Havemeyer Foundation
Monograph Series No. 3

Proceedings of the
5th International Symposium on

EQUINE EMBRYO TRANSFER

6th - 9th July 2000
Saari, Finland

Editors: T. Katila and J. F. Wade

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EDITORS' FOREWORD

The 5th International Symposium on Equine Embryo Transfer (EETV) was arranged as a satellite symposium of the 14th International Congress on Animal Reproduction (ICAR). This association with ICAR-2000 was reflected in the peripatetic venue of EETV. The meeting started on the Silja Serenade ferry in Stockholm and continued during the voyage to Helsinki. The second two days of the meeting was held in Saari, Finland.

EETV is one of a large number of symposia on equine reproduction funded by the Dorothy L. Russell Havemeyer Foundation. Sincere thanks are due to Mr Gene Pranzo, President of the Foundation, for recognising the importance and understanding the needs of international research on equine reproduction.

The scientific programme was of the highest quality, including the most novel techniques in equine embryo biotechnology. The presenters of the papers are to be thanked for their valuable contribution.

EETV was the first international equine reproduction event ever arranged in Finland. Previous meetings in this series have taken place in Cornell, Banff, Buenos Aires and Reims. Each congress has had its own particular features relating to the specific host countries. Finland is particularly well known for pure nature, lakes and sauna. Participants will get the opportunity to experience all of these.

We are confident that the meeting revealed a great deal of new information. We hope that all participants enjoyed their stay in Finland and returned home with good memories.

T. Katila
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

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

1983 **Third International Workshop on Lymphocyte Alloantigens of the Horse**
April - New Bolton Center, University of Pennsylvania, USA
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

SESSION 1:

Horses and hybrids

Chairman: D. C. Sharp

THE EQUIDS AND EMBRYOS OF J. COSSAR EWART: A GLIMPSE OF VICTORIAN AND EDWARDIAN RESEARCH IN REPRODUCTION

K. J. Betteridge

Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

James Cossar Ewart (1851–1933) lived through times of turmoil in the biological sciences, particularly those related to the understanding of heredity. In particular, there was conflict between 'men of science' and practical animal breeders, as well as among scientists, as to whether 'telegony' - the concept of one sire affecting the phenotype of a dam's subsequent offspring by different sires - is a real phenomenon. Those disputes, which preceded the rediscovery of Mendel's observations, spawned 2 different experimental approaches to the study of heredity, both of which were significant to the development of equine embryo transfer. First, there was the development, in rabbits, of the transfer procedure itself, by Walter Heape and George Romanes, independently. Ewart, on the other hand, undertook a large-scale crossbreeding experiment with horses and zebras at Penycuik, near Edinburgh. He tested the evidence for telegony that had been based on the famous case of Lord Morton's mare. She had supposedly been 'infected' by a quagga sire and produced a striped foal when subsequently bred to an Arab stallion. Ewart's studies also led to the first descriptions of the chorionic girdle (commemorated in a previous Havemeyer Symposium), of equine embryos and foetuses from the third week of gestation, and their relevance to the phylogenetic history of the horse, and of the value of comparative observations in reproduction. In considering Ewart's contributions to our science, published information will be supplemented with material gleaned from his papers and correspondence held at the University of Edinburgh. On the positive side, the latter material documents interesting exchanges between Ewart and several giants in our field (Heape, Marshall, Hill, Hammond and Catchpole). Letters tell familiar tales of the difficulties of financing research, and less familiar ones of contact with royalty, Downing Street and the aristocracy. Against this must be balanced disturbing tracts on eugenics. Overall, however, the material offers instructive insight into the life and times of:

"He who had talked with Darwin and Huxley, who had witnessed the change in human thought consequent upon the promulgation of evolution theory, [and] was notable amongst his fellows for the reason that he, a professional biologist and a University professor, left the laboratory for the farm to use animals of economic importance as his experimental material and to attack problems which not only possessed a scientific interest, but which, in their solution, might confer advantage on the community."

(From the obituary of J. Cossar Ewart by F.A.E. Crew, Animal Breeding Abstracts, 1934).

HYSTEROSCOPIC UTEROTUBAL INSEMINATION OF MARES WITH LOW NUMBERS OF FROZEN-THAWED EJACULATED AND EPIDIDYMAL SPERMATOZOA

L. H. A. Morris, C. Tiplady, S. Wilsher and W. R. Allen

Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK

INTRODUCTION

First cycle conception rates for mares inseminated with frozen-thawed ejaculated spermatozoa have been reported to range from 32% (Vidament *et al.* 1997) to 58% (Samper 1995). These figures have been achieved after performing repeated ultrasound examinations of follicular development at 4–8 h intervals to ensure that a minimum of 200–500 million spermatozoa are deposited in the uterus close to the time of ovulation. The present study sought to inseminate mares hysteroscopically (Morris *et al.* 2000) with low numbers of frozen-thawed ejaculated and epididymal spermatozoa at a fixed time after administration of an ovulation-inducing dose of human Chorionic Gonadotropin (Chorulon, Intervet, Milton Keynes, UK).

MATERIALS AND METHODS

Semen was collected by artificial vagina from two identical twin Pony stallions of known high fertility, and by flushing the epididymes of testicles recovered during routine castration of young Thoroughbred colts. Both the ejaculated and epididymal samples were extended in an equal volume of a skim milk – glucose diluent before being centrifuged at 300 *g* for 10 min. The supernatant was decanted and the sperm pellet was resuspended in either a Tris-based or a HEPES-buffered extender supplemented with glycerol. The resulting sperm suspensions were then frozen in liquid N_2 vapour in 0.5 ml straws. At thawing the straws were placed in a 37ºC water bath for 30 s.

The ovaries of the oestrous mares to be inseminated were monitored ultrasonographically once daily and, when a dominant follicle of ≥35 mm diameter was observed an ovulation-inducing dose of 1,500–3,000 iu hCG was administered iv. Between 30 and 32 h later, the mares were inseminated as follows: Group 1: 25 million spermatozoa in 0.5 ml diluent by conventional artificial insemination (n = 12); Group 2: 25 million spermatozoa in 0.5 ml diluent, deposited hysteroscopically on the uterotubal papilla at the tip of the uterine horn ipsilateral to the impending ovulation (n =14); Group 3: 5 million spermatozoa in 0.1 ml diluent, deposited hysterscopically on the ipsilateral uterotubal papilla (n = 34); Group 4: 5 million spermatozoa in 0.1 ml diluent deposited hysteroscopically in the posterior body of the uterus to simulate the site of conventional artificial insemination (n = 8); Group 5: 5 million spermatozoa in 0.1 ml diluent deposited hysteroscopically on the uterotubal papilla contralateral to the impending ovulation (n = 12); Group 6: 300 million epididymal spermatozoa in 0.5 ml diluent deposited hysterscopically on the uterotubal papilla ipsilateral to the impending ovulation (n = 25); Group 7: 5 million epididymal spermatozoa in 0.1 ml diluent deposited hysteroscopically on the uterotubal papilla ipsilateral to the impending ovulation (n = 19).

RESULTS

Of all the 148 inseminated mares, 124 (83.8%) had ovulated within 15 h after the insemination in response to the gonadotropin injection and these animals were therefore included in the results, which are summarised in Figure 1.

DISCUSSION

The results demonstrate clearly that high conception rates are achievable in mares

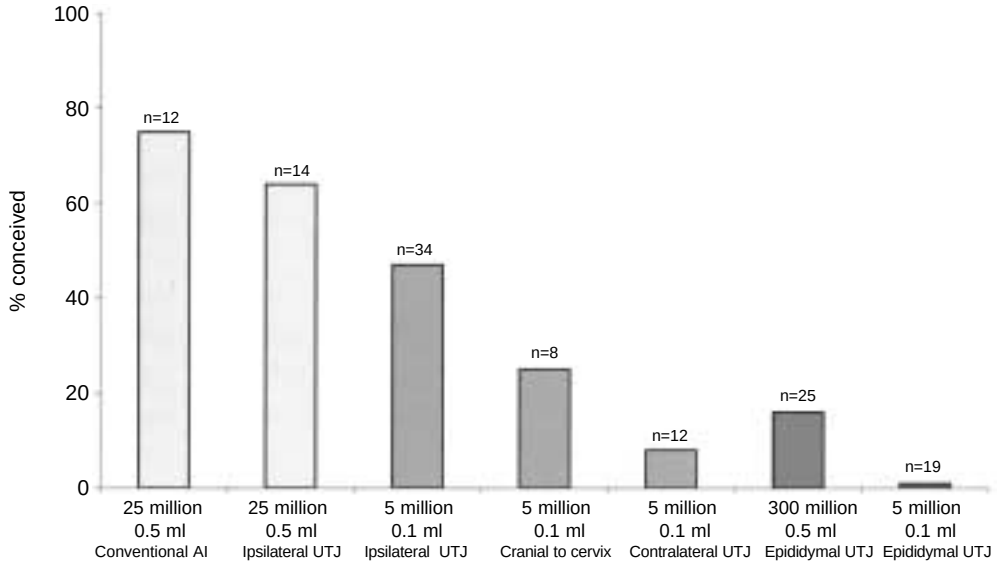

Fig 1: Conception rates achieved in mares inseminated hysteroscopically and conventionally with low numbers of frozen-thawed ejaculated and epididymal spermatozoa.

inseminated once with frozen-thawed ejaculated spermatozoa from fertile stallions at a fixed time after an ovulation-inducing dose of gonadotropin. Not only were high conception rates obtained using both conventional uterine body and hysteroscopic uterotubal insemination techniques, the number of frozen-thawed spermatozoa inseminated was only 5–10% of the minimum number of frozen thawed spermatozoa used conventionally in commercial insemination programmes.

When the insemination dose was 25 million motile spermatozoa, the hysteroscopic uterotubal method of insemination held no advantage over the conventional uterine body insemination technique. When the insemination dose was reduced to only 5 million spermatozoa in 0.1 ml extender, however, the hysteroscopic uterotubal method showed a definite advantage over conventional deposition of the inseminate just cranial to the cervix.

Not surprisingly, only one mare conceived after depositing the low dose of frozen-thawed spermatozoa onto the uterotubal papilla contralateral to the side of ovulation. This reflects the fragility of the frozen-thawed cells and their poor ability to migrate around the uterus to achieve a satisfactory sperm reservoir at the site of fertilisation.

The low number of pregnancies obtained in mares inseminated with frozen-thawed epididymal spermatozoa was disappointing. The epididymal spermatozoa showed an appreciably lower motility than the ejaculated spermatozoa, both before and after freezing, and they exhibited a higher proportion of acrosome-reacted spermatozoa after freezing. Thus, it appears that the longevity, viability and fertilising potential of epididymal spermatozoa is much less than ejaculated spermatozoa after freezing and thawing, and this deficiency cannot be overcome by hysteroscopic deposition of the sample close to the site of fertilisation.

The most important outcome of the study was that high conception rates can be achieved in mares inseminated with low numbers of frozen-thawed ejaculated spermatozoa at a fixed time after the administration of an ovulation inducing agent.

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Loomis, P.R., Amann, R.P., Squires, E.L. and Pickett, B.W. (l983) Fertility of unfrozen and frozen stallion

HYSTEROSCOPIC INSEMINATION OF NON FROZEN AND FROZEN UNSEXED AND SEXED EQUINE SPERMATOZOA

A. C. Lindsey, L. H. A. Morris*, W. R. Allen*, J. L. Schenk†, J. K. Graham, J. E. Bruemmer and E. L. Squires

*Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA; *Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK; †XY, Inc., ARBL Building, Foothills Research Campus, Fort Collins, Colorado 80523, USA*

A safe and reliable method for preconceptual sex selection of offspring has been sought for decades in humans, livestock, and even companion animals. With a method developed some 10 years ago (Johnson *et al*. 1989), effective preselection of sex has been accomplished in many species of livestock, as well as in humans (Johnson 1991; Cran *et al.* 1997; Seidel *et al.* 1997; Fugger 1999). Sex preselection has also been successful in horses (Buchanan *et al.* 2000), but due to the limited number of spermatozoa available after the sorting process, traditional breeding doses are not available. Therefore, low-dose insemination techniques must be improved in order to maximise the efficiency of sex-sorted spermatozoa. Hysteroscopic insemination has recently been shown to produce acceptable pregnancy rates when using only one million freshly collected motile spermatozoa (Morris *et al.* 2000). With these encouraging results, it has been hypothesised that hysteroscopic insemination could be an effective and practical method to achieve pregnancies using low numbers of sex-sorted spermatozoa. The objectives of Experiment 1 were: 1) to compare pregnancy rate with 5 x 10^6 spermatozoa inseminated deep in the uterine horn aided by ultrasonography, or deposited onto the uterotubal papilla with the use of a flexible video-endoscope; and 2) to determine if hysteroscopic insemination of sexed spermatozoa can result in satisfactory pregnancy rate.

Semen was collected from 2 stallions of acceptable fertility. Oestrus was synchronised (June–July) in 40 mares, ages 3–10, by administering 10 ml of altrenogest orally for 10 days, followed by 250 µg cloprostenol im on Day 11. Mares were given 3,000 iu hCG iv at the time of insemination and assigned to one of 3 groups: Group 1 mares (n=10) were inseminated with 5 x 10^6 Percoll-washed spermatozoa deposited deep into the uterine horn with the aid of ultrasonography. Group 2 mares (n=10) were inseminated with 5 x 10^6 Percoll-washed spermatozoa deposited onto the uterotubal papillae via hysteroscopic insemination. Group 3 mares (n=20) were inseminated using the hysteroscopic technique with 5 x 10^6 sex-sorted spermatozoa. Spermatozoa were stained with Hoechst 33342 and sorted into X and Y chromosome-bearing populations based on DNA content using an SX MoFlo sperm sorter. Pregnancy was determined ultrasonographically at 16 days post ovulation.

Hysteroscopic insemination resulted in more pregnancies than did the ultrasound-guided

TABLE 1: Pregnancy rate resulting from hysteroscopic or deep intrauterine insemination

Treatment	Mares inseminated	Pregnant (16 d)	Pregnancy rate (%)
Non-sorted sperm, ultrasound guided	10	0	0^a
Non-sorted sperm, hysteroscopic	10	5	50^b
Sex-sorted sperm, hysteroscopic	20	5	25^{ab}

^{a,b} Values without common superscripts differ (P<0.05)

TABLE 2: Pregnancy rate resulting from insemination of fresh/frozen non-sorted/sorted sperm

Treatment	Mares inseminated	Pregnant (16 d)	Pregnancy rate (%)	Pregnant/inseminated E	Γ
Fresh non-sorted	10	4	40		
Fresh sex-sorted	16	6	38	2/8	4/8
Frozen/thawed non-sorted	16	6	38		
Frozen/thawed sex-sorted	15	2	13	0/7	2/8

technique when non-sorted sperm were inseminated. Pregnancy rates were not significantly lower when hysteroscopic insemination was used for sorted vs non-sorted spermatozoa.

The lack of pregnancies in Experiment 1 following deep intrauterine insemination was unexpected. One cause for these poor results could be the small insemination volume used (100 µl). Buchanan *et al.* (2000) was able to achieve 35% pregnancy rates with a similar method, while using a larger volume for insemination. Another significant difference between the 2 studies lies in the treatment of sperm prior to insemination. In the previous study, non-treated sperm were inseminated within 1 h of collection. In the present experiment, sperm were inseminated 8 h or more following collection, and were subjected to numerous treatments, including 2 centrifugation steps. Further studies are needed to determine the applicability of this method of horn insemination. Based on the results of Experiment 1, deep uterine insemination with the aid of a video-endoscope is the preferred method for low-dose insemination of non-sorted, as well as sex-sorted spermatozoa.

Experiment 2 was conducted to compare effects of sexing and freezing stallion sperm on fertility using a 2 x 2 factorial design: sexed or unsexed vs fresh or frozen. Oestrus was synchronised in 41 mares as presented in the first experiment. Mares were administered 3,000 iu hCG iv either 6 h (fresh sperm) or 30 h (frozen/thawed sperm) prior to insemination. Hysteroscopic insemination was performed on all mares with 5×10^6 motile sperm (230 µl). Mares were randomly assigned to one of 4 treatment groups: Group 1 (n=10): inseminated with fresh, non-sorted spermatozoa; Group 2 (n=16): inseminated with fresh sex-sorted spermatozoa. Group 3 (n=16): inseminated with frozen non-sorted spermatozoa. Group 4 (n=15): inseminated with frozen, sex-sorted spermatozoa. Concentrations of sperm were adjusted before freezing, based on predetermined average post thaw motilities, so that each dose contained approximately 5×10^6 motile sperm post-thaw. Mares in Groups 1 and 2 were inseminated approximately 8 h after collection, based on the time needed to process and sort one insemination dose. From collection to insemination the spermatozoa were protected from light and held at room temperature in an adapted HEPES-buffered tyrodes solution (Parrish *et al.* 1988).

No difference was found ($P>0.1$) in pregnancy rate for mares inseminated with fresh non-sorted, fresh sex-sorted or frozen non-sorted spermatozoa (Table 2). Fewer mares became pregnant following insemination of frozen, flow-sorted spermatozoa compared to the other treatments, but this difference was not significant, likely due to low group numbers.

These studies demonstrated that hysteroscopic insemination can be used to obtain pregnancies with low numbers of fresh and frozen equine spermatozoa, as well as low numbers of sex-sorted spermatozoa. Further studies are needed to improve pregnancy rate with sex-sorted, frozen equine spermatozoa.

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UTERINE BLOOD FLOW DURING OESTROUS CYCLE AND EARLY PREGNANCY IN MARES

H. Bollwein, R. Mayer and R. Stolla

Gynaecological Animal Clinic, University of Munich, Germany

INTRODUCTION

While there are several studies on uterine blood flow during early pregnancy in other species (Greiss and Anderson 1970; Ford and Christenson 1979; Ford *et al.* 1989), until now there are no information about uterine perfusion during oestrous cycle and early pregnancy in mares. In a preliminary study we could demonstrate that transrectal Colour Doppler sonography is a useful method to measure impedance to uterine blood flow in mares (Bollwein 1998). In this study we aimed to use this technique for the examination of uterine blood flow during oestrous cycle and early pregnancy in mares.

MATERIAL AND METHODS

Five Trotter mares with a mean age of 9.2 years (range 6–13 years) were examined as described below. Two of them were multiparae and 3 were nullipareae. Each mare underwent transrectal Doppler investigations by the same operator over 2° oestrous cycles and 2 early pregnancies. The time of ovulation was determined from daily real-time ultrasound examinations, the last day on which the dominant follicle was visible being defined as Day 1, and the first day on which the ovulatory follicle was gone being Day 0. Examinations were carried out daily between Day °0 (=° ovulation) and Day °3, then every second day until Day 29 of pregnancy. During oestrous cycle investigations were performed daily again between Day °3 and Day 0 of the next oestrous cycle.

Both the left and right *Aa. uterinae* were investigated transrectally as published earlier (Bollwein 1998). The transrectal pulsed Doppler ultrasound examinations were always carried out at the same time (ie from 16.00–22.00 h) and lasted about 20 min for each mare. All obtained blood flow velocity waveforms were were displayed online and recorded on videotapes. Following the collection of all data, the Doppler calculations were performed offline by using 2 similar consecutive flow velocity waveforms with a maximum enddiastolic frequency shift. The analysis was based on the resistance index (RI), which is calculated as the ratio of the difference between peak systolic frequency shift (PSF) and end-diastolic frequency shift (EDF) to peak systolic frequency shift: RI = (PSF-EDF)/PSF). The RI increases if the proximal conditions remain constant and the distal vascular bed constricts. Conversely, a low value for the RI indicates decreased impedance to blood flow in the distal vasculature. The RI values of the two uniform consecutive pulse waves were averaged. To study the intraobserver reproducibility the *A. uterina* was examined 2 times, the interval between each measurement in the same vessel being approximately 5–15 min.

Statistical analyses were carried out using the Statview II+Graphics statistical software package (Abacus Concepts, Inc, California, USA, 1992) and the Statistical Analysis System (SAS Institute, North Carolina, USA, 1996).The resistance index of the left and right *A.°uterina* and of the pregnant and non pregnant *A. uterina* were compared using correlation coefficient and paired Student's *t*-test. Measurements were subjected to analysis of variance of replicate measurements, taking into account the between animals variance component and the between cycles within animal component. Intraobserver reproducibility of Doppler measurement results were expressed as intraclass correlation coefficient (Intra-CC).

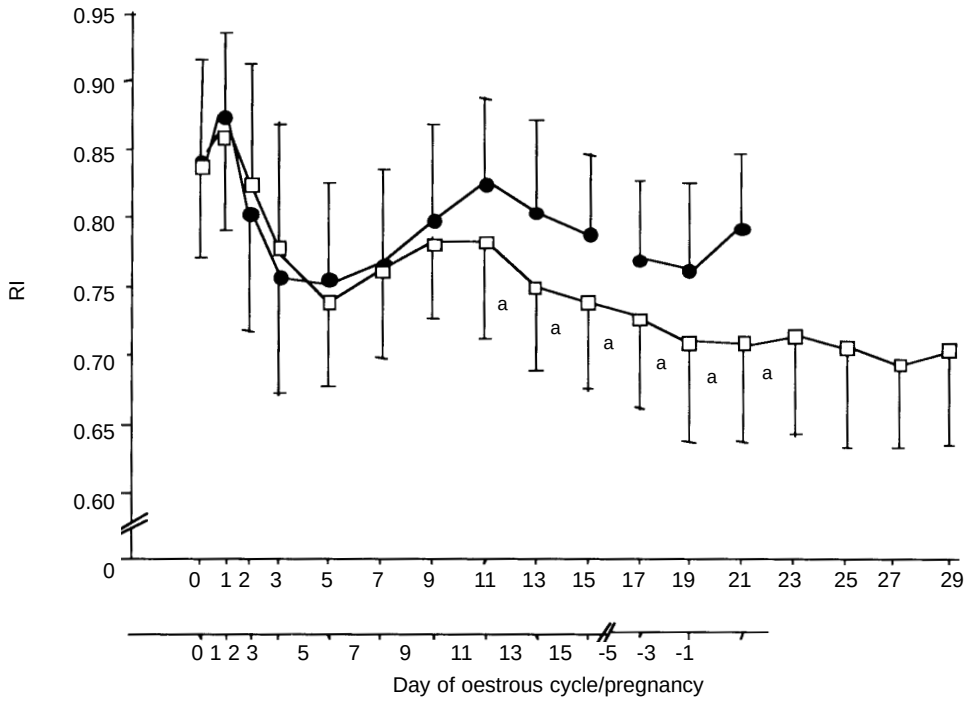

Fig 1: Resistance Index values (RI) from the Aa. uterinae *during oestrous cycle (●) and early pregnancy (□). Values are means ± sd of 2 oestrous cycles and 2 pregnancies of 5 mares. RI values during oestrous cycle with letter 'a' are different from the RI values on the corresponding days of pregnancy (P<0.05).*

RESULTS

The intra-class correlation coefficient concerning the intraobserver repeatability of RI measurements was 0.98. As during the oestrous cycle the changes of RI values in the left and right uterine arteries were correlated (RI:°r°=°0.86; P°<°0.0001) the mean of the 2 sides were used in analyses. In order to facilitate comparison of blood flow parameters between oestrous cycles with different cycle lengths, the measurement data from Day 0 to Day 15 and from Day -°5 until next ovulation were used for analyses.

Uterine blood flow of all oestrous cycles followed a consistent biphasic pattern (Fig 1). The highest RI occurred on Day 1 and Day 11. Minimum RI values were observed between Days 3 and 7 and between Day -5 and Day -1.

Comparison of the mean RI values of the oestrous cycles and early pregnancies revealed no differences until Day 9 (Fig 1). From Day 11 on, the mean RI values of the pregnancies were significantly lower compared to the values on the corresponding days of the oestrous cycle (paired *t*-test, I°<°0.05). Starting on Day 15 the resistance to uterine blood flow was significantly lower and showed a more distinct decline up to Day 29 of pregnancy at the uterine horn ipsilateral to the embryonic vesicle (paired *t*-test, P°<°0.05).

