUCTION

During the initial steps of fertilisation, capacitated sperm bind to the *zona pellucida* (ZP) of the oocyte and undergo the acrosome reaction (Yanagimachi 1994). Molecular mechanisms of gamete interaction are not completely understood, but they appear to be mediated by carbohydrates (Nixon *et al.* 2001; Wassarman *et al.* 2001; Wassarman and Litscher 2001). Although sperm-zona binding is not completely species specific, as demonstrated by inter-specific hybrids, it appears to be largely species-restricted (Schmell and Gulyas 1980; Moller *et al.* 1990; Rankin and Dean 2000). Therefore, most studies on gamete binding have used sperm and ZP of the same species. In the horse, few ZP are available for *in vitro* assays; therefore, the ability of stallion sperm to bind to bovine ZP was investigated (Coutinho da Silva *et al.* 2004). Similar numbers of stallion sperm bound to bovine and equine ZP, demonstrating that bovine ZP can be used for stallion sperm-zona binding assays.

Procedures that affect gamete interactions could impair fertilisation. Therefore, our laboratory has investigated effects of semen diluents on the interaction between sperm and ZP (Coutinho da Silva *et al.* 2003; Coutinho da Silva *et al.* 2004). Gametes were incubated in milk-based extenders [modified Tyrode's medium (TALP) containing 2.4 mg/ml of skim milk, EZ-Mixin® (Animal Reproduction Systems, Chino, USA), and INRA96® (IMV Technologies, L'Aigle, France)] or in diluents without milk [TALP, Lactose-EDTA, and EmCare® holding medium (ICP, Auckland, New Zealand)]. Results demonstrated that milk-based extenders significantly increased the number of sperm bound to ZP. Interestingly, INRA96® extender contains only one milk protein, the native phosphocaseinate, suggesting that this protein may have enhanced sperm binding to the *zona pellucida*. Therefore, the objective of this experiment was to determine the effects of casein (CS), native phosphocaseinate (NP) and caseinoglycopeptide (GP) on binding of stallion sperm to bovine ZP.

MATERIALS AND METHODS

Semen was obtained from one ejaculate from each of 6 fertile stallions. Semen was diluted 1:3 with TALP (Rathi *et al.* 2001) and centrifuged at 300 g for 5 min. Sperm pellets were suspended in TALP and stained with Hoechst 33342 (35 ìg/ml) for 15 min at 37°C. After incubation, sperm were centrifuged at 300 g for 5 min to remove excess stain and suspended in TALP to 2 x 10^6 sperm/ml.

Bovine oocytes were collected from ovaries obtained from a slaughterhouse. They were vortexed in TALP for 1–2 min to remove cumulus cells. Denuded oocytes were washed in TALP and stored in hyperosmotic salt solution (1.5 M $MgCl_2$, 40 mM Hepes, 0.1% PVP) at 5°C for no longer than 3 months before use.

On each day of the experiment, oocytes were removed from the salt solution, washed twice and incubated in TALP for 1 h at 38.5°C. After incubation, oocytes (n=10/treatment) were placed in droplets of 45 µl of TALP containing: no additions (TALP); 1 or 3 mg/ml of CS (CS1, CS3); 1 or 3 mg/ml of NP (NP1, NP3); and 1 or 3 mg/ml of GP (GP1, GP3). Sperm (5 µl) were added to the oocytes to a final concentration of 2 x 10^5 sperm/ml and incubated for 2 h at 38.5°C.

After incubation, oocytes were washed in 4 droplets of TALP using a small-bore, fire-polished, glass pipette to remove loosely bound

TABLE 1: Mean numbers of sperm bound per ZP ± sem (n = 60/trt)

Treatment	TALP	CS1	CS3	NP1	NP3	GP1	GP3
Sperm/ZP	27±2.0[a]	78±3.4[b]	102±5.8[c]	83±3.9[b]	97±7.1[c]	41±3.1[d]	61±3.8[e]

[abcde] Means with different superscript differ (P<0.01, ANOVA and Tukey's hsd)

sperm. They were placed on a glass slide and a cover slip was placed on top, supported by a mix of paraffin and petroleum jelly. Oocytes were observed under a fluorescence microscope (400x), and numbers of sperm bound to oocytes were recorded. Data were analysed by analysis of variance (ANOVA), and means were compared using Tukey's hsd test.

Results

Addition of milk proteins (CS, NP, and GP) to TALP enhanced (P<0.01) binding of stallion sperm to bovine ZP in a dose-dependent manner (Table 1). The number of sperm bound to the ZP was similar for CS and NP; however, a significant, but smaller, increase in sperm binding was observed for GP. Stallion by treatment interaction was significant (P<0.01).

Discussion

The sperm-*zona pellucida* binding assay has been used in several species to estimate the fertilising capacity of sperm. In this study, milk proteins (CS, NP, and GP) enhanced the ability of equine sperm to bind to bovine ZP. CS and NP, in particular, were very effective in enhancing sperm binding. NP is a component of INRA96® semen extender, which has been used successfully to store goat and equine semen for long periods (Batellier *et al.* 1997, 1998, 2000; Leboeuf *et al.* 2003). Batellier *et al.* (1997, 1998) demonstrated a beneficial effect of NP on equine sperm motility and fertility, and Batellier *et al.* (2000) observed that NP did not bind to the intact sperm membrane and had no indirect protective effect on equine sperm. In addition, they observed no effects of NP on sperm intracellular calcium levels after 24 h of incubation. Therefore, the mechanism by which NP preserves sperm during long term storage remains unknown.

Because capacitated sperm bind to the ZP of the oocyte and undergo the acrosome reaction (Yanagimachi 1994), the increased number of sperm bound to the ZP observed suggests an effect of milk proteins on sperm capacitation. Nagai *et al.* (1994) investigated the effects of casein phosphopeptides (CPP) on fertilisation of pig oocytes. Semen from boars with low penetration rates were incubated in fertilisation medium containing caffeine with or without CPP. They observed a significant increase in penetration rates when sperm were incubated in medium containing CPP compared to medium without CPP (100% vs. 46%). In addition, an increase in intracellular calcium concentrations were observed in sperm incubated with CPP. The authors suggested that CPP were acting as a calcium transporter and delivering calcium to those sperm that had poor ability to utilise it. Therefore, NP could be acting in the same manner to increase intracellular calcium levels on equine sperm, resulting in capacitation changes and increase in sperm binding to ZP. However, CS and GP are structurally different from NP and may increase sperm binding to ZP by a different mechanism. Further studies are necessary to determine the effects of milk proteins on equine sperm physiology.

Conclusion

In conclusion, the presence of milk proteins (CS, NP and GP) during co-incubation of sperm and ZP resulted in increased sperm binding. Mechanisms by which milk proteins enhance the ability of sperm to bind to the ZP need further investigation.

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FAILURE OF EMBRYO DEVELOPMENT AFTER INTRAFOLLICULAR INSEMINATION IN THE HORSE: DUE TO THE SPERM VIABILITY?

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Intrafollicular insemination (IFI) has successfully been used to obtain pregnancies in humans. To date, no pregnancies have resulted via IFI in the horse. The role of the spermatozoa in the failure of IFI has not been investigated. Therefore, the objective was to evaluate the percent of live/dead and the acrosomal status of spermatozoa after IFI *in vivo* incubation and to determine spermatozoa recovery rate. The study was performed with approval of the LSU Animal Care and Use Committee.

Ten reproductively sound mares had IFI performed with either 50 million (n=5) or 500 million (n=5) motile spermatozoa 36 h after hCG (Chorulon®, 2,500 iu, iv) administration (mare in oestrus with a follicle ≥35 mm). Four h after IFI the pre-ovulatory follicular fluid was aspirated. The spermatozoa were evaluated for motility using light microscopy and recovered spermatozoa were stained for live/dead with 5 µl of 20 µM Syto 24 and 5 µl of 1 µM propidium iodide and acrosomal status was evaluated after staining an airdried smear with 20 µl FITC-PNA.

In 7 of 10 samples a few motile spermatozoa were detected, however when stained for live/dead only 5 of 10 samples had live spermatozoa. Sperm recovery was 0.53% ± 0.38 (mean ± SEM) and 9.02% ± 4.41 for 50 and 500 million IFI groups, respectively and varied from 0.0022 to 24.8%, which was independent of initial sperm concentration (Student T-test, P>0.05). All spermatozoa were acrosome reacting or reacted.

These data suggest that the intrafollicular environment in the pre-ovulatory follicle might be unfavourable for sperm viability or fertilisation. Further research is necessary to detect incongruous maturation of the oocyte and spermatozoa.

INTRODUCTION

Intrafollicular insemination (IFI) may be an attractive, less technically complex approach to achieve a pregnancy than conventional IVF (*in vitro* fertilisation) or ICSI (intracytoplasmatic sperm injection). In humans, Lucena *et al.* (1991) reported a pregnancy after IFI with 200,000 sperm cells, and successful treatment for male factor infertility in the USA has been reported by Zbella *et al.* in 1992. However, in another study, a total of 50 infertility patients with normal tubal function were stimulated with clomiphene citrate/human menopausal gonadotrophin. Washed spermatozoa were injected into pre-ovulatory follicles via vaginal puncture 12–30 h after human chorionic gonadotrophin administration. Natural progesterone was given for luteal support. Only one normal intra-uterine pregnancy resulted (Nuojua-Huttunen *et al.* 1995).

In the horse no pregnancies after IFI have been reported. Meintjes *et al.* (2001) treated with or without hCG (3,300 iu, iv) before IFI and injected spermatozoa treated with or without calcium ionophore A32187 for induction of capacitation, into the pre-ovulatory follicle. However, none of the mares became pregnant. Eilts *et al.* (2002) performed IFI with concomitant artificial insemination (AI) in cyclic mares and were unsuccessful in obtaining pregnancies. The effect of many factors, such as the IFI procedure itself, sperm dose, sperm oocyte binding, sperm longevity

and influence of the procedure on the oocyte maturation are unknown (Parlevliet *et al.* 2004).

The role of the spermatozoa in the failure of IFI has not been investigated. In several studies spermatozoa could not be found in the recovered follicular fluid (Personal Communication Alvarengo and Squires). Therefore, the objective of this study was to evaluate sperm recovery rate (%), motility, the percent of live/dead and the acrosomal status of spermatozoa after IFI *in vivo* incubation. The study was performed with approval of the LSU Animal Care and Use Committee.

Materials and methods

Mares

Ten reproductively sound mares with normal oestrus cycles were used in a study during June and July. During oestrus, the uterus, cervix and ovaries were palpated per rectum and transrectal ultrasound was performed daily, until 2 days after ovulation. Oestrus mares with a follicle ≥35 mm received hCG (Chorulon®, 2,500 iu; Intervet Inc. Millsoboro, DE 19966, USA) for induction of ovulation.

Sperm preparation

From one fertile stallion semen was collected using a Hannover artificial vagina on a phantom. The gel was removed and spermatozoa were centrifuged (5 min, 300 *g*). After removing the supernatant, spermatozoa were allowed to swim up for 5 min, and motility and sperm concentration, using a hemocytometer, were evaluated with a 40 x phase objective on a microscope equipped with a stage warmer.

IFI-procedure

IFI was performed 36 h after ovulation induction, under transvaginal ultrasound guidance (6.5 MHz transvaginal, sector probe) (Universal Ultrasound, Bedford Hills, NY 10507, USA) using a 17 G double lumen needle (Cook, Vet Prod, Spencer, IN, USA) and a 5 ml syringe. For the IFI procedure each mare was administred detomidine HCl (0.01mg/kg, IV) (Domosedan®, Orion Corp, Espoo, Finland) and 0.1 mg/kg butorphanol (Torbugesic®, Fort Dodge Animal Health, Overland Park, KS, USA). After removing an equivalent volume of follicular fluid, the semen was deposited in the follicular lumen. Either 50 million (n=5) or 500 million (n=5) progressive motile spermatozoa (90%) in 0.6–1.4 ml seminal plasma, were inseminated. At 4 h after IFI, the pre-ovulatory follicular fluid was aspirated using the same system as for IFI.

Evaluation of spermatozoa

The recovered spermatozoa were evaluated for motility using a phase contrast microscope with a stage warmer (37°C) at a 10 or 40 x objective). Recovery rate (%) of spermatozoa was calculated using a hemocytometer and the spermatozoa were stained for live/dead with 5 µl of 20 µM Syto 24 and 5 µl of 1 µM propidium iodide (Molecular Probes Inc., Eugene, OR 97402, USA). The numbers of live dead cells were counted using a microscope equipped with an epi-iluminator or fluorescence microscopy (20 x fluor objective with a FITC-filter). Cells (n=200) were evaluated for acrosomal status after staining an airdried smear with 20 µl FITC-PNA (1 mg/ml stock diluted 1:1000; Sigma-Aldrich) using a microscope equipped for fluorescence microscopy (Nikon, USA) with a FITC-filter (40 x objective).

Statistics

A Student's T-test was performed to detect differences in sperm recovery rate between the 2 sperm concentration groups.

Results

One pre-ovulatory follicle did ovulate in the time between IFI and recovery of spermatozoa. In 7 of

TABLE 1: Recovery rate (%) of spermatozoa in follicular fluid, 4 h after IFI with different sperm numbers

	50 million spermatozoa (RR50)	500 million spermatozoa (RR500)
Follicle 1	1.66	7.28
Follicle 2	0.10	11.18
Follicle 3	0.01	24.8
Follicle 4	0.36	1.84
Follicle 5	ovulation	0.002
Mean ± SEM	0.53 ± 0.38	9.02 ± 4.41

No significant difference between columns (P>0.05)

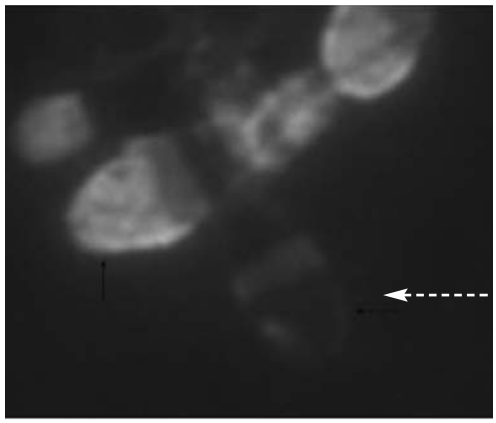

Fig 1: Acrosome reacting (→) and acrosome reacted spermatozoa after IFI (- ->)

10 samples a few motile spermatozoa were detected, however when stained for live/dead only 5 of 10 samples had membrane intact spermatozoa. No oocytes were found in the recovered follicular fluid. Sperm recovery was 0.53% ± 0.38 (mean ± SEM) and 9.02% ± 4.41 for 50 and 500 million IFI groups, respectively, and varied from 0.0022 to 24.8% (Table 1), which was independent of initial sperm concentration (Student T-test, P>0.05). All spermatozoa were acrosome reacted or reacting (Fig 1).

Discussion

In the human, although successful pregnancies have been reported using IFI there are no reports of babies born (Lucena *et al.* 1991; Zbella *et al.* 1992; Nuojua-Huttunen *et al.* 1995). The disappointing results in these early attempts at IFI in the horse might be due to several factors. The data from our experiment suggest that the intrafollicular environment in the pre-ovulatory follicle might be unfavourable for sperm viability or fertilisation. After intrafollicular injection, crude equine gonadotrophins (CEG) and epidermal growth factor (EGF) seem to influence maturation of the oocyte by enhancing meiosis by increasing cumulus expansion rate (Martoriati *et al.* 2002). Induction of ovulation by hCG might induce nuclear maturation of the oocyte but not necessarily maturation of the cytoskeleton. Follicular fluid from pre-ovulatory follicles contains high concentrations of progesterone which induces the acrosome reaction in stallion spermatozoa (Rathi *et al.* 2003). As the spermatozoa were all acrosome reacting or reacted, they might be ahead in maturation of the oocyte and not be able to fertilise the oocyte. Sperm cells were also acrosome reacted when bound to granulosa cells if IFI was performed in mares before euthanesia for other reasons (unpublished results). Further research is necessary to detect incongruous maturation of the oocyte and spermatozoa.

