Havemeyer Foundation
Monograph Series No. 5

Proceedings of the Second Meeting of the

EUROPEAN EQUINE GAMETE GROUP (EEGG)

26th–29th September 2001
Loosdrecht, The Netherlands

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

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EDITORS' FOREWORD

In September 1998, the inaugural meeting of the European Equine Gamete Group (EEGG) was held in Lopuzna, Poland. Conceived by Professors Twink Allen and Marian Tischner, the over-riding aim of this group was to act as a forum for bringing together various individuals and groups working within Europe in the field of equine assisted reproduction; and thereby to encourage them to discuss recent scientific and practical advances and to foster future collaborative efforts.

The initiation of EEGG coincided with a period of dramatic advances within the field of reproductive biology, including cloning by nuclear transfer and germ-line transplantation by spermatagonial transfer, and within the world of equine reproduction in particular. Recent years have at last seen the development of a reliable means of achieving *in vitro* fertilisation of horse oocytes (intra-cytoplasmic sperm injection) and much improved responses to superovulatory treatment, topics that have caused considerable consternation and tearing of hair over a number of years. Indeed, it is a time of rapid advance in many aspects of reproductive biology and, with the development of ever more sophisticated analytical machinery, and inroads being made into the characterisation of the equine genome, there seems to be considerable scope for it to continue.

The second meeting of EEGG confirmed the need to provide an ongoing forum for exchange of ideas in this area. The programme included a number of 'state-of-the-art' presentations, many of which represented collaborative efforts between the different groups represented in EEGG, and some of which are directly attributable to the first meeting. Given that the large funding bodies prefer to back international and interdisciplinary projects, this spirit of cooperation is essential to ensure that the current rate of progress is maintained.

The group is indebted to Mr Gene Pranzo and the Dorothy Russell Havemeyer Foundation for their support in the organisation of this meeting which was convivial and enjoyable as well as informative and interesting. Finally, we are grateful to our hosts, the veterinary school at Utrecht. Its reproductive team has long been renowned for its work on parturition and obstetrics but now, with a large group of scientists and veterinarians, it is increasing its reputation in the field of gamete biology.

T. A. E. Stout
J. F. Wade

HAVEMEYER SCIENTIFIC WORKSHOPS

1981	**First International Workshop on Lymphocyte Alloantigens of the Horse** October - New York City, USA *Organiser: Dr D. F. Antczak*
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1983	**Third International Workshop on Lymphocyte Alloantigens of the Horse** April - New Bolton Center, University of Pennsylvania, USA *Organiser: Dr D. F. Antczak*
1984	**First International Symposium on Equine Embryo Transfer** October - Cornell University, Ithaca, New York, USA *Organisers : Drs D. F. Antczak and W. R. Allen*
1985	**Fourth International Workshop on Lymphocyte Alloantigens of the Horse** October - University of Kentucky, USA *Organisers: Drs D. F. Antczak and E. Bailey*
1986	**Workshop on *Corynebacterium equi* Pneumonia of Foals** July - University of Guelph, Canada *Organiser: Dr J. F. Prescott*
1987	**Fifth International Workshop on Lymphocyte Alloantigens of the Horse** October - Louisiana State University, USA *Organisers: Drs D. F. Antczak and J. McClure*
1989	**Second International Symposium on Equine Embryo Transfer** February - Banff, Alberta, Canada *Organisers : Drs D. F. Antczak and W. R. Allen*
1990	**International Workshop on Equine Sarcoids** April - Interlaken, Switzerland *Organisers: Dr D. F. Antczak and Professor S. Lazary*
1992	**Workshop on Equine Neonatal Medicine** January - Naples, Florida *Organisers: Drs D. F. Antczak and P. D. Rossdale*
	Third International Symposium on Equine Embryo Transfer February - Buenos Aires, Argentina *Organisers : Drs D. F. Antczak, W. R. Allen, J. G. Oriol and R. Pashen*

1995

Equine Perinatology
July - Cambridge, England
Organiser: Dr P. D. Rossdale

Second International Equine Leucocyte Antigen Workshop
July - Lake Tahoe, California, USA
Organisers : Drs D. F. Antczak, P. Lunn and M. Holmes

First International Workshop on Equine Gene Mapping
October - Lexington, Kentucky, USA
Organisers: Drs D. F. Antczak and E. Bailey

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

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

1997

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

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

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

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

1998

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

Third International Workshop on Perinatology: Genesis and Post Natal Consequences of Abnormal Intrauterine Developments: Comparative Aspects
February - Sydney, Australia
Organiser: Dr P. D. Rossdale

Horse Genomics and the Genetic Factors Affecting Race Horse Performance
March - Banbury Center, Cold Spring Harbor, New York, USA
Organisers: Drs D. F. Antczak, E. Bailey and J. Witkowski

Allergic Diseases of the Horse
April - Lipica, Slovenia
Organisers: Drs D. F. Antczak, S. Lazary and E. Marti

Equine Placentitis Workshop
October - Lexington, Kentucky, USA
Organisers: Drs D. F. Antczak, W. R. Allen and W. Zent

Septicemia II Workshop
November - Boston, Massachusetts, USA
Organiser: Dr M. R. Paradis

1999

Equine Genome Project
January - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Third International Equine Genome Workshop
June - Uppsala, Sweden
Organisers: Drs D. F. Antczak, E. Bailey and K. Sandberg

Fourth International Meeting of OIE and WHO Experts on Control of Equine Influenza
August - Miami, Florida, USA
Organiser: Dr J. Mumford

European Equine Gamete Workshop
September - Lopuszna, Poland
Organisers: Drs W. R. Allen and M. Tischner

Fetomaternal Control of Pregnancy
November - Barbados, West Indies
Organisers: Drs T. Stout and W. R. Allen

2000

Equine Genome Project
January - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Uterine Infections in Mares and Women: A Comparative Study
March - Naples, Florida, USA
Organiser: Dr M. M. LeBlanc

5th International Symposium on Equine Embryo Transfer
Saari, Finland
Organiser: Dr T. Katila

2001

USDA Internationl Plant & Animal Genome Conference
San Diego, California

Equine Immunology in 2001
Sanata Fe, New Mexico
Organiser: Dr D. P. Lunn

Second Meeting of the European Gamete Group (EEGG)
Loosdrecht, The Netherlands
Organiser: Dr T. A. E. Stout

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SESSION I:

Semen preservation

Chairman: W. R. Allen

MOTILITY AND VIABILITY OF FRACTIONATED STALLION EJACULATES AFTER 24 H OF COOLED STORAGE

T. Katila, M. Karlsson, T. Reilas*, M. Andersson, R. Kaikkonen and E. Koskinen†

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INTRODUCTION

It has been shown that the presence of seminal plasma decreases sperm motility during cooled storage (Jasko *et al.* 1991; Pruitt *et al.* 1993). Therefore, semen is usually extended to concentrations between 25 and 50 x 10^6 spermatozoa/ml to reduce the proportion of seminal plasma (Varner *et al.* 1987). Seminal plasma fractions of the ejaculate are not uniform, but the contribution of accessory glands is different during the ejaculation (Magistrini *et al.* 2000). The first sperm-rich fractions seem to tolerate preservation better than the whole ejaculate (Varner *et al.* 1987).

The purpose of this study was to investigate if different fractions of seminal plasma would be beneficial or detrimental during storage of cooled semen.

MATERIALS AND METHODS

Four adult stallions (3 Finnhorses, one Standardbred) were used to collect semen for the project. Following a one-week period of daily ejaculations, semen was collected twice a week during 2 weeks with the automated phantom, Equidame (Lindeberg *et al.* 1999). The volumes of the cups were adjusted as follows: 10 ml in Cup 1, 20 ml in Cup 2, 20 ml in Cup 3, 30 ml in Cup 4 and 30 ml in Cup 5. The time to the first mount and ejaculation were recorded, as well as the number of mounts.

After collection, the volumes in the 5 cups of the phantom were recorded and their sperm concentration determined. Because Cups 4 and 5 had very low total sperm numbers, they were combined in Cup 4. The content of each cup was extended with skim milk extender 1:1. If needed, semen was extended further to give a concentration between 25 and 100 x 10^6 spermatozoa/ml, but the ratio of semen to extender was always kept at 1:1-3. A 1.5 ml volume in a 1.5 ml vial was transferred into the Equitainer. The remaining semen was centrifuged at 500 *g* for 15 min. The supernatant was removed and skim-milk extender added to a final concentration between 25 and 50 x 10^6 spermatozoa/ml. Because the pre-sperm fraction in Cup 1 did not contain any spermatozoa, centrifuged spermatozoa were taken from Cup 2 and added to the pre-sperm fraction to a final concentration of 50 x 10^6 sperm/ml (semen:extender = 1:1). Aliquots of 1.5 ml were transferred to 1.5 ml vials and packed into an Equitainer with the non-centrifuged samples.

After a 24 h storage, 0.25 ml samples were incubated for 5 min at 3°C and motility parameters (total motility = TOTMOT, progressive motility = PROGMOT, path velocity = VAP, % rapid cells = RAP) determined with a Hamilton-Thorn Motility Analyzer. A minimum of 250 cells were counted using Makler chambers and analysing 3 fields/chamber. Viability was assessed by staining spermatozoa with CFDA/PI (carboxyfluoresce-indiacetate/propidium ioide) and counting 200 cells in fluorescence microscopy as described by Katila *et al.* (2000). Because seminal plasma was stained also, disturbing the identification of viable cells in the microscope, the non-centrifuged samples were centrifuged for 5 min at a low speed before staining.

The raw mean and standard deviation were used for simple statistics. The stallion, the existence of separate pre-sperm Cup 1, the cup number, the centrifugation, the regression of time

TABLE 1: Semen concentrations (x 10^6) in Cups 2–5 (5 = total ejaculate)

Cup	Mean	sd	Minimum	Maximum
2	162.3	126.7	21	400
3	98.2	38.4	57	177
4	51.8	44.1	6	134
5	93.2	52.1	41	235

to ejaculation and the sperm numbers in cups served to explain the variation in viability and motility parameters examined with analysis of least squares. Because it was not possible to collect all ejaculates from the Standardbred stallion, his results were not included in all analyses.

RESULTS

The average number of mounts was 2 (min 1 and max 3). The average time to ejaculation was 2.5 min (min 10 s and max 9 min). When time to ejaculation increased by 1 min, viabilility and motility decreased by 1%. Almost 2/3 of the volume of Cup 4 was gel. Sperm concentration was highest in Cup 2 and lowest in Cup 4 (Table 1). There were significant differences between stallions in TOTMOT, PROGMOT and VAP. Centrifugation had a highly significant negative effect on all motility parameters, but the effect was not of the same magnitude in all cups. The difference between centrifuged and non-centrifuged samples was smallest in Cup 4. For TOTMOT, PROGMOT and %RAP, the values decreased in the following order: Cup 3 was always best, the total ejaculate second best, then Cup 4, Cup 2 and the worst one was Cup 1. However, these differences did not reach statistical significance. The viability was significantly lower in ejaculates where pre-sperm fraction was not separated in Cup 1.

DISCUSSION

This was a pilot study carried out using a small number of stallions. However, the results suggest that the pre-sperm fraction (Cup 1) might be detrimental to the survival of spermatozoa during long term storage. It was not possible to record the exact amount, but some of this fluid ended up in Cup 2 which may explain the lower motility in Cup 2, compared to Cup 3. During natural covering, the pre-sperm fraction probably does not gain entrance into the uterus because it precedes the actual ejaculation. In artificial insemination, we should perhaps avoid mixing pre-sperm fraction with the sperm that is going to be preserved. However, the accuracy of semen evaluation methods used and their correlation with fertility are not very well established. More stallions need to be examined and mares inseminated before any definite conclusions can be drawn.

It was expected that the post sperm fraction (Cup 4) would show lower motility than the sperm-rich fractions. The small difference between centrifuged and non-centrifuged samples of Cup 4 suggests that seminal plasma of Cup 4 decreased motility parameters to equal the centrifuged samples. Since the last part of the ejaculate also has low sperm concentration, its presence in preserved semen does not improve its quality.

It was somewhat unexpected that the centrifugation had such a strong negative effect on motility. It has been recommended as a method to remove seminal plasma and to improve motility during storage. In the study by Jasko *et al.* (1991), there was no difference in sperm motility after 24 h of cooled storage between centrifuged and non-centrifuged samples. One explanation for the discrepancy could be centrifugation for too long and at too high a speed. We used 500 *g* for 15 min, whereas others have centrifuged for 9–10 min at 400 g (Jasko *et al.* 1992; Pruitt *et al.* 1993). Also some stallions may be more sensitive than others. However, it is possible that the centrifugation did not lower motility but the absence of all seminal plasma. Jasko *et al.* (1992) noticed that when all seminal plasma was removed, motility decreased if the centrifuged sperm were diluted with skim milk extender. The addition of egg yolk improved sperm motion characteristics of centrifuged sperm over 48 h of cooled storage. The purpose of the centrifuged controls in our study was to evaluate whether seminal plasma had positive or negative effects by comparing non-centrifuged contents of cups to corresponding centrifuged samples. It would have been useful to have had another control where centrifuged spermatozoa could have been mixed again with corresponding seminal

plasma fractions.

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CRYOPRESERVATION OF STALLION SPERMATOZOA USING A DIRECTIONAL FREEZING TECHNIQUE

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**State Stud of Lower Saxony, Celle; †IMT Ltd, Merhavia, Israel; **Clinic for Horses, Veterinary School Hanover; ‡Institute for Reproductive Medicine, Veterinary School Hanover, Germany*

INTRODUCTION

According to Mazur *et al* (1972) cells can be damaged either by the formation of intracellular ice crystals when cooling takes place too rapidly or by chemical toxicity or osmotic stress as well as mechanical damage brought about by phase separation of solution and crystal growth. From this point of view cooling rate respiratory freezing velocity is an important regulatory factor during cryopreservation of spermatozoa (Mazur 1984; Watson 2000). In addition, the rate of cooling affects the morphology of the intercellular ice crystals and micro-architectural orientation of cells inbetween unfrozen channels and plates of ice (Holt 2000). Thus, maximising the survival rate of cells subjected to freezing and thawing requires careful control of the freezing process.

The conventional slow-freezing method involves lowering the temperature of the chamber a controlled stepwise manner. This method is based on changing temperature in a time unit by moving heat in multiple directions (equiaxial), depending on the thermal conductivity and geometrical shape of the container and of the biological material. The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials within the sample, and is not explicitly controllable. In addition, temperature gradient within the freezing chamber and non-reliable temperature recording measurements (Koebe *et al.* 1993) make it more difficult to achieve the optimal cooling rate. Commercially available systems to freeze stallion spermatozoa enable freezing of a large number of straws, but synchrony of sample and chamber temperature is critical. This affects the ability to characterise the freezing protocol accurately.

The work in this study introduced a new technique for rapid cooling of stallion spermatozoa under controlled thermal conditions (Arav 1999).

MATERIALS AND METHODS

A new freezing method, Multigradient Directional Cooling and Warming of Biological Samples (Arav 1999; IMT), aims to overcome the problems of commercially available freezing systems and to allow cryopreservation of large volume samples.

The IMT freezing technology is based on directional freezing in which the biological material is transferred through a linear temperature gradient so that cooling rate and ice front propagation are controlled precisely.

The technology is based on a series of copper blocks arranged in a line, with a straight track running through the blocks. Each side of a block can have different temperatures (T1, T2, T3, T4 in Fig 1), thereby imposing a temperature gradient on the portion of the track contained in the block (G1, G3). The blocks are separated by a gap (d2), and the temperature of the block on one side of the gap (T2) typically is different from the temperature on the other side of the gap (T3), thereby imposing a temperature gradient across the gap (G2). Biological samples to be frozen or thawed are placed inside straws, and the

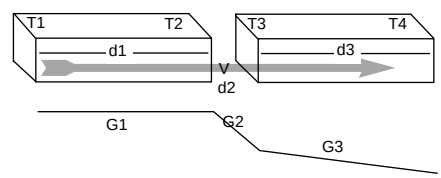

Fig 1: Directional freezing (schematic).

straws are moved along the track at velocities (V) such that the samples are frozen or thawed at rates (R) specified by protocols specific to the samples.

Cooling rate in this technology is determined by multiplying the temperature gradient by the velocity in which the sample is moved through this gradient (the velocity for each gradient must be constant):

Cooling rate (R) = Gradient (G) x Velocity (V)

The gradient (G) is a function of the difference in temperature (DT) and the distance between the 2 points (d):

Gradient (G) = Temp. difference (ΔT) / Distance (d)

This gives us the following formula for cooling rate at each freezing process:

$$R_{1,2,3} = \Delta T_{1,2,3} / d_{1,2,3} \times V_{1,2,3}$$

According to this formula, we can determine cooling rate with different combinations of (G)s, (ΔT)s and (d)s. In all cases, cooling rate will be very precise and, more importantly, identical in each point of the sample.

A total of 13 warmblood stallions with 3 ejaculates were included in the experiments outside of the breeding season. Semen was diluted in modified skim milk extender to a final concentration of 50 x 10^6 spermatozoa/ml and centrifuged for 10 min at 600 *g*. After decanting the supernatant, sperm pellets were suspended in skim milk extender containing 2% egg yolk and 2.5% glycerol. Final concentration of spermatozoa was 200 x 10^6 spermatozoa/ml. Semen was packaged in 0.5 ml straws automatically and frozen with the new technique using different temperature gradients (A; G1 5°C→G3 -50°C; B: G1 17°C→G3 -50°C; C: G1 5°C→G3 -20°C) and 5 freezing velocities (0.9 mm/s; 1.2 mm/s; 1.5 mm/s; 1.8 mm/s and 2.3 mm/s respectively 1,350°C/min to 9,246°C/min. Thawing was done at 37°C for 30 s using a waterbath.