DISCUSSION

The results of our study show that transrectal flow imaging can be used to obtain blood flow velocity waveforms from the uterine arteries at any time during the oestrous cycle and early pregnancy in mares. With the complementary use of colour Doppler, the uterine artery could be readily located and an optimal angle for insonation with pulsed Doppler could be provided. Due to the limited size of the uterine arteries, a reliable measurement of vessel area was not possible; therefore the volume flow could not be established accurately. However, assuming that factors such as cardiac performance, vessel compliance, blood viscosity and perfusion pressure in the uterine artery remain constant, a decrease in the resistance index would be associated with a proportional increase in flow

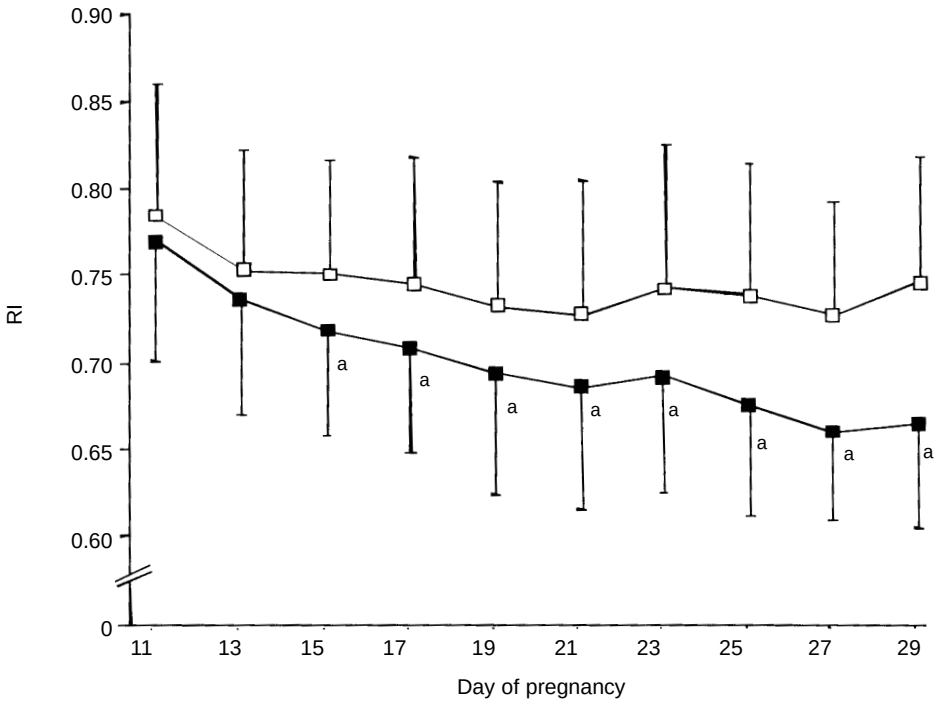

Fig 2: Resistance Index values (RI) from the Aa. uterinae *ipsi- (■) and contralateral (□) to the embryonic vesicle. Values are means ± sd of 2 oestrous cycles and 2 pregnancies of 5 mares. RI values with letter 'a' during oestrous cycle are different from the RI values on the corresponding days of pregnancy (P<0.05).*

(Scholtes 1989). The close proximity of the probe to the A. uterina enabled us to use ultrasound at a high frequency, thus producing a great Doppler shift. This advantage was reflected in the high intra-class correlation coefficients of the resistance index. The intraclass correlation coefficient indicated, which proportion of the variability in measurement results could be explained by real differences. As a rule, values for intra-class correlation coefficients above 0.75 are said to be satisfactory (Burdock 1963).

During the oestrous cycle we found similar changes in uterine blood flow as in women (Goswamy and Steptoe 1988; Sladkevicius 1993). The lowest impedance to uterine blood flow could be measured during early dioestrous and during oestrous, while the highest RI values were observed immediately after ovulation and around Day 11 of the oestrous cycle. The mechanism regulating the blood flow in the uterine arteries is complicated and multifactorial (Ford 1989). Oestrogens have a vasodilatatory effect, but the mode of intracellular signaling for the induction of this effect is still not understood (Greiss *et al.* 1986). A direct vasodilatory effect is possible, because both the oestrogen receptores in the tunica media of uterine arteries and oestrogen are known to decrease calcium uptake through potential sensitive channels (Ford 1989).

While during the first days after ovulation uterine blood supply was similar in cyclic and pregnant mares, from Day 11 on uterine perfusion was significantly higher in pregnant mares. Starting on Day 15, already prior to fixation of the conceptus, we observed a significantly higher blood flow in the uterine artery supplying the uterine horn ipsilateral to the embryonic vesicle. These findings indicate a local control of uterine blood flow by the early equine conceptus. Ford et al. (Ford and Chenault 1979) observed a similar rise in blood supply of the uterine horn ipsilateral to the embryo in cows. These authors suggested that these local changes in uterine perfusion may have resulted from oestrogens produced by the embryo. Oestrogens are produced by the equine embryo (Ginther 1992), too and might therefore be

one reason for the increase in uterine blood supply. But there are also other factors of embryonic origin like prostaglandin E2 (Weber and Woods 1993) which could improve uterine blood flow.

In conclusion, the results of this study show that there is a cyclic pattern of uterine blood perfusion in mares measurable by transrectal colour Doppler sonography. As in other species, the blood supply of the pregnant uterus compared to the non-pregnant uterus increases already during early stages of pregnancy. The factors regulating the blood flow in the uterine arteries in cycling and pregnant mares need to be investigated in further studies.

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SESSION 2:

Oocytes – maturation and fertilisation

Chairmen: E. L. Squires and H. Lehn-Jensen

FOLLICULAR WAVES AND SELECTION OF FOLLICLES IN MARES

O. J. Ginther, D. R. Bergfelt and F. X. Donadeu

Animal Health and Biomedical Sciences, 1656 Linden Drive, University of Wisconsin, Madison, Wisconsin 53706, USA

Follicle selection in mares is the mechanism whereby only one of many available follicles of a wave becomes the ovulatory follicle. Part of this report is a synopsis of a review on selection that was written in July 1999 and the references up to that month are cited (Ginther 2000a). The other part discusses studies completed from August 1999 to August 2000.

Follicular waves in mares can be classified as major waves (characterised by dominant and subordinate follicles) and minor waves (largest follicle does not attain the diameter of a dominant follicle). Both types of waves develop in association with a surge in FSH concentrations. In major waves, the future dominant follicle is detected earlier, on average, than the other follicles, and the follicles grow at a similar rate for several days (parallel growing phase). The difference in mean diameter between the 2 largest follicles at first detection and during the parallel phase is 2–3 mm, which is equivalent to a growing period of approximately one day. When the largest follicle reaches a mean of about 22.5 mm, the parallel phase ends and follicle deviation begins. Deviation is recognised by a continuing growth rate of the largest or developing dominant follicle and a decreasing growth rate of the subordinate follicles. The mean diameter differences between the 2 largest follicles at the beginning of deviation suggests that the largest follicle becomes established as the dominant follicle within one day or before the next largest follicle reaches a similar diameter (Fig 1).

The FSH surge that stimulated emergence of the wave begins to decline in concentrations when the largest follicle reaches a diameter of about 13 mm. The FSH decline continues during the remaining parallel growing phase and for several days after follicle deviation. The necessity for low concentrations of FSH for deviation is consistent with the formation of multiple dominant follicles following administration of FSH or a substance (anti-inhibin) that raises the endogenous concentrations of FSH.

According to studies in cattle, all of the follicles of the wave continue to utilise the declining FSH concentrations during the parallel growing phase. Results of a recent study in mares (Donadeu and Ginther 2000) demonstrated that more than one follicle of the parallel phase contributes to the FSH decline. The effect of FSH on the follicles and conversely the effect of the follicles on FSH during the parallel phase have been described as a 2-way functional coupling, involving multiple follicles. The FSH/multiple-follicle coupling becomes an FSH/single-follicle coupling at the end of the parallel phase or the beginning of diameter deviation. Two-way FSH/follicle coupling has been postulated to be the basis of follicle selection (Ginther 2000a,b). Ironically, the pool of growing follicles directs a continuing decline in available FSH, despite the follicles continuing requirement for FSH. By the time the FSH concentrations reach a precarious level at the end of the parallel phase, only the most developed or largest follicle is able to utilise the low levels and to direct a continuing decline. The ability of the developing dominant follicle to utilise the low FSH concentrations, at least initially, has been demonstrated in cattle; a similar study has not been done in mares.

Results of a recent study in mares indicate that inhibin is the substance that is secreted by the multiple follicles and causes the FSH decline during the parallel growing phase (Donadeu and Ginther 2000). All follicles 6 mm or larger were

ablated 10 days after ovulation. Circulating FSH concentrations increased and inhibin decreased within one or 2 days after ablation. Either the largest, 3 largest, or all follicles of the subsequent new wave were retained and the remainder ablated before they reached >10 mm. Data were normalised to the day the largest follicle of the post-ablation wave reached 13 mm (expected beginning of the FSH decline). Concentrations of FSH decreased and inhibin increased during the remaining parallel growing phase. In the one-follicle group, the increase in inhibin concentrations after the beginning of the FSH decline was delayed and was temporally associated with a slower decrease in FSH than in the groups with three or all follicles retained. The interrelationships among FSH, follicles, and inhibin were also demonstrated in a group in which all follicles of the new wave were ablated at <10 mm during sequential daily sessions. The FSH concentrations increased to about 2- to 5-fold higher than in the groups with retained follicles, and inhibin remained at low basal concentrations. After the end of the ablation sessions, inhibin concentrations increased and FSH concentrations decreased when the largest follicle of the new wave reached about 13 mm. The hypothesis was supported that the magnitude of the FSH decline was proportional to the number of follicles >13 mm from the beginning of the FSH decline until the end of the parallel phase. This effect was attributable to the secretion of inhibin, based on the temporal relationships between increasing inhibin concentrations and decreasing FSH concentrations and on the positive relationship between the number of retained follicles and the extent of the increase in inhibin. Near the expected end of the parallel growing phase or beginning of deviation, concentrations of FSH were no longer different among groups with various numbers of retained follicles, and inhibin appeared to reach maximum concentrations. At the beginning of deviation, the FSH concentrations apparently are already too low in most waves for survival of the less-developed smaller follicles.

In a recent cattle study (Ginther *et al.* 2000b), an increase in FSH concentrations occurred immediately following ablation of the largest follicle at the beginning of deviation and a minimal single dose of oestradiol at the time of ablation delayed the FSH increase. This finding indicated that oestradiol was one of the FSH-depressing factors that was lost when the largest follicle was ablated. Thus, oestradiol contributes to the continued depression in FSH concentrations after the expected beginning of deviation. A similar study has not been done in mares. However, increasing oestradiol concentrations are secreted from the largest follicle in mares just before the beginning of deviation. The continuing decrease in concentrations of FSH at the beginning of deviation likely reflects the synergistic effect (Miller *et al.* 1979) of high concentrations of inhibin and the increasing concentrations of oestradiol. However, the relative contributions of inhibin and oestradiol in the suppression of FSH at various times during deviation have not been determined adequately. The role of the continuing decline in FSH after the beginning of deviation is not known. It may serve as an assurance that FSH is adequately depressed for deviation to occur in all waves or may prevent the emergence of another wave.

Increasing or transiently elevated LH concentrations encompass deviation in both mares and cattle (Ginther *et al.* 2000c). Concentrations of LH have been experimentally reduced by doses of progesterone that did not alter the concentrations of FSH. In initial studies in mares, the reduced LH was associated with a smaller maximum diameter of the dominant follicle. However, this effect occurred well after the beginning of follicle deviation; the growth profile of the second-largest follicle was not altered. In a more recent study in mares (Bergfelt *et al.* 2000), experimental reduction in concentrations of LH beginning before deviation was associated with reduced circulating concentrations of oestradiol. However, the LH and oestradiol reduction did not alter FSH concentrations until 2 days after the expected beginning of deviation. At that time, an increase in FSH concentrations occurred as well as a reduction in diameter of the dominant follicle. These results indicated that the increased concentrations of LH that encompass deviation are utilised by the developing dominant follicle for the secretion of oestradiol. The oestradiol, as noted above, contributes to continued FSH suppression and may also be important for facilitating growth of the dominant follicle and a change from primarily FSH to LH dependency.

The research model that our laboratory is currently using for the mechanism of follicle selection in mares is shown (Fig 1). It is recognised that good research models are modified and poor models fade away.

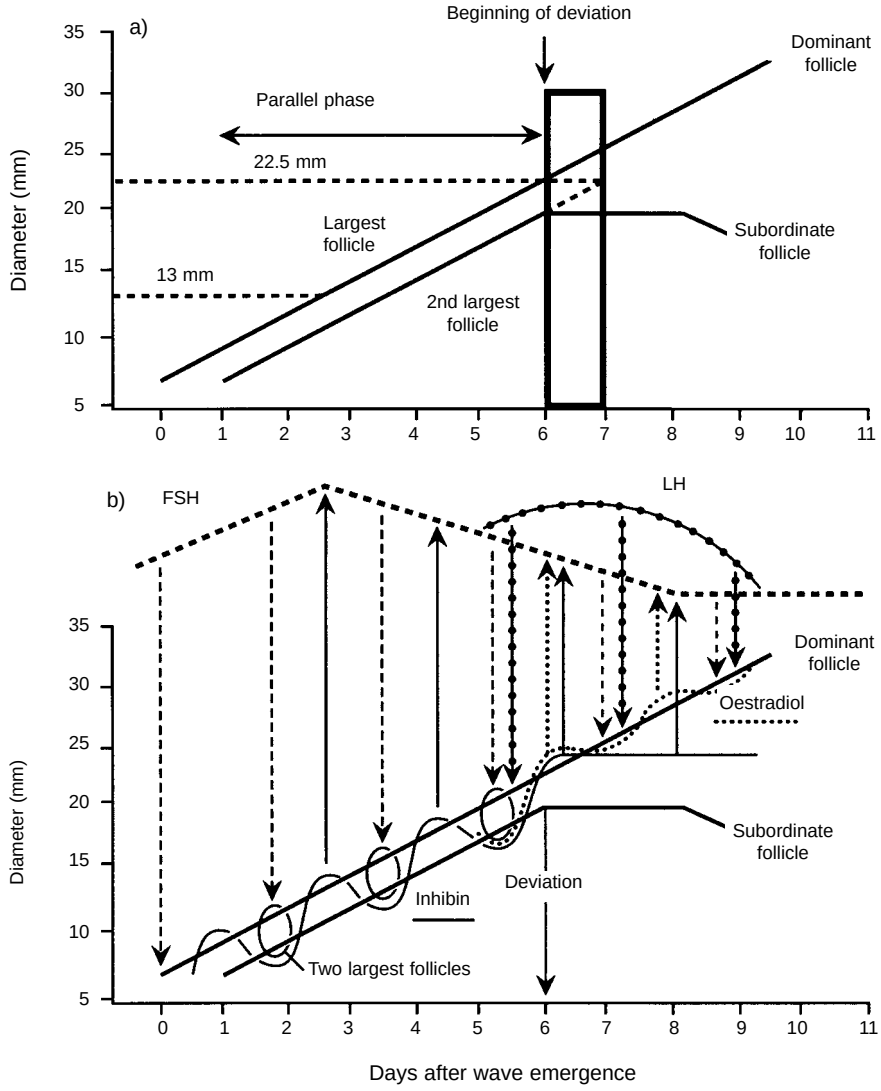

Fig 1: Schematic 2-follicle model of the size advantage of the largest follicle (a) and the hormonal aspects (b) of follicle deviation in mares. The largest follicle emerges, on average, about one day before the second-largest follicle, and the 2 follicles grow in parallel (a). When the follicles reach 13 mm, they begin to secrete a biologically active form of inhibin. As a result, the FSH surge that stimulated emergence of the follicles, begins to decline (b). Both follicles secrete increasing inhibin causing the continuing FSH decline, and the declining FSH is utilised by both follicles (2-way functional coupling). The parallel phase ends and follicle deviation begins when the largest follicle reaches 22.5 mm (a). Deviation is characterised by continued growth rate of the largest or developing dominant follicle and decreasing growth rate of the smaller or developing subordinate follicle. Deviation is established in less than one day or before the second-largest follicle can reach a similar diameter (represented by the width of the vertical bar in panel a). During this time, the FSH/follicle coupling changes from multiple-follicle to single-follicle. The more-developed larger follicle continues the FSH/follicle coupling because it is able to utilise the reduced concentrations of FSH. However, the less-developed smaller follicle is unable to maintain follicle coupling because the declining concentrations of FSH have reached a level below its requirements. Just before the beginning of deviation, oestradiol is secreted by the largest follicle under the influence of increased concentrations of LH (b). The increasing oestradiol acts synergistically with plateaued inhibin to continue the reduction in FSH concentrations after deviation. The elevated LH continues to stimulate the production of oestradiol by the developing dominant follicle and exerts a trophic effect on the dominant follicle within 2 days after the beginning of deviation.

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ORGANISATION OF THE CYTOSKELETON DURING *IN VITRO* MATURATION OF HORSE OOCYTES

J. L.Tremoleda, E. J. Schoevers*, T. A. E. Stout, B. Colenbrander and M. M. Bevers*

*Department of Equine Sciences, *Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

INTRODUCTION

Meiotic maturation is a complex process during which the oocyte must undergo a series of nuclear and cytoplasmic changes in order to produce a viable, fertilisable and developmentally competent ovum (Albertini *et al*. 1993). This process involves the breakdown of the germinal vesicle and reorganisation and segregation of the chromosomes with formation of the meiotic structures and further extrusion of the polar body. These changes are associated with a complete reorganisation of the cytoskeleton of the oocyte which in other species has been described in terms of changes in the distribution of the microtubules and microfilaments (mouse: Messinger *et al.* 1991; pig: Kim *et al.* 1996; man: Kim *et al.* 1998). Despite this important role in oocyte development, little information is available with regard to the cytoskeletal changes that take place during the meiotic maturation of equine oocytes. The aim of this study was to examine the changes in the distribution of microtubules and microfilaments and the relationship of these cytoskeletal elements to chromatin configuration, during *in vitro* maturation of horse oocytes.

MATERIAL AND METHODS

Cumulus oocyte complexes (COCs) were recovered from the ovaries of slaughtered mares by aspirating follicles smaller than 30 mm in diameter. Once recovered, COCs were washed in HEPES-buffered Tyrodes medium containing 0.1% polyvinylalcohol and 0.2% BSA and then evaluated under a stereomicroscope. Only oocytes with a complete, compact, multilayered cumulus investment were selected for culture. These oocytes were incubated in M199 medium supplemented with 10% FCS, 0.01 units/ml porcine FSH and 0.01 units/ml equine LH at 39ºC in a humidified atmosphere of 5% CO_2 in air. After 0, 12, 24 and 36 h of culture, COCs were denuded by vortexing in a calcium-free 0.25% solution of trypsin in EBSS. The oocytes were then washed in PBS and permeabilised, for 1 h at 39ºC, using medium M, a glycerol-based microtubule-stabilising solution (Simerly and Schatten 1993). Next, the oocytes were fixed for 30 min in 2% paraformaldehyde in PBS at room temperature and they were then maintained at 4ºC for 2–5 days prior staining. With regard to the staining techniques employed, first the microtubules were labelled by incubating fixed oocytes for 90 min at 37ºC with a monoclonal anti-tubulin antibody (Sigma) diluted 1:250 in PBS. After incubation, the oocytes were washed several times in PBS containing 0.1% BSA (Sigma) and then incubated for 1 h in a blocking solution (Simerly and Schatten 1993). Then the oocytes were exposed to a secondary antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC) for 1 h at 37ºC. Once the microtubules had been thus labelled, the oocytes were incubated for 1 h with Alexa Fluor 488 phalloidin to enable detection of the microfilaments and for 15 min with TO-PRO_3 (Molecular Probes) to allow visualisation of the DNA. Finally the stained oocytes were mounted on glass microscope slides with an antifade suspension. The oocytes were examined using a laser scanning confocal microscope, equipped with a krypto-argon ion laser which was able to simultaneously excite TRITC for the visualisation of the microtubules, Alexa Fluor 488 for the microfilaments, and TO-PRO_3 for the DNA, respectively. The images were recorded digitally

TABLE 1: Changes in nuclear stage during IVM of equine oocytes*

Time (h) in culture	Number of oocytes	GV (%)	Prometaphase (%)	M-I (%)	M-II (%)	Degenerate (%)
0	50	35(70)	-	-	-	15 (30)
12	48	11(23)	20(42)	2 (4)	1 (2)	14 (29)
24	49	1 (2)	-	14 (28)	17 (35)	17 (35)
36	54	1 (2)	-	5 (9)	23 (43)	24 (46)

* GV= germinal vesicle; M-I= metaphase –I; MII= metaphase –II

and archived on an erasable magnetic optical diskette.

RESULTS

In total, 201 oocytes were analysed using the CLSM and Table 1 shows the number of oocytes examined at the different times during *in vitro* maturation. At the onset of culture, most of the oocytes (70%) were in the germinal vesicle stage, shown as diffuse chromatin pattern localised within an organelle free area in the ooplasm. At this stage of development, microfilaments and weakly stained microtubules, were distributed throughout the ooplasma. After 12 h of IVM, the largest proportion of oocytes was in promethaphase (42%) and individual chromosomes were visible as aggregated dots of already condensed chromatin, around which the microtubules had concentrated. By contrast, the microfilaments were observed more near the cortical region of the oocyte. After 24 h of IVM, the oocytes were predominantly in Metaphase I (28%) or Metaphase II (35%) and by 36 h an even greater proportion had reached Metaphase II (43%). In Metaphase I oocytes, the microtubules were seen to have organised into elongated asters which formed the meiotic spindle supporting the already aligned chromosomes. In Metaphase II, the spindle was observed as a symmetrical, barrel-shaped structure with 2 anastral poles and it was now located in the periphery of the cytoplasm with the chromosomes aligned along the metaphase plate. Microtubules were only ever detected as these elongated asters in the spindle and they were not detected in any other areas of the cytoplasm. During both Metaphases I and II, microfilaments were concentrated in the oocyte cortex, and especially microfilament-rich domains were found overlying the meiotic spindle, and also around the area of the polar body formation and subsequently extrusion. Labelling consistent with the presence of microfilament labelling was also detected within the zona pellucida of the evaluated oocytes to varying degrees of intensity. A high proportion of oocytes were (30% at 0 h), or became (46% at 36 h), degenerate during maturation (Table 1) as evidenced by their aberrant chromatin and cytoskeletal patterns. In these degenerate oocytes, the DNA was often not visible at all or was visible only as hairlike strands or scattered small drops while the microtubules and microfilaments were distributed in clusters of either one or both, scattered throughout the ooplasma.

DISCUSSION

The present study enables the first description of cytoskeletal organisation, and its relationship to chromatin configuration, during the process of *in vitro* maturation of horse oocytes. In summary, we showed that the distribution of both microfilaments and microtubules, the 2 major cytoskeletal components of a mammalian ovum, change in parallel with the process of chromosomal alignment and segregation during the meiotic maturation process. After the germinal vesicle breakdown, the microtubules coalesced to form the spindle apparatus and thereafter played a clear role in chromosomal segregation and formation of the first polar body. The aggregation and accumulation of microfilaments in the oocyte cortex, initially distributed throughout the ooplasm, may suggest that they may play a significant role in the migration of other organelles during cytoplasmic maturation, a range of processes that appears to be critical in enabling an oocyte to achieve full developmental competence.

During this study, we found that a large proportion of oocytes were (30% at the onset of maturation), or became (46% after 36 h), degenerate during maturation *in vitro.* The

presence of a number of degenerate oocytes may be expected when we consider the heterogeneous population of healthy and atretic follicles from which the oocytes were drawn. Nevertheless, the rates of degeneration were much higher that those recorded for the oocytes of other species collected and incubated under similar conditions (eg 5% during IVM of bovine oocytes).

In vitro fertilisation (IVF) of horse oocytes has proved a difficult and not repeatable method. Hinrichs (1998) suggested that the primary problems reside in our inability to ensure that equine sperm adequately undergo capacitation *in vitro* and a similar inability to ensure that oocytes achieve developmental competence during maturation *in vitro*, where the latter problem is compounded by our inability to objectively assess this parameter. Although a number of studies have examined the effects of culture conditions on equine oocyte maturation, most have focussed exclusively on changes in nuclear configuration (Willis *et al.* 1991; Brück *et al.* 2000). It is increasingly clear that nuclear maturation alone is not sufficient to support normal fertilisation and embryo development. In conclusion, further studies on the structural changes that occur within the cytoplasm of the equine oocyte during *in vitro* maturation, and the relevance of these changes to the acquisition of developmental competence, are required if we are to understand the reasons for the relative failure of IVM and IVF in this species.

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EFFECT OF TEMPERATURE AND HOLDING TIME ON EQUINE OOCYTE CHROMATIN CONFIGURATION

H. G. Pedersen, E. E. Telfer* and E. D. Watson

*Department of Veterinary Clinical Studies, University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian EH25 9RG; *Institute of Cell and Molecular Biology, University of Edinburgh, Darwin Building, West Mains Road, Edinburgh EH9 3JG, UK*

INTRODUCTION

Equine oocytes are often obtained from abattoirs and a considerable period of time often elapses from excision of the ovaries until recovery of the oocytes. The effect of time and temperature could potentially affect the resulting *in vitro* maturation rates but differing results have been obtained in the different species studied. In a study in horses, storage of ovaries for 6–8 h was found not to affect oocyte maturation rates compared to oocytes from ovaries stored for 1.5–4 h (Guignot *et al.* 1999). Another study reported no effect on maturation rates after storage time of up to 15 h compared to 3–9 h (Delcampo *et al.* 1995). The same conclusion was reached after comparisons were made between storing the equine oocytes at 5–6, 6–7 and 7–8 h, and furthermore, no difference was found in cumulus morphology (Shabpareh *et al.* 1993). By contrast, storing ovine oocytes for 4, 8 and 24 h at 5ºC, 22ºC or 37ºC, resulted in a negative effect on the oocytes' capacity to reach Metaphase II. Storing ovine oocytes at 22ºC affected the oocytes less than at 5ºC and 37ºC (Moodie and Graham 1989) but another study found that exposing bovine germinal vesicle oocytes to temperatures below 23°C reduced their viability as determined by membrane integrity (Zeron *et al.* 1999). The developmental capacity of murine oocytes to the 2 cell stage embryo following storage in the ovaries for up 6 h was not different from control oocytes, but storage for 9–12 h was detrimental to development (Schroeder *et al.* 1991).

The aim of this study was to investigate the effect of holding temperature and time on equine oocyte chromatin configuration and cumulus morphology immediately after recovery from the follicle.

MATERIALS AND METHODS

Ovaries were obtained from horses and ponies after slaughter or after ovariectomy. The exteriorised ovaries were kept at 20–30ºC or 35–37ºC in M199 with Hanks salts and 25 mM Hepes for 0.5–24 h. Processed follicles ranged from 2–60 mm in diameter. The inner wall of the follicle was scraped with a bone curette to release the cumulus oophorus with the oocyte. After examination of the cumulus oophorus, the oocyte was denuded, fixed and stained with 2.5 µg/ml Hoechst stain 33258 (bisBenzimide). Cumulus oophorus morphology was classified either as compact (CP), expanded (EX), or denuded using the criteria of (Hinrichs *et al.* 1993a). The oocyte chromatin configurations were classified as fluorescent nucleus (FN), loosely condensed chromatin (LCC), condensed chromatin (CC), metaphase, or abnormal according to criteria of (Hinrichs *et al.* 1993b). The study consisted of 4 experiments.