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FACTORS INVOLVED IN FAILURE OF TRANSVAGINAL INTRAFOLLICULAR INSEMINATION IN MARES

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INTRODUCTION

Intrafollicular insemination (IFAI) has been successful in women (Nuojua-Huttunen *et al.* 1995) but failed in mares (Eilts *et al.* 2002). It would be useful for inseminations with a low number of spermatozoa and in mares with persistent mating-induced endometritis. The procedure involves direct introduction of sperm into a pre-ovulatory follicle and diluting the semen in the follicular fluid, although it is known that follicular fluid affects such sperm functions as motility (Landim-Alvarenga *et al.* 2001), acrosome reaction (Yao *et al.* 1999) and spermatozoa-oocyte fusion). Yao *et al.* (1996) Human follicular fluid (hFF) reduces zona binding capacity of spermatozoa *in vitro* (Siegel *et al.* 1990. Two glycoproteins in hFF, zona-binding inhibitory factor-1 (ZIF-1) and ZIF-2, inhibit spermatozoa-zona pellucida binding (Yao *et al.* 1998).

MATERIALS AND METHODS

Experiment I

A trial was designed to confirm the inefficiency of IFAI. When the dominant follicle reached 35 mm, 20 mares received 2,500 iu of hCG (Vetecor, Callier, São Paulo-SP, Brazil). After 30 h of ovulation induction, follicular control was performed every 6 h and inseminations were performed when proximity of ovulation was detected by pain reflex during palpation and ultrasound images (thickness of follicular wall and follicle shape). Inseminations were performed with 20 x 10^6 motile spermatozoa (0.5 ml) from frozen stallion semen, by transvaginal ultrasonography. The ovary was manipulated per rectum and the dominant follicle positioned on the biopsy line, from the ultrasound monitor; the vaginal and follicular wall were punctured and the semen was injected inside the pre-ovulatory follicle. Mares ovulated 6 h after insemination.

Experiment II

A trial was conducted to evaluate the interference of IFAI on oocyte viability after conventional artificial insemination (Eilts *et al.* 2002). Ten mares received 2,500 ui of hCG, when the dominant follicle reached 35 mm diameter and were inseminated by IFAI, with 20 x 10^6 motile spermatozoa from frozen semen, when ovulation was detected. At the same time they were inseminated with 500 x 10^6 motile spermatozoa from fresh donkey semen in the uterine body (Group: IFAI-UB). A further 18 mares were inseminated with 500 x 10^6 motile spermatozoa from fresh donkey semen in the uterine body (Group: UB). These mares were inseminated every 48 h until ovulation after detection of a 35 mm follicle. The mares in Experiments I and II were examined by ultrasound 15 days after ovulation for pregnancy diagnosis. Data from the experiments were analysed by the Goodman test.

Experiment III

An *in vitro* experiment was designed to study sperm survival in follicular fluid. Twenty samples of frozen-thawed semen from 10 stallions (2 from each stallion) were evaluated without dilution (GI) and diluted 1:1 with follicular fluid (GII). The fluid was aspirated from a dominant follicle 36 h after administration of 2,500 ui hCG, centrifuged and stored at –10°C. The percentage of total motility (TM) and progressive motility (PM) were evaluated at 0 (T0), 1 (T1), 2 (T2) and 3 (T3) h of incubation at 37°C, using CASA. The plasma

membrane integrity (FLU%) was evaluated in T0 by fluorescence staining with propidium iodide and carboxyfluoresceine. The acrosomal status was evaluated in T1 by FITC-PNA. Data from this experiment were analysed by variancy analysis.

Results and discussion

No mare became pregnant in Experiment I, corroborating other reports (Meinties *et al.* 2000). In Experiment II, in spite of a numerical difference, no statistical difference was seen between the pregnancy rate of IFAI-UB (50%) and UB (83.3%). Although this indicates some interference in oocyte viability, it is not uniquely responsible for the failure of IFAI, based on the fact that 50% of the mares of IFAI-UB become pregnant. These results disagree with Eilts *et al.* (2002) who performed concomitant IFAI and conventional insemination in 9 mares and obtained a pregnancy in just one mare with a double ovulation. The authors could not affirm if the pregnancy was from the follicle on which IFAI was performed or the other follicle.

In Experiment III, total motility (TM) was significantly higher in GI than GII, just in T0 (TM/T0/GI=58.9±7.9; TM/T0/GII=53.7±10.0), but in T1, T2 and T3 this difference disappeared (TM/T1/GI=52.3±9.9 x TM/T1/GII=47.7±8.9; TM/T2/GI=43.9±10.1 x TM/T2/GII=36.4±11.8; TM/T3/GI=35.3±13.8 x TM/T3/GII=30.2±10.6). Landim-Alvarenga *et al.* (2001) observed a significantly higher percentage of motile spermatozoa after 2 h incubation when spermatozoa from fresh stallion semen were incubated with follicular fluid compared to spermatozoa incubated in the absence of follicular fluid. In our experiment no difference in progressive motility (PM) was observed between GI and GII after incubation in follicular fluid (PM/T0/GI=19.1±5.7 x PM/T0/GII=19.4±4.1; PM/T1/GI=13.7±4.3 x PM/T1/GII=15.6±5.4; PM/T2/GI=10.2±4.7 x PM/T2/GII=9.6±4.3; PM/T3/GI=7.6±4.5 x PM/T3/GII=7.4±3.8). The FLU % did not differ (P>0.05) between groups (GI=46.6±7.4, GII=46.0±7.6), and the percentage of live spermatozoa with intact acrosome did not differ (P>0.05) between groups at T1 (GI=33.7±10.8 x GII=29.5±9.5). These results are in disagreement with Yao *et al.* (1999) using human semen, where a significantly higher incidence of reacted acrosome was observed in the group that was treated with human follicular fluid.

Conclusion

These results confirm the previous reports of failed IFAI in mares. We conclude that the problem does not seem to relate to semen survival in follicular fluid but with disturbed oocyte viability. Further studies are needed to explain why the technique failed.

Acknowledgements

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REFROZEN SEMEN: SPERM CHARACTERISTICS IN HORSES AND *IN VITRO* EMBRYO PRODUCTION IN RUMINANTS

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INTRODUCTION

Cryopreserved semen may be available in limited quantities from genetically valuable sires. Equine semen is commonly frozen in straws that contain 100–800 million spermatozoa. Assisted reproduction techniques routinely performed in livestock species such as *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) use only a small percentage of the spermatozoa in a straw of frozen semen. Consequently a large percentage of the spermatozoa are wasted.

The goals of this study were to: 1) determine if frozen equine semen could be thawed, diluted and refrozen in multiple straws at a lower concentration without a significant decrease in spermatozoal motility or viability, 2) compare cleavage and blastocyst formation rates following IVF of *in vitro* matured sheep ova using standard frozen ram semen versus semen that had been frozen, thawed and refrozen, and 3) evaluate the effect of dilution and refreezing of bull semen on fertilisation and blastocyst development rates following *in vitro* fertilisation.

MATERIALS AND METHODS

Experiment 1

Semen from 6 stallions was collected, evaluated and frozen in lactose-EDTA in 0.5 ml straws. Semen from each stallion was subsequently thawed and evaluated for motility and viability. Thawed semen was held at room temperature for 10 min and then refrozen in 0.25 ml straws. Additional semen from the initial freeze was thawed, diluted in the original extender and refrozen at concentrations of 40×10^6, 4×10^6, 4×10^5, and 4×10^4 spermatozoa per ml. Refrozen semen was evaluated for motility visually and by computer-assisted semen analysis (CASA). Viability of refrozen semen was determined by flow cytometry following staining with propidium iodide (PI).

Experiment 2

The second experiment was designed to test the effect of thawing and subsequent refreezing of ram semen on cleavage and blastocyst formation rates following IVF. Sheep ovaries were collected from an abattoir and oocyte-cumulus complexes (OCCs) were collected by follicular aspiration. OCCs were incubated in IVM medium for 24 h before being exposed to either frozen or refrozen ram semen for 24 h in IVF medium. Putative zygotes were subsequently cultured in IVC medium. Cleavage rates were determined after 24 h of culture and embryonic development rate was assessed 7 days after the day of IVF.

Experiment 3

The third experiment evaluated the effect of diluting thawed cryopreserved semen prior to refreezing using a bovine IVF model. Oocytes were harvested from cow ovaries collected from a local abattoir and matured *in vitro* for 24 h. Ova were fertilised with bull semen frozen at a concentration of 20×10^6 spermatozoa per 0.25 ml straw or with semen from the same bull that had been thawed, diluted to a concentration of 2×10^6 spermatozoa per straw and refrozen. Cleavage and blastocyst rates were determined 2 days and 7 days, respectively after IVF.

TABLE 1: Effect of freezing and refreezing on motility and viability of equine spermatozoa

Evaluation time	Total motility (%)	Progressive motility (%)	Viability (%)
Prefreezing	91.8 ± 3.1[a]	47.8 ± 12.0[a]	N/A
After 1st Freezing	64.2 ± 7.7[b]	38.5 ± 10.5[a]	31.4 ± 7.9[d]
After 2nd Freezing	45.7 ±10.4[c]	14.5 ± 6.6[b]	19.5 ±13.3[e]

[a,b,c] Values within a column with different superscripts are significantly different (P<0.0001); [d,e] values within column tended to be different (P=0.09); N/A data not available

TABLE 2: IFV success rates using frozen and refrozen ram semen

Semen type	# oocytes	% cleaved	%blastocysts
Frozen	259	88.0[a]	61.0 [a]
Refrozen	288	47.9 [b]	41.3 [b]

[a,b] Values within columns with the same superscript are not significantly different (P>0.001)

Results

In Experiment 1 total motility of equine semen decreased from 91.8 ± 3.1% prior to freezing, to 64.2 ± 7.7% after the first freezing and 45.7 ± 10.4% after the second freezing (Table 1). However, only a tendency (P=0.09) toward a decrease in sperm viability following refreezing as measured by PI staining was noted. Dilution of sperm did not compound the effect of refreezing on motility or viability parameters.

In the second experiment, blastocysts were created by IVF using refrozen semen, but cleavage rates (88.0% vs 47.9%) and blastocyst development rates (61.0% vs 41.3%) were both significantly higher (P<0.001) when frozen ram semen was used compared with refrozen semen (Table 2).

In the third experiment, post thaw motility was significantly higher (P<0.05) for bull semen frozen at 20 x 10^6 spermatozoa per straw (80.0 ± 1.8%) than for semen that had been thawed, diluted and refrozen at a concentration of 2 x 10^6 spermatozoa per straw (61.3 ± 6.2%). Cleavage rates following IVF were also higher with bull semen frozen at 20 x 10^6 spermatozoa per straw (89.9%) and lower with semen refrozen at 2 x 10^6 spermatozoa per straw (38.7%) (P<0.05), whereas blastocyst development rates were not significantly different (57.0% and 44.8%, respectively).

Discussion

Fertility of semen that has been frozen, thawed and subsequently refrozen has been examined *in vivo* in cattle and *in vitro* in sheep. Insemination of cows with refrozen bull semen yielded a similar 60 day non return rate (65.3%) as that of semen that had been frozen a single time (66.9%) (Filseth and Vatn 1979). Recently, Arav *et al.* (2003) noted that pregnancy rates for bull semen that had been frozen, thawed and refrozen were 44%, while pregnancy rates from a control group were 45.5%. Hollinshead *et al.* (2003) reported high blastocyst rates (64.8%) in an IVF system using ram semen that was frozen, thawed, sex-sorted and then refrozen. In a subsequent study, O'Brien *et al.* (2004) reported that *in vivo* development of sheep embryos produced by IVF using refrozen semen was not adversely affected.

In the present study, the first experiment demonstrated that refrozen equine semen retained approximately 70% of the initial post thaw motility and that dilution prior to refreezing had no significant effect on spermatozoal motility or viability. Ruminants were used subsequently as models to determine if refrozen semen could be used for *in vitro* embryo production, as IVP techniques are not routinely successful in the horse. Results of the second experiment confirmed that ram semen that had been frozen, thawed and refrozen could still be used successfully for *in vitro* fertilisation. However, cleavage and blastocyst development rates were lower when refrozen semen is used compared with traditional frozen semen. The third study showed that blastocysts could be produced *in vitro* using diluted, refrozen bovine semen. As with the sheep study, overall efficiency of IVF was decreased when refrozen bull semen was used.

Refreezing of frozen-thawed equine semen may be an option to preserve genetic material for

future assisted reproduction from valuable sires if semen quantity is limited.

Dilution prior to refreezing would allow for the reallocation of a single 0.5 ml straw into hundreds or thousands of smaller straws for future use in assisted reproduction. It is hypothesised the optimal technique for *in vitro* production of equine embryos using refrozen stallion semen will be intracytoplasmic sperm injection (ICSI).

Conclusions

These studies confirmed that frozen semen from stallions, rams and bulls can be thawed and refrozen and still be used for *in vitro* embryo production, albeit at lower success rates. Refreezing of frozen-thawed semen may be an option to preserve and optimise the use of valuable genetic material for future assisted reproduction.

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SESSION 5:

Superovulation

Chairman: M. Alvarenga

ADVANCES IN EQUINE SUPEROVULATION

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Induction of multiple ovulation could be used to increase embryo recovery and thereby make embryo transfer more efficient. Typically, embryo recovery from a single-ovulating mare averages approximately 50% per cycle. However, mares that spontaneously double ovulate or are induced to ovulate multiple follicles have higher embryo recovery rates. With increased embryo recovery, recipients' costs prior to embryo recovery would be reduced. Furthermore, if multiple embryos could be obtained on a given cycle, then embryos could be frozen for transfer at a later time.

The majority of cattle are superovulated with either porcine FSH or ovine FSH. However, even when large doses of FSH-P were given twice daily, the ovulation rate ranged from 1.5–1.7 ovulations per cycle (Irvine 1981; Squires *et al.* 1986). The ovaries of mares seem to be relatively insensitive to porcine FSH. Only recently has an equine FSH product been made commercially available (eFSH; Bioniche Animal Health, Athens, GA). eCG has been used successfully for years to superovulate ruminants. Unfortunately, very large doses of eCG have had no effect on follicular development or ovulation in the mare (Allen 1982). Administration of GnRH has been reported to induce ovulation of multiple follicles in seasonally anoestrous mares (Johnson 1987). In contrast, administration of GnRH to cycling mares was ineffective in inducing multiple ovulation (Squires *et al.* 1989). Secretion of inhibin by the dominant follicle is responsible for suppression of smaller follicles through the inhibition of FSH secretion. Therefore, attempts have been made to develop or provide inhibin antibodies. Active immunisation of mares against inhibin-α subunit resulted in a doubling of ovulation rate. However, multiple inoculations over several weeks were required. Furthermore, adverse reactions at the injection site were noted (McCue *et al.* 1992).

The most successful means of superovulation in mares has been the use of an equine pituitary extract (EPE) preparation. In the earlier studies, crude gonadotropin preparations were made from equine pituitary glands using the technique reported by Braselton and McShan (1970). Most recent investigations used an equine pituitary extract preparation reported by Guillou and Combarnous (1983). The first studies on induction of multiple ovulation using EPE were done at the University of Wisconsin (Douglas *et al.* 1974; Lapin and Ginther 1977). Ovulation rates of 1.7 to 3.8 ovulations per cycle have been documented after the administration of EPE (Squires *et al.* 2003). A summary of studies conducted at Colorado State University using EPE for induction of multiple ovulation in 170 cycling mares revealed 3.2 ovulations and 1.96 embryos per cycle in treated mares compared to 1.2 ovulations and 0.65 embryos per cycle in control mares.