Evaluation parameters were sperm motility (determined by computervideomicrography by MIKA-Motion-Analyser, Montreux, CH) and plasma membrane integrity evaluated by CFDA-PI fluorescence staining. All statistical comparisons were made using SAS statistics package. Split sample model was used to compare means (± sem) by paired t-test. P-values ≤0.05, ≤0.01 and ≤0.001 were considered significant.

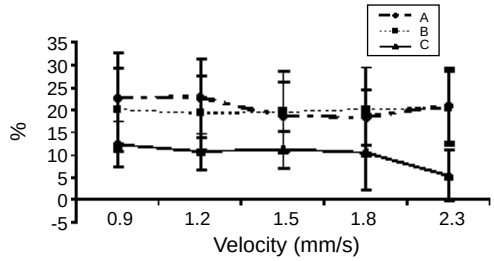

Fig 2: Progressive motility (%) of frozen/thawed stallion spermatozoa with the use of IMT-Multigradient Directional Cooling technique (Arav 1999). Results demonstrate 3 temperature gradients and 5 freezing velocities within each gradient (A: G1 5°C→G3 -50°C; B: G1 17°C→G3 –50°C; C:G1 5°C→G3 –20°C) (A:C, B:C, P≤0,001).

RESULTS

There was an increased tendency towards slower velocities in post thaw motility of spermatozoa using Gradient A and C. No differences were detected in the post thaw motility of the spermatozoa of the Gradient B and its 5 freezing velocities (Fig 2).

Comparing 3 temperature gradients with regard to the sperm motility of the spermatozoa there were only small differences between Gradient A and Gradient B. When compared with Gradient C, both wider range temperature gradients showed significant better results.

No difference in morphology of the spermatozoa was seen between the semen samples frozen with the 5 freezing rates of the Gradient A.

There were significant intra-individual differences between the 3 ejaculates per stallion, as well as significant inter-individual differences.

DISCUSSION

Systems for freezing semen using computerised nitrogen vapour freezers with specific pre-programmed freeze rates, as well as styrofoam boxes freezing semen in static nitrogen vapour, have been commercially available for years. Both systems were developed to handle large amounts of samples by a standardised method, and both should allow freezing at pre-determined cooling rates. Unfortunately, with the use of these systems, sample temperature does not follow chamber

temperature synchronously during the freezing process. This phenomenon is described as the freezing point plateau period between freezing and reappearance of cooling as a result of heat release and subsequent temperature increase of sperm samples (Holt 2000). Bwanga *et al.* (1991) observed that the freezing point plateau is detrimental to sperm survival in boars. Reduction of freezing point plateau, which lasts normally 2–3 min, improved the fertility of bull semen (Parkinson and Whitfield 1987). The method demonstrated with stallion spermatozoa may solve the problem of freezing point plateau by both automatically induced seeding of sperm samples and specific directional cooling. Directional cooling vs commercially available freezing systems may optimise synchrony of sample and 'chamber' temperature during the freezing process.

It has been empirically determined that stallion spermatozoa are generally frozen in the range of 15–60°C/min. Cryobiological research indicates that if cell water permeability and its activation energy were known, it should be possible to predict the maximal cooling rate; these theoretically calculated rates are much higher than is known empirically to be optimal (Curry *et al.* 1994). Watson (2000) explains this discrepancy by errors in the assumptions on which the theoretical calculations are based. Nevertheless, the laterally varying gradient in IMT's technology allows cooling at different rates in different temperature regimes, thereby allowing fully controlled nucleation at the freezing front. Using this technique, the problems listed below are solved. With respect to thermal history, cooling rate and thermal gradient through every point in the sample are very similar or almost identical. Temperature is constant and pre-determined and therefore the cooling rate resolution is dependent on the resolution of the sample propagation (V) through the linear thermal gradient. The apparatus controls ice crystal propagation by changing thermal gradient or interface velocity.

To summarise, the new technique is of interest for establishing optimal crypreservation protocols through sytematic studies of rapid cooling of spermatozoa and may play a role in commercial cryopreservation of stallion spermatozoa.

Acknowledgements

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THE ROLE OF LIPID DYNAMICS IN EQUINE SPERM PLASMA MEMBRANE FUNCTION

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The sperm plasma membrane plays a critical role in regulating sperm-egg interaction and, for this reason, it is an extremely dynamic structure. Sperm-oocyte interaction can be subdivided into a sequence of events. First, ejaculated sperm must be activated in the female genital tract in a process called capacitation because only capacitated sperm can bind to the zona pellucida. This binding of sperm to oocyte in turn induces changes in the sperm, collectively termed the acrosome reaction It is only relatively recently that the heterogeneity of the sperm plasma membrane has become clear (for review see Flesch and Gadella 2000). The dramatic differences in different regions clearly relate to physiological specialisations of the plasma membrane: i) the apical tip of the sperm cell is the unique site which initiates sperm-zona binding, whereas, such binding should not take place at other sites of the sperm sutrface because it prevents effective zona penetration; ii) the zona pellucida induced acrosome reaction is a result of multiple fusions between the sperm plasma membrane and the outer acrosomal membrane. The secretory event required for digestion of the zona pellucida is restricted to the apical plasma membrane area, whereas the equatorial area remains intact; and iii) exactly this equatorial sperm head surface area is responsible for the final binding and fusion with the oolemma (ie the fertilisation of the oocyte). Therefore, the composition and topological organisation of the plasma membrane regulate its affinity for adhesion factors, its permeability to hydrophilic solutes and its role in cell signalling and cell fusion events (Flesch and Gadella 2000; Gadella and Harrison 2000). This abstract will focus on the major structural and compositional changes of lipids in the plasma membrane of capacitating equine sperm cells.

Equine sperm cells (like other mammalian sperm cells) have an unusual composition and organisation of lipids in the plasma membrane. Stallion sperm contains approximately 57% phospholipids, 37% cholesterol and 6% glycolipids (see Table 1). Most components do not change upon capacitation, with the exception of

TABLE 1: Lipid class composition in stallion sperm cells

Cholesterol	37.4%[2]
Phosphatidylcholine (PC)	23.2%[1]
Phosphatidylethanolamine (PE)	8.0%[1]
Phosphatidylserine (PS)	7.5%[1]
Sphingomyeline (SPH)	6.4%[1]
Seminolipid (SGG)	6.2%[1]
Phosphatidylglycerol (PG)	2.5%[1]
Phosphatidylinositol (PI)	1.5%[1]
Unknowns	7.3%[1]

Capacitation in presence of albumin resulted in:

[1]No reduction in absolute amounts of phospholipids and seminolipid
[2]Cholesterol depletion of up to 45%

TABLE 2: Phospholipid species composition in stallion sperm cells[1]

	PC (%)	PE (%)
16:0-22:5 Alkylacyl	30.5	57.8
16:0-22:5 Plasmalogen	18.2	5.8
16:0-22:5 Diacyl	9.6	6.4
14:0-16:0 Diacyl	8.4	tr
16:0-20:4 Plasmalogen	4.8	3.5
18:0-20:4 Diacyl	0.6	5.8
16:0-20:4 Diacyl	0.5	4.6
Others	27.4	27.4

[1]No reduction in absolute amounts of phospholipid species after capacitation

cholesterol, which is depleted from the plasma membrane during this process. The plasma membrane is overloaded with polyunsaturated phospholipids (see Table 2) and basically these phospholipid species can compensate such loss of cholesterol; the polyunsaturated phospholipids are more flexible than saturated phospholipids and can therefore maintain the dynamic lipid bilayer characteristics of this membrane. The composition of phopsholipid species in the capacitating sperm cells does not change significantly (Table 2).

The release of cholesterol from the equine sperm plasma membrane could be visualised using probes that complex with unesterified sterols (analogous to porcine sperm; see Flesch *et al.* 2001). In freshly ejaculated sperm these complexes are distributed over the entire sperm head, but a lower density is apparent in the post equatorial segment. Following capacitation in the absence of albumin, the post equatorial region becomes devoid of these complexes whereas the amount in the apical region increases slightly. However, inclusion of albumin in the capacitation medium resulted in a dramatic decrease of cholesterol-filipin complexes (Gadella *et al.* 2001), which is due to the albumin mediated depletion of surface cholesterol in the responsive cells (ie only in the cells with apical clustered cholesterol).

As in somatic cells, the lipids of the sperm plasma membrane are distributed asymmetrically across the lipid bilayer. More specifically, choline phospholipids, sphingomyelin and, to a lesser extent, phosphatidylcholine are found predominantly in the outer lipid leaflet (facing the extracellular fluids), whereas the amino-phospholipids phosphatidylethanolamine and, especially, phosphatidylserine are located in the inner leaflet (ie in contact with cytosol; Gadella *et al.* 1999). Bicarbonate, a potent capacitation factor which is absent from seminal plasma (<1mM) but highly enriched in the oviduct (>15mM), is known to induce a cAMP mediated activation of protein kinase A which, in turn, affects the plasma membrane fluidity (Harrison 1996). Bicarbonate induced scrambling of the above mentioned phospholipid asymmetry in stallion and other mammalian sperm (ie mixing of outer and inner leaflet lipids; Gadella and Harrison 2000, 2001; Gadella *et al.* 2001; Rathi *et al.* 2001) occurs only in the apical plasma membrane and is a pre-requisite for the above mentioned cholesterol efflux (Flesch *et al.* 2001; Rathi *et al.* 2001).

Mammalian sperm are prone to oxidative stress. The resultant oxidative attack is believed to regulate sperm function in 2 ways: i) beneficial - mild peroxidation; and ii) detrimental - excessive peroxidation. Unfortunately, although the detrimental effects of lipid peroxidation for sperm have been described extensively, the importance of peroxidation for normal fertilisation remains to be investigated. A specific fluorescent probe was used to follow lipid peroxidation in the plasma membrane of living equine sperm using flow cytometry and confocal microscopy. The probe in its intact state fluoresces red but, after peroxidation, it fluoresces green (Pap *et al.* 2000). Preliminary results showed that lipid peroxidation was very low in ejaculated as well as capacitated sperm samples. This observation is supported by the fact that the phospholipid species composition did not alter upon capacitation (Table 2), whereas the polyunsaturated species are notorious for their extreme sensitivity for peroxidation. However, after freeze-thawing, a substantial subpopulation of stallion sperm showed probe peroxidation. The probe was applied randomly over the entire sperm surface but peroxidation was restricted to the mid-piece region. This suggested that sperm lipid peroxidation is a result of freeze-thaw damage to mitochondria which, in turn, produces reactive oxygen and attacks the closest available substrates (the mid-piece polyunsaturated fatty acid chains). The effects of these detrimental reactions is under investigation but are likely to impair the capacitation process as well as the fertility potential of sperm cells. A better understanding of how the sperm plasma membrane is organised will help greatly in understanding how we can protect the sperm cell against functionally disastrous damage during semen preservation.

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SESSION II:

Stallion fertility

Chairman: T. Katila

SOME OBSERVATIONS ON THE PUBERTY OF STALLIONS AFTER LONG TERM ADMINISTRATION OF hCG

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In human and veterinary medicine, hCG is used to treat delayed puberty and cryptorchismus.

The aim of this work was to determine the possible effects of long term administration of hCG on the reproductive functions of stallions during puberty. Nine Polish pony stallions, which were half-brothers, aged 5–7 months, were used in the study. All stallions from one stud were divided into 2 groups (5 stallions – Experimental Group; 4 stallions – Control Group). The stallions from the Experimental Group were given hCG (2,000 iu, Biogonadyl, Polfa, Poland) 3 times a week for 16 weeks. During this time, the sexual behaviour of all 9 stallions was checked with a mare in oestrus. Attempts at collecting semen by artificial vagina were made if the stallion showed copulative behaviour. The following parameters were determined in the collected semen: quantity of the ejaculate; total motility; progressive motility; concentration; total number of spermatozooa per ejaculate; and morphology. While hCG was being given to the stallions, plasma testosterone was measured once a week for the period of 16 weeks and after that once a month until the stallions reached 24 months of age. Then the daily sperm production was compared between stallions from the Experimental and Control Groups. The semen was collected once a month until the stallions reached the age of 30 months when they were castrated. Testes and other parts of the epididymis were homogenised to determine testicular and epididymal sperm reserves.

The symptoms of sexual behaviour occurred sooner in the stallions from the Experimental Group. There was no significant difference between the experimental and control stallions in quantity and quality of the ejaculate, daily sperm production or testicular and epididymal sperm reserves. However, in the treated stallions a tendency towards increased concentration and total number of spermatozooa in first ejaculates was observed. Testosterone concentration increased significantly in the first 2 weeks of hCG treatment but this difference disappeared 3–5 weeks after the first injection of hCG.

PROGESTERONE INDUCTION OF THE ACROSOME REACTION IN STALLION SPERMATOZOA IS MEDIATED BY A PROTEIN TYROSINE KINASE DEPENDENT PATHWAY

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INTRODUCTION

Progesterone (P_4) is a physiological inducer of the acrosome reaction (AR) in stallion spermatozoa. However, the capacitation-dependent changes that enable progesterone to bind to the sperm plasma membrane and the nature of the P_4-triggered pathway that results in the AR are poorly understood. The aim of the current study was to investigate the role of protein kinases in P_4-mediated induction of the AR in stallion spermatozoa and to determine whether bicarbonate, an inducer of sperm capacitation, acts via the same pathway as P_4 or otherwise synergises the effects of P_4.

MATERIALS AND METHODS

To determine whether protein kinases are involved in P_4 and/or bicarbonate-mediated induction of the AR, we examined the effects on AR progression of specific inhibitors and stimulators of protein kinase A (PKA), protein kinase C (PKC), and protein tyrosine kinase (PTK), in the presence and absence of 15mM bicarbonate and 1 µg/ml P_4. The basic incubation medium used was Tyrode's medium (Tyr: Parrish *et al.* 1988) with the osmolality maintained at around 300 mOsm and pH at around 7.2. The status of the acrosomal membranes was assessed using FITC-PNA; sperm whose caps fluoresced brightly were considered to be acrosome-intact and those with a non-fluorescent cap were classified as acrosome-reacted.

Effect of PKA stimulators and inhibitors on the AR

A portion of one ejaculate from each of 5 stallions was diluted in Tyr and divided into 2 sets of 4 x 1 ml samples. One sample from each set served as a control and the other 3 were treated respectively with H89 (50 nM; a specific PKA inhibitor), DDA (100 µM; a specific adenylate cyclase inhibitor) and 8Br-cAMP (100 µM; a potent PKA stimulator). The samples were incubated at 38°C for 5 min after which 20 µl of a 750 mM bicarbonate solution was added to one set (which became Tyr+bic samples). The samples were incubated further for 5 h at 38°C in humidified air containing 5% CO_2. At the end of the incubation, 500 µl aliquots of each sample were transferred into tubes containing 5 µl of 0.318 mM P_4 (final [P_4] = 1 µg/ml: Harrison *et al.* 2000) thus yielding a total of 8 samples with and 8 without P_4, per ejaculate. All 16 samples were incubated for a further 20 min before staining and assessment of the AR.

Effect of PTK and PKC inhibitors on the AR

A portion of one ejaculate from each of 5 stallions was diluted with Tyr and split into 5 x 1 ml samples. One sample served as a control and 3 others were treated respectively with Erbstatin (10 µM; a PTK-inhibitor) and Staurosporin at concentrations of 50 nM, when it is solely a PKC inhibitor (Breitbart *et al.* 1992), and 2 µM, when it inhibits most serine/threonine kinases (Wang *et al.* 1997). As in Experiment 1, samples were incubated for 5 min before the addition of bicarbonate and then for a further 5 h at 38°C in humidified air containing 5% CO_2. Thereafter, 500 µl aliquots of each sample (except the controls) were transferred into tubes containing 5 µl 0.318 mM P_4. The 9 resulting samples were incubated for a further 20 min before staining and AR assessment.

RESULTS

P_4 induces the AR in a PKA independent manner

During a 5 h incubation in control medium (Tyr), 36 ± 4% of sperm cells underwent AR. The presence of 15 mM bicarbonate (Tyr+bic) in the incubant resulted in significantly more (12%) sperm acrosome reacting, whereas the addition of 1 µg/ml P_4 increased the rate of AR in both Tyr (by 5%) and Tyr+bic (by 18%; $P<0.05$) media relative to the control. The PKA-pathway blockers (H89 and DDA) suppressed the stimulatory effect of bicarbonate, but not that of P_4, on the AR. Conversely, the PKA stimulator 8Br-cAMP induced a 20% increase in the percentage of sperm undergoing AR in both Tyr and Tyr+bic; this stimulatory effect was slightly (but not significantly) increased by the addition of P_4.

Involvement of other kinases in P_4-induced AR

Similar to Experiment 1, 39 ± 5% of sperm in control samples (Tyr) acrosome reacted. The presence of bicarbonate (Tyr+bic) increased the AR rate by 12% and the addition of P_4 resulted in a further 23% increase in the number of acrosome reacted sperm. Erbstatin (a PTK inhibitor) had no effect on bicarbonate induction of the AR but suppressed the stimulatory effect of P_4. Similarly, staurosporin at PKC blocking concentrations only partially reduced the stimulatory effect of bicarbonate on the AR rate (from 12% to 4% above control values) but completely blocked the effect of P_4. At higher concentrations, when it blocks a wide range of kinases, staurosporin completely suppressed the stimulatory effects of both bicarbonate and P_4.