Experiment 1: The effect of temperature on chromatin configuration

Oocytes from ovaries stored at 20–30ºC (n=14) or 35–37ºC (n=59) were used. To avoid the confounding effect of time, only oocytes that had been sitting in the follicles for less than 3 h were selected.

Experiment 2: The effect of time on chromatin configuration

Oocytes (n=222) were stored in the follicles for 0–1, 1–2, 2–3, 3–4, 4–6, 6–8 and 8–12 h after exteriorisation of the ovary.

Experiment 3: The effect of temperature on cumulus morphology

Cumulus oocyte complexes were recovered from follicles within 2 h of exteriorisation. The follicles had been stored at 20–30ºC (n=34) and 35–37ºC (n=40).

Experiment 4: The effect of time on cumulus morphology

Cumulus oocyte complexes (n=298) were left in follicles kept at 35–37ºC for varying lengths of time ranging from 0–1, 1–2, 2–3, 3–4, 4–6, 6–8 and 8–10 h.

STATISTICAL ANALYSIS

The effects of temperature and time on oocyte chromatin configuration and cumulus morphology were analysed by a Chi-square test or a Fisher test in case of small sample numbers. The null hypothesis of the statistical test assumed that there was no difference. A P-value of 0.05 or less was considered significant.

RESULTS

Experiment 1: The effect of temperature on chromatin configuration

Storing ovaries at either 20–30ºC or 35–37ºC did not affect (P>0.05) chromatin configuration in oocytes that were fixed within 3 h of the ovary leaving the animal. In the 20–30ºC group, 64.3% of oocytes were LCC and 35.7% were CC, whereas in the 35–37ºC group, 1.7% were FN, 78% were LCC and 20.3% were CC.

Experiment 2: The effect of time on chromatin configuration

There was no difference (P>0.1) in oocyte chromatin configuration within the first 6 h of storage in the follicle. The configurations started to change from 4 h onwards, but were not significantly affected until after 6 h. There was a difference in distribution of chromatin configurations between 0–6 and 6–12 h (P<0.001). There was no effect of time between 6–8 and 8–12 h (P> 0.1). The major changes between 0–6 h and 6–12 h were detected in the FN configuration (P< 0.01), which increased with time whereas LCC decreased (P<0.05). Numbers of oocytes with condensed chromatin, metaphase stages and no chromatin were unaffected by time, but significantly more abnormal configurations were seen as time passed (P<0.01, see Fig 1).

Experiment 3: The effect of temperature on cumulus morphology

Storing the ovaries for less than 2 h at 20–30ºC compared to 35–37ºC affected cumulus morphology (P<0.001). Cumulus cells held at temperatures lower than 30°C were more

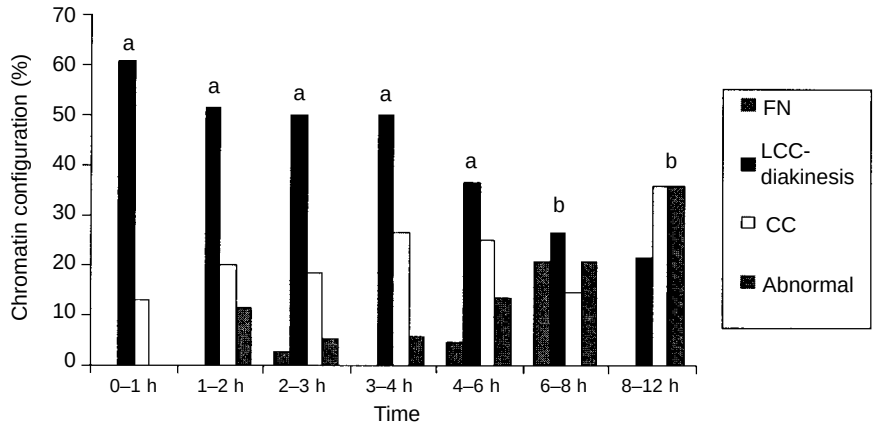

Fig 1: The effect of time on chromatin configuration. There was a significant change between 0–6 and 6–12 h. Numbers of oocytes: 0–1 h (n=23), 1–2 h (n=35), 2–3 h (n=38), 3–4 h (n=34), 4–6 h (n=44), 6–8 h (n=34), 8–12h (n=14). Letters a and b denote difference (P<0.001).

TABLE 2: Effect of temperature on cumulus morphology

	n	Compact cumulus (%)	Expanded cumulus (%)	Denuded oocytes (%)
20–30°C[a]	34	23.5	41.2	35.3
35–37°C[b]	40	5.0	67.5	27.5

Letters [a] and [b] denote difference (<0.001)

TABLE 3: The effect of time on cumulus morphology

	n	Compact cumulus (%)	Expanded cumulus (%)	Denuded oocytes (%)
0-2 h[a]	111	49.5	28.8	21.6
2-6 h[b]	169	40.2	46.2	13.6
6-10 h[b]	15	33.3	60.0	6.7

Letters [a] and [b] denote difference (P<0.05)

expanded and more oocytes had lost part of the cumulus than those held at 35–37°C (see Table 2).

Experiment 4: The effect of time on cumulus morphology

Because temperature had an effect on cumulus morphology, only cumuli kept at 35–37°C were studied. There was a difference (P<0.05) between the cumulus cells at 0–2 and 2–4 h, suggesting that cumulus expansion started almost immediately. There was no difference (P>0.1) between the cumulus morphology at 2–4, 4–6 and 6–10 h. From 0–2 h onwards there were decreasing numbers of compact cumuli, and increasing numbers of expanded cumuli, with the most expansion in the 6–10 h group.

DISCUSSION

The present study indicated that the temperature at which the ovaries were held did not influence oocyte chromatin, but did influence cumulus morphology. Lower temperature resulted in more denuded and expanded cumuli. These findings were unexpected, as lower temperatures would decrease metabolism and any influence of enzymes, which may be involved in the production of hyaluronic acid from the cumulus cells. Perhaps factors other than enzymes were involved in the cumuli becoming denuded.

There was more condensed chromatin in the group of oocytes, which had been held at 20–30ºC, but this was not statistically significant. The apparent difference in the effect of temperature on cumulus and chromatin may have been caused by slower nuclear reaction to a suboptimal environment than the time taken for cumulus expansion. Pig oocytes at the germinal vesicle stage did not survive cooling to 15°C or below (Didion *et al.* 1990) and sheep oocytes were sensitive to cooling to 20°C at various stages of meiosis with cooling inducing chromosomal abnormalities (Moor and Crosby 1985).

In the present study, chromatin configuration changed gradually with time and was significantly altered after 6 h. This was in agreement with a study on bovine oocytes, where frequency of germinal vesicle stage oocytes was unaffected by storage for up to 6 h (Richard and Sirard 1996). The majority of oocytes at 8–12 h contained condensed chromatin and abnormal configurations, whereas this was the case in few of oocytes at 0–1 h, clearly showing that time had an effect.

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EFFECT OF FETUIN ON THE ZONA HARDENING AND CORTICAL GRANULES DISTRIBUTION IN EQUINE OOCYTES MATURED *IN VITRO*

F. C. Landim-Alvarenga, S. E. A. Boyazoglu*, G. E. Jr. Seidel† and E. L. Squires†

*Department of Animal Reproduction and Veterinary Radiology, F.M.V.Z. - UNESP, Botucatu, SP, 18618.000, Brazil; *Sport Horse Research Center, Faculty of Veterinary Medicine – Perugia, 06126, Italy; †ARBL, Colorado State University, Fort Collins, Colorado 80523, USA*

Fully grown oocytes from many mammals can undergo spontaneous meiotic maturation when they are liberated from their follicles and cultured *in vitro*. However, the zona pellucida becomes resistant to chymotrypsin digestion or 'hardened', when spontaneous maturation occurs in serum-free medium (Zhang *et al.* 1991). Schroeder *et al.* (1990) described that fetuin, a component of Fetal Bovine Serum (FBS), inhibits zona pellucida hardening during oocyte maturation. Dell' Aquila *et al.* (1999) studied the effect of addition of fetuin in maturation media of equine oocytes in presence of equine follicular fluid, oestrous mare serum, equine fetal serum and BSA. The results showed that fetuin reduces zona hardening, but it does not influence sperm penetration after IVF. However, there is a lack of information on zona-hardening of *in vivo* matured oocytes. Also, there is no paper correlating zona hardening and cortical granules distribution during different culture periods in equine oocytes. So, the objective of this study was to compare zona hardening of *in vitro* and *in vivo* matured oocytes and to determine whether exposure of equine oocytes to serum-free medium containing fetuin would affect ZPH and cortical granule (CC) distribution.

MATERIAL AND METHODS

Oocytes were collected from slaughterhouse ovaries according to the method described by Choi *et al.* (1993) and matured in TCM 199 with Earle's salt (Sigma M7529, Montana, USA) supplemented with pyruvate, FSH and Oestradiol 17ß in one of the following treatments:

Group 1 (control): maturation medium containing 10% FCS;

Group 2: maturation medium + 1 mg/ml polivinylalcohol (PVA) and

Group 3: maturation medium + 1 mg/ml Fetuin.

Fifteen to 20 oocytes were cultured in 100 µl drops covered with washed, equilibrated paraffin oil. All oocytes were incubated for 36–40 h at 38.5°C in 5% CO^2 in air. Five replicates were used.

In vivo (n=12) matured oocytes were obtained from preovulatory follicles. Normal cycling mares were treated with hCG when one follicle reached 35 mm in diameter. After 30 h a transvaginal aspiration was performed to collect the oocyte.

After maturation the oocytes were striped of cumulus cells using a fine bore pipette in 1% BSA modified Dulbecco's PBS (DPBS) + hyaluronidase type V (300 iu/ml). Totally denuded oocytes were washed once in DPBS and transferred to a solution containing 0.3% protease (Pronase – Sigma, Montana, USA) in DPBS. The hardening of the zona pellucida was estimate by the time necessary for digestion of 50% of the ZP (d_{50}). The zona free oocytes were then stained with 45% acetic orcein for evaluation of nuclear status. Effects of maturation systems were analysed by ANOVA.

Cortical granules distribution was analysed using Transmission Electron Microscopy in samples of 5 oocytes taken just after collection and after 15, 24 and 36 h of maturation in each treatment. A group of 4 *in vivo* matured oocytes was also used.

RESULTS AND DISCUSSION

The addition of FCS to the maturation media significantly increase cumulus cell expansion of

equine oocytes matured *in vitro* (Table 1). However, the number of oocytes that reached metaphase II (MII) *in vitro* was not different when FCS, PVA and fetuin was added to the medium (Table 1). This is in agreement with our previous results showing similar maturation rates for equine oocytes matured in presence or absence of serum and hormones (Landim-Alvarenga and Choi 1999).

In this experiment the time necessary for digestion of 50% of the zona pellucida (d_{50}) just after collection was 12.04 min. There was no change in ZP hardening during the culture period when PVA was used. When FCS and Fetuin were added to the maturation media the time necessary for digestion of 50% of the zona (d_{50}) decreased in longer periods of culture, however no statistic difference were noted (Table 2). After 36 h of maturation the d_{50} of equine oocytes matured in presence of FCS and fetuin was lower compared with the group with PVA (Table 2). This is in agreement with the results of Schroeder *et al.* (1990) who showed that fetuin inhibited zona pellucida hardening of mouse oocytes in a dose dependent way. Also Dell' Aquila *et al.* (1998) observed that bovine fetuin inhibited zona pellucida hardening of equine oocytes during IVM with a dose effect.

Oocyte maturation *in vitro* or *in vivo* is accompanied by a slow release of approximately 30–40% of the cortical granules (Dulcibella *et al.* 1990). However, *in vivo*, this precocious release of cortical granules does not result in zona pellucida modifications. In fact, the d_{50} measured in 12 i*n vivo* matured oocytes aspirated from preovulatory follicles 30 h after hCG injection was 9.22 min.

The ultrastructural analysis of *in vivo* matured oocytes showed the highest number of CC beneath the oolema. Similar pattern was observed when oocytes were matured *in vitro* for 36 h in presence of Fetuin and FCS. However, when maturation occurs in absence of serum, the number of CC was smaller. These indicate that in this group a precocious release of CG may happened, justifying the high d_{50} value observed.

We conclude that equine ZP became resistant to lisys by protease following 36 h incubation in serum free media. The addition of fetuin or FCS to the maturation media reduced the d_{50} to values similar to the ones observed *in vivo* matured oocytes. It is likely that the problems related to IVF of equine oocytes matured in presence of serum are not related with ZPH.

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POLYVINYLALCOHOL IS SUPERIOR TO BOVINE SERUM ALBUMIN IN EQUINE IVF MEDIUM

Y. H. Choi, G. E. Seidel, Jr., S. Boyazoglu and E. L. Squires*

*Centro de Reproducción Equina Doña Pilar, Lincoln, Argentina; *Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas, USA*

INTRODUCTION

Several studies on predicting stallion sperm fertility have been done using hamster or equine oocytes. However, expenses of hamster and equine oocytes for investigating stallion sperm fertility are high compared with bovine oocytes, which often can easily be obtained from local slaughterhouses. Bovine serum albumin is widely used to supplement capacitation and fertilisation media. However, BSA is contaminated by other serum components including enzymes, hormones, lipids, and organic and inorganic ions (Bavister 1995). To make a defined culture system for examination of mechanisms of capacitation or fertilisation, polyvinylalcohol (PVA) was used as a substitute for BSA in one 2 x 2 factorial study using penetration of zona-free bovine oocytes, and in a second study using equine oocytes collected from slaughterhouse-derived ovaries.

MATERIALS AND METHODS

For Experiment 1, Bovine oocytes were collected from slaughterhouse-derived ovaries and matured in TCM-199 with hormones (FSH, LH and E_2) and 10% oestrous cow serum. After 22 h of culture, oocytes were denuded of cumulus cells by gentle pipetting in m-PBS plus hyaluronidase and placed in Tyrode's medium (pH 2.1) for 1–2 min to remove zonae pellucidae. Washed, fresh ejaculated sperm from 3 stallions were capacitated at 5×10^7 sperm/ml in TYH medium (modified Krebs-Ringer bicarbonate) plus 0.5 mM 8-bromo-cAMP for 3.5 h with or without an additional 15 min in 100 nM ionomycin, and supplemented with polyvinylalcohol (PVA; 1 mg/ml) or BSA (4 mg/ml). Intraspecies gametes were co-incubated in TYH/PVA or TYH/BSA for 18–20 h at 10^6 sperm/ml. For Experiment 2, equine ovaries were sliced near a slaughterhouse, and oocytes were collected and transported to the laboratory in sealed 15 ml conical tubes filled with TCM-199 plus 10% FCS covered with paraffin oil. Oocytes were matured as described for bovine oocytes. After 28–30 h maturation, cumulus cells were removed and oocytes with a 1st polar body were used for fertilisation after approximately one-third of the zona pellucida was removed with a fragment of razor blade in protein-free PBS supplemented with 0.3 M sucrose (Choi *et al.* 1994). Fresh semen from 2 stallions was mixed and washed twice (330 x *g* for 5 min) in TYH/PVA or TYH/BSA. Capacitation of stallion sperm was with 8-bromo cAMP and ionomycin as described for Experiment 1, as was IVF. Experiment 2 was replicated 3 times. For both experiments, oocytes were fixed and stained 18–20 h after the start of IVF and examined for enlarged sperm heads or male pronucleus formation.

RESULTS

Treatment of stallion sperm with cAMP and ionomycin in TYH/PVA resulted in a higher penetration rate ($P<0.05$) of zona-free bovine oocytes 36/104 (35%) than treatment in TYH/BSA using cAMP with 6/95 (6%) or without ionomycin, 5/96 (5%); the penetration rate for PVA plus cAMP alone was 12/110 (11%). There were significant differences in penetration rates among the 3 stallions (78, 14, 9%), when PVA plus the combination of cAMP and ionomycin were used. In Experiment 2, the penetration rate of equine oocytes was also higher ($P<0.05$) in PVA,

23/43 (53%) than BSA, 5/46 (11%). Six of the 23 oocytes were polyspermic in the PVA group vs 1 of 5 oocytes in the BSA group.

Conclusions

Stallion sperm penetration rates of bovine zona-free oocytes and partial zona-removed equine oocytes were superior with PVA compared to BSA supplementation of capacitation and fertilisation media. The reason/mechanism for PVA superiority to BSA for capacitation and fertilisation by stallion sperm in the system used requires further experimentation. However, the chemically defined medium used in this study will simplify investigation of detailed mechanisms of capacitation. Further, bovine zona-free oocytes were useful for assaying *in vitro* capacitation and fertilisation of stallion sperm.

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THE DEVELOPMENT OF BLASTOCYSTS AFTER INTRACYTOPLASMIC SPERM INJECTION OF EQUINE OOCYTES

X. Li, L. H. A. Morris and W. R. Allen

Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK

INTRODUCTION

Horse oocytes obtained from abattoir ovaries and cultured *in vitro* can complete nuclear maturation by releasing the first polar body of Metaphase II (MII) after 15–40 h of culture (Zhang *et al.* 1989; Hinrichs *et al*. 1993). Although these MII oocytes have completed nuclear maturation, it remains difficult to fertilise them by either IVF or ICSI and to induce their further development to the blastocyst stage during subsequent culture *in vitro* (Choi *et al.*1994; Dell'Aquila *et al.* 1997; Li *et al.* 2000).

The importance of the oviduct in gamete maturation and fertilisation has been established (Hunter 1994), and co-culture with oviduct epithelial cells is used increasingly to support oocyte maturation and early embryonic development *in vitro*. In the present study we investigated the influence of oviduct epithelial cells (OEC) on the maturation, fertilisation and subsequent development potential of abattoir-derived equine oocytes cultured *in vitro* after ICSI.

MATERIALS AND METHODS

Horse ovaries were obtained from two slaughterhouses and transported to the laboratory within 24 h. Cumulus oocyte complexes (COCs) were matured *in vitro* for 28–30 h, with or without OEC, in TCM199 medium containing 20% FBS (v:v), 10 µg/ml FSH, 5 µg/ml LH and 1 mg/ml oestradiol, at 38ºC in 5% CO_2-in-air. Three types of spermatozoa were prepared for ICSI; washed, acrosome-reacted and pre-decondensed spermatozoa.

The injection pipette containing the spermatozoon was pushed through the zona pellucida and plasma membrane so that the spermatozoon could be injected directly into the cytoplasm. The injected oocytes were then activated by immersing them in STALP solution containing 5 µM ionomycin and 1% ethanol for 5 min. After both ICSI and the activation procedure, groups of 5–10 oocytes were cultured for 7–9 days in DMEM medium with cumulus cells monolayer (CCM) at 38ºC in an atmosphere of 5% CO_2-in-air.

Two-cell stage embryos were stained with Hoechst 33342 (Sigma) in PBS solution (5 µg/ml) for 10 min at 38ºC before being examined under ultraviolet light (excitation wavelength 330–380 nm) using a fluorescent microscope.

CONCLUSIONS

The mammalian oviduct normally provides the optimum environment for the final maturation of

TABLE 1: Nuclear maturation of horse oocytes cultured *in vitro*, with and without oviduct epithelial cells (OEC)

Maturation medium	Total number of oocytes	Number and (%) of	
		MII≠	MII
IVM-1	145	74(51)	71(49)
IVM-1/OEC	140	77(55)	53(45)

TABLE 2: Cleavage and normal fertilisation rates in 2-cell stage embryos following the injection of 3 types of spermatozoa into the cytoplasm of MII oocytes matured with or without OEC

Injected spermatozoon	2-cell stage/total (%)	Development pattern** (%)		
		NF	PD	Others
Type I	12 / 30 (40[b])	9 (75[a])	0b	3 (25[b])
Type II	58 / 92 (63[a])	47(81[a])	6 (10[a])	5 (9[a])
Type III	10 / 24 (42[b])	4 (40[b])	1 (10[a])	5 (50[b])
Type II*	27 / 40 (68 [a])	23 (85[a])	0 b	4 (15[a])

Values in the same column with different superscripts are significantly different (P<0.05)
* Oocytes matured with OEC
** NF: normal fertilisation; PD: Parthenogenetic development; Others: abnormal cleavage and degenerated oocytes

TABLE 3: *In vitro* development potential of 2-cell horse embryos obtained from *in vitro* matured oocytes fertilised by ICSI

Medium and condition	Development results (%)*			
	2-cell	≥8-cell	≥16-cell-morula	blastocyst
Oocytes matured without OEC				
TCM199	28	2 (7)	0	0
TCM199/CCM	23	11 (48)	7 (30)	0
DMEM/CCM	20	10 (50)	10 (50)	0
Oocytes matured with OEC				
DMEM	15	6 (40)	2 (13)	1(7)
DMEM/CCM	33	23 (70)	17 (52)	7(21)
DMEM/CCM**	13	4 (31)	0	0

*The 2-cell stage embryos were derived from injection of acrosome-reacted spermatozoa into the oocytes
** Parthenogenetic embryos derived from sham-ICSI without sperm injection

oocytes, the capacitation of spermatozoa, the sperm-oocyte interaction, completion of fertilisation and embryonic development to the late morula or early blastocyst stage before the latter migrates into the uterus for development (Hunter 1994). In many species, the contribution of oviduct to the fertilisation process, and also to early embryonic development, is more important than its contribution to oocyte maturation. In the present study we demonstrated the beneficial influence of OEC for maturation of the ooplasm of the equine oocyte. The results revealed that OEC co-culture during oocyte maturation creates a environment *in vitro* that enables the zygote to develop at least to the blastocyst stage after ICSI.

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EMBRYONIC DEVELOPMENT OF EQUINE OOCYTES FERTILISED BY ICSI

C. Galli, G. Crotti, G. Mari* and G. Lazzari

*Laborotorio di Tecnologie della Riproduzione, C.I.Z.- A.I.A. Via Porcellasco 7/f, 26100 Cremona, Italy; *Dipartmento Clinico Veterinario, Universita degli Studi di Bologna, Via Tolara di Sopra 50, Ozzano Emilia, 40064 Bologna, Italy*

INTRODUCTION

The application of *in vitro* embryo production technology in the horse is very limited because the overall procedure is much less efficient as compared for example to the bovine where at present many thousands of calves are born worldwide following the transfer of *in vitro* produced embryos. In the horse successful birth of foals following assisted reproductive techniques are still occasional (Palmer *et al.* 1991; Squires *et al*. 1996; Cochran *et al*. 1998; McKinnon *et al*. 1998), anatomical and physiological peculiarities are the main constraint to the development of assisted reproductive techniques in this species. The potential application in the horse will be for assisted reproduction of infertile or subfertile mare and stallions rather than for the increase of the number of offspring obtained from any given donor.

The 3 steps involved in the production of embryos *in vitro* (maturation, fertilisation and culture) have been studied by several laboratories. *In vitro* maturation is the more efficient step as assessed by the transfer of *in vitro* matured oocytes to the oviduct for fertilisation and development (Zhang *et al*. 1989). *In vitro* fertilisation is the less reproducible step and it is now overcome by the use of intracytoplasmic sperm injection (ICSI). Embryo culture is the less studied aspect because the embryos are usually transferred to the oviduct as soon as possible after fertilisation. An efficient system that would allow the development of *in vitro* produced embryo to blastocyst it would be highly desirable both because it would allow the non surgical transfer and possibly the freezing of such embryos.

The aim of this study was to improve the basic knowledge on the influence of follicle size and cumulus morphology on the embryo development of horse oocytes fertilised by ICSI and cultured in the surrogate oviduct of the sheep.

MATERIALS AND METHODS

Oocytes were collected from ovaries of slaughtered mares during the period December 1999–February 2000. The ovaries were collected in plastic bags with saline solution and transported to the laboratory in thermostatic boxes kept at 24–26°C within 4 h from slaughter. The connective tissue capsule was removed from the ovaries to help locate the follicles. A slit was made in each follicle with a scalpel blade and a Volkman spoon was used to scrape the inside of each follicle large than an estimated 0.5 cm. The oocytes recovered were divided in two groups according to the size of the follicle above (large follicles) or below (small follicles) 2 cm of diameter. Within each group the oocytes were divided according to the cumulus morphology, compacted and expanded. The oocytes belonging to the 2 classes of follicle size were matured separately *in vitro* for 26 h (oocytes with expanded cumulus) or 30 h (oocytes with compacted cumulus). Maturation medium consisted of TCM 199 with 10% FCS, ITS (insulin transferring and selenium, Sigma), epidermal growth factor analogue (50 ng/ml, Sigma), 0.5 IU of FSH LH (Pergovet, Serono), 17ß oestradiol (2 µg/ml). Incubation was performed at 38°C in 5% CO_2. At the end of maturation oocytes were decumulated mechanically before microinjection.

One straw of frozen semen of proven fertility from the same batch was used for ICSi and prepared immediately before use. After thawing motile spermatozoa were separated on 45–90%

TABLE 1: Developmental capacity of horse oocytes following ICSI and according to follicle size and cumulus morphology

Type of cumulus-oocyte complexes	No. ICSI	No. survived (% of injected)	No. cleaved (% of survived)	N. of morula/blastocyst on Day 7 (% of cleaved)
Compacted (large follicles)	11	9 (82[a])	7 (78[a])	2 (29[a])
Compacted (small follicles)	45	43 (96[a])	36 (84[a])	11 (31[a])
Expanded (small follicles)	34	32 (94[a])	19 (59[a])	2 (11[b])
Total	90	84 (93)	62 (74)	15 (24)

Numbers within columns with different letters are significantly different (P<0.05, chi square test)

Percoll gradient and were resuspended in SOFaa BSA (6 mg/ml) containing 1 µg/ml heparin at 4 x 10^6 ml. The semen suspension was further diluted 1:1 with a 12% solution of PVP in PBS. Sperm injection was performed in microdrops of H-SOF on the warm stage of a Nikon inverted microscope equipped with Normarsky optics and Narishige micromanipulators. A motile sperm was selected in the microdrop of PVP, immobilised and aspirated in the micropipette subsequently moved to the drop containing the oocytes. Each oocyte was injected by holding it with a holding pipette with the polar body positioned at 6 or 12 o'clock. Sham injected oocytes were injected with the PVP solution only.