The response of cycling mares to EPE is dependent upon the follicle population at the onset of treatment. Ideally, EPE or eFSH should be started at the beginning of a follicular wave, before the appearance of a dominant follicle. Woods and Ginther (1983) reported that EPE treatment on Days 15–19 of the oestrous cycle resulted in a higher ovulation rate and a greater percentage of mares exhibiting multiple ovulation than treatment on Days 19 and 23. Dippert *et al.* (1992) compared the ovulation rate of mares in which treatment was begun either on Day 5 or 12 post ovulation. A higher ovulation rate was obtained with mares started on treatment 5 days post ovulation.

Attempts have been made to control follicular development prior to EPE treatment. These

include injection of progestins and oestradiol for 7–10 days prior to initiation of treatment and the use of a transvaginal ultrasound probe to eliminate follicles greater than 25 mm. In women, a GnRH agonist often is given as a means of down-regulating LH during FSH stimulation. This has been attempted in at least 2 studies in the mare with no clear advantage demonstrated (Dippert *et al.* 1992; Scoggin *et al.* 2002).

One common clinical approach is to initiate EPE or eFSH treatment at the time of embryo recovery, 7 or 8 days post ovulation. It is important that follicles greater ≥30 mm are not present on the ovary at the time of initiation of treatment, since these dominant follicles already have suppressed development of smaller follicles.

In non-stimulated mares, embryo recovery is between 50 and 60% per ovulation. Whether or not embryo recovery per ovulation is reduced in superovulated mares remains controversial. There are some studies that have demonstrated 50–60% embryo recovery per ovulation (Squires *et al.* 1986; Alvarenga *et al.* 2001), whereas other studies have reported a much lower embryo recovery rate per ovulation in superovulated mares (Douglas 1979; Palmer *et al.* 1993). There are a few studies that have directly compared pregnancy rates after transfer of embryos from superovulated mares versus single-ovulating mares. Squires *et al.* (1986) reported similar pregnancy rates between embryos from superovulated and single-ovulating mares. Based on clinical data, there appears to be no reduction in viability of embryos from superovulated mares.

Frequency of injection also has been shown to affect ovulation and embryo recovery rates after EPE or eFSH injection. EPE initially was used twice to induce ovulation of multiple follicles (Douglas *et al.* 1974), but in a subsequent study, similar success was reported with once per day injections (Lapin and Ginther 1977). Studies conducted in our laboratory compared twice daily injections of EPE versus once a day injection and reported higher ovulation and embryo recovery rates for mares injected twice per day (Alvarenga *et al.* 2001). Clinical impressions from many veterinarians using eFSH would suggest that once daily injection provides a similar response to twice daily injections. Based on preliminary data from a study ongoing in our laboratory, a similar response was obtained in mares given eFSH at 25 mg once per day versus 25 mg twice per day.

Several studies, as well as clinical trials, have been conducted during the past 2 years with eFSH (Bioniche Animal Health). In the first study, conducted at Colorado State University (Niswender *et al.* 2003), mares were given either no eFSH, 25 mg of eFSH bid, 12.5 mg eFSH bid and 12.5 mg eFSH bid plus hCG. Mares in the first 3 treatment groups received deslorelin acetate instead of hCG to induce ovulation. eFSH treatment was initiated 5 or 6 days after ovulation and continued until most follicles reached 35 mm. The number of follicles >35 mm was 1.1, 6.7, 3.8 and 3.4 for Groups 1, 2, 3 and 4, respectively. The number of ovulations was greater for Groups 2 and 4 than for controls and number of pregnancies per mare was greater for Group 4 than controls.

The 12.5 mg dose given twice daily plus hCG was selected for a subsequent study conducted in Brazil (Squires *et al.* 2003). Mares were given eFSH twice daily until the majority of follicles were >35 mm, and then hCG was administered. Mean ovulation rate and embryo recovery were 3.6 ovulations and 1.9 embryos per mare during the treatment cycle and 1.0 and 0.5, respectively, during the control cycle.

Currently, 40 light-horse, cycling mares are part of a project at Colorado State University designed to enhance the response to equine FSH. On the day of ovulation, mares are randomly assigned to one of 4 groups: Group 1 (controls), 12.5 mg eFSH bid; Group 2, 12.5 mg eFSH bid (coast); Group 3, 12.5 mg eFSH bid for 3.5 days followed by eFSH enriched with LH for an additional 3.5 days; Group 4, 25 mg SID (coast). In Groups 1 and 3, hCG was given once the majority of follicles were 35 mm, whereas in Groups 2 and 4, administration of hCG was delayed for 36 h once mares obtained 35 mm follicles (coast). The number of ovulations and embryos recovered in each group were 3.6 and 1.9 (Group 1); 3.6 and 1.1 (Group 2); 2.7 and 1.6 (Group 3); and 2.9 and 1.5 (Group 4). Embryo recovery per ovulation was 0.5, 0.3, 0.6 and 0.5 per ovulation Groups 1–4, respectively.

Data were obtained from a commercial breeding farm in Texas for 2 consecutive years. During Year 1, on 30 cycles of eFSH treatment, 45 embryos were recovered (1.5/mare) versus 54 embryos from 74 control cycles (0.73/mare). After nonsurgical transfer, the number of pregnancies for control and eFSH-treated mares was 0.65 and 1.3, respectively. In Year 2, 62 mares were treated with eFSH and 85 embryos were

obtained after one cycle (1.37 embryos/mare). After transfer, the number of pregnancies per donor was 1.05.

One of the potential problems with eFSH is the possibility of overstimulation (>5 ovulations). Generally, when mares have more than 5 ovulations, embryo recovery is zero or extremely low. Ways to enhance the response to eFSH may include selection of mares with more appropriate numbers of follicles at the time of treatment, use of progesterone and oestradiol to suppress follicular development prior to treatment, customise the dose for each mare and the possibility of stopping eFSH treatment earlier and allowing the follicles to 'coast'.

Another potential use of eFSH may include induction of multiple follicles for oocyte collection. Maclellan *et al.* (2002) attempted to collect oocytes from mares that had been eFSH-treated. Although oocyte recovery was enhanced in treated mares, the number of oocytes per follicle aspirated was lower in eFSH-treated mares than untreated controls.

Studies have been conducted to test the hypothesis that increasing the ovulation rate with EPE or eFSH would increase pregnancy rates in conditions of reduced fertility. McCue (1996) bred 15 older mares with shipped, cooled semen or frozen semen during one oestrous cycle without EPE stimulation. During the subsequent cycle, EPE was given to induce multiple ovulations and the mares were bred and flushed. Ovulation rates for the untreated and EPE cycles were 1.1 and 2.4, respectively. Embryos were collected on 12.5% of the flushes during the non-stimulated cycle and 31.2% of the flushes during the EPE-treated cycle. In a second study, 9 reproductively normal mares were treated on alternating oestrous cycles with either EPE or a saline placebo and inseminated with frozen-thawed semen containing only 300 million progressively motile spermatozoa. Ovulation rate for the EPE- and saline-treated cycles was 2.6 and 1.1 ovulations, respectively. Pregnancy rates for mares ovulating multiple follicles was 67% versus 33% for single-ovulating mares.

Hastening the first ovulation of the year is important for those breeders that desire foals born early in the year. Equine pituitary extract and eFSH have both been used to hasten the first ovulation of the year. Squires *et al.* (2003) reported a mean interval to ovulation of 7.6 days for eFSH-treated mares and 39.5 days for untreated control mares. The number of ovulations in eFSH-treated mares was 2.5 versus 1.0 for control mares. A similar hastening of the first ovulation of the breeding season also was reported in a Brazilian study (Peres *et al.* 2005).

In summary, eFSH has many applications in broodmare reproduction. Additional studies are needed to define the protocols for administering eFSH to both transitional and cycling mares that result in the greatest number of embryos being recovered. Furthermore, studies are needed to evaluate the benefit of administering eFSH prior to transvaginal oocyte aspiration.

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FOLLICULAR DYNAMIC, SUPEROVULATORY RESPONSE AND EMBRYO RECOVERY RATES IN MARES TREATED WITH CONSTANT AND DECREASING DOSES OF EQUINE PITUITARY EXTRACT (EPE) AND PURIFIED EQUINE FSH

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INTRODUCTION

Several methods have been studied to superovulate mares. Equine pituitary extract (EPE) has been the compound most used to induce multiple ovulation in mares, however despite the consistent superovulation response the embryo recovery rate is lower than expected (McCue 1996). In this laboratory, it has been observed that twice daily administration of EPE increases superovulatory response, but there is still low embryo recovery rate (Alvarenga *et al.* 2001).

Studies in cows have shown that high LH contamination in the FSH base prepared, adversely affects ovarian response, embryo quality and fertility rate. Administration of purified FSH twice daily in decreasing doses increased the quality of the embryos and also the embryo recovery rates in cows (Monniaux *et al.* 1983).

This experiment was conducted to compare the follicular dynamic superstimulatory (SO) response and embryo production in mares treated with 2 different EPE protocols (constant and decreased doses) and purified equine FSH (Bioniche, Canada).

MATERIALS AND METHODS

Six crossbred horse mares were used for this study. All mares had at least one normal oestrous cycle prior to the initiation of the study and were between 4 and 12 years of age. All mares were maintained in outdoor paddocks and had free access to hay and water. Reproductive cycles were monitored via transrectal palpation and ultra-sonography.

The same animals were used in all experimental groups to avoid individual variability. Group I: All mares received an IM injection of 10 mg cloprostenol (Prostaglandin F2α) on the first and second day of treatment and the application of 25 mg of EPE twice a day was started. Group II: Application of EPE in decreasing doses (40, 35, 30, 25, 20, 15, 10 mg) twice daily. Group III: The mares received 12.5 mg of eFSH injected IM twice daily. Group IV: Control group without treatment.

Follicular growth and ovulation were monitored daily by transrectal palpation and ultrasonography. Ovulations were defined as echogenic ovarian structures where a pre-ovulatory follicle was noted the day before. All treatments were discontinued when the majority of follicles reached 35 mm of diameter and 3,000 iu of hCG was administered. Results were analysed by Pearson Correlation Coefficients and Analysis of Variance.

RESULTS AND DISCUSSION

In Group II, a positive correlation was observed between the number of initial follicles of 16–20 mm and embryo production. However, 48 h after the start of the treatment a positive correlation ($P<0.05$) was observed between populations of follicles 16–20 mm diameter and the number of ovulations in all treated groups was similar to that reported previously (Woods and Ginther 1985).

In GI and GII after one day of treatment a higher number of follicles ($P<0.05$) between 16–20 mm in diameter was observed. On other treatment days no differences were observed in follicle population among groups. Follicular growth rate per day was similar between treated and non-treated mares.

TABLE 1: Follicular dynamic in mares treated with EPE in constant and decreasing doses and with purified equine FSH

Parameter	Groups			
	GI	GII	GIII	GIV
Number of follicles that reached 30 mm	3.5 ± 1.2^{ab}	4.2 ± 1.6^{b}	5.0 ± 1.5^{b}	1 ± 1.0^{a}
Pre-ovulatory follicular diameter (mm)	35.4 ± 4.4^{b}	35.1 ± 4.2^{b}	38.4 ± 1.3^{ab}	41.8 ± 1.7^{a}
Follicular growth (mm/day)	2.2 ± 0.5^{a}	2.2 ± 0.8^{a}	2.6 ± 0.4^{a}	2.5 ± 0.2^{a}

TABLE 2: The ovulation rate and number of embryos recovered in mares treated with EPE in constant and decreasing doses and purified eFSH

Parameter	Groups			
	GI	GII	GIII	GIV
Ovulation/mare	3.3 ± 1.6^{ab}	5.0 ± 2.1^{a}	4.8 ± 1.3^{a}	1.0 ± 0^{b}
Embryo/mare	1.2 ± 1.3^{ab}	1.8 ± 1.3^{ab}	2.6 ± 0.5^{a}	0.5 ± 0.5^{b}
Embryos per ovulation	0.36 ± 1.2^{b}	0.36 ± 1.8^{b}	0.54 ± 1.3^{a}	0.5 ± 1.0^{a}

$^{a, b}$ Numbers within columns with different letters are signicantly different (P<0.05)

The number of follicles that reached 30 mm was higher on Group I, II and III in relation to the control group. The pre-ovulatory follicular diameter was similar in the Groups III and IV and higher than others groups. There was a large variability in the size of pre-ovulatory follicles in Groups II and I (Table 1). Ovulation/mare number was similar (P>0.05) between the SO treatments (GI=3.3±1.6; GII=5.0±2.1; GIII=4.8±1.3) and significantly higher (P<0.05) than the control group (1.0±0).

The ovulation rates observed in our study with the superovulated mares were smaller than those reported by Alvarenga e*t al.* (2001). The number of embryos recovered per mare tended to be statistically higher (P=0.067) in FSH treated mares. A total of 1.2±1.3; 1.8±1.3; 2.6±0.5; 0.5±0.5 embryos per flush were recovered from mares in GI, GII, GIII and GIV, respectively (Table 2).

Conclusion

Equine FSH allowed a 5-fold improvement in embryo production when compared with non-treated mares. Based on the results of the present study it can be concluded that the differences in follicular dynamics between treated and non-treated mares were more evident after Day 4 of treatment when an increase in the number of follicles of 21–30 mm and ≥30 mm diameter is observed. The SO response can be predicted on Day 2 of treatment by evaluation of the 16–20 mm follicle population. Equine purified FSH showed a tendency to be superior to EPE in the ability to improve embryo production in SO mares. Embryo recovery rate per ovulation was similar between non-treated and FSH treated mares. The rate of follicular growth (mm/day) observed in the treated mares with purified eFSH was similar to the non-treated mares. The purified eFSH induced a better follicular maturation, the animals presented as improved rate of embryo recovered per ovulation, similar to the control group. The results indicate that Equine FSH induces improved follicle and oocyte maturation when compared with EPE. Studies are in progress to determine oocyte and follicle maturation of SO mares.

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OVARIAN RESPONSE OF MARES IN TRANSITIONAL PHASE TREATED WITH EQUINE FSH

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INTRODUCTION

The economic impact of seasonality on the horse industry requires the development of techniques to stimulate the early onset of the breeding season. These methods include: artificial photoperiod, and treatment with gonadotropin releasing hormone, equine pituitary extract and dopamine antagonists. Coy *et al.* (1999) compared the follicular development obtained with administration of eqüine pituitary extract (EPE) among mares in anoestrus and in transition. In this work, EPE induced the ovulation in just 2 of 9 anoestrus mares, however, 8 of the 9 mares treated in the transitional period ovulated in a average period of 11.8 ± 5 days after the treatment beginning, with a rate of 1.6 ± 0.7 ovulations/mare. The purified FSH of equine origin has been described as an effective inductor of multiple ovulation in mares during the breeding season, but its ability in promote the follicle development and ovulation in transitional mares was tested only recently (Niswender *et al.* 2004). However, no studies have been performed to examine if, the embryos collected form FSH-treated mares in the spring season were viable. This experiment aimed to evaluate the use of equine FSH (eFSH) for inducing follicular development and multiple ovulation in transitional mares in tropical conditions. Furthermore, the occurence of multiple ovulations and the number of embryos recovered were analysed.

MATERIAL AND METHODS

Twenty-seven crossbred mares aged between 3–15 years were used in the spring of 2003 (in Brazil). All mares were scanned with ultrasound for 2 weeks prior to the beginning of the experiment to determine the lack of cyclicity (no follicles >25 mm and no CL present). After this period, when the first follicle ≥25 mm was detected, mares were assigned to one of 2 groups: 1) Untreated controls (n= 13); and 2) eFSH (Bioniche, Canada) treated - 12.5 mg im, bid until at least half of all follicles >30 mm had reached 35 mm (n= 14). Once mares achieved a ≥35 mm follicle, they received 2,500 iu of hCG, iv (Vetecor®, Calier, Brazil), and were inseminated every other day until ovulation, with 500 x 10^6 (Group 1) and 1000 x 10^6 (Group 2) fresh motile spermatozoa from just one stallion diluted in a skimmed milk extender (1:1).