CONCLUSIONS

As anticipated, the addition of bicarbonate to the incubation medium significantly increased the proportion of stallion sperm that acrosome reacted during a 5 h incubation. Because this bicarbonate-induced stimulation of the AR was blocked completely by PKA inhibitors, it is concluded that PKA plays an important role in mediating the effect of bicarbonate on the AR in stallion sperm. However, because the PKA stimulator 8BrcAMP induced a greater increase in the proportion of sperm undergoing the AR than bicarbonate, it appears that the bicarbonate concentrations used in the current study did not maximally stimulate the PKA pathway. On the other hand, PKC and PTK do not appear to play significant roles in bicarbonate-mediated induction of the AR because neither erbastatin (a PTK inhibitor) nor PKC-blocking concentrations of staurosporin markedly inhibited the effect of bicarbonate. And although higher concentrations of staurosporin did prevent the stimulatory effects of bicarbonate, at these levels staurosporin blocks the activity of a range of protein kinases (Wang *et al.* 1997).

Against expectations, P_4 had a stimulatory effect on the AR that was independent of bicarbonate. Furthermore, the combination of bicarbonate and P_4 induced the AR in a significantly greater proportion of sperm than either alone. These findings suggest that the effects of P_4 and bicarbonate on the AR in stallion sperm are mediated primarily by independent signalling pathways. Further proof that P_4 exerts its AR stimulating effects via a different intracellular signalling pathway than bicarbonate was provided by the findings that PKA-blockers did not inhibit P_4-mediated induction of the AR but that PTK and PKC blockers did; the opposite was true for bicarbonate. The apparent involvement of protein kinases in P_4-mediated induction of the AR is very similar to that reported for ZP-mediated induction of the AR. In the case of ZP, Breitbart and Naor (1999) concluded that ZP3 activates a sperm PTK that is coupled to phospholipase C (PLC) and which, in turn, stimulates a PKC by generating diacylglycerol (DAG) from phosphoinositol-bisphosphate (PIP_2). It is proposed that P_4 acts in a similar fashion.

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VIDEOENDOSCOPIC LOW-DOSE UTEROTUBAL INSEMINATION IN THE MARE

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At natural mating the average fertile stallion ejaculates 2–12 billion spermatozoa directly into the body of the oestrous mare's uterus via the very relaxed cervix. Yet fewer than 100 spermatozoa pass through the uterotubal junction at the tip of the uterine horn to reach the site of fertilisation at the ampullary-isthmic junction of the oviduct to give per cycle conception rates of 60–70%. Sex selection at the time of fertilisation has been a Holy Grail of animal reproduction for centuries and it is now a reality by means of high speed fluorescence-activated cell sorting (FACS) of spermatozoa into X- and Y-chromosome bearing populations. However, the maximum rate of sorting still-viable stallion spermatozoa is well below the 300 million motile spermatozoa required for conventional uterine body insemination in the mare. Therefore, minimum dose insemination methods are needed to bridge the gap.

Our earlier studies demonstrated high per cycle conception rates when depositing stallion spermatozoa directly onto the uterotubal papilla. Rates of 65–70% were achieved when 10, 5 or 1 million spermatozoa suspended in 80–150 µl TALP medium were placed at the tip of the uterine horn ipsilateral to the ovary containing a ripening pre-ovulatory follicle. The conception rate fell sharply when the dose of spermatozoa was reduced to below 1 million. Subsequent experiments have yielded satisfactory per cycle conception rates of 55–70% when inseminating mares with 5 million frozen-thawed spermatozoa and 30–50% with 5 million FACS-separated sex-sorted spermatozoa. On the other hand, disappointing rates of <20% have resulted from uterotubal insemination of pre-ovulatory mares with 5–300 million epididymal spermatozoa recovered from the testes of 18–30-month-old maiden Thoroughbred colts undergoing routine standing castration for management reasons.

The videoendoscopic low-dose uterotubal insemination technique is a major advance in equine stud medicine. It enables satisfactory conception rates to be achieved in mares inseminated with much lower numbers of fresh or cooled stallion spermatozoa than was hitherto possible, with low numbers of relatively poor quality frozen-thawed spermatozoa and with low numbers of FACS-sorted gender selected spermatozoa. Further technological improvements are required to increase the simplicity, and reduce the size and price of, the videoendoscope, thereby enabling the method to be transposed directly to the studfarm.

EFFECTS OF SOMATOSTATIN ON GROWTH HORMONE RELEASE AND SEMEN PARAMETERS IN PONY STALLIONS

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In males and females, the somatotrophic axis influences fertility, and growth hormone (GH) has been shown to affect gonadal function. GH release from the pituitary is regulated via the neurohormones GH releasing hormone and somatostatin. GH release is influenced by age, reproductive status and such environmental factors as physical activity, stress and nutrition. In the male, GH may stimulate testicular steroid biosynthesis and gametogenesis. These effects can be attributed to a GH-induced release of LH and FSH and to an increase in the synthesis and release of insulin-like growth factor-1 (IGF-1). In men, certain forms of gonadal dysfunction with reduced spermatozoal motility can be attributed to low GH concentrations and have been treated successfully with exogenous GH.

MATERIALS AND METHODS

This study was performed in 6 Shetland stallions, aged 5–7 years. Seasonal variations in GH and testosterone release and the effects of a 10 day treatment with the somatostatin analogue octreotid (Novartis, Basle, Switzerland; 100 mg twice daily) on GH release, testicular response to hCG and semen parameters was investigated. Stallions served as their own controls and were treated with saline and octreotid at different time periods 10 weeks apart. Half of the animals received octreotid first and half of them saline first. Blood was taken twice daily for determination of GH and semen was collected at daily intervals. To assess testicular response to LH, an hCG stimulation test (3,000 IU iv) was performed on the last day of octreotid administration. GH was determined by RIA with an antiserum raised against porcine GH and cross-reacting 95% with equine GH. Testosterone was measured with a commercially available EIA (Serono, Vienna, Austria). Statistical comparisons were made with the SPSS statistics package using non-parametrical tests (Friedman test, Wilcoxon test).

RESULTS

Seasonal variations

No seasonal variations in plasma GH concentrations were found, whereas marked variations were found for testosterone (Table 1)

Effects of octreotid on GH release

Octreotid did not affect GH release significantly, although plasma GH concentrations tended to increase. There was no difference between GH concentrations in the morning and evening. During octreotid treatment, GH concentrations showed pronounced variations, whereas in saline treated animals GH values were stable.

TABLE 1: Plasma GH and testosterone concentrations (ng/ml) in stallions during different months

	15th January	15th April	15th July	15th October
Testosterone (ng/ml)	0.2 ± 0.1^{a}	1.4 ± 0.3^{b}	0.7 ± 0.2^{c}	0.7 ± 0.3^{c}
GH (ng/ml)	1.0 ± 0.1	0.9 ± 0.3	0.9 ± 0.2	1.2 ± 0.3

$^{a, b, c}$: values differ significantly ($P<0.05$)

TABLE 2: Semen parameters in stallions before and after 10 days of octreotid or saline treatment

		Day 0 (before treatment)	Day 1 after treatment	Day 3 after treatment	Day 5 after treatment
Progressive motility (%)	Octreotid	50.0 ± 3.7	28.7 ± 6.8	18.5 ± 5.4	35.0 ± 8.5
	Saline	46.7 ± 9.2	38.3 ± 6.5	41.7 ± 5.4	41.7 ± 7.5
Concentration (10^6/ml)	Octreotid	255.2 ± 33.2	376.5 ± 45.9	310.3 ± 51.8	195.3 ± 37.7
	Saline	303.0 ± 44.5	332.8 ± 42.0	274.2 ± 21.8	212.0 ± 15.6
Total sperm count (10^9)	Octreotid	3.2 ± 0.7	3.2 ± 0.4	2.2 ± 0.4	1.6 ± 0.4
	Saline	3.5 ± 0.7	3.4 ± 0.5	2.0 ± 0.4	1.8 ± 0.3

Effect of octreotid on semen parameters

Following the 10 day octreotid treatment, sperm motility was decreased significantly compared to pre-treatment values and saline-treated controls ($P<0.01$, Table 2). The lowest percentage of progressively motile spermatozoa (18.3 ± 5.4%) was found on Day 3 after the 10 day octreotid treatment. Motility had returned to pre-octreotid levels approximately 10 days after the end of treatment. Sperm motility did not change significantly in saline-treated stallions. No differences between octreotid and saline-treated animals were found for ejaculate volume, sperm concentration, total sperm count, daily sperm production and percentage of morphologically normal spermatozoa.

Effect of octreotid on hCG-induced testosterone release

After both saline and octreotid pre-treatment, injection of hCG was followed by a significant ($P<0.05$) increase in plasma testosterone concentrations. However, testosterone release in response to exogenous hCG was lower when animals had been pre-treated with octreotid than after saline pre-treatment (area under the curve for the time period 0–120 min after hCG: octreotid 8.7 ± 2.6 ng/ml 15 min, saline 16.0 ± 5.8 ng/ml 15 min; testosterone concentration 45 min after hCG: octreotid pre-treatment 2.8 ± 1.1 ng/ml, saline pre-treatment 4.0 ± 1.0 ng/ml, $P<0.05$).

DISCUSSION AND CONCLUSIONS

No seasonal variations in GH release were found in stallions. GH, in many species, is stimulated by gonadal steroids. Although there were seasonal variations in plasma testosterone concentrations, testosterone at all times was sufficient to stimulate a high rate of GH synthesis and release.

Octreotid did not cause a consistent reduction in GH concentrations. In man, octreotid is used to decrease a pathologically stimulated GH release. In the ponies, GH levels were normal which might explain why no reduction occurred. However, octreotid induced marked variations in plasma GH with occasionally high GH concentrations. Thus, octreotid does not always reduce GH release. Our data do not exclude the possibility that testicular GH levels were affected in a way not reflected by peripheral plasma levels.

Although GH concentrations in plasma were not reduced, octreotid had clear effects on testicular function. Sperm motility immediately after the treatment period was reduced and increased within a few days thereafter. These rapid changes indicate that octreotid influenced epididymal function but without major effects on spermatogenesis. Octreotid treatment for 10 days did not change basal plasma testosterone concentrations but reduced hCG-induced testosterone release. Effects on steroid biosynthesis can be caused either directly by GH acting on the testes, or via GH-induced changes of the IGF-1 system.

In conclusion, although the somatostatin analogue octreotid at the dose used had only minor effects on plasma GH levels, there were marked inhibitory effects on testicular function with a reduction in sperm motility and hCG-induced testosterone release. GH should not be considered as a routine treatment for subfertile stallions. However, it might improve semen quality in stallions where a partial GH deficiency exists. This may be the case in otherwise healthy ageing stallions with decreasing semen quality.

SESSION III:

Production and transfer of oocytes/embryos

Chairman: M. Tischner

THE USE OF A PROGESTERONE RELEASING INTRAVAGINAL DEVICE TO INDUCE OVARIAN ACTIVITY IN MARES

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INTRODUCTION

A primary goal of reproductive management of mares is to maximise production of live foals. Yet, only 56% of the 90.455, Thoroughbred mares bred in 1987 produced live foals (Bowen 1989).

It is clear that mares represent a seasonal species and they are naturally ready to breed from about the time of the spring solstice. The most effective approach to improving their breeding efficiency during March and April is to take advantage of the mare's response to light (Burkhard 1947; Okólski, 1981). Another approach involves the use of exogenous progesterone or synthetic progestagens. Early experiments by Loy *et al.* (1981, 1982) indicated that ovarian steroid withdrawal therapy hastened ovulation for mares in shallow transitional anoestrus. A number of studies have supported these findings and demonstrated that a range synthetic progestagens are effective in hastening the onset of oestrus. A variety of routes of administration have been used including im injection, oral and intra vaginal (Grimmett 1992).

The long and variable length of oestrus, and variation in the time when ovulation occurs during oestrus, has made reproductive management of mares time consuming, expensive and often unsuccessful. Therefore, an accurate and convenient method of controlling oestrus and ovulation in mares is of considerable importance to the equine breeding industry. It is now accepted that daily im injections of 150 mg progesterone and 10 mg estradiol for 10–15 days, combined with $PGF_{2\alpha}$ on the last day of the regime, produces accurate control of ovulation in cyclic (Loy *et al.* 1981), post partum (Loy *et al.* 1982), maiden and barren mares that have been maintained under increased photoperiods for at least 60 days (Taylor *et al.* 1982). Unfortunately, however, daily injections of progesterone and estradiol are inconvenient and time consuming.

In mares the growth of ovarian follicles and ovulation is often induced early in the breeding season by administering progesterone or its synthetic analogues in the form of insertions or saturated intravaginal sponges. In both mares and ruminants, the intravaginal preparation may cause inflammation and therefore horse breeders are reluctant to use it.

The purpose of the present study was to determine fertility of the mares after applying a progesterone releasing intravaginal device (PRID, Farmaceutici Gellini, Rome, Italy) early in the season. The PRID is a silicon spiral which consists of 1.55 g progesterone and 10 mg estradiol benzoate. It is licensed for use in cows but not in horses.

MATERIALS AND METHODS

Investigations were carried out on 35 trotter mares aged 4–23 years. In this group were 20 maiden, 12 barren and 3 foaling mares. The mares were checked routinely by rectal palpation, scanning, vaginal examination and also, when necessary, uterine swabs and uterine cytology. The devices were placed in the vagina of the mares in which several small ovarian follicles were found during the first weeks of the season but no dominating follicle occurred. Before the device was placed, each mare was palpated and scanned, the diameter of the largest follicle measured and the perineal area washed thoroughly. The PRID was removed after 10 days and the vagina was rinsed repeatedly with physiological saline and 2 ml $PGF_{2\alpha}$ analogue (Gabbrostim, Vetem) iu were given. The mares were inseminated with fresh semen and the insemination was repeated every 48 h until ovulation occurred. Ovulation was induced with 3,000 im HCG (Vetecor) given iv.

TABLE 1: Effects of treatment of anoestrus mares with PRID

No of mares	Follicle diameter (mm)		No. of days until	
	before inserting spiral	after removing spiral	AI	ovulation
35	20.2 ± 5.6	30.4 ± 4.7	7.0 ± 8.4	10.9 ± 10.3

TABLE 2: Number (%) of pregnant mares after applying PRID

Mares	Maiden 20	Barren 12	Foaling 3	Total 35
In first cycle	11 (55%)	5 (41.6%)	3 (100%)	19 (54%)
End of season	19 (95%)	11 (92%)	3 (100%)	33 (94.3%)

RESULTS

The average size of the largest follicle was 20.2 ± 5.6 mm on the day of spiral insertion. The dominating ovarian follicle was an average size of 30.4 ± 4.7 mm when the device was removed. Table 1 shows the mean number of days to first insemination and to ovulation. On average, ovulation occurred 10.9 days following PRID removal. The shortest interval from removal to ovulation was 2 days (one mare). Six mares ovulated after 3 days, 12 after 4–6 days, 10 after 7–10 days and 6 over 20 days after spiral removal.

In 29 of the 35 mares, the first AI was performed 3.7 ± 2.3 days and ovulation occurred 5.7 ± 2.4 days after removal of PRID. No side effects were observed.

Table 2 presents pregnancy rates in mares inseminated after PRID. During the first oestrus, pregnancy occurred in 11 (55%) maiden, 5 (41.6%) barren and 3 (100%) foaling mares. Ultrasound examinations at the end of the season confirmed pregnancy in over 90% of the treated mares.

DISCUSSION

Seasonal anoestrus in mares presents several managerial and economic difficulties for the horse breeding industry. Any method which reliably induces ovulation in mares during deep or shallow anoestrus could have significant commercial implications for the horse breeding industry.

The results of these experiments demonstrate that introduction of a PRID to mares during seasonal anoestrus induced follicular growth and ovulation in over 80% of mares. On the other hand, in 6 mares ovulation took place 26–41 days after spiral removal. In these mares, the dominant follicles were smaller at PRID insertion and removal than the average for other mares. It seems that the PRID is much more active in seasonal shallow anestrus than in deep anoestrus. Also Hyland *et. al.* (1987) clearly demonstrated that mares in shallow seasonal anoestrus are more likely to respond to GnRH infusion than those in deep seasonal anoestrus.

It is interesting that intravaginal spirals were also effective in suckling anestrous mares which did not show follicular growth for over 3 months after foaling. All 3 mares following PRID removal presented a dominant follicle. After insemination all became pregnant in the first cycle.

It is concluded that PRID is useful for those wishing to breed from mares early in the season.

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OOCYTE TRANSFER: PREGNANCY RATES USING OOCYTES COLLECTED AT 24 AND 36 HOURS AFTER INDUCTION OF OVULATION

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Since the first successful oocyte transfer in horses reported by Allen and Rowson in 1972, several groups have developed this technique for research and clinical applications. Oocytes are recovered by follicular aspiration through the flank or the vagina. Surgical transfer of the oocyte by ventral midline approach under general anaesthesia and flank laparotomy have been described. Studies comparing the pregnancy rate following transfer of oocytes collected 24–26 h after induction of ovulation, with or without oocyte culture between collection and transfer, have generated variable results.

In the present study, we compared pregnancy rates for oocytes collected 35 h after induction of ovulation and cultured 1–2 h before transfer (Group 1; n=24) and oocytes collected 24 h after induction of ovulation and cultured 20 h before transfer (Group 2; n=15). Recipient mares were synchronised with the donor mares and ovulation was induced at the same time for both. Transfer was performed by standing laparotomy and recipients were inseminated 24 h before, and immediately after, transfer. The oocyte from the pre-ovulatory follicle of recipients for Group 1 was collected during surgery and for Group 2 at 24 h after induction of ovulation.