At the end of the injection procedure oocytes were activated by exposing them for 6 min to ionomycin 5 µM. After 2 washings they were transferred to microdrops of SOFaa with 16 mg/ml BSA for 48 h culture. Cleaved embryos were embedded into agar chips and transferred to ligated sheep oviducts for 5 days. On Day 7 the embryos were collected and evaluated. Overall results are shown in Table 1. The experiment was replicated 3 times. After collection the embryos that developed were removed from agar, Grades 1 and 2 were frozen in 10% glycerol and Grade 3 were fixed and stained with lacmoid to determine cells number.

RESULTS

In total 72 ovaries were processed and 180 oocytes were collected. All the oocytes from the large follicles and 50% of the oocytes from the small follicles had a compact cumulus. After maturation 114 oocytes out of 180 were viable (63%); 90 oocytes that had the first polar body were injected and subsequently activated. Survival rate and cleavage rate was not different between the groups. However, embryo development was higher in the groups of the compacted oocytes (Table 1). Embryos assessed on Day 7 (Day 0 is the day of ICSI) were mainly compacted morula or early blastocysts. Four embryos that were not judged of freezable quality and were fixed and stained had 60–70 cells with several cells in mitosis.

In a second series of experiments possible parthenogenetic development induced by the activation protocol was assessed. None of the sham injected oocytes underwent cleavage after activation and culture, instead 22% of the sperm injected oocytes (Table 2). In this experiment compacted and expanded oocytes were pooled.

DISCUSSION

Nearly 40% of the immature oocytes collected from ovaries are degenerated at the end of maturation. Some of them are already degenerated

TABLE 2: Development of horse oocytes after ICSI or sham injection

	No. injected	No. cleaved (% of injected)	No. mor./blast (% of injected)
Sperm injected	39	27 (69)	6 (22)
Sham injected	28	0	0

Three replicates, June 2000

before maturation but it is difficult to visualise them because of the presence of the cumulus and some more are damaged during the removal of the cumulus that it is particularly difficult in this species. Seventy nine per cent of the surviving oocytes had the first polar body. Our data indicate that oocytes with a compacted cumulus have a higher developmental competence as compared to oocytes surrounded by an expanded cumulus. Although the numbers of viable oocytes per ovary is small, the percentage of them developing into transferable embryos is comparable to what can be obtained in a bovine *in vitro* production system.

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EFFECT OF INVASIVE ADENYLATE CYCLASE DURING OOCYTE MATURATION ON DEVELOPMENT OF EQUINE EMBRYOS FOLLOWING ICSI

L. J. Maclellan, M. M. Sims and E. L Squires

Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA

Invasive adenylate cyclase (iAC), a toxin produced by the bacterium Bordetella pertussis, can be internalised by mammalian cells resulting in increased production of cAMP from the cells own pool of adenosine 5-triphosphate (ATP) (Confer *et al.* 1984). During oocyte collection procedures there is a significant decrease in intracellular levels of cAMP [cAMPi] in cumulus oocyte complexes. Including iAC in the collection medium can prevent this [cAMPi] decrease. Culturing bovine oocytes with low concentrations of invasive adenylate cyclase during IVM can stimulate nuclear maturation while high concentrations inhibit nuclear maturation. In addition, low concentrations of iAC in maturation media result in increased rates of cleavage and increased rates of development to the blastocysts post IVF (Luciano e*t al.* 1999).

The objective of the experiment was to determine the effect of invasive adenylate cyclase during *in vitro* maturation of equine oocytes on nuclear maturation and embryo development following ICSI.

Equine oocytes were collected within 3 h of slaughtering mares in three replicates. Oocytes were obtained by slicing ovaries and washing in TCM-199 plus 0.1% BSA with or without 0.01 mM iAC and 0.5 mM 3-isobutyl 1-methylxanthine. Cumulus oocyte complexes (COC) were characterised as: 1) compact cumulus (CP) – having 3 or more layers of cumulus cells tightly adhered together, completely surrounding the oocyte; or 2) expanded cumulus (EX) – having a completely expanded cumulus with a cellular gelatinous cloud around the oocyte. Oocytes were transported in a Minitub portable incubator to the laboratory in 6 ml Falcon tubes at 38.5ºC in media equilibrated under 6% CO_2 in air. Oocytes were then transferred to 4-well plates for the remainder of the maturation period. There were 4 treatment groups: control compact (n=104) and control expanded COCs (n=56) cultured in TCM-199 with 0.6% BSA and hormones (1 µg/ml b-LH, 15 ng/ml o-FSH, 1 µg/ml E2 and 500 ng/ml progesterone); iAC compact (n=95), and iAC expanded COCs (n=52), cultured in TCM-199 with 0.6% BSA and 0.01 mM iAC.

A sample of oocytes was removed from each group at 24 h for nuclear staining with aceto-orcein. After maturation for 34–36 h oocytes were denuded by manual pipetting in the presence of 300 iu hyalauronidase/ml. Frozen thawed sperm was prepared over a discontinuous 90/45% Percoll gradient and re-suspended in hepes-buffered SOF

TABLE 1: Effects of iAC during oocyte maturation on subsequent development

	Cleaved n (%)	2-8 cell n (%)	Morulae n (%)	Blastocyst n (%)	Fragment n (%)
Control compact	24/68 (35)	20(30)	4(6)	0	7(10)
Control expanded	7/32 (22)	7(22)	0	0	4(13)
0.01mM iAC compact	18/57(32)	12(21)	4(7)	2(4)	4(7)
0.01mM iAC expanded	8/31(26)	7(23)	1(3)	0	6(19)

containing 10% polyvinylpyrrolidone (PVP, MW 360,000). Oocytes were injected with a single spermatozoon. All oocytes were activated with 10 µM calcium ionophore A23187 for 5 min and de-activated in SOF containing 20 mg/ml BSA before being transferred to culture dishes containing modified synthetic oviduct fluid (Lane and Gardner 1997).

Averaged over compact and expanded cumulus groups, oocytes in the iAC group tended to be more advanced in nuclear maturation at 24 h; 32/49 (65%) in MI or MII for iAC vs 12/30 (40%) for control oocytes ($P<0.05$). Oocyte degeneration rates were lower in the iAC groups 9/49 (18%) compared to controls, 11/30 (37%). Cleavage and development rates tended to be higher for the oocytes with compact than expanded cumulus in each treatment. Culturing expanded oocytes for 34–36 h may be too long, and further experimentation would be required to establish the optimum time of maturation for these oocytes. There was no significant difference overall between iAC and controls in cleavage or subsequent developmental rates (Table 1).

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CHROMATIN CONFIGURATION, MEIOTIC COMPETENCE AND SUCCESS OF INTRA-CYTOPLASMIC SPERM INJECTION IN HORSE OOCYTES COLLECTED BY FOLLICULAR ASPIRATION OR SCRAPING

M. E. Dell'Aquila, M. Masterson*, F. Maritato and K. Hinrichs†

*Department of Animal Production, Section of Reproduction, University of Bari, Valenzano, Bari, Italy; *Department of Clinical Studies, Tufts University School of Veterinary Medicine, Grafton, Massachusetts, USA; †Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas, USA*

Reported rates of oocyte maturation and fertilisation vary greatly among laboratories. This may be related to the populations of oocytes selected for use, as the maturation rate of horse oocytes is dependent on diameter and the degree of viability of the follicle from which the oocyte originates (Hinrichs and Williams 1997; Hinrichs and Schmidt 2000). Higher maturation rates are found in oocytes from large follicles, and in oocytes from atretic follicles. Cumulus expansion is a marker for oocyte atresia, therefore assessment of cumulus morphology is an important parameter in selection of oocytes for study. Germinal vesicle chromatin configuration also differs with follicle atresia and follicle size, and is strongly associated with the meiotic competence of the oocyte (Hinrichs and Williams 1997; Hinrichs and Schmidt 2000).

Methods for collection and selection of horse oocytes have not been standardised among laboratories. The 2 most common methods for horse oocyte collection are aspiration of follicular fluid or opening follicles and scraping the granulosa layer. Oocytes in viable follicles may be more closely attached to the follicle wall, whereas oocytes from atretic follicles may be loose within the follicular fluid (Hawley *et al*. 1995; Mlodawska and Okolski 1997). Thus, it is possible that these 2 methods of oocyte recovery result in collection of different populations of oocytes, with aspiration favouring collection oocytes from atretic follicles and scraping favouring collection of oocytes from viable follicles. The type of oocytes obtained may affect the observed results after maturation or fertilisation, and may be a factor in the reported differences in results using these procedures among laboratories. This study was conducted to determine if the meiotic and developmental competence of horse oocytes could be dependent on the method of oocyte collection.

Horse oocytes were recovered from slaughterhouse ovaries by aspiration or scraping. Only follicles visible on the surface of the ovary and >10 mm diameter were used. Aspiration was performed using an 18 ga needle attached to a vacuum pump. For scraping, follicles were opened with a scalpel blade and the granulosa cell layer was removed using a bone curette. Recovered oocytes were classified as having compact, expanded or partial cumuli; obviously degenerating oocytes were discarded. In Experiment 1, oocytes were denuded and fixed immediately after collection, and were stained with Hoechst 33258 to assess whether the collection method influenced the initial chromatin configuration of oocytes. This was done both in May and in October. In Experiment 2, *in vitro* maturation rates of oocytes recovered by aspiration or scraping were compared. IVM was performed as previously described (Dell'Aquila *et al*. 1996), with the medium supplemented with 20% (v/v) oestrous mare serum. Oocytes were cultured for 28–30 h at 38.5°C under 5%CO_2 in air. In Experiment 3, oocytes were matured *in vitro* as for Experiment 2, but with 20% follicular fluid in place of the mare serum, which increases the rate of male pronucleus formation in oocytes having compact cumuli (Dell'Aquila *et al*. 1997).

Oocytes in metaphase II were submitted to intracytoplasmic sperm injection (ICSI), as previously described (Dell'Aquila *et al.* 1997). Frozen-thawed sperm, prepared by swim-up, were used for injection. Oocytes were not chemically activated after ICSI.

The oocyte recovery rate was significantly higher for scraping than for aspiration (83% vs 48%). Oocytes collected by scraping had a significantly higher proportion of intact compact cumuli (72/107, 67% vs 69/209, 33%, respectively), and a significantly lower proportion of partial cumuli (16/107, 15% vs 111/209, 53% for scraping and aspiration, respectively). Oocytes collected by scraping during the breeding season (May) had a higher proportion of diffuse chromatin within the germinal vesicle than did oocytes collected by aspiration (10/43, 23% vs 8/113, 7%, respectively), but this difference was not seen in oocytes collected in October. Experiment 2 (maturation) was conducted in the fall and winter. The rates of maturation to metaphase II were 56/101, 55.4 % and 65/106, 61.4% for oocytes collected by scraping and those collected by aspiration respectively. These rates were not significantly different. The rates of pronucleus formation after ICSI for oocytes recovered by scraping or by aspiration were 50/99, 52.6% vs 50/85, 68.5% respectively; these rates were not significantly different.

These findings demonstrate that follicle aspiration results in loss of the cumulus in the majority of oocytes, which agrees with previous reports (Hinrichs 1991; Alm *et al.* 1997). During the breeding season, aspiration and scraping result in collection of populations of oocytes having differing germinal vesicle chromatin configurations, with scraping resulting in more diffuse chromatin, a configuration which is associated with follicle viability (Hinrichs and Williams 1997). The observed difference in chromatin configuration in scraped oocytes between seasons is likely due to the increase in prevalence of the diffuse chromatin configuration during the breeding season (Hinrichs and Schmidt 2000). These findings also indicate that when follicles >10 mm diameter are used, similar maturation and fertilisation results may be obtained when oocytes are collected by aspiration or by scraping. Selection against small follicles (<10 mm diameter) may have reduced potential differences between collection methods. The similarity in results obtained from oocytes collected by aspiration or by scraping reduces the possibility that collection technique is responsible for variations in meiotic and developmental competence observed among laboratories working with horse oocytes. This study also demonstrates that ICSI can be a valuable tool to assess the developmental capacity of horse oocytes in the absence of a repeatable method for *in vitro* fertilisation.

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TRANSVAGINAL INTRAFOLLICULAR SPERM CELL INJECTION IN THE CYCLIC MARE

M. Meinjtes*, B. Eilts†, R. Cochran**, K. Graff‡, R. Denniston, D. Paccamonti* and R. Godke

Department of Animal Science; †Louisiana State University Agricultural Center, Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803, USA

Intrafollicular insemination (IFI) is an assisted reproductive technique that has been successfully used in human reproduction (Lecena *et al*. 1991; Zbella *et al.* 1992). However it has not been reported in domestic animals. Intrafollicular insemination has potential applications for circumventing the uterine inflammation in mares with persistent mating induced endometritis, breeding with reduced numbers of sperm cells from oligospermic ejaculates or frozen semen, and research applications to further understand fertilisation. Intrafollicular insemination may also be an attractive, less technically complex approach to achieve the same goal as with conventional IVF. The objective of this study was to perform transvaginal ultrasound-guided intrafollicular insemination to evaluate the effectiveness of IFI to establish a pregnancy.

In this experiment 10 light horse mares with normal length oestrous cycles were used in a 2 x 2 factorial arrangement. The mares were teased, palpated per rectum and had the ovaries evaluated by transrectal ultrasonography daily. All IFI procedures were performed 9–13 h before ovulation. Ovulation was verified by hourly transrectal ultrasound examinations of the ovaries starting at the time of IF. Mares in Treatment A (n=3) and Treatment B (n=2) received 3,300 iu of hCG iv at the time of IFI, and mares in Treatment C (n=3) and Treatment D (n=2) were allowed to ovulate naturally after IFI. The sperm used for each mare was freshly collected from the same fertile stallion. The sperm cells used for IFI were washed and resuspended in 1.5 ml of Hams F-10 to obtain an insemination concentration of 120 x 10^6 progressively motile sperm per ml. The motility of different ejaculates ranged between 40 and 80%. In addition, the sperm cells in Treatment A and C were treated with a 3 µM concentration of calcium ionophore A32187 for 5 min to induce capacitation. The sperm cells for Treatment B and D were not treated with calcium ionophore A32187, but only were washed. All IFI were performed with the sperm cell concentration adjusted to 120 x 10^6 progressively motile sperm per ml. The IFI sperm injection procedure was performed under transvaginal ultrasound guidance with a 22 g needle connected to a 2.5 ml syringe containing the 1.5 ml sperm-cell suspension for IFI. The sperm cells remaining in the needle after each insemination were used to calculate the total motile sperm cells inseminated. The results are summarised in Table 1. All mares in Treatment A and B ovulated, however only one of 3 in Treatment C and one of 2 in Treatment D ovulated. None of the 10 mares having IFI were found to be pregnant when they were examined for pregnancy using transrectal ultrasonography.

It is not clear from this study if the sperm number per follicle was adequate, the sperm cells remained unbound in the follicular fluid, or if the sperm cells could survive in the follicular environment long enough to achieve *in vivo* fertilisation in the follicle or oviduct. Human patients have become pregnant after IF insemination with 200,000 cells (Lucena *et al.*

*Present address: Presbyterian Hospital, Assisted Reproductive Technology Services; **Present address: Reproductive Medicine and Fertility Center, 615 E. Princeton Street, Suite 225, Orlando, Florida 32806 USA; ‡Present address: Arnold Palmer Hospital, Fertility Center, 23 West Copeland Drive, Orlando, Florida 32806, USA*

TABLE 1: Ovulation and pregnancy results in mares administered or not administered 3,300 iu hCG iv before intrafollicular insemination with 180 x 10^6 motile sperm cells treated with calcium ionophore A 32187 (capacitated) or not treated with calcium ionophore A 32187 (not capacitated)

	Treatment A (hCG and capacitated)	Treatment B (hCG and not capacitated)	Treatment C (No hCG and capacitated)	Treatment D (No hCG and not capacitated)
N	3	2	3	2
Ovulated	3	2	1	1
Pregnant	0	0	0	0

1991) so it seems that enough cells were inseminated. However, the disparity of follicular fluid volume between the human and mare follicle results in a greater dilution of cells in the mare follicle. Therefore, the dose of sperm cells is still questionable. Binding of the sperm cells to granulosa cells may have prevented the sperm cells from being carried into the oviducts at ovulation. Sperm cells are known to bind to mare oviduct epithelial cells (Thomas *et al.* 1994) so the sperm cells also may have the ability to bind to granulosa cells in the follicle. It was reported that follicular fluid recovered shortly after IFI contained no sperm cells, perhaps indicating that binding of the sperm cells took place. (Alvarenga, EETV Discussion) If too many sperm cells are bound to granulosa cells, then insufficient sperm cells may enter the oviduct at ovulation to achieve fertilisation. Perhaps cryopreserved stallion sperm cells, which have a decreased adherence to mare oviduct epithelial cells (Ellington *et al.* 1999) may actually result in higher pregnancy rates because of decreased adhesion to granulosa cells. The aspiration of the small volume of follicular fluid does not inhibit ovulation or fertilisation in mares bred by normal artificial insemination procedures (Duchamp *et al.* 1996) and only a small amount of follicular fluid is actually needed to enter the oviduct at ovulation (Bezard *et al.* 1996) Since only a small volume of follicular fluid actually enters the oviduct at ovulation, an insufficient number of sperm cells may have been carried into the oviduct with a low volume of follicular fluid. Sperm cells may not retain sufficient motility in follicular fluid to allow normal fertilisation rates after IF insemination. Unpublished work in this laboratory has shown sperm cells have 63, 49, 36, 16, 7, 2 and 1% motility after incubation *in vitro* for 0, 1, 2, 3, 4, 5 and 6 h, respectively in follicular fluid at 37°C (Eilts *et al.* unpublished data). This is in contrast to motility of semen extended in skim milk extender which had 80, 72, 48, 32, 25, 16 and 12% after 0, 1, 2, 3, 4, 5 and 6 h, respectively. Therefore, if ovulation did not occur until 9–13 h after IF insemination in the present study, sperm motility after IFI may not have been adequate to attain optimal fertilisation rates. It appears that the use of hCG will be desirable in future studies of IFI to ensure ovulation.

Although IFI has been reported to be successful in human (Lecena *et al*. 1991; Zbella *et al.* 1992), a more comprehensive study attained only one pregnancy in 50 attempts (Nuojua-Huttunen *et al.* 1995). In the mare, other researchers also reported that no pregnancies were achieved after intrafollicular insemination (Alvarenga and McCue, EETV discussion). Perhaps the follicular fluid could be removed and replaced entirely with extended sperm cells. Preliminary work to determine if IFI may be detrimental in establishing pregnancy when normal artificial insemination is performed concomitant with IFI has resulted in no pregnancies (Eilts *et al.* unpublished data). In conclusion, more research is needed to determine if *in vivo*-matured oocytes can be fertilised using IFI.

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FERTILISATION RATES OF *IN VITRO* MATURED OOCYTES TRANSFERRED INTO THE OVIDUCT OF INSEMINATED MARES

C. C. Love, S. P. Brinsko*, D. D. Varner* and K. Hinrichs

*Department of Physiology and Pharmacology; *Department of Large Animal Medicine and Surgery, College of Veterinary Medicine, Texas A & M University, College Station, Texas 77843-4475, USA*

In vitro-matured (IVM) oocytes are commonly used for experimentation involving *in vitro* fertilisation (IVF). However, the fertilisation potential of IVM oocytes is unknown. The major barrier to IVF in the horse appears to be penetration of sperm through the zona pellucida. If the zona is opened by microdissection or zona drilling, IVF rates are high (Choi *et al.* 1994; Li *et al*. 1995). It is possible that *in vitro* maturation of oocytes is associated with hardening of the zona pellucida which precludes penetration of sperm. Hardening of the zona due to premature release of cortical granules has been documented during IVM in other species under specific culture conditions. The ability of IVM oocytes to allow sperm penetration must be ascertained before they may be used effectively for research in IVF. Only one previous study (Zhang *et al.* 1989) has documented fertilisation and development of IVM oocytes after transfer to mares. In that report, 4 transfers were performed. Of 29 oocytes transferred, 5 confirmed blastocysts were recovered. The objective of our study was to determine the *in vivo* fertilisation rate of oocytes matured *in vitro* in 3 different media and transferred to the oviduct of recipient mares.

Oocytes were obtained from slaughterhouse specimens by opening follicles with a scalpel blade and scraping the contents using a bone curette. Only oocytes having expanded cumuli were used. Oocytes were cultured in one of 3 treatments: 1) M199 with 10% fetal calf serum, 5 µU FSH/ml and 25 µg/ml gentamycin (mM199) for 24 h; 2) 100% follicular fluid, obtained from a preovulatory follicle aspirated 24 h after hCG administration, with 25 µg/ml gentamycin (FF) for 24 h; or 3) mM199 with 10 µg/ml cycloheximide (CH) for 24 h followed by washing and maturation in mM199 for 24 h. Cycloheximide was used to suppress meiosis in order to manipulate the time of onset of maturation (Alm and Hinrichs 1996). Oocytes were cultured in microdroplets of 10 ml medium/oocyte under oil in a humidified atmosphere of 5% CO_2 in air. After culture, oocytes were transferred into both oviducts of each of 4 mares (a separate treatment per side) via standing flank laparatomy. Mares were inseminated 6 h prior to transfer. Oocytes/embryos were recovered 40–44 h later, following euthanasia of the mare and removal of the ovary and oviducts.

A total of 130 IVM oocytes were transferred. Oocytes were transferred with an intact cumulus, thus the state of oocyte maturation or degeneration could not be determined before transfer. Of 100 oocytes/embryos recovered, 13 appeared to be the recipient mare's oocytes from previous cycles (ie were flattened, oval and had a pale cytoplasm without an intact cytoplasmic membrane) therefore, 87 oocytes/embryos recovered were considered to have been from transferred oocytes, giving a 67% recovery of transferred oocytes. One replicate (9 oocytes) was found to be contaminated after recovery, as evidenced by presence of bacteria in the cytoplasm and white blood cells attached to the zona pellucida, and this replicate was not included in the analysis. Thus, 78 oocytes were used for evaluation of fertilisation rates.

All recovered oocytes/embryos were fixed in buffered formal saline and stained with Hoechst 33258 to determine their chromosomal and nuclear status. A fertilised oocyte was considered to be an oocyte in any stage from decondensing sperm head to multiple-cell embryo. The fertilisation rate was determined in 2 ways: as a percentage of the potentially fertilisable oocytes (ie fertilised oocytes plus oocytes in MII,

disregarding degenerating oocytes) and as a percentage of the total oocytes recovered.

The fertilisation rates for potentially fertilisable oocytes for the mM199, FF, and CH groups (3, 3 and one replicates, respectively) were 11/15 (73%); 22/30 (73%) and 6/12 (50%), respectively. The fertilisation rates based on the total oocytes recovered for each treatment were 11/25 (44%), 22/37 (59%) and 6/16 (38%), respectively. The overall fertilisation rate for potentially fertilisable oocytes was 68% (39/57) and the fertilisation rate for all oocytes recovered was 50% (39/78).

One recovered oocyte was in Anaphase II with a decondensing sperm head, and one was in mitosis of the first division; these were grouped with the cells having 2 pronuclei. Thus, of the fertilised oocytes recovered, 20/39 (51%) had 2 pronuclei, 13/39 (33%) were 2-cell embryos, and 6/39 (15%) were 3- to 4- cell embryos. There was a significant difference in distribution of embryo stage among maturation treatments. The proportions of fertilised oocytes that had cleaved to 2 or more - cell embryos in the mM199 and CH group were both significantly higher than that for the FF group (9/11, 82%; 5/6, 83%; and 6/22, 27%, respectively; $P<0.05$). Of the 6 embryos in the 3- to 4- cell stages, 4 were from the mM199 group and 2 from the CH group.

The findings of this study demonstrate that IVM oocytes have a high potential for fertilisation *in vivo*, indicating that irreversible hardening of the zona pellucida *in vitro* is not responsible for the low IVF rates found in the horse. While fertilisation rates did not differ among maturation media, the cleavage rate of fertilised oocytes was significantly lower for oocytes matured in FF. Even in treatments with high cleavage rates, however, the majority of oocytes were in the 2-cell stage. This may represent a slower rate of cleavage than would be expected *in vivo* at this time, as embryos recovered 1–2 days after ovulation are typically at the 3- to 4- cell stage (Betteridge *et al.* 1982). Fertilisation and cleavage occurred in oocytes suppressed with cycloheximide and then matured, offering a potential means of manipulating the timing of maturation and transfer of oocytes. Future studies should be directed toward determining the developmental competence (ability to develop to morula and blastocyst, and produce a fetus) of IVM horse oocytes.

Acknowledgements

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COMPARISON OF BOVINE AND EQUINE OOCYTES AS HOST CYTOPLASTS FOR EQUINE NUCLEAR TRANSFER

K. Hinrichs, T. Shin, C. C. Love, D. D. Varner and M. E. Westhusin

College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843-4466, USA

Transfer of adult somatic cell nuclei to enucleated oocytes has resulted in production of offspring in many species, including sheep, cattle and mice. The basic steps involved in nuclear transfer are maturation of oocytes to MII, enucleation of MII oocytes to form a host cytoplast, placement of the somatic cell into the perivitelline space, fusion of the cell membranes to introduce the somatic cell nucleus into the cytoplast, and activation of the reconstructed oocyte. No information is currently available on nuclear transfer in the horse, however, our preliminary studies suggested that many steps involved in nuclear transfer may be more difficult in the horse than in other species. To define the areas which may present problems in work with horse oocytes, we compared the efficiency of nuclear transfer procedures using either enucleated bovine or equine oocytes as host cytoplasts. We evaluated their ability to decondense a transferred horse somatic cell nucleus and to undergo cleavage and development after nuclear transfer. Bovine oocytes have been used successfully as host cytoplasts for the transfer of nuclei from a variety of species, including sheep, pigs, monkeys and rats (Dominko *et al.* 1999). While development to the blastocyst stage was reported for interspecies nuclear transfer embryos, in our experience and that of others, these embryos rarely progress past the 16-cell stage.