Embryo recovery was performed 7–8 days after ovulation and all mares received 7.5 mg of Dinoprost Trometamina (Lutalyse, - Rhodia Mérieux Veterinary Ltda, SP, Brazil), IM. After collection, the embryos were traced and immediately classified, in a stereoscopic microscope, according to their development and quality (McKinnon and Squires 1988). The statistical analyses were performed using t-test.

On the day of embryo recovery, jugular blood samples were collected. The serum was used to determine progesterone concentration though radioimmunoassay (Kit de Progesterone Coat-A-Count - Medlab, SP, Brazil). A correlation study was performed between ovulation number and blood concentration of progesterone through Pearson's correlation.

RESULTS AND DISCUSSION

The results presented on Table 1 showed that the treatment with eFSH hastened the onset of the first ovulation of the year and promoted multiple ovulation in 12 of 14 treated mares (85.7%). Additionally, 10 of 14 mares (71.43%) produced one to 5 embryos. Six oocytes from treated mares

TABLE 1: Ovulatory response and embryo recovery rate of mares in transitional phase treated with eFSH and non-treated (control)

Parameters	Control	Treatment
Number of mares (n)	13	14
Treatment days	-	4.8 ± 1.1
Interval from 1st follicle ≥ 25mm to 1st (days) pre-ovulatory follicle	14.9 ± 10.8[a]	4.1 ± 0.9[b]
Interval from 1st follicle ≥ 25mm to 1st ovulation (days)	18.0 ± 11.1[a]	6.6 ± .2[b]
Ovulation/mare	1.0 ± 0.0[a]	5.6 ± 4.5[b]
Number of mares with ≥ 2 ovulations (%)	0 (0%)	12 (85.7%)
Embryos recovered		
per mare	0.7 ± 0.5[a] (0–1)	2.0 ± 1.8[b] (0–5)
per ovulation	69%[a]	36%[a]

(t-test.) [a,b] means within rows with different superscripts differ (P<0.05)

TABLE 2: Effect of ovulation number on the concentration of serum progesterone on the day (D7-8) of the embryonic recovery in mares-not treated (control) and treated with eFSH in the spring transition period

Ovulations (n)	Mares (n)	Concentration of progesterone (ng/ml)
1 (control)	13	7.39 ± 2.11
1 (treatment)	1	8.02 ± 0.00
2	2	14.53 ± 2.46
4	3	18.44 ± 2.57
5	3	24.38 ± 11.03
6	1	44.00 ± 0.00
10	1	24.90 ± 0.00
12	1	38.49 ± 0.00
17	1	45.00 ± 0.00

Correlation r= 0.855 P< 0.001

were also recovered.

The mean number of ovulations per mare was higher than that reported by Rosas *et al.* (1998), Machado *et al.* (2003) and Alvarenga *et al.* (2003) (4.6, 4.2 and 4.0 ovulation/mare, respectively); but the mean number of embryo recovery per mare was similar (2.0, 2.2 and 2.0, respectively). Niswender *et al.* (2004) also administered eFSH for transitional mares, but achieved only 2.5 ± 1.7 ovulation/mare after 5.2 ± 1.3 days of treatment, probably because the ovulation was induced as soon as the first pre-ovulatory follicle was detected. It seems that, during the ovulatory season, the experiments with purified equine FSH needed a longer treatment period (7–8 days) and consequently a longer interval for the ovulations in comparison to the treatment with eFSH during the transitional phase. This might mean that the mares in transitional period are more receptive to the treatment. Although the number of embryos recovered per mare increased, the recovery rate per ovulation was still low and is similar to single ovulations: 0.36 (Rosas *et al.* 1998), 0.47 (Machado *et al.* 2003) and 0.5 (Alvarenga *et al.* 2003).

Eight embryos were recovered in the control group. From those, 2 (22.2%) were initial blastocysts (Bi), 3 (33.3%) were blastocysts (BI) and 4 (44.4%) were expanded blastocysts (Bx). Among the 28 embryos recovered from the treated mares, 5 (17.9%) were Bi, 8 (28.6%) Bl and 11(39.3%) Bx. Also, one (3.6%) morula and 3 (10.7%) non-identified structures were collected from the treated group. Additionally, 8 (88.9%) embryos from the control group and 21 (75.0%) from the treated group were classified as Grade 1–1.5, and one (11.1%) from the control group and 4 (14.3%) of the treated group as Grade 2–2.5.

The progesterone concentration was positively correlated with the ovulations, as demonstrated in Table 2. However, the correlation was not linear. After the first dose of prostaglandin F2α, the control mares ovulated earlier (P=0.029) than those from the treated Group (10.9 ± 3.5 versus 22.4 ± 17.5 days) that received 2 doses. However, 11/14 treated mares (78.6%) continued to cycle and ovulated after 12 to 17 days.

CONCLUSIONS

In conclusion, eFSH hastened the onset of the first ovulation of the year with the advantage of obtaining multiple viable embryos. Furthermore, most of the mares continued to cycle after the treatment.

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ULTRASTRUCTURAL ANALYSIS OF EMBRYOS OBTAINED FROM SUPEROVULATED MARES

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INTRODUCTION

Several protocols have been studied, in the equine species, in an attempt to achieve multiple ovulations. Equine pituitary extract (EPE) has classically been used to superovulate mares, but the results are still inconsistent. Recently the use of purified equine FSH (eFSH) was described. Hofferer *et al.* (1993) pioneered the use of eFSH purified by chromatography. Palmer *et al.* (1993) obtained 2.8 ± 0.4 ovulations per cycle using eFSH, but the embryo recovery rate was similar to that obtained in non-superovulated mares. Better results were obtained by Machado *et al.* (2003) and Alvarenga *et al.* (2003), using 2 applications per day of 12.5 mg purified eFSH. In those experiments, the embryo recovery rate per mare was 3 times higher than that obtained with the use of EPE.

Although these promising results were achieved, there have been no studies concerning the morphological quality of the embryos obtained after superovulation. This experiment aimed to compare the ultrastuctural features of equine embryos collected after superovulation and single-ovulation.

MATERIALS AND METHODS

Twenty-seven crossbred mares aged between 3–15 years were used. They were scanned with ultrasound for detection of follicles ≥25 mm and were assigned to one of 2 groups: 1) untreated controls (n= 13) and 2) eFSH treated (n= 14). To the treated group, 12.5 mg eFSH (Bioniche, Canada) was administrated im, bid until at least half of all follicles >30 mm had reached 35 mm. At this stage, they received 2,500 iu of hCG, iv (Vetecor®, Calier, Brazil), and were inseminated daily until ovulation, with 1000 x 10^6 fresh motile spermatozoa. Embryo recovery was performed 7 to 8 days after ovulation. Embryos from both groups were incubated in DPBS containing 125 mg/ml propidium iodite and 10 µg/ml HOECHST 33342 and examined in a fluorescent inverted microscope (UV filter 535/617 nm). For ultrastructural analysis, 5 embryos from each group were selected randomly, fixed in 2.5% glutaraldehyde and prepared following a standard electron transmission microscopy protocol.

RESULTS AND DISCUSSION

In the control group, 8 embryos were collected after 11 flushings (0.73 embryos per mare) and after eFSH treatment 28 embryos were obtained after 13 flushes (2.15 embryos per mare). All collected embryos were blastocyst (B) or expanded blastocyst (Bx).

Reports show that superovulation alters the normal maturation of an oocyte (Hyttel *et al.* 1988; Blondin *et al.* 1996). It is possible that superovulation forces the follicles into an accelerated growing phase, leaving the oocyte with insufficient time to acquire competence. This may be the one of the reasons for the low recovery rate/ovulation observed in experiments that use EPE in the superovulation protocol (Palmer *et al.* 1993; Machado *et al.* 2003). However, the recent results with purified eFSH showed a much higher recovery rate/ovulation, with good quality embryos (Alvarenga *et al.* 2003; Machado *et al.* 2003). In fact, in this experiment, the percentage of embryos classified as Quality 1 was 89% (8/9) in the control group, and 75% (21/28) in the treated group. Moreover, all embryos in Group 1 (control) and 90% of embryos in Group 2 (treated)

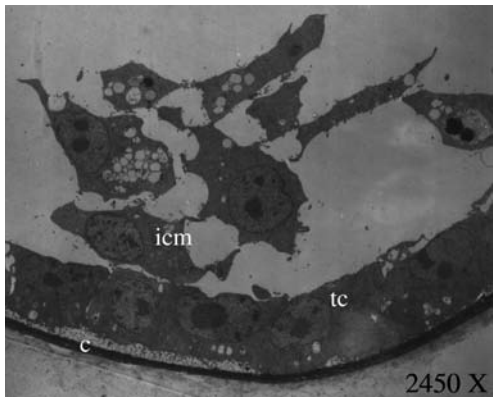

Fig 1: General view of a superovulated equine embryo showing the aspect of the trophoblast cells (tc) and the inner cell mass (icm). c= capsule.

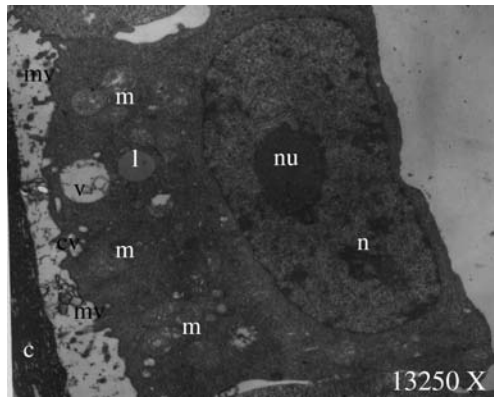

Fig 2: Ultramicrography of a trophoblastic cell, showing the apical surface, toward the acellular capsule (c), covered with microvilli (mv). cv = coated vesicle, l = lipid droplet, m = mitochondria, n = nucleus, nu = nucleoli, v = vacuole.

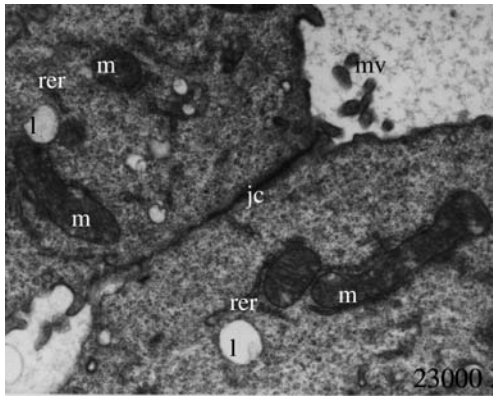

Fig 3: Electromicrography showing the junctional complexes (jc) between the trophoblastic cells. Notice the microvilli (mv) in the apical surface. l = lipid droplet, m = mitochondria, rer = rough endoplasmic reticulun.

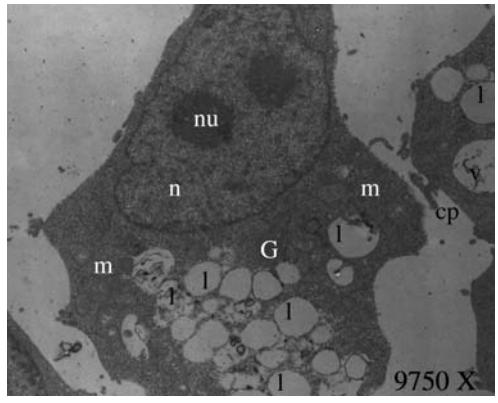

Fig 4: Electromicrography of the inner cell mass of a superovulated equine embryo. Notice the irregular shape of the cell, the presence of cell prolongaments (cp) and the amount of lipid droplets (l) into the cytoplasm. G = Golgi complex, M = mitochondria, n = nucleus, nu = nucleoli, v = vacuole.

presented more than 95% of viable cells.

Indeed, the morphology of studied embryos was similar. In both groups the embryos were surrounded by an acellular capsule consisting of fine fibrils circumferentially arranged (Figs 1 and 2). In some blastocysts a very thin *zona pellucida* was still seem. The perivitelinic space, between the capsule and the trophoblastic cells, was filled with microvilli (Figs 2 and 3). Trophoblastic cells formed a columnar epithelium adjacent to the capsule. At the apical region, the trophoblastic cells presented junctional complexes, characterised by *zonula ocludens* and tight junctions (Fig 3). Desmosomes were seen just after the junctional complexes, at the lateral membrane. Irregular shaped cells linked by long cellular prolongaments formed the inner cell mass (Figs 1 and 4). No junctional complexes were seen between them or between the inner cell mass and the trophoblastic cells.

The trophoblastic cells presented a spherical nucleus, centrally located. In the surface toward the capsule, many coated vesicle were seen (Fig 2). Those vesicles appeared to join other cytoplasmic vesicles and the smooth endoplasmic reticulum. Apparently, the material carried by

these vesicles was stored in electron dense granules that migrate to the lateral or blastocoele surface.

In the inner cells as well as in the trophoblast a great amount of lipid droplets was observed (Fig 4). Small droplets seemed to fuse, forming bigger ones. Mitochondria and smooth endoplasmic reticulum were seen in association with those droplets forming metabolic units that seemed to be responsible for steroidogenesis. In both groups mitotic figures were seen frequently.

CONCLUSIONS

Although the treatment of mares with exogenous gonadotrophins may be associated with certain deviations in the process of oocyte maturation, reducing fertilisation and embryo recovery rate, embryos collected from superovulated mares had normal viability and ultrastructure. A fertility trial confirmed this observation because 12 embryos recovered from 4 superovulated mares were transferred and resulted in 8 pregnancies.

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SESSION 6:

Artificial control of reproductive function in embryo transfer

Chairman: L. Losinno

COMPARISON BETWEEN SHORT AND LONG ACTING PROGESTERONE FOR TREATMENT OF NON-CYCLING EMBRYO RECIPIENT MARES

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Introduction

The successful use of progestins to prepare ovariectomised (Hinrichs *et al.* 1985; Hinrichs *et al.* 1986; McKinnon *et al.* 1988) and transitional intact (Carnevale *et al.* 2000) mares for receiving embryos has been reported and has focused on the use of short rather than long acting preparations. Utilisation of non-cycling progestin-treated mares as embryo recipients is an interesting practice in commercial programmes, especially at the beginning of the breeding season, when few cycling mares are usually available. Different progestin preparations have been given to mares, but only a few seem to be effective (McKinnon *et al.* 2000). Recently, a long acting injectable progesterone preparation was given (1,500 mg every 7 days) to cycling mares after prostaglandin administration and effectively maintained normal luteal phase levels of progesterone (Bringel *et al.* 2003).

The aim of the present study was to compare, retrospectively, pregnancy and early embryonic death rates among cycling and non-cycling recipient mares given short or long acting progesterone before embryo transfer.

Materials and methods

From late autumn to early spring in a commercial embryo transfer programme in Brazil, Quarter Horse and Paint Horse donor mares were monitored by transrectal palpation/ultrasonography and inseminated with fresh or cooled shipped semen every 48 h from detection of a 35 mm follicle until ovulation. Light-horse cycling (control; n=152) and non-cycling (n=112) recipient mares between 5 and 15 years of age and weighing 350–600 kg were used.