The recovery rate of oocytes in the donor mares was 24/34 (71%) in Group 1 and 15/23 (65%) in Group 2. In the recipient mares in Group 1, 8/9 oocytes were recovered during surgery and 8 mares had ovulated at the time of exteriorisation of the ovary during surgery. In Group 2, 8/10 oocytes were recovered. In Group 1, 18 oocytes were transferred to 16 recipients and 6 mares were pregnant from transferred oocytes (38%) as determined by parentage determination on the 14-day-old embryo. In Group 2, 14 oocytes were transferred in 10 recipients and only one pregnancy was obtained (10%). In summary, 6 embryos were obtained from 18 transferred oocytes in Group 1 (33%) and one embryo from 14 oocytes in Group 2 (7%).

In conclusion, the high incidence of ovulation before oocyte recovery remains an important limiting factor for Group 1. More efficient systems for oocyte maturation need to be identified to increase the efficacy of transfer of oocytes collected 24 h after induction of ovulation.

MICROSURGICAL TRANSFER OF HORSE EMBRYOS

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INTRODUCTION

Artificial insemination techniques have radically influenced horse breeding and the method of selecting stock. Breeding of sport horses, in particular, is largely based on insemination with fresh or frozen semen. Embryo transfer, however, despite its potential benefit to selection programmes, has played a limited role so far.

This is due partly to the high costs involved and partly to unsatisfactory results following non-surgical embryo transfer. Surgical methods have resulted in improved rates of embryo survival but the technique is complicated and more traumatic for the recipient mare.

The aim of our work is to try to establish a technique of equine embryo transfer suitable for wide application within breeding practice.

MATERIALS AND METHODS

Warmblood mares aged 1.5–8 years, some of which were in active competition were used as donors. The recipient mares were Czech warmbloods aged 3–16 years and without reproduction problems. Their oestrous cycles were synchronised by a single administration of the synthetic prostaglandin F_{2a}. Folicular development and ovulation were monitored twice daily in donors, and once daily in recipients, by ultrasoud scanning of the ovaries. Donors were usually inseminated with frozen semen, although fresh semen was used on some occasions.

TABLE 1: Comparison of 3 microsurgical techniques with surgical and non-surgical methods

Method	No. of transfers	No. of pregnancies (%)
Manual	11	6 (55)
Endoscopy	7	3 (43)
Ultrasonography	4	2 (50)
TOTAL	**22**	**11 (50)**
Surgical	31	18 (72)
Non-surgical	43	14 (33)

Embryo recovery was carried out 180–204 h (non-surgical and surgical) or 168–192 h (microsurgical) after ovulation. Dulbecco's phosphate buffered medium was used for embryo flushing.

Embryos were transferred within 2 h of recovery by surgical, non-surgical or microsurgical methods.

The surgical method was carried out via the flank of standing mares treated by local anaesthesia. The wall of one uterine horn was perforated and an embryo introduced into the lumen via a glass capillary.

Non-surgical transfer was performed transcervically using a proprietary transfer gun under vaginal or rectal control.

Three methods of microsurgical technique were used: endoscopic, ultrasonographic and manual.

Using local anaesthesia and tranquilisation, a 60 cm non-flexible, long optical section of endoscope, tongs and a needle were introduced through the flank of standing mares into the abdominal cavity.

Ultrsasound guided transfer was carried out transvaginally. A transducer-needle guide was introduced by means of puncture of the vaginal wall to the abdominal cavity and, under manual control via the rectum, through the wall of the uterine horn to the lumen of the uterine horn.

Manual transfer used a long needle inserted into a metal guide with a handle at the cranial end. The technique of needle introduction into the

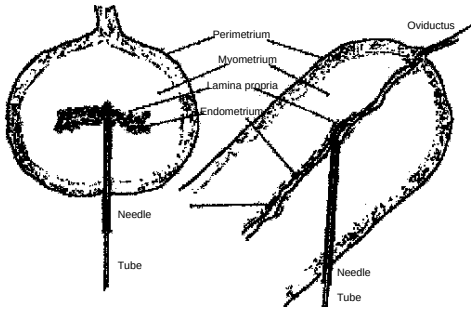

Fig 1: Microsurgical embryo transfer to mare's uterine horn.

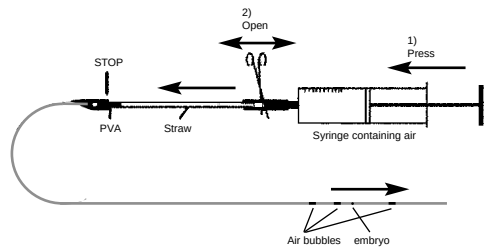

Fig 2: Schema of microsurgical set used for transfer of embryo into uterus horn lumen.

uterus horn lumen was carried out as for ultrasound guided transfer, although control was manual.

Crucial to any chosen method is the correct introduction of the embryo into the uterus lumen. A simple technique enabling double control of needle tip position in the lumen was designed.

Figure 1 illustrates this. A Teflon 1.2/0.8 mm tube containing the embryo is placed in the transfer needle. Smooth tube movement 2–3 cm over the needle mouth indicated needle tip position in the horn lumen.

Figure 2 shows a simple device designed for regulating embryo deposition into the uterus horn lumen. The embryo is ejected pneumatically from the tube by pressure of air in the syringe connected to the tube. An 0.25 ml straw containing a PVA stopper was inserted between the syringe and the Teflon tube. The syringe contained a small volume of liquid. If the tube mouth was located in the lumen, the syringe pressure ejected the embryo and medium. The liquid then passed through the inserted straw and closed the PVA stopper hermetically. The volume of the straw determined the volume of medium deposited in the uterus horn lumen.

Results

Results achieved by all 3 methods were compared with surgical and non-surgical methods of embryo transfer. The microsurgical techniques were tested on a limited number of mares and achieved pregnancy rates of 43–55%. This compares with 33% following non-surgical and 72% following surgical embryo transfers.

Discussion

Despite the fact that microsurgical methods of embryo transfer have been carried out on only a small number of animals so far, all 3 methods appear to be potentional alternatives to the currently used surgical and non-surgical methods.

LUTEAL ACTIVITY IN MARES AFTER NON-SURGICAL EMBRYO TRANSFER

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INTRODUCTION

Pregnancy rates attained after non-surgical transfer of equine embryos vary greatly between practitioners but, in most hands, the technique is less successful and less consistent than surgical transfer (see Squires *et al.* 1999 for review). Nevertheless, the results obtained in large-scale embryo transfer (ET) programmes in Argentina (80% pregnancy rate: eg Losino *et al.* 2000) demonstrate clearly that non-surgical transfer can produce high rates of success, given a degree of operator experience and careful selection and management of recipient mares. The most commonly proposed explanation for the relative failure of non-surgical ET is luteal insufficiency resulting from $PGF_{2\alpha}$ release induced by cervical and uterine trauma during transfer, or from contamination introduced into the uterus at this time. However, other aspects of embryo and recipient quality undoubtedly affect the success of ET. In the latter respect, there is good evidence in farm animal species that the maternal steroid hormone environment affects conceptus development and *vice versa.* For example, higher maternal blood progesterone concentrations enhance conceptus growth in cattle (Mann and Lamming 2001) and lower circulating oestradiol concentrations facilitate maternal recognition of pregnancy by weakening the luteolytic drive (Mann and Lamming 1995). Conversely, maternal recognition of pregnancy is itself a clear demonstration that the growing conceptus can dramatically influence maternal steroid hormone concentrations. In horses, it has been suggested that exogenous progesterone administration enhances conceptus growth (Weithenauer *et al.* 1986) and that the growing conceptus stimulates an increase in maternal luteal activity from as early as Day 8 after ovulation (Sevinga *et al.* 1999). However, neither of these hypotheses has been supported by previous or subsequent studies and both conclusions remain questionable. The aim of the current experiment was to examine the relationship between the success of non-surgical ET and luteal activity; and to determine whether ultrasonographic measurment of corpus luteum (CL) size is a reliable indicator of luteal function, given that it is often used as such in clinical practice.

MATERIALS AND METHODS

Fifty Dutch Warmblood mares maintained at grass were synchronised repeatedly in groups of donors and recipients. When the mares were in oestrus, their reproductive tract was examined daily using transrectal ultrasonography to monitor follicle development and to determine the day of ovulation. Donor mares were inseminated on alternate days during oestrus and, 7 days after ovulation, embryos were recovered from the uterus of these mares by non-surgical lavage with Dulbecco's PBS supplemented with 0.5% fetal calf serum. The embryos were transferred non-surgically using a sheathed embryo transfer pipette to the most suitable recipient available. Ideally, recipients had ovulated between 1 day before and 2 days after the donor because previous reports demonstrated that this 4 day period represents a window of acceptable synchronisation during which the likelihood of establishing pregnancy is highest (see Squires *et al.* 1999 for review). Luteal function was measured in terms of both peripheral plasma progesterone concentrations and maximum ultrasonographic cross-sectional surface area of the corpora lutea. For this, jugular vein plasma samples were collected daily during oestrus, until

TABLE 1: Mean (± sem) plasma progesterone concentrations and luteal surface areas in mares pregnant or non-pregnant after non-surgical embryo transfer

Stage	Progesterone concentrations (ng/ml)		Total luteal surface area (cm^2)	
	Pregnant (n=29)	Not pregnant (n=23)	Pregnant (n=29)	Not pregnant (n=23)
Day of transfer	8.9 ± 1.4	8.2 ± 1.3	11.7 ± 0.7	10.2 ± 0.6
Day 11 after ovulation	8.7 ± 0.7	8.0 ± 0.6	6.5 ± 0.8	6.7 ± 0.8
Day 15 after ovulation	7.9 ± 0.5	1.90 ± 0.5	5.4 ± 0.8	2.7 ± 0.6
Day 19 after ovulation	6.6 ± 0.5	0.5 ± 0.6	6.0 ± 0.6	1.5 ± 0.4

Progesterone concentrations did not differ between non-pregnant mares and pregnant mares until the onset of cyclical luteolysis. Luteal size was a good indicator of luteal function but tended to 'overestimate' progesterone concentrations at the time of transfer and during luteolysis.

Day 3 after ovulation and thereafter on alternate days until Day 19 after ovulation; progesterone concentrations were subsequently measured by radioimmunoassay. Corpus luteum surface area (cm^2) was measured on the day of embryo transfer and on Days 11, 15 and 19 after ovulation. In the case of multiple ovulation, all CLs were measured and the values were summed.

RESULTS

In total, 52 embryos were recovered and transferred to recipient mares that had ovulated between 2 days before and 4 days after their respective donor; 29 of the 52 (56%) recipient mares became pregnant. Mares that did not conceive were less likely to have been 'properly' synchronised with their donor; 27 of the 29 (93%) pregnant mares ovulated -1 to 2 days after their donor compared to 15 of 23 (65%) non-pregnant mares. These proportions were not altered if synchronisation was calculated on the basis of plasma progesterone concentrations rather than using ultrasound data. Only 2 of the non-pregnant mares showed evidence of premature luteolysis after ET; the remainder had a mean dioestrous length of 15.0 ± 0.4 days. One mare lost her pregnancy between Days 15 and 19, apparently as a result of failed maternal recognition of pregnancy because progesterone concentrations dropped below 1 ng/ml on Day 17. Peak progesterone concentrations varied greatly between mares, but there were no significant differences in the rate of the plasma progesterone rise or in the mean plasma progesterone concentrations between pregnant and non-pregnant mares until Day 13 after ovulation, when the non-pregnant mares began to undergo cyclical luteolysis. Similarly, mean luteal surface area did not differ between pregnant and non-pregnant mares until Day 15 after ovulation. Although luteal size and progesterone concentration on the day of ET tended to be higher in mares that became pregnant than in those that did not (Table 1) the differences were not statistically significant. Luteal surface area was a reasonably good indicator of systemic progesterone concentrations but it tended to 'overestimate' luteal activity early in dioestrus (ie at the time of ET) and during luteolysis when the fall in CL size was slower than that in plasma progesterone concentrations. Indeed, the CL was still clearly visible for 2–3 days after progesterone concentrations had reached baseline values.

CONCLUSIONS

In summary, luteal function, measured as plasma progesterone concentration or luteal surface area, did not differ between pregnant and non-pregnant mares until the onset of cyclical luteolysis. Therefore, transfer-induced luteolysis was not a significant cause of failed ET in this study. More often, failure to establish pregnancy appeared to result from inadequate donor-recipient synchrony (35% of failed transfer attempts). In the remainder of non-pregnant mares, poor embryo quality *per se* or damage inflicted to the embryo during the transfer procedure are the most likely causes of failure to establish pregnancy. Furthermore, we found no evidence that the presence of a viable conceptus affected progesterone concentrations other than by preventing the onset of cyclical luteolysis. Conversely, higher maternal progesterone concentrations did not improve the likelihood of a mare becoming pregnant after ET. Finally, although luteal surface area was a good estimator of peripheral progesterone concentration

in mares, it tended to over-estimate this parameter during luteolysis and must therefore be considered an unreliable indicator of luteal function in the instance of impending pregnancy loss.

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SESSION IV:

Oocyte maturation *in vitro*

Chairman: P. Daels

DISTRIBUTION, MORPHOLOGY AND ULTRASTRUCTURE OF PREANTRAL FOLLICLES IN THE OVARY OF THE MARE

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INTRODUCTION

The ovaries of each species include hundreds of thousands of oocytes enclosed in follicles, at different stages of follicular development. Most are preantral follicles (Van Wezel and Rodgers 1996). They consist of the primordial follicles - oocytes surrounded with a single layer of flattened epithelial cells which may include some granular cells; the primary follicles - oocytes surrounded with a single layer of granulosa cells; the secondary follicles - oocytes surrounded with 2 either complete or incomplete layers of granulosa cells (Braw-Tal and Yossefi 1997). These follicles constitute a potential source of gametes which could be useful for IVM/IVF procedures. Research on the isolated preantral mouse follicles resulted in follicle growth, antrum formation, ovulation *in vitro* and even in a few live young after IVM/IVF (Eppig and Schoeder 1989). To date no experiments on horses have resulted in progeny following IVM/IVF. There are no reports in the literature of distribution and morphology of the pre-antral follicles of the mare. Therefore, our research focused on examining the localisation and histomorphological characteristics of the oocytes and preantral follicles in the ovary of this species.

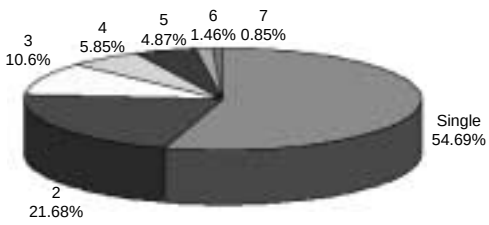

Fig 1: The percentage of follicles that occurred singly or in groups of 2 to 7.

MATERIALS AND METHODS

Light microscopy

Ovaries were recovered from the local abbatoir 10 min after the mares were slaughtered. They were fixed in 10% formalin for 6 weeks. The portions of ovaries were dehydrated, embeded in paraffin wax and sectioned at 10 µm. The slides were stained with hematoxilin and eosin (Miyamura *et al.* 1996). The follicles were counted, measured and evaluated by light microscopy (LM). Selected follicles and groups of follicles were photographed.

Transmission electron microscopy

Small pieces (1 x 2 mm) of ovary were collected and fixed in 3.5% glutaraldehyde and 1% OsO_4. The samples were dehydrated by passing through an ethanol series, embedded in Epon and finally serially sectioned into semi-thin sections (2 µm). These were stained with toluidyne blue and examined by LM for the presence of preantral follicles containing an intact oocyte. Those sections presenting an oocyte were re-embedded and ultra-thin (65 nm) sections were collected on copper grids, stained, dried and examined by

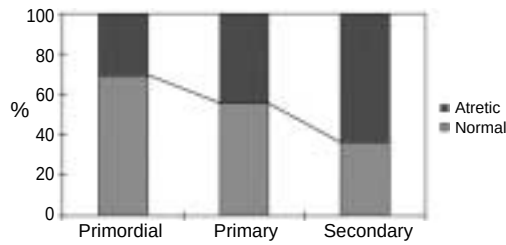

Fig 2: The precentage of atretic follicles in preantral follicles.

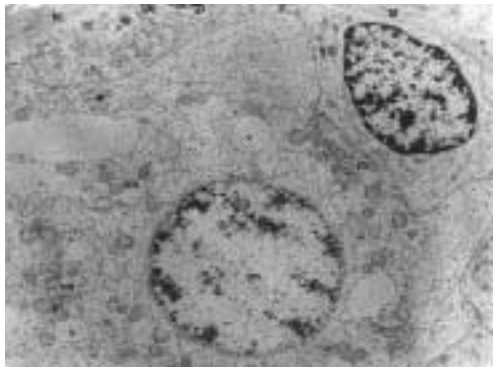

Fig 3: Electron micrograph from a primordial follicle of the mare. Note the oocyte nucleus (on), granulosa cells (gc), granulosa cell nucleus (n), basal lamina (bl), vacuoles (v), round mitochondria (rm). 10560x.