Mature bovine oocytes were obtained from a commercial source. One hundred fifty-nine *in vitro*-matured Metaphase II bovine oocytes underwent enucleation, of which all (100%) survived intact. Micromanipulation was performed as previously described (Hill *et al*. 2000). The enucleated cells were recombined with equine cumulus or fibroblast cells and were subjected to 2 x 25-µs 1.2 kv/cm DC pulses. The proportion of cells successfully fused was 109/159 (69%). Oocytes were activated using ionophore A23187 or ionomycin followed by culture in 6-DMAP. Ninety-three fused oocytes were cultured further *in vitro* on Vero cell monolayers; of these, 49 (53%) cleaved and 36 (39%) developed to the 8 to 16-cell stage. No morula or blastocyst development was observed. There were no significant differences in fusion or cleavage rates between oocytes fused with cumulus cells (35/51, 69% and 17/35, 49%, respectively) or fibroblasts (74/108, 69% and 32/58, 55%, respectively).

Horse oocytes were recovered from slaughterhouse-derived ovaries and were harvested and matured *in vitro* as previously described (Hinrichs and Schmidt 2000) or were matured in 100% equine follicular fluid derived *in vivo* from a dominant preovulatory follicle 24 h after hCG administration. Of 86 *in vitro*-matured Metaphase II horse oocytes used for NT, 59 (69%) were intact after enucleation. The difficulty of enucleation was increased because of various factors. The consistency of the zona pellucida was more pliable than that of the bovine oocytes, and thus was more difficult to penetrate with the large pipette needed for enucleation and for transfer of somatic cells. The metaphase plate and polar body were often widely separated, necessitating 2 zona punctures to fully enucleate the oocyte. In addition, the polar body frequently appeared to be attached to the zona pellucida, making it difficult to remove without damage to the oocyte. No obvious differences in ease of oocyte manipulation were noted between maturation treatments. The enucleated oocytes were recombined with equine cumulus or fibroblast cells. Of recombined cells, 28/59 (48%) successfully fused. In unfused oocytes, either the somatic cell lysed after the

electric pulse, or both oocyte and somatic cell membranes were found to be still intact. Oocytes were activated using either ionomycin or calcium ionophore A23187, followed by cycloheximide treatment. Subsequent culture was performed on a Vero cell monolayer. Only 3/28 (11%) of the fused recombined cells cleaved; no significant differences were noted between maturation treatments or donor cell source. These recombined cells developed to the 2-, 3-, and 5-cell stage but did not develop further. Presence of nuclei was confirmed in these embryos using Hoechst 33258.

To our knowledge, this is the first report documenting the production of equine nuclear transfer embryos. The results of this study indicate that rates of enucleation, fusion and subsequent cleavage are lower when equine IVM oocytes are used as cytoplasts than when bovine IVM oocytes are used. The difficulty in performing the procedures necessary for nuclear transfer in the horse is unfortunate in light of the the low efficiency of methods for harvest of equine oocytes (by follicular slicing and scraping of the granulosa cell layer or by follicle aspiration), relatively low maturation rates of equine oocytes *in vitro*, and labour-intensive methods needed for denuding of the equine cumulus (by individual pipetting, as mass vortexing is not effective). These factors combine to increase the difficulty of using horse oocytes as host cytoplasts, in comparison with bovine oocytes. The success obtained with bovine host cytoplasts demonstrates that the use of equine somatic cells can result in successful fusion and cleavage. Further work is needed to determine optimal parameters for *in vitro* maturation and nuclear transfer in horse oocytes.

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NUCLEAR TRANSFER EMBRYOS IN THE HORSE

B. C. Reggio, M. Sansinena, R. A. Cochran, A. Guitreau, J. A. Carter, R. S. Denniston and R. A. Godke

Department of Animal Science, Louisiana State University, Baton Rouge, Louisiana 70803, USA

INTRODUCTION

After the recent births of healthy foals produced from intracytoplasmic sperm injection by this laboratory (Cochran *et al.* 1998), an attempt was made to produce cloned horses embryos using similarly harvested oocytes from live mares (Meintjes *et al.* 1995) (Part I). Oocyte availability is one of the major limitations in conducting nuclear transfer studies in many species. In the horse, oocyte collection is cumbersome and often seasonal, thus potenially limiting to the success of a nuclear transfer program. In a study conducted by Dominko *et al.* (1999) bovine oocytes were used as recipients of nuclear transfer of various mammalian species (rat, cow, sheep, pig, monkey and rat). Regardless of the species of donor fibroblasts used in this study, all couplets progressed through the first cell cycle, and some reconstructed embryos developed to the blastocysts stage. Since the timing of the first two cleavage divisions in bovine *in vitro*-produced embryos corresponded more closely to the timing of cleavage in other the donor nucleus species, bovine oocytes were used in the second part of this study (Part II).

Part I. A group of 6 mixed breed mares were treated daily with 0.044 mg/kg altrenogest (Regumate) then subjected to a follicle reduction via transvaginal ultrasound-guided aspiration on Day 21. Oocytes were then collected every 10 d ays beginning on Day 31 and matured *in vitro* in TCM-199 supplemented with oestrous mare serum for 36 h. Mature oocytes were enucleated by removing the first polar body and second metaphase plate using Hoechst 33342 stain (5 µg/ml) and epifluorescent microscopy to confirm the complete removal of maternal DNA. A single donor cumulus cell recovered from the pool of mature oocytes was then inserted into the perivitelline space of each cytoplast. Enucleation and reconstruction were conducted in 2 µg/ml of cytochalasin B (CB) using a Nikon Diaphot microscope equipped with Hoffman objectives and Narishige micromanipulators. Donor cells and recipient cytoplasts were fused with 2 electrical pulses (3.5 kV/cm, 30 µs) in a 0.3 M mannitol fusion buffer containing 0.1 mM MgSO4, 0.5 mM HEPES and 1 mg/ml of BSA (pH 7.0; 25°C).

Immediately following the electrofusion procedure, couplets were incubated for 1 h in 7.5 mg/ml of CB, washed and held in P-1™ culture medium (Irvine Scientific) for 9 h at 38°C. Couplets (n = 36) were then randomly assigned to one of two treatments: activation (A) or no activation (B). Activation was performed at 45 h post-maturation with 5 µM ionomycin followed by a 4 h treatment in 2 mM 6-dimethylaminopurine. Nonactivated oocytes were maintained in P-1 medium supplemented with 10% FBS.

From 142 follicles aspirated (n = 5 aspirations) (Table 1), 74 cumulus enclosed oocytes were

TABLE 1: Aspiration, recovery and maturation of equine oocytes of live mares

Aspirated	Recovered	Matured*	Reconstructed*
142	74 (52%)	48 (65%)	36 (75%)

*Percentage of oocytes matured and reconstructed based on the number of oocytes recovered.

TABLE 2: Fusion of equine reconstructed oocytes

Treatment	n	Fused
Activated	18	14 (77%)
Nonactivated	18	10 (56%)

TABLE 3: Enucleation, reconstruction and fusion of bovine oocytes with equine fibroblast donors

Treatment	Enucleated	Reconstructed	Fused
Proliferating	26	25	13 (52%)
Quiescent	17	16	13 (81%)
Total	43	41	26 (63%)

TABLE 4: Cleavaged equine reconstructed embryos and nuclei staining

Treatment	Cleaved (day 3)	Nuclei staining
	10-cell	8-cell
	10-cell	6-cell
Proliferating	10-cell	6-cell
10 (78%)	8-cell	4-cell
	8-cell	2-cell
	10-cell	4-cell
	6-cell	4-cell
Quiescent	5-cell	3-cell
10 (78%)	5-cell	cf*
	5-cell	cf*
Total 20 (77%)		

*cf: = cytoplasmic fragments.

recovered (52%), 48 were matured *in vitro* (65%) and 36 were successfully reconstructed (75%). A total of 24 reconstructed oocytes were successfully fused (67%) but failed to divide in culture, regardless of the activation treatment schedule (Table 2).

Part II. The objective of second study was to evaluate the ability of the cow oocyte to support mitotic cycles following interspecies nuclear transfer, and to explore the potential of the bovine oocyte as recipient for nuclear transfer in species where oocyte collection is often limited. Oocytes will be obtained from a commercial source (BOMED; Madison,WI) and enucleated at 18 h post-maturation. Quiescent (starved) or proliferating donor cells will be immediately injected into the perivitelline space of enucleated oocytes. In this study, couplets were electrofused with one single electrical pulse (1.80 kV/cm, 30 ms). Activation was performed with ionomycin (4 min) followed by a 3 h treatment in 6-methylaminopurine. Parthenogenic oocytes (control) were activated in similar fashion. Nuclear transfer couplets were cultured in glucose-free P1 medium, and cleavage was assessed at 72 h post-reconstruction (the end point). Couplets were exposed to a Hoechst 33342 stain to determine the presence of nuclei.

In summary, a total of 43 oocytes were enucleated resulting in 41 successfully reconstructed (Table 3). A total of 26 reconstructed couplets successfully fused (63%), and 20 (77%) of these had cleaved when assessed on Day 3 of culture. From a total of 20 fused couplets, stained with Hoechst 33342 on Day 3 of culture, revealed that four embryos were at the 10-cell stage, three were at 8-cell stage, three were at 6-cell stage, three were at 5-cell, three were at 4-cell, one was at 3-cell, and one at 2-cell stage (Table 4). In the remaining two couplets, staining revealed cyto-plasmic fragmentation and no nuclei.

Although acceptable rates of fusion were obtained in these preliminary trials, more work is needed to elucidate the factors necessary for effectively activating equine oocytes prior to nuclear transfer. Although this was a preliminary study, the bovine cytoplast was able to support mitotic division and give indication that they could serve as a cytoplasmic recipient for future interspecies nuclear transfer in the horse. Certainly more research is needed to develop this methodolgy, and normal live nuclear transfer offspring are needed validate the technology.

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EFFECTS OF OOCYTE MATURITY AND METHODS OF COLLECTION, CULTURE AND INSEMINATION ON EQUINE OOCYTE TRANSFER

E. M. Carnevale, L. J. Maclellan, T. J. Scott, M. A. Coutinho da Silva, C. F. Scoggin and E. L. Squires

Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA

Assisted reproductive techniques, such as oocyte transfer, are currently being used to obtain pregnancies from subfertile mares. For oocyte transfer, preovulatory oocytes are usually collected 24 h after hCG, and maturation is completed *in vitro* prior to transfer. Development of techniques that would eliminate *in vitro* culture and allow the use of immature oocytes and low numbers of sperm would permit assisted reproductive techniques to be used when an incubator and/or culture expertise are not available, when preovulatory oocytes are not available (eg euthanasia of mare), or when sperm numbers are low (eg subfertile stallions, sexed sperm, frozen sperm). Objectives of 2 experiments were to compare embryo development rates for: 1) oocytes completing maturation *in vitro* or within the oviduct; 2) intraoviductal versus intrauterine insemination of recipients; 3) oocytes matured within the follicle versus *in vitro*; and 4) oocytes collected from live mares versus from slaughterhouse ovaries.

In Experiment 1, oocytes were recovered using transvaginal, ultrasound-guided follicular aspirations (TVA) from naturally cycling donor mares 24 to 26 h after hCG when follicles reached 35 mm. Multiple oocytes (1–4) were transferred surgically into oviducts of 4 or 5 recipients per group. Recipient's oocytes were collected prior to transfers. Three groups of transfers were compared: 1) transfers of oocytes cultured *in vitro* for 12 to 14 h post collection with uterine insemination of recipients 2 h post surgery; 2) transfers of oocytes into oviducts within 1 h of collection, with completion of oocyte maturation occurring within oviducts, and uterine insemination of recipients 14–16 h post surgery; and 3) transfers of sperm and oocytes (cultured 12 to 14 h *in vitro*) into oviducts. Oocytes, cultured *in vitro*, were placed in culture medium (TCM 199 with 10% FCS, 0.2 mM pyruvate and 50 µg/ml gentamicin sulphate) in an atmosphere of 5% CO_2 and air at 38.5°C. Recipients were inseminated into the uterine body with 2×10^9 progressively motile sperm from one of 2 stallions of known fertility. Approximately 1 h prior to intraoviductal insemination (Group 3), semen was collected from a stallion and separated through a density gradient (90/45% Percoll centrifuged for 10 min at 300G). The sperm pellet was washed and resuspended in Hepes-buffered synthetic oviduct fluid (H-SOF with 0.6% BSA); 500,000 progressively motile sperm were transferred with oocytes into each recipient's oviduct. In Experiment 1, numbers of embryos detected on Day 16 of gestation were not different ($P>0.1$) for Groups 1, 2 and 3 (8/14, 57%; 6/14, 43% and 3/11, 27%). Therefore, maturing oocytes successfully completed final stages of maturation within the oviduct, and sperm deposited within the oviduct were capable of fertilising oocytes.

In Experiment 2, oocytes were collected by TVA from live mares or from slicing slaughterhouse ovaries. Four groups of oocytes were transferred into oviducts of recipients: 1) oocytes matured *in vivo* and collected by TVA from preovulatory follicles of oestrous mares 32–36 h after administration of hCG; 2) immature oocytes collected from dioestrous mares between 5 and 10 days after aspiration or ovulation by TVA and matured *in vitro* for 36–38 h; 3) immature oocytes collected from dioestrous mares between 5–10 days after aspiration or ovulation by TVA and transferred into a recipient's oviduct <1 h after

collection; and 4) immature oocytes collected from slaughterhouse ovaries containing a corpus luteum and matured *in vitro* for 36–38 h. Recipients in Group 1, 2 and 4 were inseminated 12–14 h prior to oocyte transfer; recipients in Group 3 were inseminated 34–36 h after transfer. Recipients were inseminated with 2×10^9 progressively motile sperm from one of two stallions. Oocytes, matured *in vitro*, were placed in maturation medium (TCM 199 with 0.2 mM pyruvate, 1 mM glutamine, 25 mM bicarbonate, 10% FCS, 15 ng/ml FSH, 1 µg/ml LH, 1 µg/ml E_2, 50 µg/ml gentamicin) and incubated for 36–38 h at 38.5°C in 5% CO_2 and air. Oocyte recipients were allowed to ovulate the preovulatory follicle; subsequently, the number of recipient ovulations were subtracted from the number of embryonic vesicles imaged with ultrasound.

In Experiment 2, oocytes matured *in vivo* (9/11, 82%) resulted in higher ($P<0.001$) embryo development rates than oocytes matured *in vitro* (TVA, 2/29, 7% and slaughterhouse, 4/40, 10%) or within the oviduct (0/27). However, method of maturation or source of oocytes did not affect ($P>0.1$) embryo development rates after transfer of immature oocytes.

In conclusion, oocyte transfer was a repeatable method for testing oocyte competence after IVM. Embryo development rates were high after transfer of oocytes matured *in vivo*. The final stage of oocyte maturation successfully occurred within the oviduct; however, immature oocytes did not complete maturation within the oviduct. Pregnancies were obtained from oocytes collected by TVA or from slaughterhouse ovaries, matured *in vitro*, and transferred into recipients; however, embryo development rates were low. Transfer of sperm and oocytes into the oviduct resulted in embryo development, suggesting this technique (GIFT) has a potential use in equine assisted reproduction.

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SESSION 3:

Embryo cooling and freezing

Chairman: E. Palmer

NOVEL METHODS OF EMBRYO CRYOPRESERVATION

S. P. Leibo

Audubon Center for Research of Endangered Species, Department of Biological Sciences, University of New Orleans, New Orleans, Louisiana, USA

The capability to cryopreserve mammalian embryos is a powerful adjunct to methods of assisted reproduction. Tens of thousands of mice and hundreds of thousands of cattle have been produced from cryopreserved embryos. In contrast, only about 50 equine pregnancies and fewer than 10 foals resulting from cryopreserved conceptuses have been reported in the literature. Because of innumerable investigations of the cryobiology of embryos, especially those of mice and cattle, considerable mechanistic understanding has been derived of the factors that determine ultimate survival and full-term development of cryopreserved embryos. However, there have been many fewer studies of the basic cryobiological aspects of equine embryos that may influence their response to freezing and thawing. Any such investigation must take into account unique features of the equine conceptus, and also the unusual reproductive biology of the mare compared to the female of many other species.

Cryopreservation of embryos of all species consists of the following steps:

1. Exposure to molar concentrations of one or more cryoprotective additives (CPAs);
2. Cooling to sub-zero temperatures under conditions that result in efflux of virtually all intracellular water from the embryos;
3. Immersion in liquid nitrogen (LN_2) at -196°C, permitting unlimited storage with no loss of biological function;
4. Warming to physiological temperatures;
5. Removal of the CPAs from the embryos.

Cryopreservation is a unidirectional process; consequently, all the steps of the sequence must be performed properly if an embryo is to survive cryopreservation. But each of these steps has the potential to damage or destroy mammalian embryos. For example, common CPAs are glycerol, dimethyl sulphoxide, ethylene or propylene glycol. Concentrated solutions (10–50% by volume) of those CPAs may damage embryos, either by direct toxic effects or by osmotic shock, during the initial exposure or during its removal. Very few studies of the effects of CPAs on equine embryos have been conducted. Another factor that may affect embryo development is exposure to non-physiological temperatures. Although embryos may 'tolerate' exposure to temperatures near or below 0°C, it is becoming increasingly evident that embryos may undergo subtle injury caused by chilling. There are significant differences in chilling sensitivity among embryos of different species, and among various developmental stages of a given species. Little is known about the susceptibility of equine embryos to chilling injury. A third factor known to have a major effect on embryo survival is the rate at which samples are cooled from ~0°C to sub-zero temperatures below -30°C. Only one very brief study of this variable in the horse conceptus has been reported. Most investigations have used a single cooling rate of 0.3°C/min to freeze equine embryos; this may or may not be the optimum rate. Yet another potentially damaging event may occur during removal of the CPA from the embryo after its cryopreservation. The effect of CPA removal on embryo survival depends on the permeability of that specific embryonic stage to the CPA itself and the temperature coefficient of permeability. There are significant differences among species with respect to embryo permeability; little is known about the relationship between permeability to various CPAs and temperature of the equine conceptus.

In summary, systematic studies of cryobiological variables known to affect survival of embryos of other mammalian species, as well as studies that allow for exceptional characteristics of the equine conceptus may yield improved methods for their cryopreservation.

COMPARISON OF GLYCEROL AND ETHYLENE GLYCOL IN EQUINE EMBRYO FREEZING USING CONFOCAL MICROSCOPY, DAPI-STAINING AND NONSURGICAL TRANSFER

M. Huhtinen, A. Sjöholm and J. Paranko*

*MTT, Equines, Ypäjä, Finland; *Department of Biology, Laboratory of Animal Physiology, University of Turku, Finland*

INTRODUCTION

After reaching a certain diameter and developmental stage, equine embryos are severely damaged during freezing. Several cryoprotectants have been tested in an attempt to overcome this problem, but so far, only glycerol has given reasonable results in conventional cryopreservation procedures (for review see Seidel 1996). In theory, the higher permeability of ethylene glycol versus glycerol (Pfaff *et al.* 1993) would favour the use of the former. The availability of reliable techniques to study damages induced by freezing and thawing of embryos have been limited. One such tool could be confocal fluorescence microscopy because it enables a 3-dimensional analysis of otherwise difficult intact biological specimens, like fluorescently labelled multicellular embryos. The aim of the present work was to compare cryodamage in equine embryos after freezing in glycerol or ethylene glycol using confocal microscopy, DAPI-staining, and nonsurgical transfer.

MATERIALS AND METHODS

Thirty-seven embryos, 140–190 µm in diameter, were frozen in a programmable freezer after addition of the selected cryoprotectant. Glycerol and 50 mM glutamine were added in 4 steps for 18 embryos. Nineteen embryos were placed into 1.5 M ethylene glycol in Emcare. All embryos were thawed by holding the straws in the air for 10 s and then in a water bath at 30ºC for 20 s. In the glycerol group, the cryoprotectant was removed in six steps with sucrose (Huhtinen *et al.* 1997). Half of the embryos in each treatment group were stained with DAPI (1 µg/ml) for 15 min in room temperature, washed and transferred nonsurgically to the uteri of recipient mares that had ovulated four days before transfer. The remaining half were fixed in 3.7% formaldehyde for 15 min in room temperature. After three washes in PBS, they were extracted with the solution of 0.1% Triton X-100 in PBS for 5 min. After 3 washes in PBS, the embryos were treated with RNAse (1 mg/ml) in PBS for 15 min. After short washes in PBS, the embryos were incubated for 15 min in PBS containing 1% BSA. Then the embryos were incubated in the presence of Oregon Greed 514 phalloidin and propidium iodide (0.5 µg/ml) for 30 min in room temperature. After 3 washes in PBS, the embryos were mounted on a slide in 1% agar and analysed with Leica TCS NT confocal microscope.

TABLE 1: Number/percentage of DAPI-stained cells in frozen-thawed embryos before transfer

	Embryo number									
	1	2	3	4	5	6	7	8	9	10
Gly	0*	1	6*	10	10*	11	25	20%*	30%	-
EG	4*	10*	14	15	30	35	35	50	30%*	40%

* = The embryo continued its development after transfer

Results and discussion

Of 9 embryos frozen with glycerol, 4 continued developing in the uteri of the recipient mares. In the ethylene glycol group, 3 out of 10 transfers were successful. The number/percentage of DAPI-stained cells is presented in detail in Table 1. No statistical differences in pregnancy rates or in the percentages of DAPI-stained cells were noted between the 2 freezing protocols. Double labelling for actin and nuclei indicated well preserved cytoskeleton in the outer embryonic cell layers. More diffuse actin staining in the inner parts of the embryo was considered as an indication of improper cryopreservation.

This study compared 2 different freezing methods for equine embryos: glycerol + L-glutamine and Emcare ethylene glycol. The effects of L-glutamine or Emcare media itself cannot be differentiated from the effects of glycerol and ethylene glycol. However, both methods seemed to be able to protect the embryos equally well to produce pregnancies after transfer. The thawing method might also have to be selected according to the freezing protocol, although the same thawing methods have been used in the previously published papers irrespective of the cryoprotective agent (Bruyas 1997).

The developmental stage of the embryo influences the rate of cryoprotectant passage to the inner parts of the embryo. The capsule, in particular, seems to impede the successful freezing of equine embryos (Bruyas 1997). Therefore, the time that embryos are equilibrated in the cryoprotectant solutions should vary according to the developmental stage of the embryo. The embryo should be carefully monitored during cryoprotection, and the freezing should not be started equilibrium has been reached in the solution and the cryoprotective agent has entered also the inner cell mass cells.

The study of whole-mount equine embryos indicates that confocal microscopy with multi-labelling technique is a promising method for the analysis of cellular damage induced by cryopreservation. However, further work is required to elucidate if the cytoskeletal damages seen in the thawed embryos are severe enough to prevent further development of the embryos.

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CRYOPRESERVATION OF EQUINE EMBRYOS BY OPS, CRYOLOOP, OR CONVENTIONAL SLOW COOLING METHODS

N. Oberstein, M. K. O'Donovan, J. E. Bruemmer, M. Lane, G. E. Seidel, Jr., E. M. Carnevale and E. L. Squires

Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA; Colorado Center for Reproductive Medicine, Englewood, Colorado 80110, USA

Cryopreservation of equine embryos has been only marginally successful with conventional slow-cooling procedures. The objective of this experiment was to compare the efficacy of 2 vitrification procedures to conventional slow-cooling procedures for freezing equine embryos.

Small (≥ 300 µm) embryos were recovered from mares on Day 6 or 7 post ovulation. Twenty-seven Grade 1 or 2 embryos were randomly assigned to one of 3 cryopreservation treatments.

For Treatment 1, embryos were placed in 1.8 M ethylene glycol (EG) and 0.1 M sucrose and equilibrated on a warm plate at 30°C for 10 min. Individual embryos were loaded into 0.25 ml French straws as follows: 5 µl of H-SOF, air bubble, 50 µl H-SOF with 1.8 M EG and 0.1 M sucrose containing the embryo, air bubble, and 150 µl of H-SOF. Straws were heat-sealed on one end and sealed with the PVC cotton plug on the other end. Straws were placed in a programmable freezer at 0°C, cooled at 0.5°C/min to -6°C and seeded. The temperature was held for 1 min at -6°C, and then dropped to -35°C at 0.3°C/min after which straws were plunged into liquid nitrogen. Embryos were thawed by holding straws in air for 10 s and then placing them in a 37°C waterbath for 20 s. Cryoprotectant was removed by transferring embryos directly into H-SOF. Embryos were subsequently washed through three 150 µl drops of H-SOF for 5 min each.

Embryos in Treatment 2 were cryopreserved in an open pulled straw (OPS) (Hochi *et al.* 1996). After washing, embryos were placed into a droplet of H-SOF containing 7.5% DMSO and 7.5% EG for 3 min. Embryos were transferred in approximately 1–2 µl of solution into a 20 µl droplet of H-SOF with 16.5% EG, 16.5% DMSO and 0.5 M sucrose. Approximately l µl of medium containing the embryo then was drawn into the fine end of an open pulled straw by capillary action. The fine end was immediately submerged into liquid nitrogen. The interval from embryo contact with concentrated cryoprotectant solution and freezing was 20–30 s. Straws were stored in liquid nitrogen until viability testing. Embryos were thawed by immersing the pulled end of the straw in 1.2 ml H-SOF containing 0.15 M sucrose for 5 min and moved twice into fresh H-SOF for 5 min.