After a donor mare ovulated, unless a cycling recipient was available, an anovulatory recipient was treated hormonally before receiving an embryo. After 2 days of oestradiol cypionate administration (10 mg/day, im; ECP; Pharmacia and Upjohn Co., MI, USA), anovulatory recipients were supplemented with either short or long acting progesterone (P4) for 5–8 days before embryo transfer. Mares receiving short acting P4 (Northside Pharmacy, Lexington, USA) were treated intramuscularly with either 200 mg/day (n=54) or 400 mg every other day (n=13). Recipients given long acting P4 (P4 LA 150; B.E.T. Pharm, Lexington, USA) received 1,500 mg im every 7 (n=30) or 6 (n=15) days.

Embryos (n=264) were recovered 7–9 days post ovulation and transferred non-surgically by a single technician into cycling recipients 4–8 days after ovulation or into non-cycling recipients after 5–8 days of P4 supplementation. Pregnancies were checked by transrectal ultrasonography on Days 12, 25 and 50.

Pregnancy and embryonic death rates were compared using Chi-square analysis. Significance was indicated by a probability of $P<0.05$.

Results

Pregnancy rates on Days 12 and 50 and embryonic death rates were similar ($P>0.05$) for cycling and P4-treated recipients, irrespective of the type of progesterone supplementation given (Table 1). There were no significant differences ($P>0.05$) in these end points when data of all P4-treated groups were combined and compared to cycling recipients. Embryonic death rates in the intervals studied (Days 12–25 and 25–50 of pregnancy) for cycling and anovulatory recipients were similar ($P>0.05$) within each group and between groups. Embryo transfers into anovulatory recipients 5–8

TABLE 1: Day 12 and 50 pregnancy rates and embryonic death (ED) rates following transfer of equine embryos into cycling and non-cycling recipients receiving different progesterone treatments

Group	Transfers	Pregnant 12d (%)	Pregnant 50d (%)	ED (%)
Cycling	152	114/152 (75.0)	94/152 (61.8)	20/114 (17.5)
P4 200 mg/day	54	41/54 (75.9)	33/54 (61.1)	8/41 (19.5)
P4 400 mg/day	13	10/13 (76.9)	08/13 (61.5)	2/10 (20.0)
P4 LA 1,500 mg/7 days	30	23/30 (76.6)	16/30 (53.3)	7/30 (30.4)
P4 LA 1,500 mg/6 days	15	11/15 (73.3)	09/15 (60.0)	2/11 (18.2)
Total	264	199/264 (75.4)	160/264 (60.6)	39/199 (19.6)

Results within the same column are not different (P>0.05)

days after the start of P4 supplementation resulted in similar (P>0.05) pregnancy and embryonic death rates as did transfers into anovulatory recipients during spring and autumn or during the winter months.

Discussion

All progesterone treatments evaluated in this study were equally effective for preparation of anovulatory mares as embryo recipients. Pregnancy rates obtained after non-surgical embryo transfer in the present study were similar to those reported previously (Squires *et al.* 1999). The comparison between data combined for all P4-treated mares and cycling mares reinforce the suitability of anovulatory hormone-treated mares as embryo recipients, as demonstrated previously (McKinnon *et al.* 1988; Carnevale *et al.* 2000).

Although embryonic death rates did not differ significantly (P>0.05) among groups, the number of embryo losses in mares receiving P4 LA every 7 days appeared to be higher. The disparity in the number of animals among groups and the reduced number observed in some of them possibly prevented the occurrence of any significant effect of group. Further studies are necessary to conclude if administration in 6 day intervals is a more suitable protocol. Combined data for all P4-treated mares was similar (P>0.05) to control and figures are in agreement with the 5–24% range in embryo loss rates in fertile mares reported previously (Ball 1988).

Embryonic death rates at different intervals after embryo transfer have been studied previously (Carnevale *et al.* 2000). The percentage tended to be higher (P=0.06) between Days 17 and 25 of pregnancy. We have compared embryo loss at 2 intervals (12–25 and 25–50 days of pregnancy) among cycling and P4-treated recipients and no significant (P>0.05) differences were found within or between groups.

Previous studies on the administration of progestagens to mares have not focused on the use of long acting injectable P4 preparations. The use of P4 LA is extremely advantageous due to the great labour and animal stress associated with the daily injections required by the short acting forms. We have also demonstrated that P4 in oil can be administered every other day without altering pregnancy or embryonic death rates in non-cycling embryo recipients. Anovulatory mares given exogenous progesterone might be used as embryo recipients during their transitional or deep anoestrus periods, as far as no significant (P>0.05) differences in pregnancy and embryonic death rates were observed after transfers performed in the spring and autumn or during the winter months.

Equine embryos have been transferred into cycling mares within a large range of days after ovulation. Data from several breeding seasons in commercial embryo transfer programmes in Brazil have been examined and no significant (P>0.05) differences were found in pregnancy rates for recipients that had received embryos from 3 to 8 days after ovulation (Fleury *et al.* 1989; Jacob *et al.* 2002). The data from the present study show

that hormone-treated recipients allow almost the same flexibility, yielding similar (P>0.05) results when given P4 for 5–8 days before transfer, which is quite interesting due to its practical implications in commercial programmes.

In conclusion, non-cycling mares in deep anoestrus or transitional phase given short or long acting progesterone for 5–8 days before transfer can be successfully used as embryo recipients.

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PREGNANCY CAN BE MAINTAINED IN MARES GIVEN $PGF_{2\alpha}$ DURING THE PERIOVULATORY PERIOD BY BIORELEASE LAP4-150 (BRT-LAP4-150)

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A previous report by the authors demonstrated that BRT-LAP4-150 which is native progesterone (150 mg/ml) in a proprietary BRT non aqeuous vehicle, kept serum levels of progesterone elevated above 4 ng/ml in mares for approximately 8 days when given im at a dose of 1,500 mg. BRT- LAP4-150 is presently being used at a dose of 1,500 mg approximately every 7 days to maintain pregnancy in ET recipients without endogenous P4 and in pregnant mares with low levels of endogenous P4. An additional use would be to overcome the luteolytic effects of $PGF_{2\alpha}$ or its analogues when used during the periovulatory period to stimulate uterine clearance. Previous reports show that cloprostenol is effective for this use when given for 1–3 days on Days 1–2 post ovulation. However, there is controversy among researchers on the effects of such treatment on luteolysis and pregnancy. The present experiment was conducted in Brazil with 27 mixed-bred barren and foaling mares during January–May to determine if LAP4-150 could overcome the luteolytic effects and negative impact on pregnancy of 4 days of dinoprost tromethamine ($PGF_{2\alpha}$) given im from Days 1–4 post ovulation. Four groups were studied: 1) controls; 2) $PGF_{2\alpha}$; 3) $PGF_{2\alpha}$+LAP4-150; and 4) LAP4-150. $PGF_{2\alpha}$ was given at a dose of 10 mg/day, LAP4-150 was given at a dose of 1,500 mg every 7 days beginning on Day 0 (day of ovulation) through 21 days of the experiment. All mares were bred by one fertile stallion via AI and serum was collected for P4 assay by RIA. Pregnancy was detected by ultrasound beginning at 14 days. Results were analysed by 2-way analysis of variance and Tukeys test was used to test for differences among days in P4 concentrations. 1,500 mg LAP4-150 given every 7 days overcame the luteolytic effects of 4 days of $PGF_{2\alpha}$ and resulted in normal pregnacy rates (Table 1).

TABLE 1: Serum P4 and pregnancy rates

Days	Control	$PGF_{2\alpha}$	$PGF_{2\alpha}$ + LAP4150	LAP4-150
0	0.16^{a}	0.99^{a}	0.17^{a}	0.04^{a}
2	2.53^{a}	1.31^{a}	6.63^{a}	5.04^{a}
5	5.02^{a}	0.28^{a}	2.39^{a}	7.97^{b}
7	5.48^{a}	0.36^{b}	2.08^{a}	7.39^{a}
10	5.54^{a}	0.98^{b}	4.93^{a}	12.62^{c}
14	5.72^{ab}	1.96^{a}	4.23^{ab}	7.28^{b}
21	5.06^{a}	5.24^{a}	3.06^{a}	4.31^{a}
No pregnant	6 of 6^{a}	0 of 9^{b}	4 of 5^{a}	5 of 7^{a}

Different superscripts denote differences ($P<0.05$) among rows

INTERVALS TO OVULATION AFTER TREATMENT WITH OESTRADIOL CYPIONATE (ECP) OR BIORELEASE DESLORELIN (BRT-DES)

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A preliminary study found that when deslorelin was injected im in oestrous mares with ovarian follicles 35 mm or greater at a dose of 1.0, 1.5 or 2.0 mg/ml in BRT deslorelin vehicle, ovulation occurred in 44.1, 49.8 and 48.7 h, respectively, as compared to 100 h in controls. The BRT deslorelin vehicle was designed to release deslorelin for approximately 6–36 h. The present trial was designed to test efficacy of a lower dose of BRT-Des and, because oestrogens induce LH release in the mare, ECP and a combination of these 2 drugs on intervals to ovulation was also studied. Two doses of BRT-Des (0.5 vs 1.0 mg) and 10 mg of ECP were evaluated in Brazil in a total of 142 barren mares, which were either M. Paulista or M. Marchador, and between 7 and 15 years of age from November to February. Effects of follicular size were evaluated such that mares were assigned further to one of 2 follicular size groups: 30–35 mm or greater than 35 mm in diameter. Ultrasonic examinations of each ovary every 12 h following treatment detected the occurrence of ovulations. Data for intervals to ovulation were analysed by one-way analysis of variance for a 2 x 6 factorial experiment and Dunnetts test was used to make mean comparisons. Treatment effects were significant ($P<0.05$) and data are shown in Table 1.

Results indicate that a low dose of BRT-Des (0.5 mg) was as effective as 1.0 mg in significantly reducing the interval to ovulation as compared to controls or ECP treatment. The interval was reduced by approximately 45 h. ECP actually prolonged the interval to ovulation. The interval to ovulation in mares with follicular diameters of 30–35 mm was about 5 h longer than in mares

TABLE 1: Intervals to ovulation after treatment with ECP, BRT-Des or ECP+BRT-Des in mares with ovarian follicles that were 30–35 or greater than 35 mm in diameter

	Control		10 mg ECP		DES 0.5 mg		DES 1.0 mg		DES 0.5 mg + ECP		DES 1.0 mg + ECP	
	30–35 mm	>35 mm	30–35 mm	>35 mm	30–35 mm	>35 mm	30–35 mm	>35 mm	30–35 mm	>35 mm	30–35 mm	>35 mm
X	104.4[a]	81.0[b]	123.1[c]	99.6[d]	49.0[e]	45.8[e]	44.8[e]	40.8[e]	57.6[e]	48.0[e]	57.3[e]	45.6[e]
sem	6.4	3.7	4.2	3.6	3.2	4.5	2.4	2.0	9.6	3.1	7.4	3.0

Means with different superscript letters are significantly different ($P<0.05$)

THE EFFECT OF TRANSVAGINAL FOLLICULAR ASPIRATION ON CORPUS LUTEM FORMATION IN MARES

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A reliable method to induce a corpus luteum (CL) in recipient mares would be beneficial in embryo transfer programmes. It has been suggested that luteal tissue may form following aspiration of mature follicles, but the effects of the size of follicles has not been determined. We hypothesised that aspiration of follicles <35 mm during oestrus results in the formation of hormonally functional CL. Aspirated follicles were grouped as A (<25 mm), B (26–30mm) and C (31–34 mm). One follicle from each group was aspirated in 7 mares, ie a total of 21 aspirations. All aspirations were performed during oestrus using an ultrasound guided transvaginal technique. Serum progesterone concentrations were measured on Days –1, 0, 1, 2, 5 and 8 after aspiration and compared between groups; and ultrasonographic images of CL were compared to serum progesterone concentrations in all mares. Data were analysed by Kruskal-Wallis ANOVA. Significance was set at P<0.05. Follicles >25 mm were more likely to form a hormonally active CL (progesterone > 1 ng/ml) than follicles <25 mm (90% vs 0%; P<0.05). Hormonally active CL formation after aspiration showed no difference between Groups B and C (100% vs 80%). Ultrasonographic appearance of luteal tissue did not relate to serum progesterone concentrations. These results suggest that a hormonally functional CL can be induced in mares by aspiration of follicles >25 mm. Future studies will determine if the induced CL is able to maintain pregnancy.

INTRODUCTION

Equine embryo and oocyte transfer programmes require ovulation synchrony between donors and recipients. Most embryo transfer programmes use a large herd of recipients to ensure accurate synchronisation. This is costly and time consuming, and alternative methods have been described. However, these use pharmaceuticals to induce oestrus and ovulation which is time consuming and may also require multiple recipients for each donor. Ovariectomy followed by progestagen treatment is available but also expensive and is more invasive (Hinrichs *et al.* 1986; McKinnon *et al.* 1988). An alternative may be to aspirate follicles in oestrous mares.

Aspiration of mature follicles in cycling mares can result in formation of luteal tissue (Hinrichs *et al.* 1991). Aspirations were performed using a standing flank surgical approach and follicles ranged from 30–44 mm. However, it was not determined if the size and maturity of the aspirated follicles were important for luteinisation. It was hypothesised that transvaginal aspiration of follicles <35 mm during oestrus would result in the formation of a hormonally functional CL. The aims of this study were to determine if luteal formation depends upon size of the aspirated follicle and if there is a critical follicular size for a hormonally active CL to be induced reliably with this technique.

MATERIALS AND METHODS

Seven mares were examined using transrectal palpation and ultrasonography to determine stage of oestrous cycle and measure follicular growth. Follicles were grouped as A (5–25 mm), B (26–30 mm) and C (31–34 mm). One follicle from each group was aspirated in 7 mares, yielding a total of 21 aspirations. All aspirations were performed during oestrus using an ultrasound guided transvaginal technique (Carnevale *et al.* 2001). Each mare was sedated with 300 mg xylazine and given 25 mg propanthelene bromide to facilitate

relaxation of the rectum. Aspirations were deemed successful if the needle could be visualised within the follicle, aspiration fluid could be seen to enter the follicle, the follicle collapsed on the ultrasonic image and a yellow, serum-like fluid was recovered in the collection flask. The day of aspiration was designated as Day 0. Each mare was examined on Days 1, 2, 5 and 8 to record further follicular growth and CL development. Blood samples were recovered on Days –1, 0, 1, 2, 5, and 8 and progesterone concentrations were measured using a commercial kit (Coat-a-Count Progesterone Solid Phase I 125 Radioimmunoassay, Diagnostic Products Corp., California, USA). Mare, follicular group and day of blood collection were compared to progesterone concentration. Luteal tissue was considered present if progesterone values were > ng/ml. Data were analysed by Kruskal-Wallis ANOVA. Significance was set at $P<0.05$. Progesterone data were excluded for mares that ovulated spontaneously.

Results

Follicular groups had a significant effect on progesterone concentrations ($P<0.05$). Follicles from Groups B (4/5) and C (5/5) were more likely to form a hormonally active CL (progesterone >1 ng/ml) than those from Group A (0/4) (90% vs 0%; $P<0.05$) following aspiration. Progesterone concentrations were elevated by Day 5 and continued to rise until Day 8. There was no individual mare effect on progesterone levels.

Ultrasonographic appearance of luteal tissue was recorded for each mare after each aspiration. Apparent luteal tissue was seen on ultrasound in 20 of 21 mares. Fourteen aspirations showed luteal tissue by Day 1. Two more had apparent luteal tissue by Day 2, and 3 more by Day 5. Group A aspirations had no increase in progesterone concentration but did have hyperechoic evidence of luteal tissue on ultrasound. However, the presence of luteal tissue was not necessarily related to progesterone concentration.

Discussion

The study demonstrates that a hormonally functional CL can be induced in mares during oestrus by aspiration of follicles >25 mm. Follicles of 26–30 mm and >30 mm formed luteal tissue following aspiration in 80% and 100% of aspirated follicles, respectively. This can be compared to hCG induced ovulations of follicles >30mm in which ovulations occur in 80% of cases (Samper 2001). Elevated serum progesterone concentrations were not detected until 5 days after aspiration, slightly delayed compared to those reported following ovulation (Nett *et al.* 1976), but similar to data reported by Hinrichs *et al.* (1991).