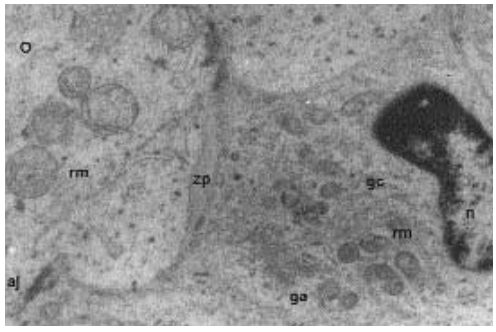

Fig 4: Electron micrograph showing a detail from a primordial follicle of the mare. Note an oocyte (O), granulosa cell (gc), granulosa cell nucleus (n), round mitochondria (rm), Golgi apparatus (ga), adhesive junctions (aj), patch of zona pellucida (zp). 26240x.

transmission electron microscopy (TEM; Philips EM - 300, Hyttel 1998).

RESULTS AND DISCUSSION

Localisation

Of the total 823 preantral follicles located and evaluated, 567 came from transverse and 256 from longitudinal sections. In a single histological slice from a transverse and a longitudinal section, an average of 70.5 and 84.7 preantral follicles, respectively were found. The preantral follicles were found in all parts of the ovary stroma and their diameters were 35.2–45.0 µm. Follicles occurred singly (54.7%) or in groups of 2–7 (45.3%, Fig 1; Szlachta and Tischner 1998).

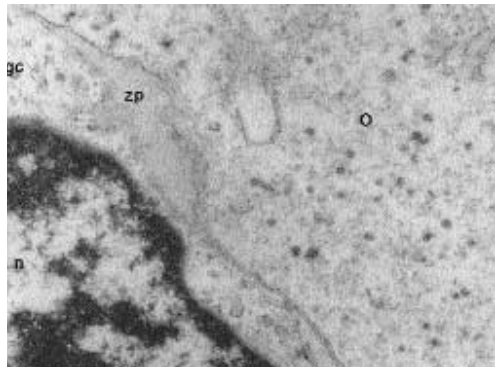

Fig 5: Electron micrograph showing detail from a primordial follicle of the mare. Note an oocyte (O), granulosa cell (gc), granulosa cell nucleus (n), patch of zona pellucida (zp). 32000x.

Follicles located within a distance of equal to, or less than, the diameter of the follicle were treated as a group. No regularity in preantral follicle location similar to that in the cow (Miyamura *et al.* 1996) was observed. This part of the study indicated that samples collected from any part of the ovarian stroma can be used for preantral follicle isolation (Szlachta and Tischner 2000).

Morphology

An attempt was made to define how many of the follicles are normal and how many atretic. The following abnormalities were indicative of atresia: disintegrated oocyte or its nucleus, irregularly shaped nucleoplasm, large vacuoles within the ooplasm, corrugated or disintegrated zona pellucida, degenerated granulosa cells surrounding the oocyte, pycnotic nuclei within these cells or scarce capillaries in the close vicinity of the follicle (Stevens and Lowe 1992). Follicles which showed at least 2 of the above abnormalities were regarded as atretic. The percentage of atretic follicles increased with the stage of follicular development and was 30.6%, 43% and 63.6% in the primordial, primary and secondary categories, respectively (Fig 2).

Ultrastructure

Among 100 semi-thin sections, only one included a primordial follicle. Ultra-thin sections and photographic documentation were made from this follicle. The follicle was evaluated as a

normal primordial follicle with no signs of atresia. Typical ultrastructures described in other species (Hyttel 1998) were observed. The primordial follicle enclosed an oocyte (O) containing a nucleus (on) located slightly off centre. The oolemma was attached to the flattened granulosa cells (gc) by adhesive junctions (aj). Numerous cytoplasmic organelles were observed (Fig 3). Round mitochondria (rm), a small number of Golgi complexes (ga) and lipid droplets indicated low activity of the oocyte (Hyttel 1997). A similar organelle distribution has been reported in the cow, rabbit and human oocytes from primordial follicles (Fair *et al.* 1997). However, a few elongated mitochondria and the first traces of zona pellucida formation (zp) in the primordial follicle were observed (Figs 4 and 5). These features in the cow were observed in a very small number of oocytes from the primary follicles (Fair *et al.* 1997; Hyttel 1997).

Conclusions

1. The preantral follicles were located in all parts of the ovary stroma.
2. The percentage of atretic follicles increased with the stage of follicular development.
3. The first traces of zona pellucida formation were observed in the primordial follicle.

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CUMULUS MORPHOLOGY AND OOCYTE CHROMATIN CONFIGURATION ON DAYS 6, 11, 15 AFTER OVULATION AND DURING OESTRUS IN THE MARE

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INTRODUCTION

Cumulus oocyte complexes are frequently recovered from slaughterhouse material for studies on *in vitro* maturation and fertilisation. In these mares, stage of cycle is not known and follicles of varying sizes often yield oocytes that vary in the maturity and morphology of the cumulus oocyte complex. This may, in part, explain the differences reported in chromatin configuration and cumulus morphology both before and after *in vitro* maturation of the oocytes (Fulka, Jr. and Okolski 1981; Hinrichs and Williams 1997; Goudet *et al.* 1998). Few studies have investigated the effect of stage of cycle on oocyte chromatin configuration. A higher maturation rate was found in equine oocytes collected in the follicular phase than during the luteal phase (Goudet *et al.* 1998). Similarly, maturation rate and subsequent development was greater in bovine and human oocytes collected during a phase of follicular growth (Cobo *et al.* 1999; Hagemann *et al.* 1999).

When the relationship between cumulus expansion and chromatin configuration in the mare was investigated, the highest proportion of prophase oocytes was found in oocytes with compact cumuli (Hinrichs and Williams 1997). In the mare, some studies indicated that more oocytes with expanded cumulus cells at the time of retrieval from the follicle matured to metaphase II and did so more rapidly than oocytes with compact cumulus cells (Hinrichs and Williams 1997), but other studies could not confirm these findings (Goudet *et al.* 1997).

Information about chromatin configuration and cumulus morphology at certain stages of the cycle is of importance in relation to selecting oocytes for IVM. The objective of this study was to investigate whether stage of cycle influenced chromatin configuration and cumulus morphology.

MATERIALS AND METHODS

Twelve cycling pony mares were ovariectomised on Day 6 (n=4), Day 11 (n=4) and Day 15 (n=4) after ovulation. Eleven additional tracts were obtained from a slaughterhouse. In these latter mares, plasma progesterone was less than 1 ng/ml, plasma oestradiol was greater than 40 pg/ml and at least one follicle larger than 40 mm was present. These mares were presumed to have been in oestrus at the time of slaughter.

The exteriorised ovaries were kept at 30–37°C in M199 with Hanks salts and 25 mM Hepes until processing. All visible follicles on the surface were processed followed by the follicles found after slicing the ovary thinly. Each follicle was cut open and the inner wall was scraped with a bone curette to release the cumulus oophorus with the oocyte. After examination of the cumulus oophorus using a stereomicroscope, the oocyte was denuded by pipetting with smaller and smaller bore pipettes (0.2–0.6 mm in diameter), fixed in buffered formalin and stained with a DNA-specific fluorescent Hoechst stain. The oocyte chromatin was classified as fluorescent nucleus (FN), loosely condensed chromatin (LCC), condensed chromatin (CC), diakinesis, metaphase, degenerated (Degen) and no chromatin. Cumulus oophorus morphology was classified either as compact (CP), slightly expanded (Sl ex), moderately expanded (M ex), very expanded (V ex), fully expanded (F ex) or denuded.

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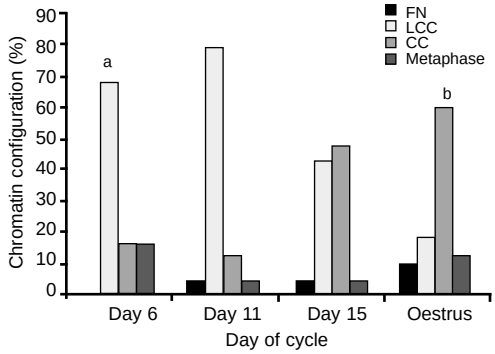

Fig 1: Chromatin configuration changes during the cycle. Numbers of oocytes on each day: Day 6 (n=25), Day 11 (n=26), Day 15 (n=31), Oestrus (n=57). Metaphase included diakinesis. Letters a and b denote a difference (P<0.001).

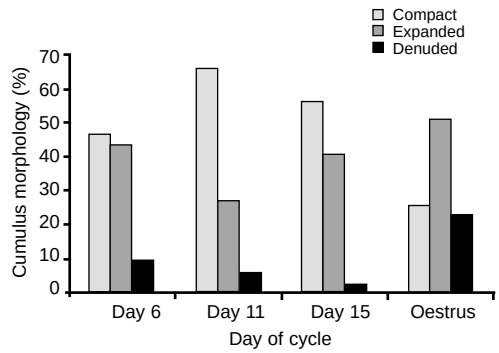

Fig 2: Cumulus morphology during the oestrous cycle. There was a significant difference between Day 11 and oestrus (P<0.01) and Day 15 and oestrus (P<0.05). Numbers of cumuli on each day: Day 6 (n=32), Day 11 (n=33), Day 15 (n=39), Oestrus (n=70).

Differences in parameters caused by stage of cycle was analysed among groups using a test of randomness (single-tailed Chi-square) where data were expressed as frequencies. Fisher's exact test was used for comparisons in which total number of measurements was less than 20 and when more than 20% of expected frequencies were less than 5.

RESULTS

A total of 293 follicles from 23 mares were processed.

Oocyte chromatin configuration during the cycle

Oocyte chromatin configuration was significantly associated with day of the cycle as determined by distribution of FN, LCC and CC (P<0.001, Fig 1). Day 6 and Day 11 were similar in chromatin configuration distribution but, after Day 11, the distribution changed. LCC and CC appeared to be in a reciprocal relationship. LCC was predominant at the beginning of the cycle whereas CC increased as the cycle progressed.

Cumulus morphology during the cycle

A total of 174 cumuli oophori were recovered. There was a tendency for follicles to contain an equal number of compact and expanded cumuli early in the cycle, followed by the greatest numbers of compact cumuli on Day 11. There was then a gradual decrease until oestrus at which time the largest proportion of expanded cumuli was seen. Despite this apparent trend, these differences were not significant other than in cumulus morphology distribution between Day 11 and oestrus, and Day 15 and oestrus. The highest numbers of denuded oocytes were found during oestrus (Fig 2).

Chromatin configuration and cumulus morphology

FN were only found in compact cumulus oocytes, and most LCC were present in this group (Fig 3). As the cumulus expanded, chromatin appeared to shift from the immature configurations through condensed chromatin and diakinesis to metaphase stages, which were most frequent in the fully expanded cumulus oocytes (P<0.001).

DISCUSSION

The present study demonstrated specific changes in chromatin configuration and cumulus morphology with the progression of the cycle.

Chromatin configuration and cumulus morphology showed a similar tendency to have most compact cumuli and immature chromatin configurations consistent with healthy follicles on Day 11, followed by signs of progression of maturation as seen by most expanded cumuli and condensed chromatin during oestrus. The highest proportion of oocytes displayed meiosis-like changes early in the cycle. This may be due to most follicles being atretic at this point (Pedersen *et al.* 1999). From Day 6 to Day 11 chromatin configurations did not differ much, but a change was obvious between Day 11 and Day 15. During oestrus the frequency of metaphase oocytes was

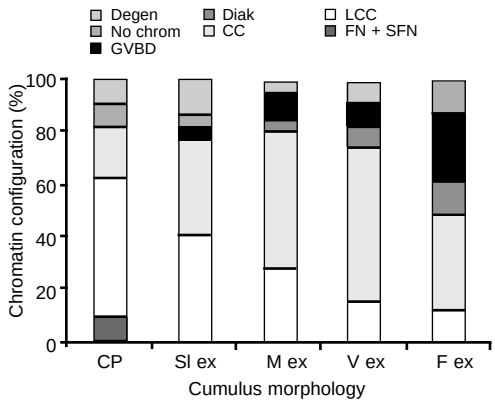

Fig 3: Chromatin distribution within cumulus groups. Chromatin configurations changed significantly with cumulus morphology (P<0.001). Numbers of oocytes within each group: Compact (Cp) (n=62), Slightly expanded (Sl ex) (n=22), Moderately expanded (M ex) (n=21), Very expanded (V ex) (n=12), Fully expanded (F ex) (n=8).

also high. The hormonal environment may have been more suitable for maturation during oestrus, which was supported by higher maturation rate of oocytes collected in the follicular phase than during the luteal phase (Goudet *et al*. 1998).

The present study demonstrated a significant correlation between changing cumulus morphology and change in distribution of chromatin configuration. Compact cumulus was the only group which contained oocytes with a fluorescent nucleus. With increasing cumulus expansion, the chromatin configurations indicated a progression of meiosis in the oocyte because there were increasing frequencies of diakinesis and metaphase oocytes. Of fully expanded cumuli, more than 40% contained a metaphase oocyte. (Hinrichs and Williams 1997) reported that more expanded than compact cumulus oocytes had condensed chromatin and that, after culture, more expanded than compact cumulus oocytes had matured to metaphase. This was in agreement with the present study, suggesting that oocytes with expanded cumulus cells had initiated meiotic maturation, which was completed during culture.

In summary, stage of cycle affected chromatin configurations and cumulus morphology. This may be due to follicles being larger during the follicular phase, but also that the hormonal environment is more suitable even for the smaller follicles. It remains to be seen whether oocytes, which have begun meiotic maturation *in vivo*, perhaps as a result of the follicle becoming atretic, can actually be fertilised and mature.

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FACTORS INFLUENCING NUCLEAR MATURATION AND CUMULUS EXPANSION IN THE EQUINE OOCYTE

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In the equine species, research and knowledge on oocyte maturation lags behind other mammalian species. Moreover, the *in vitro* maturation rate of equine oocytes is low in comparison with other domestic mammals. It remains lower than 70% (Squires 1996) whereas in the goat, the sow and the cow more than 90% of the oocytes reach metaphase II. Gonadotropins appear to be the primary regulators of mammalian oocyte maturation, but are only part of the complex sequence that regulates ovarian function. Several factors have been shown to influence nuclear maturation and/or cumulus expansion in the mammalian oocyte, such as epidermal growth factor (EGF; Lonergan *et al.* 1996), luteinising hormone (LH; Squires 1996), follicle stimulating hormone (FSH; Singh and Armstrong 1997), growth hormone (GH; Izadyar *et al.* 1996), bovine serum albumin (BSA), fetal calf serum (FCS) and L-glutamine (Furnus *et al.* 1998). Their role in the *in vitro* maturation of equine oocytes has been examined in the authors' laboratory.

In Experiments 1–3, cumulus oocyte complexes (COCs) were collected by transvaginal ultrasound-guided aspiration in the standing mare (Duchamp *et al.* 1995). All follicles from 5–30 mm were punctured at the end of the follicular phase, 24 or 34 h after induction of ovulation. After follicular fluid aspiration, follicles were flushed with PBS and heparine (50 IU/ml) at 37°C. Aspirated fluid from each follicle was examined individually for oocyte recovery. In Experiment 4, COCs were collected from ovaries recovered from mares in a slaughterhouse during the breeding season. The follicles were scraped to dislodge the oocytes from the follicular wall. COCs were then located under a stereomicroscope. In all experiments, COCs were cultured for 30 h at 38.5°C in air with 5% CO_2 and maximum humidity. After culture, cumulus expansion was assessed by stereomicroscopy. Subsequently, oocytes were denuded, stained with 1 µg/ml bis-benzimide and the nuclear stage was assessed by epifluorescent microscopy.

In Experiment 1, COCs were cultured individually in 500 µl of control medium (TCM199 + 5 mg/ml BSA + 1 µg/ml estradiol + antibiotics), control medium with eGH (0.5 µg/ml) or control medium with eLH (5 µg/ml). The nuclear maturation rate was significantly higher in the presence of eLH (54%) compared to the control medium (32%, $P<5\%$); no significant effect of eGH was observed (42%, Fig 1). The addition of eGH or eLH significantly increased the cumulus expansion rate (29% and 58% respectively vs 13% in the control medium, $P<5\%$, Fig 1).

In Experiment 2, COCs were cultured individually in 500 µl of control medium (TCM199 + 5 mg/ml BSA + 1 µg/ml estradiol + antibiotics) or control medium with EGF (50 ng/ml). The nuclear maturation rate was significantly higher in the presence of EGF (76%) compared to the control medium (33%, $P<5\%$; Fig 2). The addition of EGF significantly increased the cumulus expansion rate (60% vs 19% in the control medium, $P<5\%$; Fig 2).

The results of Experiments 1 and 2 demonstrate that EGF induces the highest nuclear maturation rate, but the cumulus expansion rate remains low. In Experiments 3 and 4, we attempted to improve the cumulus expansion rate. COCs were cultured in the medium that resulted in the highest nuclear maturation rate (TCM199 + 5 mg/ml BSA + antibiotics + 50 ng/ml EGF) and supplemented with FSH and FCS, which have been shown to increase the cumulus expansion rate in the bovine and porcine species.

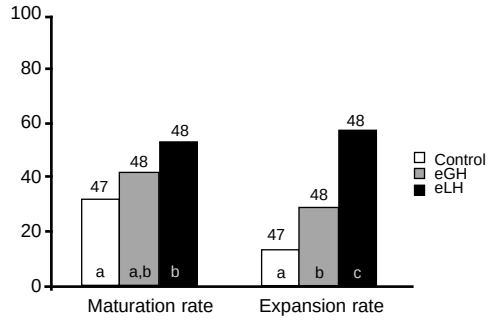

Fig 1: Influence of eGH and eLH.
a,b,c: values with different superscripts differ significantly (P<5%).