For Treatment 3, embryos were directly immersed into liquid nitrogen using a cryoloop (Hochi *et al.* 1994). Embryos were washed 4 times in H-SOF and placed into H-SOF with 7.5% DMSO and 7.5% EG for 2.5 min. Embryos were transferred into H-SOF with 17.5% DMSO, 17.5% EG, 1 M sucrose, and 0.25 µM ficoll. A nylon loop was mounted. Embryos were transferred into H-SOF with 17.5% DMSO, 17.5% EG, 1 M sucrose, and 0.25 µM ficoll. on a stainless steel cylinder inserted into the lid of a cryovial. Loops were purchased mounted and epoxied into vials. The metal insert on the lid enabled the use of a handle with a small magnet for manipulation of the loop. While the embryo was in the first cryoprotectant solution, the cryoloop was dipped into the second cryoprotectant solution to retain a thin film of medium within the loop. A single embryo was pipetted onto the film. The cryoloop containing the embryo was then immediately plunged into the cryovial, which was already submerged under liquid nitrogen. The cryovial lid was sealed under liquid nitrogen in one motion. The interval between contact with concentrated cryoprotectant and plunging was 20–30 s. Embryos were stored in

TABLE 1: Effect of cryopreservation on embryo grade, size and percent live cells

	Grade				Diameter (µm)				% live cell†
	Initial	Post thaw	20 h culture	S.E.	Initial	Post-thaw	20 h culture	S.E	
Treatment									
Slow-cooled	1.4	2.6	2.9	0.2	257	218	238	11.1	74*
Vitrified OPS	1.4	2.9	3.1	0.1	232	184	176	9.9	51
Vitrified cryoloop	1.3	2.6	3.3	0.2	211	184	196	11.4	48

*Three embryos in the slow-cooled treatment group were killed and lost during staining and are not included
†sd ± 37

liquid nitrogen until thawing in a 4-well dish at 37°C. The lid and loop was removed from the cryovial under liquid nitrogen. The loop containing the embryo was sequentially placed into wells, containing 840 µl H-SOF plus 420 ml of H-SOF supplemented with 1 M sucrose for 2.5 min, 840 µl H-SOF plus 210 ml of H-SOF supplemented with 1 M sucrose for 3 min, and 840 ml H-SOF for 5 min. After thawing, embryos were cultured for 20 h in SOFaa in the presence of 6% CO_2, 5% O_2, 89% N_2 at 38.5°C.

Embryos were graded and measured pre-freeze, post thaw, and after 20 h of culture. Embryos were then stained with propidium iodide and Hoechst 3342 to determine the proportion of live cells. Embryos were washed in a 100 µl droplet of H-SOF under oil and placed in staining solution containing H-SOF supplemented with 8 mg/ml BSA with 10 µg/ml PI and Hoechst 33342. Embryos were placed into a 100 µl droplet of staining solution at 38.5°C for 15 min under foil. After staining embryos were washed twice in H-SOF and mounted on a slide in an 11 µl drop of H-SOF, covered with a cover-slip, which mounds on four drops of a paraffin oil/vaseline mixture. Fluorescence was observed with a Nikon Eclipse E800 narrowband microscope (Filter FITC, TRITC and DAPI) with a 20X/0.75 objective (Nikon); nuclei and dead cells of embryos fluoresce with bright blue and red colour, respectively, and percent live cells were estimated. It was not possible to count the cells, due to the large numbers of cells per embryo. Three different people independently estimated the percent live cells and the average of these numbers were taken.

Results were evaluated by analyses of variance. There were no significant differences ($P>0.1$) in grade or size of embryos prior to cryopreservation. Upon thawing, an equal proportion of embryos fractured in the slow-cooled, OPS and cryoloop procedure resulting in 12.5% (1/8), 18% (2/11) and 12.5% (1/8) fractured embryos.

Because the fractured embryos in the slow-cooled method appeared viable they were included in the results. Embryos that survived cryopreservation were stained, and ranged in diameter from 140–320 µm after 20 h of culture. There were no significant differences ($P>0.1$) in embryo grade or viability post-thaw among cryopreservation treatments. Embryo grade and viability as assessed by staining were correlated $r=-0.66$ ($P<0.004$). This shows that morphological grade is a good indicator of viability. OPS and cryoloop procedures were as effective as conventional slow-cooling methods.

The present experiment demonstrated that vitrification of small (≥ 300 µm) equine embryos in OPS or a cryoloop yields similar results to controlled slow cooling ($P>0.1$) as measured by percent live cells and morphological grades. In conclusion vitrification procedures with OPS or cryoloop were similarly effective for cryopreservation of small equine embryos. These results are in agreement with those obtained in other species including bovine (Vajta *et al.* 1998) and hamsters (Lane *et al.* 1999). Further, both vitrification methods produced results which were similar to other studies where morphological grade post-thaw and subsequent transfers were performed. In those studies embryos graded 1 or 1.5 prior to cryopreservation were determined to grade 2.4 on average and result in 50 to 53% pregnancy rates, respectively (Slade *et al.* 1985; Squires *et al.* 1989). It is likely, therefore, that these techniques will eventually supercede the

slow-cooling methods because of speed, ease of technique, and reduced cost for equipment. In this study embryos were evaluated by morphological grade or staining; however, a true indicator of viability is pregnancy rates. Therefore, this study should be repeated to determine pregnancy rates following embryo transfer.

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METABOLIC ACTIVITY OF EQUINE BLASTOCYSTS CRYOPRESERVED IN ETHYLENE GLYCOL AND GALACTOSE

M. K. O'Donovan, N. Oberstein, J. E. Bruemmer, M. Lane*, G. E. Seidel, Jr., D. K. Gardner* and E. L. Squires

*Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA; *Colorado Center for Reproductive Medicine, Englewood, Colorado 80110, USA*

INTRODUCTION

The inability of expanded equine blastocysts to survive standard cryopreservation protocols is well documented (Yamamoto *et al.* 1982; Squires *et al.* 1989; Young *et al.* 1997). The objective of this study was to compare the efficacy of 3 protocols for cryopreserving equine embryos 300–600 Fm in diameter. It was hypothesised that exposing expanded equine blastocysts to galactose and ethylene glycol at various concentrations for 40 min would allow proper equilibration to occur prior to cryopreservation, thus improving post thaw survival.

MATERIALS AND METHODS

Morphological Grade 1 or 2 embryos recovered on Day 7 post ovulation, between 300–600 Fm in diameter were randomly assigned to one of 3 cryoprotectant treatments: 1) (n=8) 0.3 M galactose (10 min), 2.0 M ethylene glycol (EG) + 0.3 M galactose (40 min); 2) (n=5) 0.3 M galactose (10 min), 2.0 M EG + 0.3 M galactose (15 min), and 4.5 M EG + 0.3 M galactose (25 min); or 3) (n=8) 0.3 M galactose (10 min), 2.0 M EG + 0.3 M galactose (10 min), 4.5 M EG + 0.3 M galactose (15 min), 2.0 M EG + 0.8 M galactose (10 min). All embryos were loaded into 0.25 ml straws and cooled to -6°C at 4°C/min, held 5 min, seeded, cooled to -35°C at 0.5°C/min and plunged into liquid nitrogen. Embryos were thawed in air (15 s) followed by a 37°C water bath (15 s). Upon thawing, cryoprotectants were removed in 3 or 4 steps based on the specific treatment. Viability was assessed following the freeze/thaw procedure by grading the embryos and recording changes in diameter over time. Additionally, metabolic activity of the embryos pre-freeze and post thaw was compared. Measurements of glucose and pyruvate uptake and production of lactate were analysed using microfluorescence assays (Gardner *et al.* 1996). Embryos were stained after the last metabolic assay using Hoechst 33342 (10 µg/ml) and propidium iodide (10 µg/ml) to determine percent live cells.

RESULTS

Twenty-one embryos were randomly assigned to one of 3 treatments, n_1=8, n_2=5, n_3=8. Mean diameters of embryos were not different among treatments ($P>0.1$) but varied within treatment over time ($P<0.05$) (Table 1). Treatment differences in morphological grades were evident following 12 h of IVC, and differences within treatment were seen over time (Table 1).

Embryos that did not degenerate after thawing were stained, and percent live cells were estimated at 53%, 21% and 6% for Treatments 1, 2 and 3, respectively; differences between Treatments 1 and 3 were significant ($P<0.05$). Four of 8 embryos in treatment one stained with 90% live cells, 2 embryos had 25% live cells, and 2 embryos had 5% live cells. Three embryos in treatment 2 degenerated in culture and could not be stained; however, one of the 2 remaining embryos stained 90% live cells and the other had 15% live cells. Seven embryos in treatment 3 stained with 5% live cells, and one embryo stained with 15% live cells.

Mean values for glucose and pyruvate uptakes and lactate production are presented in Table 2. Differences in pre-freeze and post thaw values within treatments were not significant ($P>0.1$).

TABLE 1: Mean diameters and morphological scores (± sd) by time

Endpoint	Treatment	Initial	Thaw	Post thaw assay	Post IVC	Post IVC assay
Diameter (Fm)	1 (n=8)	407[a] (±90)	294[b] (±123)	247[b] (±80)	330[a,b] (±113)	345[a] (±109)
	2 (n=5)	410[a] (±97)	279[b] (±80)	275[b]* (±35)	295[b]* (±64)	302[b]* (±81)
	3 (n=8)	369[a] (±49)	303[a,b] (±56)	250[b,c] (±56)	216[c] (±27)	198[c] (±45)
Grade (1-4)	1 (n=8)	1.3[a] (±.27)	3.4[b] (±.23)	3.4[b] (±.34)	3.1[b] (±.74)	2.9[b] (±.88)
	2 (n=5)	1.2[a] (±.45)	3.6[b] (±.42)	3.5[b]* (±.35)	3.6[b,A]* (±.89)	3.7[b,A]* (±.67)
	3 (n=8)	1.4[a] (±.35)	3.3[b] (±.26)	3.8[c] (±.38)	4[c,A] (± 0)	4[c,A] (± 0)

[a,b,c] Means without common superscript within rows differ (P<0.05); [A] Column means differ from treatment 1 (P<0.05); * n=2 embryos; grade 1 = excellent, grade 4 = poor

TABLE 2: Mean values (± sd) of metabolic substrates pre-freeze and post thaw (pmol/h/mm^2)[a]

Substrate	Pre-freeze	Treatment 1	Treatment 2	Treatment 3
Glucose	734 (±540)	512 (±610)	291 (±144)	104 (±234)
Pyruvate	125 (±203)	213 (±104)	128 (±64)	110 (±160)
Lactate	939 (±588)	888 (±270)	676 (±402)	764 (±527)

[a]There were no treatment differences (P>0.1)

TABLE 3: Mean metabolic parameters (± sd) for viable and nonviable embryos[a]

Substrate	Viable	Viable	Nonviable	Nonviable
(pmol/h/mm^2)	Pre-freeze	Post thaw	Pre-freeze	Post thaw
Glucose	572 (±120)	580 (±653)	785 (±611)	225 (±323)
Pyruvate	129 (±180)	269 (±82)	124 (±215)	118 (±112)
Lactate	1384 (±767)	929 (±233)	799 (±414)	746 (±433)

[a]There were no differences (P>0.1)

Embryos were pooled across treatments into nonviable (Grade 3 to 4, 5 to 25% live cells) and viable (Grade 2.5 or better, 90% live cells) groups. There was no significant difference in post-thaw metabolic parameters between viable (n=5) and nonviable (n=16) embryos (Table 3).

DISCUSSION

In the current investigation, treatment one (low molar ethylene glycol) was more effective in cryopreserving large equine embryos than Treatments 2 (high molar ethylene glycol) and 3 (step-down equilibration). Embryos in treatment one had significantly better final morphological grades and a higher percentage of live cells. Additionally, embryos in Treatment 1 had less change in metabolic activity pre-freeze to post-thaw than embryos in other treatments, although the difference between treatments was not significant (P>0.1). Expanded equine blastocysts exposed to 2 M ethylene glycol for 40 min survived cryopreservation.

Three embryos from Treatment 2 were fractured upon thaw and degenerated in the post thaw metabolic assay. Conceivably, embryos

frozen in a cryoprotectant solution containing 4.5 Methylene glycol and 0.3 M galactose require different rates for cooling or thawing. Altering these rates may reduce problems associated with fracture planes.

Although Young *et al.* (1997) reported success with step-down-equilibration using glycerol and large equine embryos, it was clearly not repeated in this study using a different cryoprotectant plus adding galactose before adding cryoprotectant. Step-down-equilibration may have failed in the study because ethylene glycol permeates equine embryos faster and more effectively (embryos lose less volume) than glycerol (Pfaff *et al.* 1993). Moving embryos through 2 M, 4.5 M and 2 M solutions of ethylene glycol may have been toxic.

Although Gardner *et al*. (1996) successfully used microfluoresence assays to determine nutrient uptake and utilisation of *in vitro*-produced, cryopreserved Day 7 bovine embryos, it was difficult to make any strong conclusions regarding viability from metabolic data in this experiment. Although viable embryos appeared to have higher rates of glucose uptake and utilisation than nonviable embryos, significant differences were not detected, in part due to the large variation in values. The variation in all substrates in the present study may be a result of different ages of embryos; embryos could have been between 168 and 192 h of age. A larger sample size and a more homogenous population of embryo age might have lead to different conclusions.

In summary, treatment one, 2 M ethylene glycol for 40 min, was the most promising method for cryopreservation of expanded equine blastocysts. Perhaps the extended exposure to ethylene glycol allowed for greater intracellular equilibration and subsequent protection than previous studies. One caveat in these studies is that no embryos were transferred, so pregnancy rates are not available. Assessing viability with microfluorescence assays may be enhanced by creating a larger more homogeneous population of embryos.

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PRODUCTION OF CAPSULAR MATERIAL BY EQUINE TROPHOBLAST TRANSPLANTED INTO IMMUNODEFICIENT MICE

A. Albihn, J. Samper*, J. G. Oriol, B. A. Croy and K. J. Betteridge

*Department of Biomedical Sciences, Ontario Veterinary College; *The Equine Research Centre, University of Guelph, Guelph, Ontario, N1G 2W1, Canada*

The equine embryonic capsule is presumed essential to normal embryonic development (Betteridge 1989; Stout *et al.* 1997). It also seems to impede the successful freezing of equine embryos (Bruyas 1997). The capsule is considered to be produced largely by the trophoblast (Oriol *et al.* 1993) but, because production of this mucin-like glycoprotein by equine embryos has not so far been demonstrated *in vitro* (Betteridge 1989; McKinnon *et al.* 1989), this assumption is difficult to prove. The present study was therefore designed to test the hypothesis that capsular material is produced by the trophoblast, independently of maternal contributions. To do so, xenogeneic transplantation of equine endometrium and/or trophoblast into mice with severe combined immunodeficiency (SCID; *scid/scid or scid/scid.bg/bg* mice; Croy and Chapeau 1990) was used as an '*in vivo* culture system'.

To develop the procedures (Experiment 1), endometrial biopsy samples from dioestrous mares were partly infiltrated with India ink, then used to prepare multiple 1 mm^3 grafts. These were surgically transplanted into various sites in 18 mice for 4, 8 or 16 days. The results of Experiment 1 are summarised in Table 1.

Overall, 23/52 (44%) grafts were recovered in histological sections, proportionately more from the ovarian fat pad or kidney capsule than from the uterine lumen. Histomorphology was well maintained for up to 16 days and ink staining helped recovery (78% recovery for ink-stained versus 26% for non-stained grafts). Technical difficulties rather than graft rejection most probably accounted for the failure to find some grafts at the time of euthanasia. Thus, xenotransplantation into SCID mice was shown to be a useful culture system for grafted equine endometrium.

In Experiments 2 and 3, endometrial biopsy samples and conceptuses from 5 mares 13–15 days after ovulation were used to prepare grafts of endometrium (E), trophoblast (T) and capsule (C) for transplantation into 59 mice. At this stage of development, 'trophoblast' would have comprised trophectoderm, endoderm and possibly mesoderm. Grafts recovered at the time of euthanasia were examined for the presence of capsule-like material either histochemically (Experiment 2, 20 mice) or immunohistochemically (Experiment 3, 39 mice). PAS staining was used in Experiment 2, a mouse monoclonal antibody against equine capsule (MAb OC-1; Oriol *et al.* 1993) in Experiment 3.

The overall graft recovery rate in Experiment 2 was 22/49 (45%; 11/28 single grafts, and 11/21 when E and T were co-engrafted). Capsule-like extracellular glycoprotein at the graft site was identified by PAS staining of histological sections as summarised in Table 2. Strong PAS-positive reactions (5–7 mm thick) were found in

TABLE 1: Graft recovery rates in Experiment 1

No. mice	Ink infiltration	Graft recovery rates				
		Kidney	Uterine lumen	Ovarian fatpad	Total	%
12	-	3/11	3/12	3/11	9/34	26%
6	+	6/6	2/6	6/6	14/18	78%
18		9/17	5/18	9/17	23/52	44%

TABLE 2: Strong PAS-positive reactions found in grafts recovered in Experiment 2

Endometrium alone	
- in mice with T elsewhere	:0/7
- in E+T sites	:0/1
Trophoblast alone	
- in mice with E elsewhere	:2/2
- in E+T sites	:2/4
E+T	:5/6
Capsule	:2/2

TABLE 3: Mab OC-1 binding to grafts recovered in Experiment 3

Endometrium alone	
- in mice with T elsewhere	:0/2
- in E+T sites	:0/10
Trophoblast alone	
- in mice receiving no E	:12/12
- in mice with E elsewhere	:2/2
- in E+T sites	:5/5
E+T	:10/10

4/6 sites containing T alone (either originally co-engrafted with E or with E at another site in the same mouse), 5/6 successfully co-engrafted E+T sites, and 0/8 grafts of E alone (whether originally co-engrafted with T or with T at another site in the mouse). There was no discernible increase of the PAS-positive reactions with time (4–16 days).

The PAS-positive material was more closely related to T, or to the interface between T and E, than to E alone. Weak PAS staining (a <1 µm rim) occurred in 4 of the 8 E grafts. Both grafts of C recovered were PAS-positive. These results suggested that grafted trophoblast, rather than endometrium, was producing the capsule-like material. However, there remained the possibility that endometrium grafted to another site in the same mouse was influencing the production of capsule-like material.

That possibility was addressed in Experiment 3, in which immunohistochemistry was used on cryostat sections of recovered grafts. The overall graft recovery rate in Experiment 3 was 41/69 (59%; 16/35 single grafts and 25/34 when E and T were co-engrafted). In a non-manipulated Day 14.5 equine conceptus (positive control), MAb OC-1 bound to both trophoblastic cells and their coatings. A polyclonal antibody to canine parvovirus (negative control) showed no reaction with any tissue tested. As is summarised in Table 3, MAb OC-1 bound to 19/19 recovered T grafts and their secretions (12 of these in the absence of any E graft in the mouse), 10/10 successfully co-engrafted E+T sites, and 0/12 recovered E grafts, including 11 in which T had been grafted to the same or another site in the mouse.

These results support the contention that the trophoblast is the principal site of production of the equine capsule; interactions between equine endometrium and trophoblast were found not to be essential for production of capsule-like material in grafted tissues.

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DOES THE EMBRYONIC CAPSULE IMPEDE THE FREEZING OF EQUINE EMBRYOS?

E. Legrand, J. M. Krawiecki*, D. Tainturier†, P. Cornière, H. Delajarraud and J. F. Bruyas†

*Progen, 50500 Carentan, France; *Veterinary Clinic, EAABC, 49400 Saumur, France; †Department of Reproduction, Nantes Veterinary School, BP 40706, 44307 Nantes, France*

There has been no increase in the success rate of equine embryos freezing since 1982 (± 20 to 30%). A widely held view is that the younger (morula or early blastocyst) (Slade *et al*. 1985; Skidmore *et al.* 1991) and smaller (200 µm) (Slade *et al.* 1984; Lagneaux and Palmer 1991) the embryo is, the greater its chance of pregnancy after deep-freezing procedure. Previous studies indicate that the capsule plays a bigger part in the freezability of embryos in glycerol than the size or the stage (Legrand *et al.* 1999; Bruyas *et al.* 2000). In order to verify this fact 3 experiments have been conducted.

EXPERIMENT 1

The aim of the first experiment was to evaluate the relationship between the thickness of capsule and the cellular damage in embryos treated with cryoprotectant with or without freezing.

Materials and methods

Examination of histological sections of 15 embryos treated with glycerol and 15 frozen/thawed embryos from previous studies (Bruyas *et al.* 1993; Bruyas *et al.* 1995; Legrand *et al.* 1999; Bruyas *et al.* 2000) was performed. In each of these previous studies, each embryo, after a classical procedure of glycerol incorporation and removal and a classical freezing/thawing process using glycerol as cryoprotectant, was incubated during 6 h at 37°C in order to underline cellular damage. After culture, they were fixed at 4°C in glutaraldehyde 2%, post fixed in 2% osmium tetroxide, dehydrated in a graded ethanol series and embedded in epon 812. They were serially sectioned into semi-thin sections (1 µm) and every fifth section was stained with 0.5% hot toluidine blue for light microscopy.

In the present study, nuclear and capsular stages were assessed using these semi-thin sections. Each class of nuclei (pycnotic, caryorhexic and caryolytic for dead cells and interphasic and mitotic for live cells) were counted. Notation of the capsule thickness was as follows: (Fig 1): 0 = no capsule; 1 = forming capsule, a simple trace is detectable; 2 = sharp and thin capsule, sometime discontinued; 3 = sharp capsule; 4 = thick capsule (0,8 µm). Four sections of each embryo were looked at, the capsular note was determined as the mean of the 4 notes.

Results and discussion

Embryos treated with glycerol without freezing and with a capsule graded from 0–3 showed an increase (Fig 2) in the rate of dead cells, whereas this rate decreased dramatically for embryos with a Grade 4 capsule (Fig 3).

To explain that, our hypothesis is as follow. In embryos with a capsule noted 0–2, the water out flow and glycerol entry induce mild osmotic damage. In embryos with a thicker capsule (Grade 3) the water outflow cannot be compensated by the glycerol entry due to the thickness of the capsule. Therefore there is a higher degree of osmotic damage. Embryos with a thick capsule (Grade 4) presented the same morphology as fresh embryos with a very low rate of dead cells. This observation suggests that there are no fluid movements through the capsule: no glycerol entry no water outflow, and therefore no osmotic damage.

In the frozen/thawed groups (Fig 4), the rate of dead cells was directly proportional to the capsule thickness. This rate was respectively: 46.5 ± 20.7% (Grade < 2), 57.4 ± 19.8 % (Grade 3), and 85.4% ± 10.1 % (Grade 4). The embryos with thick capsule do not tolerate freezing-thawing

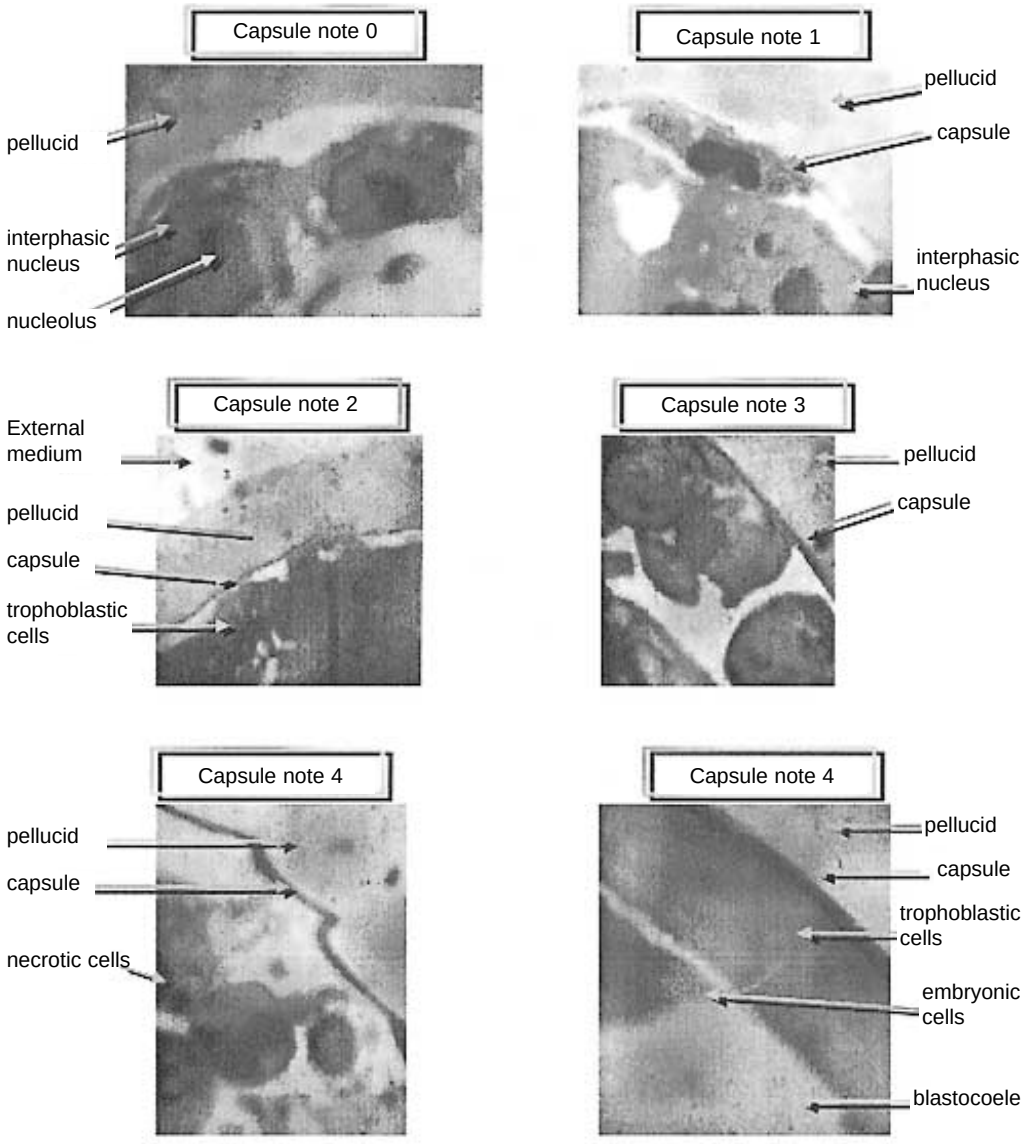

Fig 1: Embryonic capsule notation.

process, and the embryos without capsule are less damaged than others.