Although almost all aspirations resulted in a hyperechoic structure indicative of luteal formation (Pierson and Ginther 1985), only a portion of those structures produced progesterone. As luteal tissue was noted within 2 days after aspiration, there appears to be a discrepancy between ultrasonographic appearance of luteal tissue, progesterone production and the assumption of dioestrus in the mare. In the authors' experience, it would be difficult to utilise ultrasonographic architecture of luteal tissue as a measure of luteal health and productivity. This raises questions about using the grading of a CL in recipient mares prior to embryo transfer as the sole indicator of recipient viability.

In conclusion, aspiration of follicles >25 mm in oestrous mares was a reliable and rapid way to induce hormonally active luteal tissue. This may be a practical method to produce recipient mares for embryo and oocyte transfer programmes.

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EFFECTS OF A SINGLE OESTRADIOL ADMINISTRATION ON FOLLICULAR DYNAMICS AND LUTEAL FUNCTION OF CYCLIC MARES

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INTRODUCTION

The pharmacological control of oestrus and ovulation is of great importance to the breeding management of mares. The increased use of assisted reproductive technologies (artificial insemination, use of shipped cooled and frozen semen, embryo transfer, etc) makes synchronisation of oestrus and ovulation a relevant issue in the modern equine breeding industry.

Because mares have a relatively long oestrus (5–7 days) and ovulation periods (~36–48 h), synchronisation of oestrus and ovulation have been difficult in the horse. The administration of progesterone or progestogens is routinely used to suppress oestrous behavior and syncronise oestrus in cycling mares. Treating cyclic mares with progesterone and oestradiol (P & E) to control ovulation has been shown to be superior to using progesterone alone (Evans *et al.* 1982). It is thought that oestradiol treatment suppresses follicular development and consequently, ovulation (Burns and Douglas 1981).

The main objective of the present study was to evaluate what effects a single administration of oestradiol formulations (without adding exogenous progesterone) would have on follicular dynamics and luteal function of cyclic mares. In addition, pharmacokinetic parameters for a single administration of 2 different oestradiol-17-beta formulations delivered in a proprietary long-acting vehicle (BioRelease Technologies, Lexington, KY, USA) and oestradiol cypionate in the cyclic mare were determined.

MATERIALS AND METHODS

Sixteen mares were included in the study conducted during July and August at the NCSU Veterinary Equine Research Center in Southern Pines, North Carolina, USA. The project was approved by the NCSU Institutional Animal Care and Use Committee. Mares were allotted randomly to one of 3 experimental groups: Group A (n = 4; 50 mg of oestradiol-17-beta; BRT-E_2), Group B (n = 4; 50 mg of oestradiol-17-beta in biorelease agent; Biorelease-E_2), Group C (n =4; 50 mg of oestradiol cypionate; ECP) and Group D (n =4; 5 ml of 0.9% NaCl solution). All injections were administered intramuscularly starting within 48 h post ovulation as determined by transrectal ultrasonography.

Blood samples were collected at 0 (pre-treatment), 8, 16, 24 h, and thereafter, once daily until the first day of the subsequent oestrus. Blood collection tubes were kept upright and centrifuged immediately at approximately 1,000 x *g* for 10 min after blood was clotted. Sera were stored at –20°C and assayed by RIA to determine concentrations of serum progesterone and oestradiol. Transrectal ultrasonography was utilised to document ovarian and uterine changes throughout the duration of the study.

Pharmacokinetic and statistical analyses

A computer program (WinNonlin, Version 4.0, Pharsight Corporation, Mountain View, CA) was used to determine pharmacokinetic parameters according to methods described previously. Pharmacokinetic parameters were calculated for each individual horse and the results were reported as the mean ± SD. Noncompartmental analysis was used to calculate the AUC for each drug formulation using the trapezoidal rule. Experimental data were analysed by ANOVA and Kruskal-Wallis ANOVA on Ranks whenever tests

TABLE 1: Pharmacokinetic parameters for different oestradiol formulations following a single intramuscular injection (50 mg total per mare) administered during early luteal phase in cycling mares

Pharmacokinetic variable	BRT-E_2 (mean ± SD)	Biorelease-E_2 (mean ± SD)	ECP (mean ± SD)	Control (mean ± SD)
T_{max} (h)	8 ± 0	14±12	40.00 ± 6.5	24 ± 13.1
C_{max} (µg/ml)	338.73 ± 110.4	374.51±165.6	62.73 ± 62.6	34.73 ± 43.3
$t_{1/2}$ (h)	23.85 ± 14.5	18.24 ± 5.8	39.84 ± 20.6	57.22 ± 41.8
AUC (h*pg/ml)	5973.49 ± 3478.84	7776.15 ± 3986.4	3190.02 ± 3322.9	1482.72 ± 1745.4
$\lambda(h^{-1})$	0.04 ± 0.02	0.04 ±0.01	0.02 ± 0.01	0.02 ± 0.01

T_{max}: time to maximum concentration
C_{max} = Maximum plasma concentration
$t_{1/2}$ = Overall biologic half-life in relation to the elimination rate
AUC = Area under the concentration-time curve
λ = the elimination-rate constant
BRT-E_2: oestradiol-17-beta delivered in a proprietary long-acting vehicle
Biorelease-E_2: oestradiol-17-beta delivered in a proprietary long-acting vehicle different from BRT-E_2
ECP: oestradiol cypionate

for normality and equal variance failed.

RESULTS

Concentrations of serum progesterone did not differ among groups during the first 10 days following administration of treatments ($P > 0.05$). Concentrations of serum oestradiol during the first 24 h after administration of experimental drugs were 10-fold higher in mares treated with BRT-E_2 or Biorelease-E_2 than in ECP-treated mares. No significant differences on duration of oestrus or interoestrous intervals were detected among groups ($P > 0.05$); however, 2 mares treated with ECP had relatively prolonged dioestrus (32 and 38 days). No follicles ≥25 mm were detected during the first 10 days in mares following administration of BRT-E_2 or Biorelease-E_2, whereas, 2 mares in the ECP and control groups had follicles >25 mm during the first 10 days following ovulation. Ultrasonography of the uterus of treated mares did not reveal the presence of endometrial pattern following oestradiol administration.

A summary of relevant pharmacokinetic parameters for each oestradiol formulation is provided in Table 1.

DISCUSSION

A single administration of oestradiol caused suppression of follicle development during the first 10 days following ovulation without altering luteal function. Administration of oestradiol (all formulations) did not result in appreciable amount of endometrial oedema as determined by transrectal ultrasonography. Dioestrus in 2 mares that received ECP lasted more than 30 days. The authors speculate that the prolonged action of ECP administered during early dioestrus may have induced pseudopregnancy in these 2 mares. Spontaneous persistence of the corpus luteum from a normal oestrual ovulation, in absence of uterine pathology or early embryonic death, is not likely to occur (Ginther 1990). Mares used in this study were not artificially inseminated nor had any documented uterine pathology. Induction of pseudopregnancy via administration of long-acting oestradiol formulation during early dioestrus should be investigated further.

The 2 preparations containing oestradiol-17-beta were prepared in a proprietary long-acting vehicle. Both had half lives shorter than ECP; however, the measurement of total absorption as indicated by the area under the curve (AUC) and the maximum concentration detected (C_{max}) were higher for the 2 oestradiol-17-beta preparations than that of ECP-treated mares. Time to reach maximum concentration following administration of oestradiol-17-beta preparations was also shorter than that of ECP. Based on the pharmacokinetic data of the present study, the authors would recommend oestradiol-17-beta preparations over ECP as absorption and elimination of oestradiol -17-beta is more uniform than that when ECP is used. To our knowledge, this is the first report on pharmacokinetics of ECP in cycling mares.

Future studies should verify whether a simplified (and less expensive) protocol for synchronisation of oestrus and ovulation in mares using oestradiol and prostaglandin (without progesterone) could yield similar degree of synchronisation of that when progesterone and oestradiol are combined.

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PREGNANCY RATES AND PLASMA PROGESTERONE CONCENTRATIONS IN EMBRYO RECIPIENT MARES RECEIVING HORMONE TREATMENT

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Introduction

Embryo transfer (ET) is accepted as an important tool for genetic improvement of equine breeding. Increased pregnancy rates from ET are necessary to reduce the cost, which is still high. Among the factors affecting the success of the technique, the recipient plays a major role. Several studies have investigated the ideal day of cycle for the transfer and the application of drugs to increase progesterone levels, such as anti-prostaglandins, progesterone and, more recently, the hCG and GnRH analogues. The objective of this study was to observe the effects of different hormonal treatments in recipient mares on the time of ovulation, the pregnancy rate and plasma progesterone concentration.

Materials and methods

The embryo recipients were 67 Mangalarga breed mares, randomly distributed in 4 groups: G1 (n=18) - Control, saline solution, 5 ml, im; G2 (n=17) - 40 mg busereline acetate, im (Conceptal®); G3 (n=16) - 3,000 iu of hCG, im (Vetecor®); G4 (n=16) – Norgestomet implant, 3 mg, S.C. (Crestar®). The animals received the treatments on the embryo transfer day, 5 to 9 days after the ovulation was detected.

On the day of ovulation, the mares' ovaries were scanned and all the follicles and the corpus lutea measured. On the day of embryo transfer (5–9 days after ovulation), the animals received the treatment and their ovaries were scanned again. Pregnancy diagnosis was performed when the embryo was 13-days-old, with embryonic vesicle measurement and scanning of the ovaries. Three days later, they were scanned for pregnancy confirmation with embryonic vesicle measurement and scanning of the ovaries. Blood samples were taken for progesterone analysis between the day of embryo transfer and the first pregnancy diagnosis.

Effects of treatment were analysed by ANOVA and the hypothesis tested was considered statistically significant at $P<0.05$.

Results

Secondary ovulation with formation of an accessory corpus luteum was not observed in any of the animals. The mean value of progesterone plasma concentration between the day of treatment (day of embryo transfer) and first pregnancy detection (13-day-old embryo) was higher (Table 1) in group G3 (11.8 ± 0.44 ng/ml) treated with hCG ($P<0.001$) compared to the other groups (G1 = 7.8 ± 0.38; G2 = 9.3 ± 0.27; G4 = 9.9 ± 0.45 ng/ml). Although there was no significant difference in pregnancy rates among the groups ($P>0.05$), there was a numeric difference on Day 13 (G1= 66.7; G2= 70.6; G3= 87.5; e G4= 75.0%), and Day 16 (G1= 56.0; G2= 70.6; G3= 81.3 and G4= 75.0%) between the groups treated with hCG and the other groups (Table 2). Compared to the control group, the group treated with hCG showed an increase of 20.8 and 25.7% in pregnancy rate on Days 13 and 16, respectively. The embryonic vesicle diameter, measured on Day 13 and 16 of the embryo, was not statistically different among groups ($P>0.05$).

Discussion

Most follicles in all groups on the day of treatment (ET day) measured 15 – 25 mm. These follicles were not responsive to LH or hCG which could explain the absence of secondary ovulation. In agreement with an *in vitro* study performed by

TABLE 1: Pregnancy rate (mean ± SD) on Day 13 and 16 in recipient mares given different hormonal treatments on the day of embryo transfer

Treatment	N	Pregnancy rate	
		D13	D16
Control	18	66.7% ± 0.11	55.6% ± 0.12
GnRH	17	70.6% ± 0.11	70.6% ± 0.11
hCG	16	87.5% ± 0.08	81.3% ± 0.10
Progesterone	16	75.0% ± 0.11	75.0% ± 0.11

TABLE 2: Plasma progesterone concentration (mean ± SD) in recipient mares on the day of embryo transfer until pregnancy diagnosis (Day 16). Mares were given different hormonal treatments on the day of embryo transfer

Treatment	N	P4 (ng/ml)
Control	97	7.8 ± 0.38[a]
GnRH	85	9.3 ± 0.27[b]
HCG	83	11.8 ± 0.44[c]
Progesterone	71	9.9 ± 0.45[b]

Kelly *et al.* (1988), the plasma progesterone concentration was higher in Group 3 ($P<0.001$), mares treated with hCG, and this could be explained by the hCG luteotrophic action which promotes an increase in the number of large luteal cells that produce the majority of secreted progesterone by the corpus luteum (Hansel and Blair 1996). Similar results were reported by Thatcher (1989): MacMillan (1986); Drew and Peters (1994); Sheldon (1993); Milvae *et al.* (1984). In the present study, the GnRH analogue promoted a direct LH release with a consequent progesterone rise, because plasma progesterone concentration in Groups 2 and 4 was higher than in the controls ($P<0.05$). This can be explained by the rise in the number of LH receptors in the corpus luteum and greater LH receptor affinity between Days 1 and 14 of the oestrous cycle. When compared to the control group, the hCG group showed an increase of 20.8 and 25.7% in pregnancy rates on Days 13 and 16, respectively. This result could be statistically significant if the number of animals in each group was higher. According to Newcombe *et al.* (2000), hCG helps to prevent occurrence of the luteolytic signal, as it causes an increase in secretion of uterine proteins and corpus luteum progesterone secretion. The treatments increased serum progesterone, and the pregnancy rate numerically, reflecting the relationship between plasma progesterone concentration and pregnancy rate.

Conclusions

The hormonal treatments with buserelin acetate, hCG or Norgestomet in recipient mares on embryo transfer day (5–9 after ovulation) did not cause ovulation or formation of an accessory corpus luteum. However, these treatments do promote an increase in plasma progesterone concentrations between the embryo transfer day and pregnancy detection day when compared with untreated mares.

The hCG causes a greater serum progesterone rise than buserelin acetate and Norgestomet and leads to a numerical increase in pregnancy rates when compared to the other groups. The embryonic vesicle size measured on Day 13 and 16 was not increased by any of the treatments.

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EMBRYO TRANSFER IN THE DROMEDARY CAMEL (*CAMELUS DROMEDARIUS*) USING ASYNCHRONOUS, MECLOFENAMIC-ACID TREATED CAMELS AS RECIPIENTS

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Embryo transfer offers the chance to greatly increase the productivity of a particular female animal and thereby increase the overall rate of genetic improvement. In the camel, embryo transfer is particularly useful to increase the number of progeny from desirable male and female combinations whether it be for racing, milk, meat or transport (Yagill and von Creveld, 1990). There are, however, 2 essential pre-requisites of embryo transfer programmes, firstly to induce superovulation in the donor animals and secondly to be able to synchronise groups of recipient animals.

In the dromedary camel methods for the collection and transfer of fresh, hatched Day 7 blastocysts are well established (Mckinnon *et al.* 1994; Skidmore *et al.* 2002). However, as all camelids have a relatively short luteal lifespan of only 8–10 days, the time window for transferring embryos into the uterus and for them to relay the maternal recognition of pregnancy signal to the mother, so that she maintains the corpus luteum, is short (Skidmore *et al.* 1994). The exact mechanism that controls luteolysis in camels is unknown but firm evidence for the involvement of prostaglandins has been demonstrated by the oral administration of the prostaglandin synthetase inhibitor, meclofenamic acid, which has been shown to prolong the luteal lifespan in camels (Skidmore *et al.* 1998).

Results of previous embryo transfer studies in camels have shown that the best pregnancy rates were achieved if the embryos were transferred to recipients on Day 6 after ovulation, but was dramatically reduced if the level of synchrony between the donor and recipient increased to +1 (embryo transferred to a recipient on Day 8 after ovulation) or –3 days (embryo transferred to a recipient on Day 4 after ovulation) (Mckinnon *et al.* 1994; Skidmore *et al.* 2002). The present study aimed to determine whether camels treated with meclofenamic acid during the luteal phase could be used as asynchronous recipients in an embryo transfer programme.