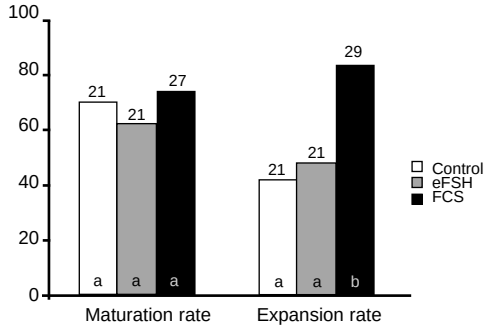

Fig 3: Influence of eFSH and FCS.
a,b: values with different superscripts differ significantly (P<5%).

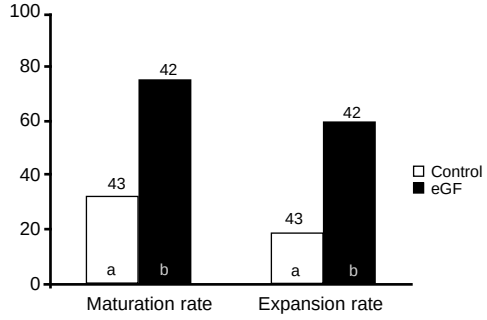

Fig 2: Influence of EGF.
a,b: values with different superscripts differ significantly (P<5%).

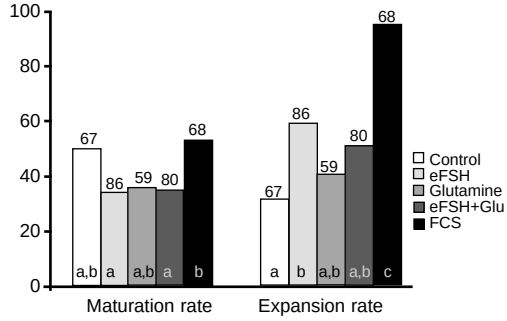

Fig 4: Influence of eFSH, FCS and glutamine.
a,b,c: values with different superscripts differ significantly (P<5%).

In Experiment 3, COCs were cultured individually in 500 µl control medium (TCM199 + 5 mg/ml BSA + antibiotics + 50 ng/ml EGF), control medium with eFSH (1 µg/ml) or control medium without BSA with FCS (20%). The nuclear maturation rates were not significantly different between the 3 media (Fig 3). The addition of eFSH did not significantly increase the cumulus expansion rate (48% vs 42% in the control medium). The cumulus expansion rate was significantly higher in the presence of FCS (83%) compared to the control medium (42%, P<5%; Fig 3).

Hyaluronic acid is the major component of the extracellular matrix of the cumulus. We hypothesised that FSH had no effect on cumulus expansion, because some precursors for hyaluronic acid were missing. Glutamine is a precursor for hyaluronic acid synthesis.

In Experiment 4, we tested the influence of glutamine on nuclear maturation and cumulus expansion rates. We used a modified TCM199 that was supplemented with $NaHCO_3$ (9.5 mM), Hepes (5.8 mM), pyruvic acid (2.3 mM) and lactic acid (4.6 mM). COCs were cultured in groups of 5–8 oocytes in 400 µl control medium (modified TCM199 + 5 mg/ml BSA + antibiotics + 50 ng/ml EGF), control medium with eFSH (1 µg/ml), control medium with L-glutamine (2 mM), control medium with eFSH plus L-glutamine or control medium without BSA with FCS (20%). The addition of FSH or glutamine tended to decrease the nuclear maturation rate compared to the control medium; the addition of FCS had no effect (Fig 4). The cumulus expansion rate was significantly higher in the presence of eFSH (60%) or FCS (94%) compared to the control medium (32%, P<5%). The cumulus expansion rate was not improved by the presence of glutamine alone (41%) or glutamine + eFSH (51%) compared to the control medium or the control medium with eFSH (Fig 4).

The results of Experiments 3 and 4 show that FSH increases the cumulus expansion rate of oocytes obtained at the slaughterhouse, but not from *in vivo* punctures. One of the 2 collection techniques may damage the cumulus, or select a population responsive to FSH. Addition of FCS induces the highest cumulus expansion rate in oocytes from slaughterhouse and *in vivo* punctures.

The results of our studies suggest that a culture medium containing EGF and FCS will generate high nuclear maturation and cumulus expansion rates. Further studies are necessary to find a chemically defined substitute for serum.

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INFLUENCE OF SERUM ON *IN VITRO* MATURATION OF HORSE OOCYTES

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INTRODUCTION

The success of *in vitro* embryo production in the horse lags behind other domestic animals. Barriers to routine *in vitro* fertilisation include oocyte maturation and sperm capacitation. Several studies have been conducted to define the appropriate culture methods for maturation of equine oocytes. Most studies indicate that the *in vitro* maturation rate does not increase after 30 h and that LH, FSH, serum and estradiol are beneficial to oocyte maturation. Observations made by Tonetta and DiZerega (1989) indicate that gonadotrophins are only part of the complex sequence of factors, such as growth factors, that regulate ovarian function and, with it, the maturation of oocytes. In addition, it is difficult to assess the effects of altered zona pellucida of oocytes matured *in vitro* and the less than adequate preparation of equine sperm cells (low efficiency of *in vitro* capacitation of stallion spermatozoa, Blue *et al.* 1989; Ellington *et al.* 1993). Other assisted reproduction techniques like nuclear transfer, intracytoplasmic sperm injection (ICSI) or cloning depend on a successful *in vitro* maturation rate. To carry out these assisted techniques, it is not only necessary that the nucleus has reached metaphase II, but that the first polar body is extruded to have an orientation where the metaphase II plate is situated. Therefore, the maturation of oocytes is the most important requirement for further manipulation.

The objective of this study was to evaluate the effect of 2 sera with different IGF I content (65 ng/ml for fetal calf serum [FCS]; 150 ng/ml for oestrous mare serum [EMS]) on oocyte maturation as assessed by polar body extrusion.

MATERIALS AND METHODS

Cumulus oocyte complexes (COCs) were obtained either by follicular scraping of slaughterhouse ovaries or by follicle aspiration from living mares (Alm *et al.* 1997). According to their cumulus morphology - compact or expanded - COCs were cultured in Hepes-buffered TCM 199 with FSH and either with 10% FCS or 10% EMS. The EMS was prepared from one mare. At the time of blood recovery, the pre-ovulatory follicle had a diameter of 48 mm detected by follicle monitoring. One day after blood recovery the ovulation could be detected. COCs were matured *in vitro* for 0, 12, 24 or 32 h. Afterwards the oocytes were fixed either with acetic alcohol (1:3 acetic acid:ethanol) for at least 24 h and stained with aceto-orcein, or in buffered formol saline (BFS) and stained with Hoechst 33258 to evaluate the stage of development.

RESULTS

A total of 430 COCs were recovered from the follicles; 220 (51.2%) with compact cumulus and 210 (48.8%) with expanded cumulus. Only 385 COCs (89.5%) were included in the study because 16 (3.7%) were lost during processing and 29 (6.7%) could not be categorised by nucleus form.

Among the treatments there were time dependent differences in the maturation rate of compact COCs and expanded COCs, and in the presence of oocytes that had extruded their first polar body. The total maturation rate (telophase 1/metaphase 2) was similar for both sera: 0, 43.3 and 66.7% for compact COCs after incubation with EMS for 12, 24 or 32 h, respectively; and 0,

37.5 and 59.3% after incubation with FCS. Using expanded COCS, we obtained maturation rates of 16.7, 66.7 and 68.9% after incubation with EMS, and 4.8, 61.5 and 42.9% after incubation with FCS for 12, 24 or 32 h, respectively.

The proportion of oocytes with degenerated chromatin was similar using the different sera. The highest degeneration rate was observed after incubation with FCS of expanded COCs over 32 h.

The number of oocytes with extruded polar body as a requirement for ICSI and nuclear transfer was significantly increased after incubation of expanded COCs with EMS for both 24 and 32 h incubation. After incubation of compact COCs the number of oocytes with extruded polar bodies increased from 9.4% and 18.5% after 24 and 32 h of incubation with FCS to 30.0 and 33.3% using EMS, respectively.

In comparison to COCs with expanded cumulus, and independent of the type of serum, COCs with compact cumulus investment required a longer maturation time to reach metaphase II, in agreement with results of previous investigations (Hinrichs *et al.* 1993; Alm *et al.* 2001).

Conclusion

It is assumed that the higher concentration (more than double) of IGF I in EMS, compared to FCS, could be responsible for the higher polar body extrusion rate.

Acknowledgements

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SESSION V:

In vitro fertilisation

Chairman: T. A. E. Stout

ASSESSMENT OF ZONA PELLUCIDA PENETRATION BY STALLION SPERM DURING *IN VITRO* FERTILISATION OF HORSE OOCYTES MATURED *IN VITRO* AND *IN VIVO*

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INTRODUCTION

To date, only 2 foals have been produced by conventional *in vitro* fertilisation (IVF) of equine oocytes, both of which were derived from *in vivo* matured oocytes recovered from pre-ovulatory follicles (Palmer *et al*. 1991; Bezard 1992). Indeed, despite numerous attempts (eg Zhang *et al.* 1990; Dell'Aquila *et al.* 1996), no offspring have been produced after IVF of horse oocytes matured *in vitro* (IVM). The most commonly proposed reasons for this dramatic lack of success are poor developmental competence of equine oocytes after IVM and inadequate induction of capacitation and the acrosome reaction (AR) in stallion sperm *in vitro*. Although such compounds as heparin, caffeine, lysophospholipids and calcium ionophore A23187 induce capacitation and the AR in stallion sperm *in vitro* (Graham 1996), they do not significantly improve IVF success rates. One further *in vitro* inducer of the AR in stallion sperm is progesterone (P4: Cheng *et al.* 1998) which is present in follicular fluid and is thought to be a physiological inducer of sperm activation during fertilisation; the effects of progesterone on equine IVF have yet to be reported. With regard to potential oocyte-related barriers to IVF, zona pellucida (ZP) dissection (Choi *et al.* 1994) and drilling (Li *et al.* 1995) both markedly improve fertilisation and embryonic development rates by removing the need for the sperm to penetrate the ZP. Yet more dramatic increases in embryo production have been recorded after intracytoplasmic sperm injection (ICSI) and, encouragingly, live foals have recently been born after ICSI of equine oocytes matured *in vitro* (Li *et al.* 2001). That the low fertilisation rates resulting from equine IVF are partially overcome by assisting sperm penetration through the ZP suggests that further examination of the interaction between sperm and oocyte/ZP are necessary. To this end, 3 experiments were carried out to investigate whether sperm penetrate IVM horse oocytes during IVF and to examine the effects on this process of cumulus morphology at the onset of IVM and the presence of cumulus cells or P4 during IVF. In addition, to determine whether IVM alters ZP binding properties or penetrability, sperm-oocyte interaction was examined during IVF of oocytes matured *in vivo*.

MATERIALS AND METHODS

Culture and fertilisation of cumulus oocyte complexes

Cumulus oocyte complexes (COCs) recovered from slaughterhouse ovaries and processed as described by Tremoleda *et al*. (2001) were cultured in M199 supplemented with 10% FCS and 0.01 units/ml of porcine FSH and equine LH (Sigma) at 39°C in a humidified atmosphere of 5% CO2-in-air. After 36 h in culture, the COCs were denuded of their cumulus by incubating them in PBS containing 0.1% hyaluronidase for 5 min at 37°C and then vortexing them. Oocytes with a visible first polar body were selected for IVF with percoll-treated, frozen-thawed sperm from a single stallion. Groups of 15–25 oocytes were incubated in 500 µl aliquots of IVF medium (fert-TALP; Parrish *et al.* 1988) containing 1×10^6 sperm/ml. In Experiment 1, COCs were classified prior to IVM as compact (C-COCs n=89) or expanded (E-COCs n=72), according to their cumulus morphology, and incubated in IVF medium with or without 150 ng/ml P4. After 6 or 20 h of IVF, oocytes from the C-COC (n=35 at 6 h; n=34 at 20 h) and E-COC groups (n=54 at 6 h; n=38 at

TABLE 1: Sperm-oocyte complexes analysed by CLSM after different fertilisation conditions, for oocytes matured *in vitro* (Experiments 1 and 2) and *in vivo* (Experiment 3)

	Type of oocyte/COC	Incubation time (h)	No. of oocytes analysed IVF + P4*	No. of oocytes analysed IVF **	No. of sperm/oocyte bound to the ZP
Experiment 1	Compact COCs	6	20	15	≈50–100
		20	28	26	
	Expanded COCs	6	17	17	
		20	19	19	
Experiment 2	Non-denuded oocytes	6	67	-	≈0–1
	Denuded oocytes		52	-	≈100
Experiment 3	*In vivo* matured oocytes	24	15	-	≈30

* IVF+P4: incubation in fertil-TALP supplemented with 150 ng/ml progesterone
** IVF: incubation in fertil-TALP without progesterone

20 h) were washed with PBS containing 0.1% BSA and aspirated through a narrow-bore pipette to remove any loosely attached spermatozoa. The sperm membranes were then permeabilised by a 1 min incubation in 0.05% Nonidet, and the sperm-oocyte complexes were fixed in 3% paraformaldehyde, for subsequent confocal laser scanning microscopy (CLSM). In Experiment 2, IVM oocytes were either denuded (n=52) or not (n=67) of their cumulus investment and incubated for 6 h with sperm in fert-TALP plus progesterone, before being permeabilised and fixed for CLSM. In Experiment 3, 15 *in vivo* matured oocytes were recovered from pre-ovulatory follicles by transvaginal ultrasound-guided aspiration 32 h after donor mares were injected iv with 2,500 iu hCG (Chorulon; Intervet International, The Netherlands). These were incubated with sperm in fertil-TALP plus progesterone for 24 h, and then prepared for CLSM.

Assessing sperm penetration through the zona pellucida

The position of sperm within the oocyte or ZP, and the acrosomal status of these bound/penetrated sperm, was analysed by labelling the acrosome membranes with FITC-conjugated peanut agglutinin (FITC-PNA; E-Y. Lab. Inc., California, USA) and counterstaining the DNA with Ethidium homodimer-1 (EthD-1; Molecular Probes Europe BV, The Netherlands). The stained complexes were then washed twice in PBS before being mounted on slides together with an anti-fade suspension (Vectashield; Vector Lab. California, USA). Finally, the sperm-oocyte complexes were examined using a CLSM (Leica TCS MP, Heidelberg, Germany) equipped with a krypton-argon ion laser to simultaneously excite FITC and EthD-1.

RESULTS

In Experiment 1, sperm did not penetrate the ZP of *in vitro* matured oocytes, irrespective of cumulus morphology at the onset of IVM or the addition of progesterone to the IVF medium (Table 1). Although large numbers of sperm bound to the ZP (≈50–100 sperm/oocyte), none showed classical signs of having undergone the AR. Instead, most bound sperm had a mottled swollen acrosomal cap and numerous detached acrosomal caps were also bound to the ZP. There were no discernible differences in sperm binding or penetration between sperm-oocyte complexes incubated for 6 h or 20 h. Zona–penetration was also absent in oocytes incubated without prior removal of their cumulus (Experiment 2). In addition, far fewer sperm bound to the ZP of cumulus-intact than of denuded oocytes (Table 1) and, as in Experiment 1, most of the bound sperm had a mottled, swollen acrosomal cap, and none showed clear signs of AR. The number of 'free' acrosomal caps bound to the ZP of non-denuded oocytes also seemed lower than for denuded oocytes, although this was not specifically investigated. Sperm also failed to penetrate the investments of *in vivo* matured oocytes during 24 h of IVF, although more sperm bound to the ZP of these COCs than to those of non-denuded IVM oocytes (≈30 vs 0–1 sperm/oocyte). Finally, all oocytes remained in metaphase of the second

meiotic division after IVF and none showed signs of activation (ie pronucleus formation: Yanagamachi 1994).

Discussion

The use of CLSM together with specific labels for acrosome status (FITC-PNA) and DNA (EthD-1) enabled detailed visualisation of sperm-oocyte interaction during IVF. Furthermore, the results of this study did not support the hypothesis that restricted zona penetration by stallion sperm is related purely to impaired maturation of the oocyte and its cellular investments during IVM. Instead, the fact that sperm did not penetrate the ZP of either *in vivo* or *in vitro* matured oocytes suggests that the problem lay more in suboptimal sperm activation conditions during IVF. In an attempt to improve these conditions, progesterone, an inducer of the AR that enhances sperm-zona binding *in vitro* (Meyers *et al.* 1995; Cheng *et al.* 1998), was added to the IVF media at a concentration (150 ng/ml) similar to that found in mare pre-ovulatory follicular fluid (Grøndhal 1994). However, this did not improve sperm penetration through the ZP. Perhaps the most striking observation was the large proportion of sperm bound to the ZP of either *in vivo* or *in vitro* matured oocytes that had a mottled, swollen acrosomal cap but no clear signs of a normal acrosome-reaction. Furthermore, because the IVF medium used (fert-TALP) contains bicarbonate and Ca^{2+}, it should have been able to initiate and facilitate capacitation and the AR (Rathi *et al.* 2001). However, although the high levels of sperm-ZP binding suggest that capacitation did occur, the AR did not. This may be because the AR in stallion sperm is dependent on binding to a specific functional domain of the zona or because it requires specific conditions that are present in the oviduct but were not adequately mimicked *in vitro*. Thus, in the present study, defective sperm-zona binding and failure of normal AR induction were the major causes of failed IVF. In man, defective sperm-zona binding is also a common problem and relates more often to abnormalities of spermatozoa than of the oocyte (Liu and Baker 2000). Further studies to improve the conditions for inducing 'physiological' capacitation and ZP-binding of stallion sperm are needed to optimise sperm-oocyte interaction during IVF.