The explanation could be that embryonic capsule interferes with the cryoprotective effects by preventing glycerol entry in embryonic cells.

EXPERIMENT 2

In order to remove this difficulty the purpose of the second experiment was to test the effects of enzymatic treatments on the capsule so as to delete or to permeabilise it.

Materials and methods

The effect on the embryo was also observed. Two enzymes, trypsin (EC 3.4.21.4) and collagenase (EC 3.4.24.3), have been used in different concentrations (Trypsin: 0.005%, 0.01%, 0.025%, 0.05%, 0.1%, 0.25%, 0.5%, 1%,

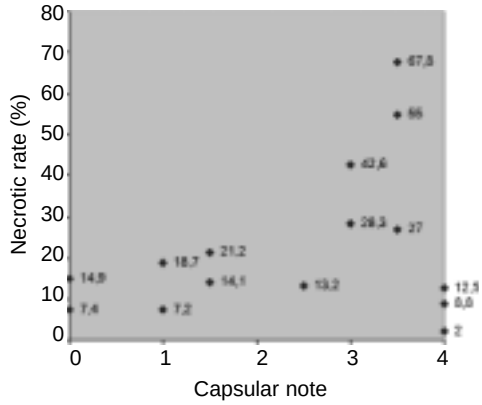

Fig 2. Relation between necrotic rate and capsular thickness in embryos without freezing procedure.

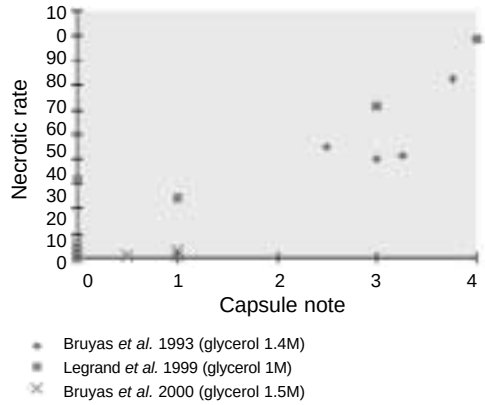

Fig 4. Relationship between necrotic rate and capsular thickness in frozen/thawed embryos.

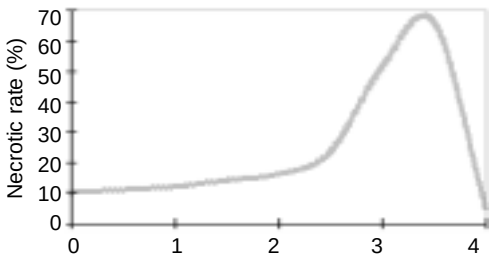

Fig 3. Model graph of the relationship between necrotic rate and capsular importance.

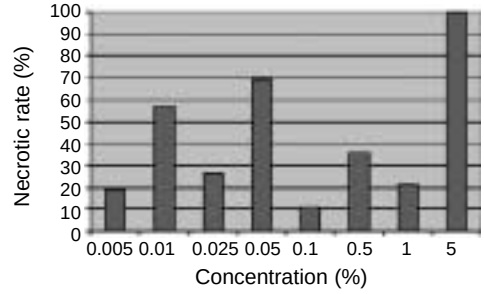

Fig 5: Effect of trypsin treatment on embryos treated with glycerol without freezing/thawing process.

TABLE 1: pregnancy rate according to the age and size of embryos

Pregnancy diagnoses	Age of embryos (days post ovulation)				Diameter of embryos (µm)			
	5.5	6.5	7.5	8.5	< 200	200–500	500–1000	> 1000
Day 14	0/1	1/1	2/2	3/4	2/3	1/1	0/1	3/3
Day 21	0/1	1/1	0/1*	2/4	1/3	0/0*	0/1	2/3
Day 28	0/1	1/1	0/1*	2/4	1/3	0/0*	0/1	2/3

* a pregnant mare was injured between Day 14 and Day 21

5% and collagenase : 0.5%, 1%, 2.5%, 5%). Thirteen Day 8 embryos were placed in PBS medium + enzyme for 15 min, then glycerol was added in 3 steps (0.5 M for 10 min; 1 M for 10 min; 1.5 M for 10 min) and immediately removed in 3 steps (Glycerol (G)1.25 M + sucrose (s) 0.25 M for 10 min; G 0.6 M + s 0.25 M for 10 min; s 0.25 M for 10 min) without freezing. Histological analysis was performed as in the first experiment.

Results and discussion

Necrotic rate with trypsin treatment is reported in Figure 5.

Data show that: i) trypsin seemed to be better to increase the permeability of the capsule than collagenase; and ii) low enzymatic concentrations (<0.05%) were inefficacious, whereas high concentrations (>1%) were toxic for embryonic cells.

EXPERIMENT 3

The aim of the third experiment was to transfer frozen/thawed embryos without capsule (Patent FR 97 073 11/PCT).

Materials and methods

Eight embryos (5.5–8.5 days old and 187–1,581 µm) were collected. After being placed in a bath of trypsin (0.2 % w/v) for 15 min, they were frozen/thawed conventionally: addition of glycerol in 3 steps (0.5 M for 15 min; 1 M for 15 min; 1.5 M for 15 min), seeding at -7°C, cooling rate of -0.3°C/min, storage in liquid nitrogen, thawing in water bath (37°C) for 1 min, removal of glycerol in 3 steps using sucrose (Glycerol (G)1 M + sucrose (s) 0.25 M for 15 min; G 0.5 M + s 0.25 M for 15 min; s 0.25 M for 15 min). Embryos were then non surgically transferred in synchronous recipients.

Results and discussion

Six pregnancies were observed by ultrasound examination at Day 14 as summarised on Table 1. For the first time pregnancies were obtained with large equine blastocysts (Day 8).

The capsular hypothesis is a simple explanation for the previous results on equine embryo freezing; why the largest embryos are particularly difficult to freeze, and why results are often erratic in this species. The capsule is specific to horse embryos. These 3 experiments indicated that: i) it is not impossible to freeze large Day 8 embryos; and ii) experiments on cryopreservation of equine embryos should take thickness and permeability of the capsule into account.

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WHEN DO EQUINE EMBRYOS ENTER THE UTERINE CAVITY: AN ATTEMPT TO ANSWER ?

I. Battut, A. Grandchamp des Raux, J. L. Nicaise, F. Fieni, D. Tainturier and J. F. Bruyas

Department of Reproduction, Veterinary School, B.P. 40 706, 44307 Nantes Cedex 03, France

A previous study (Battut *et al.* 1997) has suggested that horse embryos enter the uterine cavity between 144–156 h after ovulation.Variability in development stage was considerable between embryos recovered at the same age: from 117–417 cells at 156 h and 272–2217 cells at 168 h (Colchen e*t al.* 2000). The purpose of the present study was: 1) to determine more precisely the timing of embryonic transport between 144–156 h after ovulation, if necessary using successive uterine flushings at 3 or 6 h interval on the same mare; and 2) to evaluate homogeneity in development stage among embryos recovered at the same age.

MATERIAL AND METHODS

Seventeen mares (9 Trotter, 2 Thoroughbred and 6 Pony) were used as embryo donors, between 30th June and 3rd September. The onset of oestrus was detected by daily teasing with a stallion, follicular growth was then checked by daily ultrasound examination. When a growing follicle reached 33 mm, ovulation was induced alternatively by one iv injection of 2,500 iu human Chorionic Gonadotrophin (hCG), or 4 iv injections of 20 µg buserelin at 12 h intervals. Artificial insemination was performed 24 h after the first injection, with pooled fresh semen from 2 stallions. Ovulation was checked hourly by ultrasound examinations starting 32 h after injection, until ovulation, or until 48 h. Embryo collection was performed by uterine flushing, the moment being chosen at random; either 144, 147 or 150 h after ovulation. When no embryo was obtained, some collection attempts were repeated 3 or 6 h later (see Fig 1).

After recovery, embryos were measured under an inverted microscope, fixed in 2% glutaraldehyde, embedded in Epon 812, sectioned (1 µm) and stained with Toluidin blue for histological analysis (Bruyas *et al.* 1993).

RESULTS

Recovery rates

Forty five first collection attempts, 28 second and 7 third collection attempts were performed.

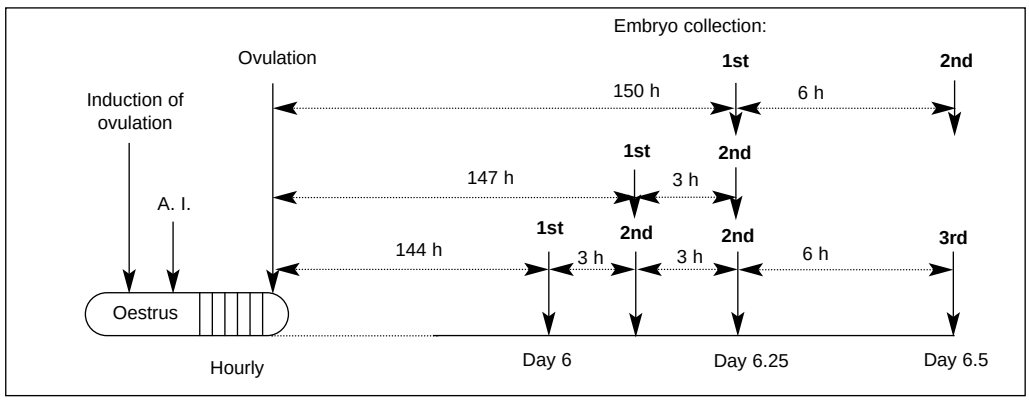

Fig 1: Experimental protocol.

TABLE 1: Rates of successful embryo collection knowing the exact age of the embryo

	First collection attempt			Second and third collection attempts				
Moment of collection (h after ovulation)	144 h	147 h	150 h	147 h (after 144)	150 h (after 144)	150 h (after 147)	156 h (after 150)	156 h (after 144 and 147)
Successful collections	2/16 (16%)	3/14 (21%)	5/15 (33%)	0/2	1/9 (11%)	0/9	0/8	0/7
No. of embryos	2	4[a]	7[b]		1			

Twin embryos were recovered in 1[a] or 2[b] cases (double ovulations were synchronous)

TABLE 2: Morphology of embryos recovered 144, 147 or 150 h after ovulation

Moment of recovery (h after ovulation)	144 h	147 h	150 h
No. of embryos	n = 2	n = 4	n = 8
Diameter (µm): mean + sd (range)	186 + 21 (171-201)	163 + 11 (146-168)	166 + 9.8 (153-183)
Cell number: mean + sd (range)	407 + 227(n = 2) (247-568)	440 (n = 1)	317 + 60 (n = 4) (274-403)
% inner cell mass	37 + 9	29	35 + 7
% mitosis	5.6 + 0.7	5.2	5.1 + 3

For the first collection attempts, rates of succes did not significantly differ according to the moment of collection. Most of the time, at second and third collection attempts, the cervix was relaxed, and embryos have probably been lost, either during flushing or in the time elapsed between 2 successive collections.

In contrast with our previous results (Battut *et al.* 1997), some embryos were recovered from the uterus 144 h (exactly 6 days) after ovulation. Recovery rates at 150 h were lower than that previously reported at 156 h: all the embryos do not seem to have reached the uterus 150 h (exactly 6.25 days) after ovulation. These data corroborate previous results (Colchen *et al.* 2000), indicating that the moment of arrival of equine embryos in the uterus after ovulation varies among embryos, within an interval about 12 h. The possible reasons explaining this variability are: i) variable delay between ovulation and fertilisation, depending on individual oocytes; ii) maternal factors: no influence of maternal breed was observed, and progesteronemia has no influence (Colchen *et al.* 2000); iii) environmental factors (other than season); and iv) embryonic factors: sex; moment of PGE_2 secretion; other individual factors.

Morphology

Under inverted microscope, all the embryos looked like morula or early blastocyst, the difference between the 2 stages being difficult to evaluate. Diameters and cell numbers are presented on Table 2. There was no significant difference according to the moment of recovery.

Only 8 embryos could be subjected to histological analysis. One was degenerated, 6 were early blastocysts, and one was a morula (150 h, 168 µm, 313 cells). Fragments of capsule were visible on all the embryos, but a continuous and very thin capsule was observed only around one embryo (150 h, 153 µm, 279 cells). Cell numbers are presented on Table 2. There was no correlation between diameter, cell number, percentage of inner cell mass, and percentage of mitosis.

These results indicate that morphology of equine embryos, between Day 6 and Day 6.25, does not depend on their age in relation to ovulation. Hypothesis of explanation are: i) variability in the interval ovulation-fertilisation; ii) variability in development rate; iii) variability in the interval : arrival in the uterus - recovery. In the uterus, capsule begins to form, and cell number

seems to increase 2-fold every 6 h (Colchen *et al.* 2000). Embryos were recovered at a fixed interval after ovulation, but the moment of transport to the uterus is variable with respect to ovulation. These reasons explain the large possible variation in embryonic morphology (cell number, aspect of capsule) according to the time spent in the uterus between arrival and recovery.

Conclusion

Monitoring ovulation hourly does not help to recover an homogenous group of embryos, for freezing or micromanipulation, for example. Factors related to individual oocytes or embryos, and responsible for this variation, have to be identified.

When ovulations are checked by ultrasound examination only once or twice a day, embryo collection must be performed 156 h after the first observation of a corpus luteum, to be sure that the embryo has reached the uterine cavity.

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SESSION 4:

Commercial embryo transfer

Chairmen: K. Hinrichs and W. R. Allen

CHANGES OF PGFM, PROGESTERONE AND LH SECRETION PATTERNS IN RELATION TO CYCLE LENGTH AFTER CERVICAL MANIPULATION IN MARES

J. Handler, M. Königshofer, H. Kindahl*, H.-O. Hoppen†, J. E. Aurich and C. Aurich

*University of Veterinary Sciences, Vienna, Austria; *Swedish University of Agricultural Sciences, Uppsala, Sweden; †College of Veterinary Medicine, Hannover, Germany*

INTRODUCTION

In horses, early embryonic loss after transcervical transfer of embryos is tremendous when compared to other species. Better results have been obtained after surgical transfer. Endocrine changes caused by cervical manipulation may be responsible for this phenomenon.

In non-pregnant horse mares the effects of cervical manipulation on cycle length were controversially discussed in previous studies (Hurtgen and Ganjam 1979; Betteridge *et al.* 1985; Wilde *et al.* 1989). While Hurtgen and Ganjam (1979) were able to shorten dioestrus by digital manipulation of the cervix, Betteridge *et al.* (1985) as well as Wilde *et al.* (1989) could not find any release of prostaglandins and influence on oestrous cycle after insertion of a transfer catheter into the cervix. Kask *et al.* (1997) induced prostaglandin release and shortened oestrous cycle length in individual mares by performing sham embryo transfers, while Betteridge *et al.* (1985) did not obtain comparable responses to this manipulation. All these conflicting results might be caused by the use of different techniques of cervical stimulation.

In the present study we tested the hypothesis that in non-pregnant mares, a standardised procedure of cervical manipulation and dilatation causes an increase in prostaglandin release leading to disturbances of luteal function and a subsequent decrease in cycle length.

MATERIALS AND METHODS

Mares (n=6) were controlled for cyclic activity during 5 consecutive oestrous cycles (oestrous behaviour, transrectal palpation and sonographic evaluation of ovaries and uterus). Daily blood samples were taken for determination of plasma progesterone and LH concentrations. Progesterone as well as LH secretion were calculated as area under the curve (AUC) for the time period from Day 1–Day 18 of the cycle (ovulation = Day 0). During the second, third and fourth cycle at Days 5 and 7 after ovulation, the following experiments were performed in every mare at random order: 1) Insertion of an embryo flushing catheter into the cervix via speculum; 2) Insertion of the catheter

TABLE 1: Oestrous cycle length, dioestrous length, maximal progesterone concentrations and total progesterone release (AUC) in mares after insertion of a catheter into the cervix (insertion), after insertion of the catheter and dilatation of the balloon (dilatation) and without treatment (control)

Treatment	Cycle length d, mean ± sd	Dioestrous length d, mean ± sd	Max P4 ng/ml, mean ± sd	P4 (AUC) ng/ml, mean ± sd
Control	22.7 ± 2.1[a]	18.8 ± 1.8[a]	8.5 ± 1.1[a]	83.1 ± 15.8[a]
Insertion	21.8 ± 2.8	17.2 ± 2.2	8.1 ± 1.8	76.6 ± 14.2
Dilatation	19.8 ± 1.2[b]	16.3 ± 1.4[b]	7.8 ± 1.4[b]	70.6 ± 14.5[b]

[a,b] – different superscripts indicate significant differences (P<0.05) based on Wilcoxon Signed Rank Test

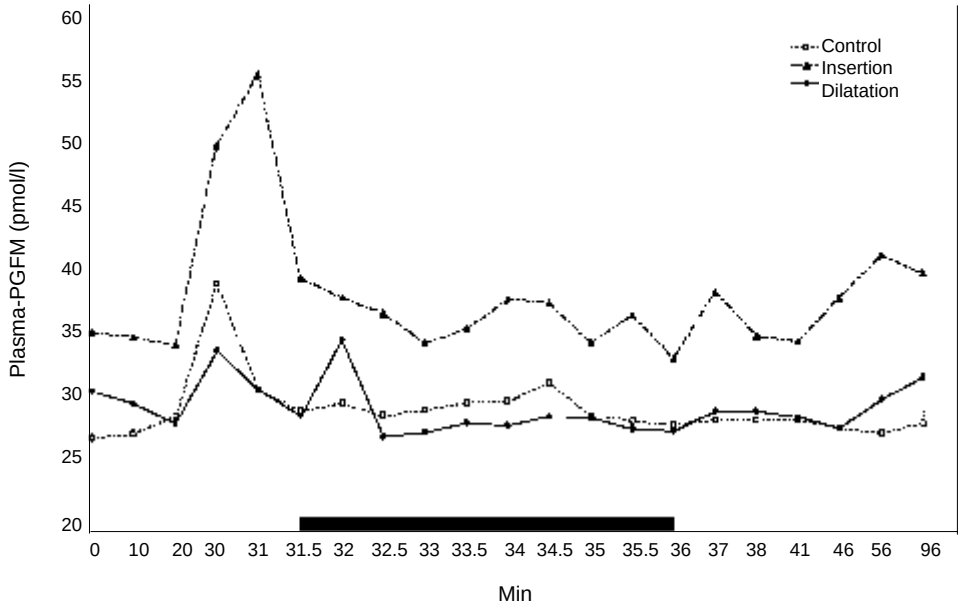

Fig 1: PGFM concentration in blood plasma of mares after insertion of a catheter into the cervix (insertion), after insertion of the catheter and dilatation of the balloon (dilatation) and without treatment (control). Black bar indicates time period of dilatation and insertion, respectively.

and stepwise dilatation of the balloon to a size of 4.5 cm in diameter; 3) No treatment (control). Blood samples for determination of prostaglandin metabolites (PGFM) were taken 30, 20, 10 min and immediately before insertion of the catheter, after insertion of the catheter and at 30 s intervals during dilatation of the balloon. Further blood samples were taken 1, 2, 5, 20 and 60 min after removal of the catheter. Hormone concentrations were analysed by radioimmunoassay methods: progesterone (Hoffmann *et al.* 1973), LH (Behrens *et al.* 1993) and PGFM (Granström and Kindahl 1982).

Results and discussion

Intracervical insertion and dilatation of the catheter caused significant changes in progesterone levels, duration of dioestrus and cycle length, respectively. Cycle length (in parentheses dioestrous length) was 21.8 ± 2.8 (17.2 ± 2.2) days in mares after insertion of the catheter, 19.8 ± 1.2 (16.3 ± 1.4) after insertion and dilatation of the catheter and 22.7 ± 2.1 (18.8 ± 1.8) days in untreated mares ($P<0.05$). Corresponding values for maximal progesterone values (in parentheses area under curve, AUC) were 8.1 ± 1.8 (76.7 ± 14.2), 7.8 ± 1.4 (70.6 ± 14.5) and 8.5 ± 1.1 (83.1 ± 15.8) ng/ml, respectively ($P<0.05$; Table 1). Total LH release was not changed by cervical manipulation. However, no significant differences in PGFM release could be detected between the treatments. An increase of PGFM could be seen in individual mares after insertion of the speculum, but insertion or dilatation of the catheter did not cause any further changes (Fig 1).

In conclusion, cervical dilatation seems to influence luteal function causing a decrease in oestrous cycle length in the mare. This might be also involved in embryonic loss after transcervical transfer. However, this seems not to be caused by a direct prostaglandin release reflected in an increase of plasma PGFM concentrations.

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THE INFLUENCES OF MATERNAL SIZE, AGE AND PARITY ON PLACENTAL AND FETAL DEVELOPMENT IN THE HORSE

S. Wilsher and W. R. Allen

Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK

Clear evidence that maternal size, and hence uterine size, profoundly effects the birthweight of the foal was demonstrated by Walton and Hammond (1938) in their classical experiment of between breed inseminations in Shetland ponies and Shire horses. Tischner and Klimszak (1989) also showed the effect of intrauterine environment on the development of the fetus in horses by transferring pony embryos to larger draft mares and comparing the birth size and subsequent development of the foals with sex-matched siblings born from their natural pony mothers. In both experiments the size differences between the foals at birth persisted into adulthood. It is hardly surprising that uterine size effects the size of the foal so dramatically since the diffuse, non-invasive, epitheliochorial equine placenta establishes a close microvillous interdigitation with the entire endometrial surface of the maternal uterus.

Endometrosis may compromise the uterine environment during pregnancy and Bracher *et al.* (1996) showed a close relationship between the health of the endometrium and the normality and density of the microcotyledons per area of placenta during the first two thirds of gestation. The condition appears to be associated more with age than parity and Ricketts and Alonso (1991) showed that aged maiden mares develop endometrosis, without the endometrium having suffered the challenges of semen, infection, pregnancy or parturition. Parity is also associated with foal birthweight and Hintz *et al.* (1979) observed that Thoroughbred mares < 7 years of age had lighter foals with smaller cannon bones than mares 7–11 years old. Furthermore, these differences persisted until at least 510 days of age. Barron (1995) surveyed racetrack performance of Thoroughbreds and concluded that foals born from parous mares aged 7–11 years were more successful than those from maiden mares or older animals. Similarly, Finocchio (1996) reported that that third foals were most likely to become Stakes winners, followed closely by foals produced in parities 5, 2 and 4, in that order.

In the present experiment the effects of maternal size, age and parity on the gross and microscopic development of the placenta and on foal birthweight were investigated. Normal term placentae were recovered at spontaneous third stage labour from 2 cohorts of mares and, after weighing the allantochorion and measuring its gross area and volume, stereological techniques were applied to 10 biopsies selected randomly from all parts of the allantochorion to measure the microscopic surface area of the microcotyledons. The microscopic fetomaternal contact per unit volume of chorion (surface density of microcotyledons) and, by multiplication with the gross volume of chorion; the total area of fetomaternal contact across the entire placenta, were recorded.

In the first cohort of mares between-breed embryo transfer was used to create 8 Thoroughbred-in-Pony (Tb-in-P) pregnancies in which the genetically larger Thoroughbred fetus experienced cramping and nutritional deprivation *in utero*, 7 P-in-Tb pregnancies in which the smaller Pony fetus was exposed to nutritional excess *in utero*, with 7 normal Tb-in-Tb and 7 P-in-P pregnancies as controls. Strong positive correlations were demonstrated between maternal weight and foal birthweight and between birthweight and both the weight and gross area of the placenta. Unexpectedly, surface density of the microcotyledons, which was also was positively correlated with foal birthweight, was the highest in

the Tb-in-Tb control and lowest in the P-in-P control placentae, and was higher in the P-in-Tb than the Tb-in-P pregnancies, thereby indicating that this parameter is influenced more by the breed of the mother than by the intrauterine experiences of the fetal foal. The very strong correlation that existed between foal birthweight and the total area of fetomaternal contact across the placental interface demonstrated convincingly that the *in utero* growth of the foal is governed by 'real' placental size and competence which, in turn, is governed by the area of available endometrium.

The second cohort of 32 normal, commercial Thoroughbred mares resident in the Newmarket area were separated into 4 groups on the basis of age and parity; primigravid mares aged 4–7 years and parous mares aged 5–9 years, 10–15 years and ≥ 16 years. The gross placental parameters and foal birthweights were all lower in the primigravid mares than the other 3 groups of parous mares. Of particular interest was the finding of a reduced surface density of microcotyledons in these primigravid animals, compared to the 5–9 year old parous mares, despite the reasonable assumption of a healthy, virginal endometrium in the maiden group. The surface density of microcotyledons was also lower in the aged (≥16 years) mares than in the other 2 groups of parous mares, presumably due to the effects of age-related endometrosis in the opposing endometrium. However, this microscopic interface deficiency was offset by a higher volume and gross area of the placenta in the aged animal which maintained a high mean foal birthweight in the group. Thus, primiparity appears to be more important in determining foal birthweight than maternal age, due to reduction in microcotyledon surface density, coupled with reduced volume of the chorion, lessening the total area available for haemotrophic exchange of nutrients and gases across the placental interface.