A total of 40 embryos were recovered from the uteri of 10 donor camels by non-surgical uterine lavage on Day 7 after ovulation. These embryos were aspirated individually into 0.25 ml straws, and loaded into a bovine/equine embryo transfer pipette (IMV Technologies, L'Aigle, France) before being transferred transcervically into the uterus of the recipient camel that had ovulated either one day (OV+8; n=10), 3 days (OV+10; n=10) or 5 days (OV+12; n=10) before the donor was mated. Each recipient received 1 g of meclofenamic acid (Arquel: Parke Davis, Gwent, UK) dissolved in 100 ml of water per os, once on Day 7 and twice on Days 8 and 9 after ovulation, and thereafter once daily until 8 days after embryo transfer. The Control group of camels (n=10) had ovulated one day before the donor was mated and each received 100 ml of water per os, daily from Day 7 to Day 16 after ovulation, and embryos were transferred into the control animals on Day 8 after ovulation.

The results showed that whereas only 1/10 (10%) of the control group of recipients conceived, a total of 8/10 (80%) Day 8, 6/10 (60%) Day 10 and 7/10 (70%) of Day 12 recipients were diagnosed pregnant by ultrasonography of the uterus on Day 20 of gestation, as described by Tinson and McKinnon (1992), and this was subsequently confirmed by detecting a fetus with a heartbeat between Days 27–30 of gestation.

Daily peripheral serum samples were also recovered from all the recipient camels from Day 0 (day of ovulation) to 7 days after the end of

meclofenamic treatment, and assayed for progesterone concentrations. These serum progesterone concentrations remained elevated (above 1 ng/ml) throughout the period of meclofenamic acid administration in all recipient camels. Thereafter, concentrations remained elevated in all the pregnant animals, whereas in non-pregnant animals concentrations had declined to baseline values (<1 ng/ml) within 48 h after the end of the treatment period.

This requirement for negative asynchrony between donor and recipient that has been shown in previous studies may be necessary for the embryo to recover from the shock of the recovery, and handling and transfer process which could cause a temporary slow down in development. Thus, using a Day 8 recipient would not allow the embryo sufficient time to recover and release enough maternal recognition of pregnancy signal to suppress the luteolytic releases of endometrial $PGF_{2\alpha}$ that are poised to start as early as Day 9 in this species. Administration of meclofenamic acid however, can prolong the lifespan of the corpus luteum, by inhibiting release of prostaglandin synthetase, and therefore gives the embryo more time to release sufficient maternal recognition signal for the corpus luteum to be maintained, even in animals where the recipient has ovulated as much as 5 days ahead of the donor. However, in the non-pregnant animal the rapid decline in serum progesterone concentrations after the end of the treatment period shows that once the inhibitory influence of meclofenamic acid has been removed $PGF_{2\alpha}$ is released and luteolysis can occur.

These results show that it is possible to use camels treated with meclofenamic acid during the luteal phase, as asynchronous recipients for embryo transfer in an embryo transfer programme.

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SESSION 7:

Commercial embryo transfer

Chairman: E. L. Squires

EMBRYO TRANSFER IN MINIATURE MARES: PRELIMINARY RESULTS

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INTRODUCTION

Embryo transfer techniques in the horse were developed in the late 1970s (Squires *et al.* 2003). Nowadays, the collection and transfer of fresh and/or cooled equine embryos is allowed in many breeds. Major candidates for embryo transfer are older, multiparous mares that have a history of infertility, or mares that compete in jumping, racing or any other sport. Embryo recovery is normally done 7 or 8 days after ovulation. Factors that affect embryo recovery include day of recovery, number of ovulations, age of donor mare and quality of the stallion's semen (Squires *et al.* 1999). Although embryo collection and transfer are commonly used in the horse industry in most breeds, there are no reports of its use in miniature horse breeds (miniature horses or mini shetland ponies). The literature on reproductive parameters in these breeds is also poor (Judd 1994; Metcalf *et al.* 1997; Paccamonti *et al.* 1999; Neves *et al.* 2000). A recent study on the evolution of equine embryo transfer in Brazil does not include any data on miniature breeds (Carmo and Alvarenga 2003). The aim of this study was to develop a protocol to collect and transfer embryos in miniature mares.

MATERIALS AND METHODS

Seven miniature mares and one stallion of proven fertility were used. The size of animals used was less than 85 cm. All mares were nulliparous, and between 2–4 years old. Oestrus was induced with $PGF_{2\alpha}$ (Lutalyse – Pharmacia Brasil Ltda. – Paulinia – SP – Brazil). Mares were examined daily through rectal palpation and ultrasound, and were inseminated when a follicle larger than 35 mm was found. Semen was collected using a Hannover model artificial vagina (Minitüb GmbH, Hauptstrasse 41 - 84184 – Tiefenbach, Germany). After collection, semen was diluted with skimmed milk. For insemination, a total of 300 $x10^6$ live spermatozoa in a total volume of 10 ml were used. After insemination, 2,500 iu hCG (Vetecor 5000IU- Laboratorios Calier S.A. – Osasco –SP- Brazil) were administrated intravenously to the mare. The day of ovulation was recorded, and mares were flushed either on Day 8, or 9 or 10 after ovulation. A Foley catheter (size 26 ch) was used for flushing (Willy Rüsch AG- 71394- Kernen – Germany), and the cuff was inflated with 30 ml of air. The solution used was lactated Ringer (Ringer com Lactato- Indústria Farmacêutica Texon Ltda. – R. José Garibaldi, 1230, Viamão –RS- Brazil), in a lower volume than that used for normal size mares. The fluid was drained out through a filter cup (Nutricell Nutrientes Celulares- Campinas – SP- Brazil), and the remainder was collected into a 1,000 ml graduated cylinder to ensure that the whole volume infused

TABLE 1: Day of flushing, volume of fluid used in each of 3 attempts, success of flushing and size of embryo retrieved

Day of flushing	Volume used	Success	Size of embryo
8	700	Y	0.5
8	650	Y	0.5
10	500	Y	3
9	650	Y	1.5
10	700	Y	3
8	300	Y	0.8
9	350	N	
9	450	N	
10	500	N	
9	500	N	
10	550	N	

TABLE 2: Mean volume and embryo size according to day of collection

	Day 8 (n=3)	Day 9 (n=4)	Day 10 (n=4)
Mean volume	550 ± 282.84 ml	562.5 ± 35.35 ml	487.5 ± 106.06 ml
Embryo size	0.6 ± 0.21 mm (n=3)	1.5 ± 0.0 mm (n=1)	3.0 ± 0.0 mm (n=2)

TABLE 3: Comparison between embryos from normal sized mares and embryos from miniature mares according to day of collection

Day of collection	Embryo size (mm)- normal mares	Embryo size (mm)- miniature mares
6	0.208	none
7	0.406	none
8	1.132	0.6
9	2.220	1.5
10	none	3.0

was recovered, and to record the volume used. This procedure was repeated at least 3 times. The embryos were then searched for, identified, graded and measured. The embryos were not transferred due to the small number of mares in the study, as the aim was to obtain as many embryos as possible.

Results and discussion

A total of 11 embryo collections were performed, with 6 embryos retrieved. The mean amount of fluid used was 531.81 ± 134.52 ml. All the embryos retrieved were at expanded blastocyst stage. All embryos were quality Grade 1 except for one that was Grade 5. The mean size of the embryos was 1.55 mm ± 1.18 mm. When flushing was performed on Day 10, 2 embryos measuring 3 mm were recovered. Some mares were initially flushed on Day 7, before the beginning of the experiment, but no embryo was obtained. Results of flushings performed are shown in Table 1. The mean amount of fluid used and size of embryo according to day of flushing are shown in Table 2. A comparison with some data of embryo size found in normal sized mares is shown in Table 3 (adapted from Squires *et al.* 1985).

Conclusions

These results show that embryo collection can be performed in miniature horse breeds, with a good recovery rate. Recovery rates in this study were similar to those described by Squires *et al.* (1987). The size of embryos seems to be smaller than in normal sized mares. Further studies are in progress to determine the ideal day of collection, method of embryo transfer and pregnancy rates.

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USE OF DEEP UTERINE LOW DOSE INSEMINATION OF FROZEN AND COOLED STALLION SEMEN IN A COMMERCIAL EMBRYO TRANSFER PROGRAMME

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INTRODUCTION

The interest in the use of artificial insemination (AI) techniques to obtain satisfactory fertility rates using a small number of spermatozoa has increased, especially for frozen and sexed-sorted spermatozoa. Recent approval of frozen semen techniques by important horse breed associations has increased the interest in the application of frozen semen by the horse industry. However, the fertility of frozen semen is still low and a large number of doses are necessary to achieve a pregnancy. One of the major obstacles to the routine use of frozen semen in horses is the low number of insemination doses produced per ejaculate. The conventional dose for artificial insemination with frozen-thawed semen in mares is about one billion motile spermatozoa that are deposited in the uterine body. Only a small number of spermatozoa will reach the fertilisation site as a result of a sperm selection during the uterine transport.

Also, it has been reported that a more severe acute inflammatory response is expected after AI with frozen semen in most of inseminated mares (Watson 2000) and a reduction of sperm number associated with the deposition of semen close to the site of fertilisation may avoid or decrease this inflammatory reaction.

New AI techniques have been developed aiming to improve the fertility rates of frozen stallion semen and for the use of a small number of spermatozoa in new breeding technologies. Recent experiments have shown that low-dose hysteroscopic insemination, where semen is deposited at the uterotubal junction (UTJ), an important sperm reservoir in horses (Scott 2000), is an effective technique for horses (Lindsey *et al.* 2001; Morris *et al.* 2000; Squires *et al.* 2000).

The aim of the present study was to evaluate the potential use of low dose deep intrauterine AI, with a simple technique, in a commercial embryo transfer programme.

MATERIALS AND METHODS

A total of 720 low dose AI were performed by only one technician, between March and October of 2003 on a stud farm located in North of Germany. Frozen (n= 473) and cooled semen (n= 247) from 33 different warmblood stallions were used. After heat detection and observation by ultrasound evaluation of a 35 mm follicle and ovulation induction with hCG (2,500 iu iv), follicular growth was monitored daily and each 6 hours for mares designated to be inseminated with frozen and cooled semen, respectively. Mares were inseminated with a special model of pipette (Minitub-Germany) that was guided by transrectal palpation to the tip of the horn, ipsilateral to the pre-ovulatory follicle or ovulation. The cooled semen was stored and transported in a special container (Equitainer), from different stud farms located in Germany, at 5°C between 24–72 h before use. Mares were inseminated pre-ovulation with 200 million cooled sperm in a volume of 7 ml or less than 6 h post ovulation with a 100 million of frozen-thawed sperm in 1–2 straws. There was a large variability in the motility of the frozen and cooled semen that was used. Embryo flushes were performed 7–9 days after ovulation and transferred non-surgically to synchronised recipient mares that ovulated on day before and 4 days after the donor mares.

RESULTS AND DISCUSSION

Embryo recovery rates from mares inseminated with cooled (104/247; 42%) or frozen semen

(209/473; 46%) were not signicficantly different (P>0.05). A similar pregnancy rate was also observed in recipient mares that were inseminated with embryos from cooled semen (74/104; 71%) when compared with embryos from frozen semen (132/209; 63%). There was no significant difference (P>0.05) in percentage of early embryonic death (EED) between embryos produced by cooled (12.5%) or frozen semen (11.36%) and this percentage of EED is considered normal for horses. A large individual variability was observed in fertility between stallions as reported by other authors.

The results of the present study showed that good embryo recovery rates could be obtained with low dose AI in the tip of the horn with a more practical method that can be used in field conditions. This technique seems to give results similar to those reported by others using hysteroscopic AI (Squires *et al.* 2000). This technique also allowed a significant economy of straws, multiplying the number of embryos produced per conventional dose. Further studies are needed to compare the low dose deep intrauterine AI using the pipette with hysteroscopic AI and also to determine if this technique can improve the fertility rates of mares susceptible to endometritis.

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USE OF MARES AS EMBRYO RECIPIENTS AFTER FIRST POST PARTUM OVULATION

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Introduction

The mare is unique among domestic animals in that the first post partum oestrus is ovulatory. This oestrus, commonly referred to as foal heat, is characterised by normal follicular development and ovulation. The onset of the foal heat occurs within 5–12 days after parturition in >90% of mares (Ginther 1992). Reduced pregnancy rates and higher embryonic death rates results from breeding mares during the foal heat (Ginther 1992; Ball *et al.* 1993). The success of breeding mares soon after foaling depends on several factors. The most important of these is correct selection of the mares to be bred. If the mare fails to become pregnant, resorbs, or aborts the fetus, nothing has been gained by foal heat-breeding.

To maximise the success of an embryo transfer programme in horses, it has become essential to know factors that affect the pregnancy rates after embryo transfer. Such factors are season, age and size of embryo, synchrony of recipient and donor, method of transfer, technician, procedures for culture and storage of embryos, age and reproductive history of the donor, management of recipients and embryo quality (Squires *et al.* 1999).

If a commercial embryo transfer farm program has few recipients, it is sometimes better to use known mares considered to be without reproductive problems, but it is possible that these mares are in foal heat. However, no studies on the fertility of mares as embryo recipients after first post partum ovulation have been conducted. The objective of this study was to evaluate the potential to use recipient mares in foal heat as embryo recipients.

Materials and methods

During 2 breeding seasons in a commercial embryo transfer (ET) programme, 131 embryos were transferred non-surgically to reproductively healthy mares (Mangalarga Marchador breed) by 2 technicians. The mares were evaluated daily by palpation *per rectum* and ultrasonography to monitor follicle development and ovulation. During the foal heat, recipient mares were also examined carefully by ultrasonography to detect the presence of intrauterine fluid. Only mares with no fluid accumulation, without difficult delivery, retained placental membranes, endometritis, or other complications associated with foaling were used.

Recipient mares (Mangalarga Marchador breed) between 3–12 years of age were distributed in 3 experimental groups: GI (n=42) cyclic mares without foals, GII (n=50) mares in foal heat, GIII (n=39) mares on the second cycle after parturition. Embryo recoveries were performed at Day 8 post ovulation. The equine uterine catheter (Bivona‘) was introduced into the vagina through the cervix into the uterine body. The cuff was then inflated with 60 ml of air and the catheter was gently pulled against the cranial opening, closing the cervix. One litre of Ringer Lactate solution (LRS) was introduced into the uterus through the catheter and then drained from the uterus into a filter cup with a 75 mm filter. This process was repeated 3 times then the cuff was deflated and the catheter was removed from the uterus. The 30 ml remaining in the filter cup was transferred to a Petri dish and observed through the stereoscopic microscopic to locate and classify the embryo. The recovered embryo went through successive

flushes (10–15 drops) in an embryo holding solution TQC TM holding plus (Nutricell-Nutrientes Celulares/SP) or DPBS with 10% bovine fetal serum. Non-surgical transfer of embryos was accomplished with the use of 2 transfer devices (French transfer gun for small embryos and artificial insemination pipette for big embryos). No hormonal synchronisation of ovulation was used between donor and recipient. Embryos were transferred to recipients on Day 4–8 post ovulation and only Grade 1 and 2 embryos were used. Embryonic vesicles were observed 7 days after ET and at 60 days of pregnancy by ultrasound image (Scanner 450 Vet, Pie Medical, Holland).

Statistical analysis

Statistical differences between the 3 groups regarding differences in pregnancy rates and pregnancy losses were evaluated by Cui-Square test. Significance was set at $P<0.05$.

RESULTS

No statistical differences were observed between the experimental groups in pregnancy rates at 15 and 60 days and in the incidence of early embryonic death ($P>0.05$). Results are shown in Table 1.