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TIMING OF PHYSIOLOGICAL CHANGES IN HORSE ZYGOTES AND SPERMATOZOA DURING *IN VITRO* FERTILISATION

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INTRODUCTION

Slow development of IVM/IVF techniques in equids has been due, in part, to limited availability of horse oocytes, and in part to the low efficiency with which sperm and oocytes form zygotes and embryos during IVF culture. To date, only 2 foals have been born following conventional methods of *in vitro* fertilisation of *in vivo* matured oocytes (Palmer *et al.* 1991; Bezard 1992). Because of this limited efficacy of conventional IVF, an intracytoplasmic sperm injection technique (ICSI) was developed in the horse, and has been used to produce 7 foals (Squires *et al.* 1996; Cochran *et al.* 1998; McKinnon *et al.* 2000; Li *et al.* 2001). Nevertheless, standard IVF of *in vitro* matured horse oocytes needs to be improved. The investigation of physiological changes of capacitated spermatozoa and matured oocytes during IVF gamete culture allows a better understanding of zygote development and their specific culture requirements during IVF.

In this study, the timing of first events 0, 2, 4, 8, 16, 24 and 32 h after insemination in the zygotes up to the first mitosis was evaluated. In parallel, the movement, morphology and integrity of IVF-spermatozoa were observed.

MATERIALS AND METHODS

Cumulus-oocyte complexes (COCs) were obtained by scraping follicles from ovaries of slaughtered mares of unknown reproductive history, as previously described (Alm *et al.* 1997). Oocytes (n=192) with a compact cumulus were selected from all recovered COCs and matured for 32 h in TCM 199 containing FSH and 10% FCS. For *in vitro* capacitation, cryopreserved semen from one ejaculate from one stallion was used; a motile sample of thawed spermatozoa for IVF was obtained by swim-up separation and subsequent treatment with 200 µg/ml heparin. After IVM, 2–5 oocytes were placed in a 50 µl droplet of TALP-medium and approximately 5 µl of the final sperm suspension were added to each droplet to give a final concentration of 1×10^6 motile sperm/ml in the fertilisation droplet. Fertilisation was carried out for 0, 2, 4, 8, 16, 24 and 32 h. Oocytes and zygotes were selected randomly and analysed for chromatin configuration (penetration, fertilisation). To characterise stallion spermatozoa after *in vitro* capacitation treatment and during IVF a computer assisted sperm motion analyser was used (Mika-Strömberg, Switzerland) and further parameters (morphological integrity, acrosomal status and membrane quality) were assessed.

RESULTS

Depending on duration of IVF (2, 4, 8, 16, 24, 32 h) the following penetration rates (%) in horse oocytes were detected: 10.7, 41.2, 23.1, 43.5, 45.8 and 46.4, respectively (2 h vs. 4, 8, 16, 24, 32 h; $P<0.05$). First events of fertilisation, eg 2 pronuclei and subsequent stages were observed as follows (%): 8 h (7.7), 16 h (17.4), 24 h (29.2) and 32 h (25.0). There was some increase in penetration between 2 and 4 h post insemination ($P<0.05$) and in fertilisation between 8 and 16 h. The following events during IVF in the fertilised eggs were representative: decondensed sperm head (4 h), one pronucleus and sperm tail (8 to 16 h), 2 pronuclei (16 to 24 h), syngamie (24 h) and mitosis (32 h). The first cleavage was observed 32 h after IVF. These results are in agreement with

those relating to first cleavage 2 days after ICSI (Kato *et al.* 1997; Guignot *et al.* 1998). Li *et al.* (2000) reported on cleavage 24 to 32 h after ICSI in activated oocytes.

Immediately after insemination, the motility of sperm decreased from 50.1 to 41.7% (4 h), 35% (8 h), 20% (16 h), 6.7% (24 h) and 3.3% (32 h). The highest percentage of spermatozoa with a hyperactive movement pattern was evaluated immediately after capacitation with heparin (14.6%) and at 2 h after insemination (12.5%). Acrosomal defects represented the highest proportion of morphological abnormalities. The percentage of total detached acrosomes rose from 12.7% before insemination to 22.6% at 2 h, and up to 54.4% 32 h after insemination. Similar changes during IVF were observed for the parameter 'membrane integrity', which decreased immediately after 2 h of insemination ($P<0.05$).

Conclusion

These results present a clear time table for the first events of penetration and fertilisation in horse eggs. This is associated with dynamic changes of motility and morphology of stallion spermatozoa during IVF. The results showed that the process of IVF substantially reduced sperm viability, as expected. These changes in stallion spermatozoa are influenced in part by oocytes during common gamete culture.

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EFFECTS OF NALOXONE ON *IN VITRO* MATURATION AND CLEAVAGE OF EQUINE OOCYTES AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI)

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INTRODUCTION

The use of isolated and *in vitro* matured oocytes is far from being applied routinely in assisted reproductive programmes in the horse, as in most other species. The knowledge of mechanisms regulating oocyte meiotic maturation is still fragmentary and improvements in this area could provide significant contributions to increasing the success of *in vitro* procedures of embryo production. In the horse, oocytes recovered from slaughtered mares and classified as compact (Cp), in relation to cumulus morphology, have lower meiotic competence, a slower rate of maturation, reduced ability to respond to activation stimuli and reduced formation of a male pronucleus after intracytoplasmic sperm injection (ICSI) than those with expanded (Ex) cumulus (Dell'Aquila *et al.* 1997, 2001). The high percentage of oocytes recovered with Cp cumulus, either by aspiration or scraping (Dell'Aquila *et al.* 2001), further reduces the availability of oocytes suitable for *in vitro* biotechnologies, that is already limited by the low number of antral follicles per ovary.

Previous studies have demonstrated that the μ-opioid receptor gene is expressed in bovine cumulus-oocyte complexes (COCs), that the opioid antagonist Naloxone (Nx) influences the *in vitro* maturation (IVM) rate with opposite and dose-dependent effects and that ß-endorphin reduces the rate of oocytes reaching the metaphase II stage after *in vitro* culture (Albrizio *et al.* 2000; Dell'Aquila *et al.* 2000).

This study examined the presence of the μ-opioid receptor transcript in equine COCs and the effects of Nx added during IVM culture, on maturation and cleavage rates after intracytoplasmic sperm injection (ICSI) of equine oocytes recovered with Cp or Ex cumulus and cultured separately.

MATERIALS AND METHODS

Methods for oocyte collection and evaluation, IVM, ICSI, RNA extraction and RT-PCR were described by Dell'Aquila *et al. (*1997, 2001) and Albrizio *et al. (*2000). Briefly, IVM culture was performed using medium TCM-199 supplemented with 20% fetal calf serum (FCS), FSH, LH, and 17 ß-estradiol. Naloxone was added at concentrations of 10^{-6}, 10^{-8} and 10^{-10} M. Control oocytes were cultured in the absence of Nx. ICSI procedure was carried out in fertilisation medium and embryo culture was performed for 72 h in cleavage medium (Cook, Sydney, Australia). Total RNA was extracted and reverse transcribed using Express Direct (Pierce, Illinois, USA).

EXPERIMENTAL DESIGN

COCs for molecular analysis were pulled and submitted to RNA extraction. Total RNA was reverse transcribed and amplified and the amplification product was analysed as described by Albrizio *et al.* (2000, 2001).

In the first set of culture trials, oocytes were cultured *in vitro* for 28–30 h (Experiment 1a; 4 trials) or 40 h (Experiment 1b; 2 trials). After culture, oocytes were denuded of surrounding cumulus cells, fixed, stained with Hoechst and observed under fluorescence microscope to evaluate the stage of nuclear maturation.

In the second set of trials (3 trials), oocytes were cultured *in vitro* in the presence of 10^{-8} M Nx, denuded of cumulus cells, evaluated for first

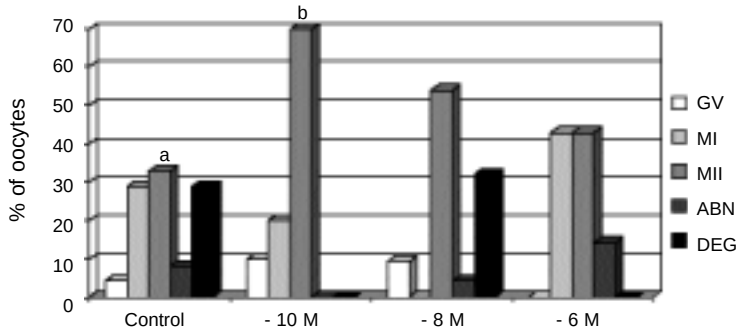

Fig 1: Effects of Nx at 10^{-10}, 10^{-8} and 10^{-6} M on the maturation rate in vitro *of equine oocytes recovered with compact cumulus. (Chi-square test: a;b P<0.001).*

polar body extrusion, submitted to ICSI and cultured *in vitro* for 72 h. The stage of embryonic development was recorded every 24 h.

RESULTS

The μ-opioid receptor gene is expressed in equine COCs as has been indicated by the presence of a fragment of expected length (441 bp).

In the set of IVM trials conducted for 28–30 h, the addition of 10^{-10} M Nx significantly improved the maturation rate of equine oocytes recovered with a Cp cumulus (56/80, 70% vs 16/48, 33% for treated and control oocytes respectively; P<0.001) whereas no effect was found in the group of Cp oocytes when cultured with 10^{-6}, 10^{-8} M Nx (12/28, 43%; 12/22, 54% respectively; NS) and in the group of oocytes issuing from Ex cumulus at 10^{-8} and 10^{-10} M Nx (18/33, 54% and 18/30, 60%, respectively vs 15/27, 55% in control conditions; NS; Fig 1). The addition of 10^{-6} M Nx significantly reduced the maturation rate of Ex oocytes (8/32, 25%; P<0.05).

In the set of prolonged IVM cultures (40 h), a trend towards increased maturation rate for Cp

TABLE 1: Effects of Naloxone on the *in vitro* maturation rate of equine oocytes recovered with compact (Cp) or expanded (Ex) cumulus and cultured for 40 h (Nx 10^{-10} M)

Conditions Nx (M)	Cumulus	N° cultured oocytes	N° evaluated oocytes	GV	MI	MII+PB	Abnorm	DEG
Control	Cp	11	10	1 (10)	0 (0)	8 (80)	0 (0)	1 (10)
Control	Exp	9	9	2 (22)	1 (11)	6 (66)	0 (0)	0 (0)
10^{-10}	Cp	12	12	0 (0)	0 (0)	12 (100)[a]	0 (0)	0 (0)
10^{-10}	Exp	10	10	0 (0)	3 (30)	5 (50)[b]	0 (0)	2 (20)

Chi-square test for %MII +PB : [a,b]P<0.05

TABLE 2: Effects of Naloxone at 10^{-8} M on cleavage rate of *in vitro* matured equine oocytes submitted to intracytoplasmic sperm injection (Chi-square test: NS)

Cumulus morphology	Compact	Expanded	Compact	Expanded
Culture condition	Control	Control	Nx	Nx
N° (%) of oocytes: Cultured	12	14	13	16
Injected (% of cultured)	5 (42)	10 (71)	6 (46)	8 (50)
Cleaved (% of injected)	3 (60)	6 (60)	3 (50)	2 (25)

Chi-square test: NS

oocytes was observed but data did not attain statistical significance. In this experiment the maturation rate of Ex oocytes was significantly reduced ($P<0.05$, Table 1).

The cleavage rate (4–8 cell stage) after ICSI for oocytes cultured in the presence of Nx 10^{-8}M, both for oocytes recovered with a Cp or an Ex cumulus, was not statistically different for treated and control oocytes (3/13; 23% vs 3/12; 25% respectively for Cp oocytes; NS); (2/16; 12.5% vs 6/14, 43% respectively for Ex oocytes; NS; Table 2). Further studies are ongoing to evaluate the effects of 10^{-6} and 10^{-10} M Nx, also in long term cultures to the blastocyst stage.

Discussion

These results provide support to the hypothesis that, in the horse, opioids work as local modulators of oocyte maturation and that the addition of Nx could be useful to improve the efficacy of IVM-ICSI-EC protocols. Further studies are ongoing on the expression of opioid receptor genes in equine oocytes at different stages and in spermatozoa, on the signalling pathways of opioid receptors in germ cells and on the effects of modulating these patterns, by using opioid antagonists, on the outcome of assisted reproductive technologies.

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IN VITRO AND *IN VIVO* CULTURE IN THE SHEEP OVIDUCT OF EQUINE EMBRYOS OBTAINED BY IVM AND ICSI

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INTRODUCTION

The technology of *in vitro* embryo production in the horse is far from established. Although several reports describe the technique of *in vitro* maturation, much less success has been obtained with conventional IVF and embryo culture. To overcome the step of *in vitro* fertilisation, intracytoplasmic sperm injection (ICSI) has been applied and has proved suitable to obtain viable early cleavage stage horse embryos (Squires *et al.* 1996; Cochran *et al.* 1998; McKinnon *et al.* 2000). By contrast, very little experimental work has been done to acquire information on the *in vitro* requirements of the horse embryo because, in most studies, the embryos have been transferred soon after cleavage to the oviduct of recipient mares.

This study investigated the effect of a completely *in vitro* culture system based on medium SOF, compared to an *in vivo* culture system in the surrogate sheep oviduct. These 2 systems are used successfully to produce IVP bovine embryos in the authors' laboratory.

MATERIALS AND METHODS

Equine ovaries were collected at the abattoir and transported to the laboratory within 4 h in PBS at about 25°C. The oocytes were collected by scraping of the follicles with a Jacobson curette and were matured in medium TCM 199 supplemented with 10%FCS, ITS supplement (insulin, transferrin, sodium selenite, Sigma), 1 mM sodium pyruvate, EGF (50 ng/ml, Sigma), IGF1 (100 ng/ml, Sigma), LH and FSH (0.1 IU each, Pergovet, Serono, Italy). Between 22 and 24 h after the onset of maturation, the oocytes were denuded by pipetting and returned to maturation medium for 4–6 h up to 28 h. At that time the oocytes with an extruded first polar body were selected for injection. One hour prior to the start of injection the semen was thawed and separated on a Percoll gradient (45–90%). The pellet was diluted at a concentration of 4 million sperm per ml and then further diluted 1:1 with a 12% solution of PVP in medium SOF supplemented with BSA, amino acids, heparin (1 µg/ml) and PHE. The frozen semen of 3 stallions was used for this study. After ICSI (Day 0) the oocytes were allowed to cleave *in vitro* in microdrops of medium SOF supplemented with BSA and amino acids. On Day 2 the cleaved embryos were either embedded in agar and transferred to the sheep oviduct or left in culture. The intermediate recipient sheep were treated with an intravaginal releasing device on the day of transfer and no attention was paid to the stage of their cycle. On Day 7 the embryos were recovered from the sheep oviduct and recovery rate was 100%. The number of viable embryos (compacted morulae and blastocysts) derived from both *in vivo* and *in vitro* culture was recorded. The experiment was done in 3 replicates.

RESULTS

A total of 332 oocytes were collected from 85 ovaries (3.9 oocytes per ovary). After IVM the oocytes were denuded. Eighty-nine degenerated oocytes (26.8%) were discarded. Of the remaining 243 oocytes, 176 (72.4% of viable oocytes, 53% of total oocytes) had extruded the first polar body and were selected for ICSI. Cleavage rate was 69.3% (122/176, Table 1).

Forty-seven cleaved embryos were cultured *in vitro* and 75 were transferred to the sheep oviduct. On Day 7, 7 embryos (14.9% of cleaved) and 34 embryos (45.3% of cleaved) developed from the *in vitro* and the *in vivo* culture respectively (Table 2).

TABLE 1: Efficiency of oocyte recovery, *in vitro* maturation and cleavage after ICSI

No. ovaries	No. oocytes (no. per ovary)	No. degenerated (% of oocytes)	No. matured-injected (% of oocytes)	No. cleaved (% of matured)
85	332 (3.9)	89 (26.8)	176 (53.0)	122 (69.3)

TABLE 2: Effect of *in vitro* versus *in vivo* culture of cleaved horse embryos

Type of culture	No. cleaved embryos	No. developed to compacted morula or blastocyst (% of cleaved)
In vitro (SOF)	47	7 (14.9[a])
In vivo (sheep oviduct)	75	34 (45.3[b])

Student *t* test: values with different superscipts are significantly different (P<0.05)

DISCUSSION

A considerable amount of experimental work has increased our understanding of the *in vitro* requirements of early embryos in several mammalian species, particularly the bovine. By contrast, very little work has been done in horses and there are only few reports on the culture of equine embryos to the blastocyst stage (Battut *et al.* 1991; Ball and Miller 1992; Choi *et al.* 1994; Dell'Aquila *et al.* 1997; Li *et al.* 2001).

This study compared a completely *in vitro* system based on medium SOF with an *in vivo* culture system using the sheep oviduct. We have shown that early cleavage stage equine embryos derived from IVM and ICSI develop to morulae and blastocysts at a much higher rate following culture in the sheep oviduct as compared to *in vitro* culture in medium SOF. Moreover, the rate of development obtained in this study after *in vivo* culture is considerably higher than that in the published reports cited above. This suggests that the culture media used to now for equine embryos are not sufficiently optimised to allow all the competent embryos to develop to the blastocyst stage.

In conclusion, this study shows that a high rate of maturation and fertilisation by ICSI can be obtained with horse oocytes and that the effect of the following culture of the cleaved embryos has a major impact on the efficiency of the whole procedure.

This work was supported by MIPAF-RAIZ.

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SESSION VI:

Manipulation of the germ line

Chairman: B. Colenbrander

IN VITRO DEVELOPMENT OF HORSE OOCYTES RECONSTRUCTED WITH THE NUCLEI OF FETAL AND ADULT CELLS

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INTRODUCTION

Since the development of embryo transfer techniques in the horse (Allen and Rowson 1975), a variety of new breeding technologies such as *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and nuclear transfer (NT) have been applied to horse breeding, both in the laboratory and on the studfarm (Palmer *et al.* 1991; Li *et al.* 2000a,b; 2001). However, the low efficiency of *in vitro* embryo production in the horse has limited its commercial application in this species. The reasons for this are numerous and include the restricted availability of oocytes, practical problems with *in vitro* oocyte maturation and fertilisation and the need to develop an effective *in vitro* culture system for early embryonic development. The present study investigated the basic conditions required for nuclear transfer in the horse using both fetal and adult fibroblasts as donor cells.

MATERIALS AND METHODS

TCM199 (Gibco BRL, #22340-012, Grand Island, New York, USA) and DMEM (Gibco BRL, #22320-014) were used as the basic media for oocyte maturation and culture of the reconstructed oocytes after nuclear transfer. A monolayer of oviduct epithelial cells (OEC) from a frozen horse OEC culture line was used in co-culture with the oocytes during their maturation and during development of the reconstructed oocytes.

Horse ovaries were obtained from 2 abattoirs and transported to the laboratory over 20 h in PBS containing 125 iu/ml penicillin and 35 iu/ml streptomycin, at a temperature of 10–20°C. COCs were recovered from the ovaries by scraping the walls of follicles 0.5–3.0 cm in diameter and groups of 20–30 COCs were matured *in vitro* by co-culture with OEC for 28–30 h at 38°C in 5% CO_2-in-air. Fetal fibroblast cells (FFC) and adult skin fibroblast cells (AFC) were used as donor cells after they had undergone 3–7 passages. FFC were derived from a 32-day-old Thoroughbred x pony fetus and AFC were obtained from subdermal biopsies recovered from a 4-year-old female pony.

MII oocytes were enucleated in EBSS-20% v:v FBS with 5 µg/ml cytochalasin B (CCB) and a selected donor cell was injected into the perivitelline space of the recipient enucleated oocyte, alone or in combination with a small volume of inactivated Sendai virus. The fibroblast-cytoplasm couplets produced from FFC and AFC were aligned manually in 0.28 M mannitol fusion buffer and subjected to 2 DC pulses of 220–250 kv/cm, each for 30 µs. Fibroblast-cytoplasm couplets that fused were then activated chemically by transfer to PBS containing 5 µM ionomycin for 5 min, followed by culture for 2, 4 or 6 h in TCM199 medium containing 5 µg/ml CCB and 10 µg/ml cycloheximide.

Groups of 5–10 reconstructed oocytes were co-cultured with a monolayer of OEC in 500 µl drops of development medium for 6–7 days at 38°C in an atmosphere of 5% CO_2-in-air. Nuclear reprogramming (nucleus 2–3 times bigger than that of the original donor cell with an obvious nucleolus or some condensed chromatin) in the reconstructed oocytes was analysed 12–18 h after fusion treatment by staining with 5 µg/ml Hoechst 33342.

RESULTS AND DISCUSSION

Fusion of fetal and adult fibroblast cells with recipient cytoplasm

The effects of method of fusion of the FFC and AFC to recipient oocytes after nuclear transfer are

TABLE 1: Fusion of horse fetal and adult fibroblast cells with recipient cytoplasm followed by treatment with DC pulses alone or in combination with Sendai virus

Donor cells	Fusion treatment DC pulses	Sendai virus	Fusion results Total number of oocytes	No. fused (%)
FFC	+	-	44	25 (57[b])
FFC	+	+	51	42 (82[c])
AFC	+	-	41	20 (49[b])
AFC	-	+	32	5 (16[a])
AFC	+	+	121	98 (81[c])
AFC Control*	-	-	15	0

DC = direct current; FFC = fetal fibroblast cells; AFC = adult skin fibroblast cells.
Values with different superscripts are significantly different (P<0.05).
*Neither DC pulses nor Sendai virus were used for attempted fusion treatment.

TABLE 2: Nuclear reprogramming and cleavage of reconstructed horse oocytes following nuclear transfer using fetal and adult fibroblast cells

Donor cells	Culture time in CCB + cycloheximide (h)	Number of fused oocytes	No. reprogrammed (%)	No. 2-cell (%)
FFC	2	16	0 (63[a])	2 (13[a])
	4	18	18 (100[b])	6 (33[ab])
	6	34	30 (88[b])	18 (53[b])
AFC	6	81	68 (84[b])	28 (35[ab])

CCB = cytochalasin B; FFC = fetal fibroblast cells; AFC = adult skin fibroblast cells.
Values with different superscripts are significantly different (P<0.05).

TABLE 3: *In vitro* development of 2-cell horse embryos obtained from oocytes reconstructed by nuclear transfer of fetal or adult fibroblast cells

Donor cell	No. 2-cell embryos	No. (%) 2-cell embryos that developed to			
		<8-cell	>8-cell	>16-cell/morula	blastocyst
FFC	26	10 (38)	8 (31)	7 (27)	1 (4)
AFC	28	14 (50)	8 (29)	4 (14)	2 (7)

FFC = fetal fibroblast cells; AFC = adult skin fibroblast cells.

presented in Table 1. The fusion rate of FFC induced by DC pulses alone was significantly lower than that obtained when DC pulses were combined with Sendai virus (57% vs 82%, P<0.05). Similarly, rates of fusion with AFC were lower when the cell-couplets were stimulated by DC pulses alone compared to the combination of DC pulses and Sendai virus (49% vs 81%, P<0.05). On the other hand, Sendai virus alone induced fusion of only 16% (5/32) of the cell-couplets in the AFC group. Our results therefore suggest that, at least in the horse, there exists a useful synergy between electrical stimulation and treatment with Sendai virus for the induction of cell fusion.

Nuclear reprogramming and cleavage of the reconstructed oocytes

The rates of nuclear reprogramming and cleavage of the reconstructed oocytes in the FFC and AFC groups are compared in Table 2. When using FFC as donor nuclei the rate of reprogramming was increased by lengthening the culture time in CCB and cycloheximide from 2 h to 4 or 6 h (63%, 100% and 88%, respectively; 2 h vs 4–6 h, P<0.05). Furthermore, higher cleavage rates were obtained after 4–6 h culture (33-53%). Similar nuclear reprogramming and cleavage rates were obtained when AFC were used as donor cells following 6 h culture in DMEM supplemented

with CCB and cycloheximide (84% and 35% respectively).

In vitro *development of 2-cell stage embryos*

The development of 2-cell stage embryos reconstructed from the transfer of either FFC or AFC is summarised in Table 3. There were no significant differences in development rates of 2-cell stage embryos obtained from FFC and AFC ($P>0.05$). Only a very low proportion (4–7%) of 2-cell embryos derived from both donor cell types developed to blastocysts after 6–7 days co-culture in vitro with a monolayer of OEC. No differences in morphology were observed in the blastocysts derived from either fetal or adult cell types. This deficiency of developmental competence in the cloned horse embryos might be related to such factors as damage to the cytoskeleton during enucleation or exposure to ultraviolet light. Alternatively, it could be due to failure of formation of the 'equine blastocyst capsule' *in vitro*. This capsule is a tough, elastic investment which develops uniquely between the zona pellucida and the trophectoderm in the Day 6.5 late morula/early blastocyst stage equine embryo (Betteridge 1989; Oriol *et al.* 1993). It is considered to be essential for survival of the young embryo in the potentially hostile maternal uterus during the period of embryonic mobility (Days 6–17 after ovulation; Ginther 1985) by providing structural protection from the strong myometrial contractions and to aid the accumulation and imbibition of essential nutrient components in the exocrine endometrial gland secretions (Allen 2001).

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DEVELOPMENT OF A NOVEL TECHNIQUE FOR TRANSPLANTING SPERMATOGONIA TO THE TESTES OF LARGE ANIMALS

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The successful transplantation of spermatogonial stem cells, and therefore the germ-line, between individuals of the same species was first described by Brinster and Avbarbock (1994) and Brinster and Zimmermann (1994). Later studies have documented transplantation between species, and spermatogonial transplantation has been accepted rapidly as a powerful tool for studying the control of spermatogenesis, for allowing genetic manipulation of the male germ-line and for preserving the germ-line of genetically valuable males. However, while transplantation in rats and mice is straightforward, by microinjecting A-spermatogonia into multiple seminiferous tubules, transplantation into the testes of larger mammals has proved problematic. This is principally because, unlike in the mouse, the rete testis, the most desirable site for injection, is entirely intratesticular. Ultrasound-guided injection into the rete testis has achieved only moderate success. The aim of the current study was to develop an accurate and repeatable method for injecting spermatogonia into the rete testis of bulls.

Preliminary studies were performed on testes recovered from slaughtered bulls. In the first trial, a needle was guided ultrasonographically into the mediastinum testis and its position confirmed by scanning in 3 perpendicular planes. Success of needle placement was examined by injecting 5 ml of blue gelatine and immediately incising the testis to determine if the dye had passed along the rete. Because placement was not 100% successful, a second trial was performed in which a small volume of an ultrasound-positive contrast medium (Levovist-4; Schering AG, Germany) was injected after placement of the needle. Rapid progression of the contrast medium along the rete and into the seminiferous tubules confirmed correct needle location in all cases and in a subsequent test on a live-anaesthetised bull. In addition, culturing bull A-spermatogonia in the presence of the contrast medium proved that the latter did not compromise the viability of the cells destined for injection. Recently initiated studies will examine the outcome of injecting frozen-thawed A-spermatogonia into the rete testis of 5–7 month old bulls, whose testes have been 'emptied' of spermatogenic cells by irradiation.

In conclusion, we have developed an accurate method for injecting into the rete testis of bulls. Given that the rete testis of stallions is ultrasonically more defined, if narrower, than that of bulls, spermatogonial transplantation by rete testis injection may offer considerable potential for basic studies of spermatogenesis and for germ-line preservation in this species.

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STALLION EPIDIDYMAL SPERMATOZOA: PRE-FREEZE AND POST THAW MOTILITY AND VIABILITY AFTER 3 TREATMENTS

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INTRODUCTION

The maturation of spermatozoa in transit through the epididymis includes the acquisition of fertilizing capacity, progressive motility and plasma membrane characterisation. Each functional zone of the epididymis is regulated by mechanisms that are under the influence of testosterone and other androgens and about which little is understood. Such regulation is essential for the secretion of certain proteins and enzymes from the epididymal epithelium which modify the structure and metabolism of the spermatozoa in preparation for ejaculation.

Spermatozoa perfused from the epididymes of stallion testes have been shown to display progressive motility equal to, or better than, ejaculated spermatozoa (Squires 2000). However, per cycle conception rates achieved when using frozen-thawed epididymal spermatozoa are currently only ± 20% (Morris *et al.* 2000b).

The present experiment investigated the effects of seminal plasma (SP) and glass wool (GW) separation upon pre-freeze and post thaw motility and plasma membrane integrity of stallion epididymal spermatozoa, with a view to improving their fertilising capacity.

MATERIALS AND METHODS

Semen was flushed from the epididymes of testicles recovered during routine castration of 2-and-3-year-old Thoroughbred colts with either a skim-milk glucose diluent (Kenney), or SP followed by 1:1 dilution with Kenney. Half of each aliquot recovered in these ways was then filtered through GW before freezing.

For cryopreservation the samples were centrifuged at 400 *g* for 10 min and the supernatant decanted. The pellet was resuspended in a lactose-egg yolk-glycerol freezing extender and frozen in 0.5 ml straws suspended above liquid N_2 vapour. At thawing, the straws were placed in a water bath at 37°C for 30 s. The percentage of total progressively motile spermatozoa in each treatment group was determined after cooling the aliquot to 4°C, and after freezing and thawing. The percentage of spermatozoa with intact plasma membranes was assessed using a fluorescence microscope after staining the sample with propidium iodide in a 0.2% sodium azide solution.

Oestrous mares were inseminated hysteroscopically as described by Morris *et al.* (2000a) at 32 h after iv injection of an ovulating dose (1,500–3,000 iu) of human chorionic gonadotropin (Chorulon, Intervet, Milton Keynes, UK) given when the oestrous mare exhibited a dominant ovarian follicle ≥35 mm diameter. The appropriate aliquot of semen (500 µl) was aspirated into a wide bore equine GIFT catheter that was passed through the working channel of the videoendoscope. The latter was then directed up the uterine horn ipsilateral to the pre-ovulatory follicle and the inseminate was deposited gently onto the clearly visible uterotubal papilla.

T-tests were performed to analyse differences between the treatment groups.

RESULTS

Addition of seminal plasma did improve the initial total progressive motility (TPM) both after equilibration to 4°C and after freezing and thawing (Fig 1). However, this increase was not significant ($P>0.05$).

There were no differences in TPM when the epididymal spermatozoa were filtered through glass wool after having recovered them by

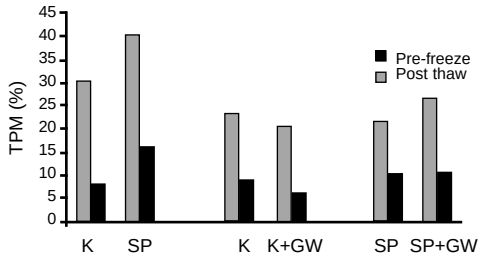

Fig 1: Total progressive motility (TPM) of epididymal spermatozoa after 3 treatments.
K = Kenney skim-milk extender; SP = Seminal plasma; GW = Glass wool.

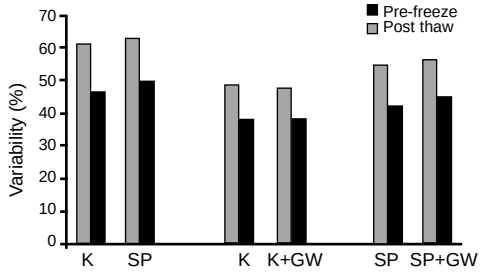

Fig 2: Viability of epididymal spermatozoa after 3 treatments.
K = Kenney skim-milk extender; SP = Seminal plasma; GW = Glass wool.

flushing the epididymes with Kenney or SP, or after having cooled, or frozen and thawed the spermatozoa. Likewise, the percentages of spermatozoa with intact plasma membranes after they had been cooled, or frozen and thawed following each treatment, were not significantly different (Fig 2).

The motility of epididymal spermatozoa was considerably lower than that of ejaculated spermatozoa, both before and after freezing. Similarly, the conception rates achieved when hysteroscopically depositing small aliquots (0.1–0.5 ml) of 2 doses (5×10^6 or 300×10^6) of fresh or frozen-thawed epididymal spermatozoa directly onto the uterotubal papilla at the tip of the uterine horn ipsilateral to the pre-ovulatory follicle were appreciably lower than those attained with similar doses of ejaculated fresh or frozen-thawed spermatozoa (Morris *et al.* 2000b). Furthermore, these conception rates were not improved by exposing the epididymal spermatozoa to seminal plasma prior to uterotubal deposition (Fig 3).

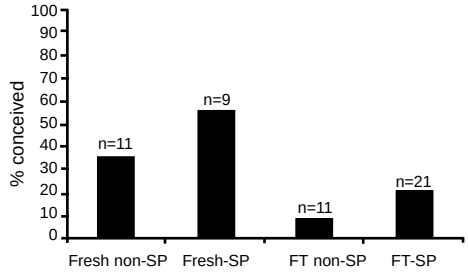

Fig 3: Conception rates achieved after hysteroscopic insemination of fresh or frozen-thawed (FT) epididymal spermatozoa exposed or not exposed to seminal plasma (SP).

DISCUSSION

In this experiment, the addition of seminal plasma to the medium used to flush the spermatozoa from the caudal portion of the epididymes of stallion testes improved the initial TPM of the spermatozoa. Six hours later, however, the motility was no better than that of the same epididymal spermatozoa that had not been exposed to SP. The complete role of seminal plasma is still not entirely understood although it is known to transport and sustain the initial motility of spermatozoa after ejaculation even though it may have detrimental effects on sperm longevity with increasing time (Baas *et al.* 1983). One of the constituents of the epididymal fluid and spermatozoa may be a 'sperm motility factor'. Such molecules have been isolated from the spermatozoa and epididymal fluid of many species (Bavister *et al.* 1978) and, it is thought, might be present in, or bound onto, the spermatozoa and may trigger motility of the spermatozoa when interacting with the components of SP. Plasma membrane integrity was not affected by the addition of SP, suggesting that lipid and protein composition of epididymal spermatozoa has a greater effect on cryosurvival than SP and cannot be correlated to initial or SP-enhanced motility. This feature has been investigated in other species with similar results (Graham 1994).

Glass wool filtration did not improve the quality or viability of epididymal spermatozoa, as it has been shown to do with fresh and frozen-thawed ejaculated spermatozoa (Samper *et al.* 1991). This result was surprising as fewer epididymal spermatozoa show intact plasma

membranes compared to ejaculated spermatozoa. However, the glass wool treatment did not appear to weed out the compromised cells or improve the overall viability of the filtered spermatozoa.

In summary, the results of this study demonstrated no beneficial effect of exposing epididymal spermatozoa to seminal plasma or glass wool filtration before cooling to 4°C, after freezing and thawing, or prior to hysteroscopic insemination.

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