DISCUSSION

These results disagree with Ginther (1992) and Ball *et al.* (1993), who reported pregnancy rates achieved by breeding during foal heat are generally 10 to 20% lower than those obtained by first breeding at subsequent oestrous periods. Controversy still exists over whether a greater incidence of pregnancy loss occurs in mares conceiving during foal heat-breeding (Ginther 1992). The study showed no significant difference in rate of pregnancy loss between groups.

Mares that foal earlier in the breeding season, young mares, mares without difficult delivery, retained placental membranes, endometritis, or other complications associated with foaling are often better candidates to be bred on the foal heat. Younger mares are generally better than older mares because they usually recover (uterine involution) sooner from foaling. Late-foaling mares are usually problem breeders and are often less fertile than mares that foal earlier in the season (Zent 2003).

Uterine contractility is necessary for delivery of the fetus, expulsion of placenta and uterine involution (rapidly reducing the size of the post parturient uterus to its pregravid state). The histologic character of the endometrium correspondingly reverts to conditions more conductive to embryonic support. The endometrium usually has a normal pre-gravid histologic appearance by Day 14 post partum (Gygax *et al.* 1979). Several studies have shown that time of ovulation is critical to the success of foal heat-breeding. Mares that ovulated before 10 days after foaling have much lower pregnancy rate than mares that ovulate at 10 days later (Zent 2003).

It has been well documented that spermatozoa, rather than bacteria, are responsible for the acute inflammatory response in the equine uterus after insemination. It seems therefore, likely that a transient post mating endometritis is physiological, with the purpose of eliminating excess spermatozoa, semen products and contaminants before the entry of the embryo into the uterus (Troedsson *et al.* 1998). Persistent breeding-induced endometritis affects approximately 15% of mares in clinical practice and is a major cause of reduced fertility. Mares

TABLE 1: Pregnancy rates and embryonic death

	Embryos transferred	15 days of pregnancy	60 days of pregnancy	Pregnancy loss
GI	42	72%[a]	66.6%	6.6%[a]
GII	50	70%[a]	64.0%	8.3%[a]
GIII	39	76%[a]	62.9%	8.3%[a]

Columns with equal superscripts are not different ($P>0.05$)
GI - cyclic mares without foals, GII - mares in foal-heat and GIII - mares on the second cycle after parturition

susceptible to this condition are unable to remove breeding-induced endometritis until 24–48 h after breeding (Troedsson 1999).

Mares bred on a foal heat, receive semen soon after giving birth (7–10 days). This process induces endometritis provoked mainly by spermatozoa and this event could damage the normal process of uterine involution and consequently, could decrease fertility rates and increase embryonic death. When using mares after foal heat (first ovulation post partum) as recipients the embryo enters the uterus later (17–22 days) so probably did not damage uterine involution, and consequently, fertility rates were not affected.

Conclusions

The results of the present study demonstrated that mares in foal heat, selected on the basis of age and absence of uterine fluid accumulation can be used as recipients without economical losses in a commercial embryo transfer programme.

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IPSILATERAL DOUBLE OVULATION IS ASSOCIATED WITH REDUCED EMBRYO RECOVERY IN MARES

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INTRODUCTION

Embryo transfer in horses is less efficient than in cattle, largely because of a lack of effective superovulation regimens. Typically, superovulation attempts in mares have resulted in an average of 3 follicles ovulating per cycle. Alvarenga *et al. (*2001) reported an average of 7 ovulations per mare with twice daily administration of equine pituitary extract (EPE), although subsequent studies using similar treatments have again reported an average of about 3 ovulations (Niswender *et al.* 2003; Scoggin *et al.* 2002).

Although the number of ovulations in mares is low compared to cattle, recovery of even one extra embryo from a mare can be very valuable in saved labour and time. Unfortunately, the recovery rate reported in superovulated mares is variable. Some authors report recovery rates per follicle ovulated equivalent to those for control (single ovulating) mares; however, some reports have low (~40%) embryo recovery in control mares (Rosas *et al.* 1998; Scoggin *et al.* 2002), making the data difficult to interpret. Other authors report a lower recovery rate when multiple ovulations are induced. This may be due to an adverse effect of superovulation treatment on oocyte viability. In support of this is the finding that higher doses of EPE or purified eFSH are associated with lower recovery rates (Alvarenga *et al.* 2001; Niswender *et al.* 2003; Briant *et al.* 2004) and that superovulation by vaccination against inhibin yielded equivalent embryo recovery rates to that for controls (McCue 1992).

Other possible explanations for reduced embryo recovery in superovulated mares are that the unique morphology of the equine ovary, in which all follicles ovulate in the ovulation fossa, hinders concurrent ovulation of more than one follicle from the same ovary, or that competition between oocytes or embryos within the oviduct may reduce viability. To look at whether ovulation of 2 follicles from one ovary is associated with decreased recovery rate, records from spontaneously double ovulating mares in our embryo transfer programme were assessed.

MATERIALS AND METHODS

Data on embryo recovery from 411 commercial embryo donor mares, aged 4–25 years, were evaluated. Most were polo ponies, with a few warmbloods, Quarter Horses and Peruvian Paso horses. Embryo donors were examined by ultrasonography *per rectum* to monitor follicle growth and inseminated with semen from a fertile stallion from 2 days pre- to 12 h post ovulation. A total of 87 stallions were used . Time of ovulation and location of ovulating follicles were recorded. Uterine flushing for embryo recovery was performed on Day 7 or 8 using the technique of Riera and MacDonough (1993). Mares ovulating 2 follicles were classified as having ovulated them from the same (ipsilateral double ovulation) or different ovaries (contralateral double ovulation). Transcervical embryo transfer was performed within 1 h of collection by using a disposable 21 inch equine embryo transfer gun, covered with a sanitary plastic sheath (Riera and MacDonough 1993).

RESULTS

A total of 964 double-ovulation cycles were recorded with all pertinent data. The proportion of double-ovulating mares that ovulated follicles from the same ovary was 528/964 (55%). This was significantly different from a 50% distribution ($P<0.05$). The embryo recovery rate per follicle ovulated was significantly lower in ipsilateral-

than contralateral-ovulating mares (574/1056, 54% vs 588/872, 67%; P<0.001). This was associated with a significantly greater proportion of flushes which yielded no embryos in ipsilateral than in contralateral-ovulating mares (147/528, 28% vs 66/436, 15%; P<0.001). The proportion of flushes yielding 2 embryos was significantly lower in the ipsilateral group (193/528, 37%) than the contralateral group (218/436, 50%; P<0.001). The pregnancy rate per embryo transferred was not different between embryos recovered from ipsilateral or contralateral-ovulating mares (63% and 66%, P>0.1), nor was there a difference in pregnancy rate for embryos recovered singly or in pairs for either ovulation group (P>0.1).

Discussion

These data show that spontaneous ipsilateral double ovulation is associated with decreased embryo recovery, compared to contralateral double ovulation. This supports the hypothesis that some mechanism at the level of the ovary, or local interaction between oocytes or embryos within the oviduct, may interfere with ovulation or embryo survival when mares are superovulated.

The significant increase in proportion of flushes yielding no embryos in spontaneously ipsilateral-double ovulating mares is puzzling. If reduced embryo recovery is related to 2 follicles being present near the ovulation fossa at the same time, these data suggest that even the first follicle to ovulate is somehow perturbed or fails to express the oocyte normally. Dippert *et al.* (1994) examined the ovaries of superovulated mares to determine whether a track to the ovulation fossa was present in each corpus lutem. There was no difference in proportion of corpora lutea with an ovulation track between control and superovulated mares (>90% in both groups). These authors also recovered early embryos from the oviduct 2 days after ovulation, and found a similar embryo recovery rate between mares superovulated with EPE and control mares (Dippert *et al.* 1994), in spite of their previous finding that EPE-treated mares had reduced embryo recovery from the uterus (Dippert *et al.* 1992). Unfortunately, Dippert *et al.* (1994) did not divide mares into ipsilateral or contralateral multiple ovulations, so it is unclear whether their findings can be applied to the current problem.

An alternative hypothesis to explain reduced embryo recovery in ipsilateral-ovulating mares is that some inter-oocyte or inter-embryo interactions occur in the oviduct, which lower embryo viability. This is unlikely, however, because transfer of multiple oocytes to one oviduct is associated with normal fertilisation and embryo development rates (Carnevale and Ginther 1995).

Further work with embryo collection from the oviducts of ipsilateral- and contralateral-double ovulating mares may help to determine the cause of reduced embryo recovery on uterine flush when follicles ovulate from the same ovary. The fact that this phenomenon occurs in spontaneously ovulating mares indicates that reduced embryo recovery rates in superovulated mares are not due entirely to effects of EPE upon embryo viability.

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A NOVEL METHOD FOR NON-SURGICAL EMBRYO TRANSFER IN THE MARE

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Since the original reports by Allen and Rowson (1972) and Oguri and Tsutumi (1972) of the recovery and transfer of Day 7 embryos between donor and recipient mares, the technique of embryo transfer has been much slower to be exploited commercially in the horse than in the cattle and other farm species. However, embryo transfer is the method of choice for breeding top class Sporthorses as it allows production of foals from superior fillies without interrupting their competitive careers and it can improve the fecundity of genetically desirable older mares suffering age-related endometritis that may cause placental insufficiency (Bracher *et al.* 1986).

Transcervical non-surgical embryo transfer is preferable to surgical transfer, via midline or flank laparotomy, for its speed, simplicity, economy and lack of post-transfer complications. However, non-surgical transfer rates have fallen consistently below those achieved by surgical transfer in all but the most experienced hands. Although recent studies comparing surgical and non-surgical transfer rates are lacking, some 20 years ago Iuliano *et al.* (1985) reported pregnancy rates of 72 vs 45% for surgical vs non-surgical transfer of a total of 83 horse embryos, while Squires *et al.* (1982) similarly reported mean pregnancy rates of 55 and 22% respectively, for surgical transfer of 64 embryos vs non-surgical transcervical transfer of 29 embryos.

These lower non-surgical pregnancy rates have variously been ascribed to: i) release of $PGF_{2\alpha}$ (Kask *et al.* 1997) and/or oxytocin (Handler *et al.* 2002) induced by manipulation and/or penetration of the cervix leading to premature luteolysis; ii) localised inflammatory responses in the endometrium coupled with reflux of the embryos through the cervix (Squires *et al.* 1999); iii) low grade endometritis established by untoward passage of commensal bacteria from the vagina into the progesterone dominated, and therefore infection-sensitive, uterus during the transfer process (Allen 1982 and subsequent unpublished observations).

Therefore, a combination of great manipulative experience and expertise, combined with rigorous culling of unsuitable mares from the recipient herd, is necessary to achieve high pregnancy rates from non-surgical transfer. This is reflected in reported pregnancy rates from more recent studies, often commercial operations, that are performing many transfers a year. For example, the non-surgical transfer of 480 embryos between 2000 and 2003 resulted in a pregnancy rate at Day 16 of 69.8% McCue and Troedsson (2003). Similarly, such conditions occur in Argentina where embryo transfer has been used annually for some years to produce multiple foals from high-goal polo pony mares and Losinno *et al.* (2001) reported an overall pregnancy rate of 81% from the non-surgical transfer of 993 embryos.

In an attempt to increase non-surgical transfer rates in the authors' laboratory, which does not have the throughput of large numbers of embryo transfers that would lead to manipulative expertise, radical changes were made to the conventional transfer method. These aimed principally to facilitate a smoother passage of the transfer pipette through the cervix of the recipient mare while maintaining a high level of cleanliness and minimal manipulative disturbance. Thus, after scrupulous cleaning of the recipient mare's perineum, a duck-billed Polansky's speculum is inserted into the vagina to visualise the cervical os with the aid of a focused pen torch. Modified Vulsellum forceps (Wilsher Forceps; Holborn Surgical Instruments, Kent, UK) are then used to grasp the ventral quadrant of the external cervical

os and pull the cervix backwards towards the vulva, thereby elongating and straightening the cervical canal and elevating the uterus within the abdomen. The embryo is loaded into a pre-warmed sterile 'rigidly-flexible' disposable transfer pipette, protected in a plastic sheath, which is passed visually through the cervix and well up one uterine horn in a single smooth movement to deposit the embryo in its larger-than-normal volume of transfer medium (Fig 1).

Using this method 17 of 20 experimental mares (85%), synchronised to have ovulated 0–2 days after the embryo donor, and to which a Grade 1 embryo was transferred, became pregnant. Measurement of peripheral serum progestagen concentrations in the recipient mares daily for 3 days after each transfer showed no significant fall in values, regardless of whether they became pregnant.

This new method of non-surgical embryo transfer in the mare holds great promise in the expanding field of commercial equine embryo transfer. First and foremost, it appears to be highly efficacious. The first trial carried out specifically to assess the practicality and accuracy of the technique yielded a high pregnancy rate of 85%. Over many years past, the authors' laboratory has never achieved better than 60% pregnancy rate when transferring Day 7 or 8 equine embryos non-surgically by the more conventional transcervical passage of a transfer gun containing the embryo in a 0.5ml straw.

This modified method of non-surgical transfer also offers other advantages over the conventional transfer method. It maintains minimal contamination by the passage of only sterile instruments into the vagina. The expanding arms of the Polansky's speculum restrain the vaginal mucosa, with its potentially contaminating commensal bacteria, from contact with both the Wilsher Forceps and the embryo transfer pipette. Accurate visual appraisal of the cervix is also possible and the external os of the cervix can be grasped easily with the forceps and causes the mare no discomfort. Furthermore, retracting the cervix straightens both its lumen and that of the uterus to allow swift, easy passage of the transfer pipette. The larger volume of transfer medium (2.5 ml) may also be advantageous both by protecting the embryo from temperature fluctuations and by carrying the embryo past and therefore well away from the tip of the pipette. Indeed Jasko (2002), when washing the tip of one type of transfer pipette after carrying out 25 non-surgical transfers, found the embryo still stuck to the tip of the transfer gun on 2 occasions (8%). McCue and Troedsson (2003) have also noted that embryos may become caught in the side-ejecting tip of some transfer guns

In summary, our new method of non-surgical embryo transfer makes it manipulatively straightforward to deposit an embryo in a larger than normal volume of medium deep into the recipient mare's uterus without the need for the operator to place an arm in either the vagina or the rectum. The technique is simple to perform, it does not require vast expertise and experience, it provides additional temperature protection for the embryo, and it can achieve high pregnancy rates.

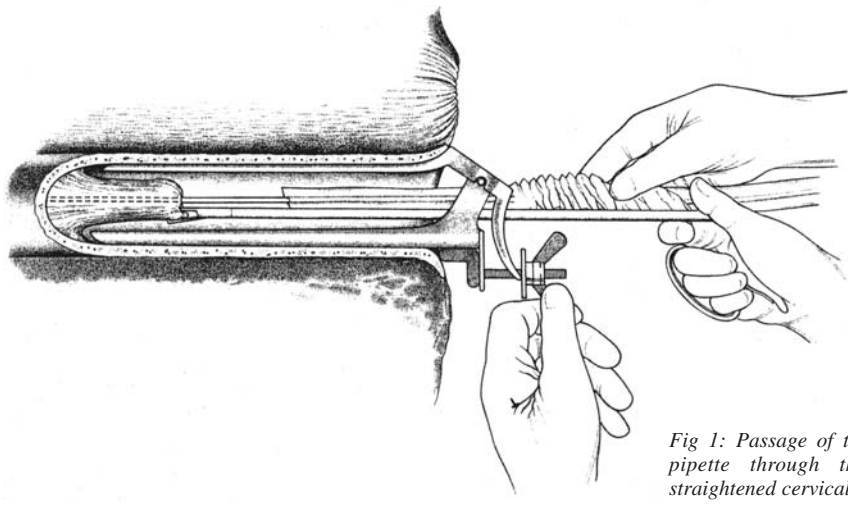

Fig 1: Passage of the embryo transfer pipette through the retracted and straightened cervical canal.

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