Accordingly, when selecting recipient mares for use in an embryo transfer programme it should be remembered that maternal parameters such as size, age and parity will all bear varying degrees of influence on health and total microscopic area of the placenta which, in turn, will govern the size and health of the foal at birth and in later life. Ideally, recipient mares should be between 5 and 9 years of age, should have already produced at least one healthy foal, and should have a body size that is equal to, or bigger than, that of the mare producing the embryo.

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USE OF BUSERELINE TO INDUCE OVULATION IN DONOR MARES

J. F. Bruyas, E. Trocherie, S. Hecht, N. Lepoutre, A. Granchamp des Raux, J.-L.Nicaise, X. Vérin, J. Bertrand, I. Barrier-Battut, F. Fiéni, R. Hoier*, A. Renault†, L. Egron† and D. Tainturier

*Department of Reproduction, Nantes Veterinary School, B.P. 40706-44307 Nantes cedex 03, France; *Department of Clinical Studies, Royal Veterinary and Agricutural University, Frederiksberg, Denmark; †Intervet, BP 17144, 49071 Beaucouzé Cedex, France*

Human chorionicgonadotropin (hCG) injected iv (2,500 ui) is commonly used for induction of ovulation in mares. However, repetitive administrations of xenogenic gonadotropin in horses were described as responsible for antibodies production. In France, implantable sustained release deslorelin for ovulation induction in mares, are not commercially available. It would be desirable to have another means of inducting of ovulation in donor mares. The only one GnRH-analogue available in France for animal treatment is busereline. Many previous studies have shown that a single injection of GnRH or GnRH-analogues were not effective in mares. Harrison *et al.* (1991) suggested that repeated injections of busereline were able to induce ovulation in mares. The purpose of these 5 studies was to test the efficacy of busereline for hastening ovulation using different protocols in donor mares.

MATERIALS AND METHODS

Five cross trials were performed to compare, in each experiment, 2 treatments alternatively injected to induce ovulation of 2 or 4 successive oestrous cycles of embryo-donor mares (Fig 1). A total of 22 mares were used. Oestrus was detected by teasing. Follicular growth and ovulation were checked by ultrasonography, daily until observation of 33 mm follicle, then every 12 h (excepted in Experiments 4 and 5) until observation of a corpus luteum.

Tested treatments started when a growing follicle reached 33 mm of diameter.

In Experiment 1 and 2, Treatment A (busereline 40 µg) and placebo were injected iv 4 times every 12 h. In Experiment 2, blood samples were taken every 15 min, until ovulation, for measurement of LH using an homologous radioimmunoassay (Hoier 1994).

In Experiment 3, Treatment A (busereline 40 µg) and B (busereline 20 µg) were injected iv 4 times every 12 h.

In Experiment 4, Treatment B (20 µg busereline injected iv 4 times every 12 h) and hCG (2,500 ui, one iv injection) were compared and time of ovulation was determined by hourly ultrasound examinations between 32 h and 48 h after the first injection or the only one of treatments.

In Experiment 5, treatment C (13.3 µg busereline injected iv 3 times every 6 h) and hCG (2,500 ui, one iv injection) were compared, and time of ovulation was determined by hourly ultrasound examinations as in Experiment 4.

In Experiments 1 and 3, mares were inseminated with fresh semen from a fertile stallion with a dose of 400.10^6 spermatozoa diluted in skim milk extender, every other day, from the start of treatment to the ovulation. Non-surgical embryo collections were performed 6 days after ovulation.

STATISTICAL ANALYSIS

Quantitative data were analysed by Student's *t* test on paired series and qualitative data were analysed by the method of McNemar, each mare being its own control.

RESULTS

In Experiment 1, busereline treatment induced significantly a higher rate of ovulation both within

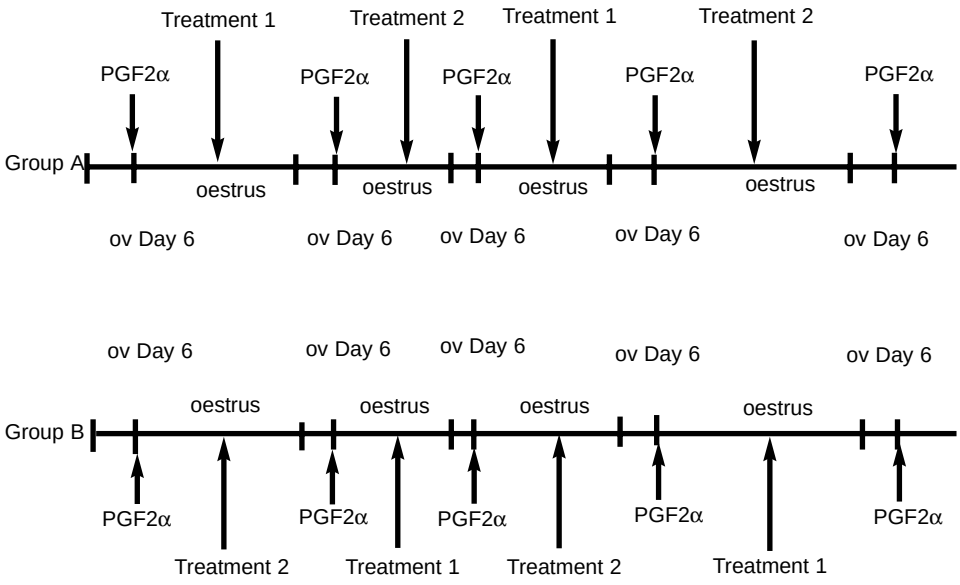

Fig 1: Experimental protocol.

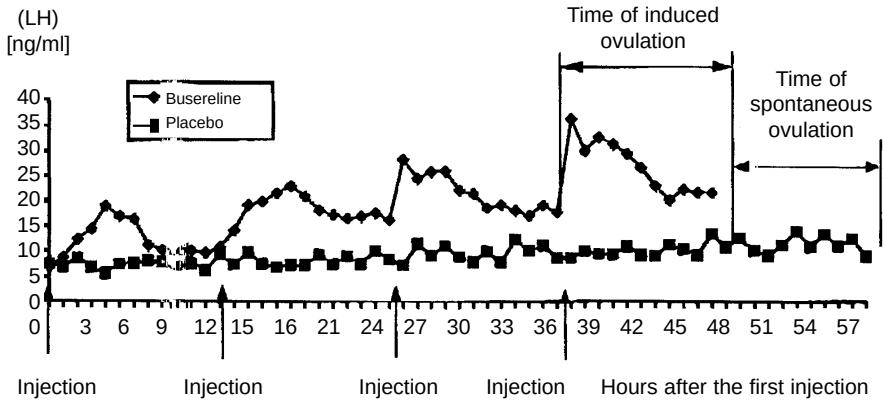

Fig 2: Variation before ovulation of LH concentrations of a mare treated in 2 consecutive cycles alternatively with busereline (40 µg) and placebo injected intravenously 4 times every 12 h.

48 h ($P<0.001$) and between 24 and 48 h ($P<0.05$) after the first injection (Table 1). There was no difference in the embryo recovery rate between the 2 treatments (respectively 35 and 44%).

In Experiment 2, mean plasma LH concentrations after the first injection were significantly higher in busereline cycles than in placebo cycles. Figure 2 shows an example of variations of LH concentrations in one mare during the 2 treated cycles with placebo and busereline.

In Experiment 3, there was no difference between the 2 treatments both in the success rate of induction of ovulations and in the recovery rate of embryos (Table 1).

In Experiment 4, success rates in the induction of ovulation did not differ significantly between the 2 treatments (busereline 4 x 20 µg or hCG 2,500 ui). When ovulation occurred between 32 and 48 h after the first or the only injection, there was no difference in time of ovulation (Table 2).

In Experiment 5, success rates in the induction

TABLE 1: Success rates of induction of ovulation in Experiments 1 and 3

	Exp 1 (72 paired oestrous cycles)			Exp 3 (60 paired oestrous cycles)		
	Busereline 40 µg x4	Placebo	P	Busereline 40 µg x4	Busereline 20 µg x4	P
Ovulation within 48 h after 1st injection	61%	22%	< 0.00	183%	90%	N.S.
Ovulation between 24 and 48 h after1st injection	47%	14%	< 0.054	7%	57%	N.S.

TABLE 2: success rates of induction of ovulation in Experiment 4 and 5

	Exp 4 (50 paired oestrous cycles)			Exp 5 (60 paired oestrous cycles)		
	Busereline 20 µg x 4	hCG 2500 ui	P	Busereline 13.3 µg x 3	hCG 2500 ui	P
Ovulation between 32 and 48 h after 1st injection	56 %	76 %	N.S.	57 %	50 %	N.S.
Mean time of ovulation ± sd	39.5 ± 3.1 h	38.4 ± 2.5 h	N.S	.43.1 ± 2.1 h	37.7 ± 2.3 h	< 0.01

of ovulation did not differ significantly between the 2 treatments (busereline 3 x 13.3 µg or hCG 2,500 ui). However, there were many early spontaneous ovulations within 32 h after the start of treatment quite only in cycles treated with hCG (11/30 versus 1/30). When ovulation occurred between 32 and 48 h after the first or the only injection, there was a significant difference (P<0.01) in time of ovulation (Table 2).

Discussion

These data show that: i) busereline (40 µg) significantly hasten ovulation, compared with placebo without altering fertility; ii) this busereline treatment significantly increase LH concentrations during a 48 h period; iii) busereline (20 µg) injected 4 times every 12 h is as effective as hCG in hastening ovulation; iv) busereline (13.3 µg) injected 3 times every 6 h seems also as effective as busereline (40 µg) and hCG to induce ovulation in mares.But, there is no explanation to understand why, in the 5th experiment, spontaneous early ovulations were more frequent with hCG than busereline. Therefore, to valid this new treatment, it would be interesting to conduct a new experiment expecting an equal proportion of early spontaneous ovulations between the 2 tested treatments.

The last busereline treatment tested (3 injections every 6 h induces ovulation later than hCG. Those data are agreed with results obtained by Mc Kinnon *et al.* (1997). They had also reported that induced ovulations occurred later with deslorelin implants than hCG. This difference would be due to a different mechanism of action between hCG and GnRH analogs. Further studies would be conducted to understand these mechanisms. In this point of view, whereas spontaneous ovulation occurs without short LH surge, an injection of hCG or equine LH induces an exogenous short LH surge and induces the ovulation. Only one injection of GnRH or GnRH analogs induces an endogenous surge of LH but not the ovulation, but repeated (or continuous) injections of GnRH induce repeated endogenous LH surges (Fig 2) and the ovulation.

However, these 5 experiments show that busereline treatments (4 injections of 20 µg every 12 h or 3 injections of 13.33 µg every 6 h are interesting alternatives to hCG when mares are immunised against hCG.

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IMPROVEMENT OF OVARIAN SUPERSTIMULATORY RESPONSE AND EMBRYO PRODUCTION IN MARES TREATED WITH EPE TWICE DAILY

M. A. Alvarenga, P. McCue*, E. L. Squires* and J. R. Neves Neto

*Department of Animal Reproduction and Veterinary Radiology-Scholl of Veterinary Medicine and Animal Science, UNESP –Botucatu –SP –Brazil-18618; *Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA*

INTRODUCTION

Equine pituitary extract (EPE) is the only compound that has been reported to consistently induce multiple ovulation in horses. However, the superovulatory response in mares treated with EPE, is far lower than the response obtained in other species. Ovulation rates of 1.7 to 3.8 ovulations per cycle have been reported after EPE treatment in mares (McCue 1996).

In cows, the superovulatory response is more consistent when FSH is injected twice daily than once daily (Monniaux *et al*. 1983). On the first report of successful superovulation in mares Douglas *et al.* (1974) used EPE twice daily in seasonally anovulatory mares, a subsequent publication from the same laboratory showed similar response using EPE once daily (Lapin and Ginther 1977). Since then, the protocol used to induce multiple ovulations in mares consist of once daily EPE administration. Attempts to improve the effectiveness of EPE for induction of supeovulation in mares have focused on the the daily dose (Woods and Ginther 1982), time of treatment initiation (Dippert *et al.* 1992) and utilisation of purified fraction of equine FSH (Rosas *et al.* 1998). Our recent study had demonstrated that more frequent daily injections of EPE increased ovarian response to EPE (Alvarenga *et al.* 1999). The purpose of the present experiment was to compare superovulatory response and embryo recovery rates of cycling mares follow EPE administration once and twice daily.

MATERIALS AND METHODS

Equine pituitary extract (EPE) was prepared at Colorado State University utilising a method purposed by Guillou and Combarnous (1983).

Seven days after ovulation mares were treated with PGF2∞ (Dinoprost 10 mg) administered once, im and then randomly assigned to one of 2 treatments: Group 1 (n=8) received 25 mg of EPE once daily and Group 2 (n=8) treated with 25 mg of EPE 2 times daily (8 am and 6 pm) given im. For both group of mares the treatment was discontinued when at least half of >30 mm detected follicles reached 35 mm and 2,500 iu of hCG was administered iv.

Mares were inseminated daily, until ovulation, with semen from a fertile Arabian stallion.

Embryo recovery was performed 8 days after ovulation. On the day of embryo recovery blood samples were collected to determine differences in the circulating levels of progesterone in mares with different number of ovulations.

RESULTS

A higher number of ovulations occurred and a larger number of embryos were recovered in mares treated twice daily than those treated once daily ($P<0.05$). The results are summarised on Table 1.

DISCUSSION

Previous studies on superovulation in mares have focused on daily administration of Equine Pituitary Extract and this regime has resulted in a modest increased on superovulatory response. Poor results were obtained when EPE was injected twice daily in anoestrus and transitional mares (Douglas *et al.* 1974) but no direct comparison was made between the 2 protocols to inject EPE in cycling mares. Based on the results of the present study twice daily injections expressively increased

TABLE 1: Superovulatory response in mares treated with EPE once and twice daily

Parameters	EPE once daily	EPE twice daily
Number of mares	8	8
Days treated	6.62 ± 1.3[a]	6.6±1.6[a]
Interval treatment - ovulation	8.1±0.9[a]	8.5±1.7[a]
% mares with multiple ovulations	62.5%	100%
Mares with > 4 ovulations	1	6
Ovulations/mare	2.4 ±1.8[a]	7.1±5.1[b]
Embryo/mare	1.6±1.0[a]	3.5±1.8[b]
% embryo/ovulation	67%[a]	49%[a]

[a-b] Means with different superscripts differ within rows (P<0.05)

the number of ovulations and embryo produced compare to those given EPE only once per day.

The number of ovulations (7.1) and embryos produced per mare (3.5) in this study with 2 daily EPE injections was higher than that reported previously in any reports (McCue 1996).

The improvement in superovulation reported in here, with more frequent EPE injections, may be related to the short circulating half-life of FSH. The other hypothesis would be that the total daily dose of EPE given (50 mg) was higher compared to a dose given once daily (25 mg). However, this is not likely because utilisation of a similar total daily dose of EPE (40 mg) in a previous study in our laboratory (CSU) resulted in only 3.4 ovulations per cycle (Dippert *et al.* 1994).

As the number of ovulations increased the percent of embryos recovered tended to decreased particularly in mares having large number of ovulations. This may have resulted from poorer quality oocytes that were not fertilised or early embryonic death.

A linear increase on blood progesterone levels was detected in mares with more than one ovulation, showing that a large number of functional CL were formed.

Based on the present study we can concluded that this new protocol to use EPE (twice daily) was able to induce multiple ovulations and an increase on embryo production with results never reported before in mares. Further studies are necessary to assess the viability of embryos recovered from multiple ovulating mares.

ACKNOWLEDGEMENTS

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IMPACT OF MULTIPLE OVULATIONS IN A COMMERCIAL EQUINE EMBRYO TRANSFER PROGRAMME

L. Losinno, J. J. Aguilar and H. Lisa*

*Producción Equina, Fac.Agr.y Veterinaria, Universidad Nacional de Rio Cuarto, (5800) Rio Cuarto, Argentina; *Equine Embryo Transfer Centre La Irenita SA, Daireaux, Buenos Aires, Argentina*

INTRODUCTION

Argentina is internationally known for its equine industry and is one of the countries with more equine embryo transfer performed per year. Most of these transfers are demanded by the Polo industry which has been greatly benefited with this technology since 1989 (Riera 1999). The Argentine Association of Polo Horse Breeders (AACCP) have no restrictions on foals produced by artificial insemination or embryo transfer.

The mare is generally consider a monovular species, but the reported incidence of multiple ovulations has ranged between 4 and 43% (Ginther 1992; Bruyas 1997). A wide variation on the reports consider breed, age, reproductive status, genetics, nutrition and season as the main factors that may affect the incidence of multiple ovulations in mares (Squires 1987; Pascoe 1987; Ginther 1992; Bruyas 1997). The incidence of multiple ovulations in Thoroughbreds and draft horses varied between 15 and 30% whereas that in ponies and light breeds appeared to be lower (2–10%) (Ginther 1992; Brujas 1997).

Despite of considerable amount of investigations to induced superovulation in mares through the use of equine pituitary extracts, FSH and immunisation against inhibin limited and inconsistent results have been obtained (Squires 1987; McCue 1999). Currently, the donor mares in commercial embryo transfer programmes are not superstimulated because of the lack of commercially available treatments.

Factors affecting the reproductive efficiency of equine embryo transfer (EET) programmes include age, selection and management of donors and recipients, degree of synchrony, embryo quality, transfer technique and pregnancy losses (Aguilar 1997; Squires 1999).

The limitations for a successful commercial operation in EET include financial considerations related to the costs of purchasing, maintaining and synchronising recipients, breed restrictions for the number of foals accepted per season and subfertility of mares and stallions (McCue 1999).

The objectives of this study were to determine the frequency, distribution, repeatability and impact of multiple ovulations of donor mares on reproductive efficiency and economic results of a large-scale commercial equine embryo transfer programme.

MATERIALS AND METHODS

Records from four natural breeding seasons (96/97–99/00) in the southern hemisphere (October–April) of an Equine Embryo Transfer Centre were analysed in this study. The facilities were located in the Buenos Aires province, Argentina, at 33°S and 64°W in a central, temperate area with daylight hours ranging from 11.5–14.5 during the breeding season.

A total of 197 Polo Argentino donor mares, mostly Thoroughbred type, were included in the study. Most of the mares were in active competition during the spring and immediately after finishing the campaign in Argentina, they were moved to the Embryo Transfer Centre. The donors and recipients mares were maintained exclusively on good quality pasture. The cycles of a 500 mares herd, mostly criollo type mares, were continuously monitored by palpation and ultrasound, to provide naturally synchronised recipient mares.

TABLE 1: Frequency, percentage of positive flushes, embryo recovery rate, pregnancy rate and efficiency of cycles with single, double, triple and multiple ovulations

	Cycles n (%)	% positive flushes	Embryos recovered (%)	Pregnancy rate	Efficiency
Single	821(71.0)	64.9	533(64.9)[a]	79.9[a]	0.52[a]
Double	313(27.1)	76.4	364(116.3)[b]	82.3[a]	0.97[b]
Triple	22(1.9)	81.8	36(163.6)[c]	80.6[a]	1.32[c]
Multiple	335(29.0)	76.7	400(119.4)[b]	82.1[a]	0.98[b]

Different superscripts indicate statistical differences

Donor mares were inseminated with at least 500 x 10^6 motile sperm of fresh extended semen when a 35 mm follicle was detected by ultrasonography. Embryo recovery attempts were performed non surgically at Days 7–8 post ovulation with PBS supplemented with 1% FCS with an in-line embryo filter system (Aguilar 1997). All transfers were carried out using the non-surgical technique to recipients selected from the available pool according the degree of synchrony with the donor (Riera 1999).

The transferred recipients were monitored for pregnancy by ultrasound at Day 7 after transfer.

STATISTICAL ANALYSES

Embryo recovery rate and pregnancy rate of single and multiple ovulations were compared by Chi Square test, whereas efficiency was compared by *t*-Test.

RESULTS

The number of flushes performed to each mare varied from 1–23 and the average number of flushes per mare was 5.8. From the total 1156 flushes, 790 (positive flushes, 68.3%) resulted with at least one embryo collected. The overall embryo recovery rate was 80.7% (933/1156) which gave on average 1.18 embryos per positive flush. The pregnancy rate at 30 days was 80.6% (752/933) and the overall efficiency in terms of pregnancies per flush was 0.65 (752/1156).

The frequency, percentage of positive flushes, embryo recovery rate, pregnancy rate and efficiency of cycles with single, double, triple and multiple ovulations are showed in Table 1.

There was a statistical tendency (P=0.1) for the monthly distribution of multiple ovulations per cycle to increase towards the end of the breeding season (April): October 0% (0/14), November 26.0% (19/73), December 29.8.% (60/201), January 30.4% (104/341), February 23.3% (39/167), March 27.8% (37/133) and April 53.3% (8/15).

Mares with at least one multiple ovulation detected during these 4 seasons were considered multiple ovulators and represented a 75.0% (148/197) of the population under study, whereas only 24.8% (49/197) of the mares were classified as single ovulators.

Multiple ovulator mares repeated this event on an average of 38% of the cycles, and this repeatability varied between 7 and 100 %. A 5.4% (8/148) of the mares repeated multiple ovulations on 100% of their cycles (n=22). The distribution of mares with different repeatabilities is showed in Graphic 1.

The benefit/cost ratio obtained from pregnancies of multiple ovulation cycles was 96% higher than the benefit/cost ratio obtained from pregnancies of single ovulation cycles (2.40 and 1.25, respectively).

DISCUSSION

It can be concluded from these results that multiple ovulations increased the embryo recovery rate. Indirectly can be assumed that oocytes from multiple ovulations are able to fertilise and develop to normal transferable embryos. These embryos once transferred were able to produce pregnancies at very similar rates as embryos from single ovulated oocytes.

Multiple ovulations clearly increased the efficiency of the embryo transfer programme because allowed the production of a higher number of pregnancies at a lower cost which had a direct impact on the economic results.

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EMBRYO RECOVERY RATES IN MARES WITH ECHOGENIC PREOVULATORY FOLLICLES

P. M. McCue, D. K. Vanderwall and E. L. Squires

Equine Reproduction Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA

INTRODUCTION

Echogenic particles may be noted in the follicular fluid of dominant follicles prior to ovulation in some mares. The goals of this study were to determine the incidence of preovulatory follicles containing echogenic follicular fluid and compare embryo recovery rates following ovulation of echogenic or non-echogenic follicles. Reproductive records of 721 mares examined by transrectal ultrasonography prior to breeding over a total of 1,694 oestrous cycles were reviewed. Embryo recovery data were recorded for 34 mares that ovulated a single non-echogenic follicle or a single echogenic follicle during oestrous cycles within the same breeding season. Non-echogenic and echogenic preovulatory follicles were noted in 93.8% and 6.2% of the cycles, respectively. Embryo recovery rate per flush was significantly higher ($P<0.05$) following ovulation of a non-echogenic preovulatory follicle (45.0%) vs an echogenic preovulatory follicle (20.5%). The echogenic particles may be granulosa cells that have been shed from the follicular wall or red blood cells and/or fibrin associated with preovulatory haemorrhage. Echogenic ovulatory follicle phenomenon may represent a mild form of the persistent or haemorrhagic anovulatory follicle syndrome in mares.

Equine follicles typically reach a diameter of 40–45 mm prior to ovulation (Ginther 1992). Follicular fluid of equine preovulatory follicles usually has a non-echogenic appearance ultrasonographically, since the follicular fluid is largely devoid of cellular material. Occasionally an increase in echogenicity within the follicular fluid may be noted during the development of large preovulatory follicles. In some mares a further increase in increase in echogenicity of the follicular fluid is followed by the formation of echogenic strands that crisscross the lumen of the follicle (McKinnon 1998). The resulting structure, often called a persistent or haemorrhagic anovulatory follicle, is filled with coagulated blood and fibrin (Meyers 1995) and may eventually luteinise without ovulating.

Preliminary observations in our laboratory suggested that pregnancy rates were decreased in mares with echogenic preovulatory follicles. Consequently, the goals of this study were to determine the incidence of preovulatory follicles containing echogenic follicular fluid and compare embryo recovery rates and pregnancy following ovulation of echogenic or non-echogenic follicles.

MATERIALS AND METHODS

Reproductive records from all privately owned mares examined prior to breeding at the Equine Reproduction Laboratory, Colorado State University during the 1994–1998 breeding seasons were reviewed. Transrectal ultrasonography was used to examine all mares. Mares were included in the study only if sequential daily ultrasound examinations were performed during oestrus and if an ovulation was detected. Echogenic ovulatory follicles were defined as preovulatory dominant oestrual follicles with more than 5 echogenic particles observed on any cross-sectional ultrasound image of the follicular lumen.

Mares were usually administered human chorionic gonadotropin (Chorulon, Intervet, Delaware, USA, hCG; 2,500 iu, iv) or gonadotropin releasing hormone (Ovuplant, Fort Dodge Animal Health, Iowa, USA, GnRH; 2.1 mg deslorelin acetate, subcutaneously) to induce

TABLE 1: Embryo recovery rates from mares following ovulation of non-echogenic or echogenic preovulatory follicles

Ultrasound appearance	(N)	No. of cycles	No. of embryos	% embryo recovery
Nonechogenic	34	111	50	45.0
Echogenic	34	44	92	0.5

ovulation when a preovulatory follicle greater than 35 mm was detected. Mares were inseminated with either fresh, cooled-transported or frozen semen prior to ovulation. A mare was considered to be pregnant following breeding to an echogenic or nonechogenic follicle if either an embryo was collected 7–8 days after ovulation or an embryonic vesicle was detected by transrectal ultrasonography 12–16 days after ovulation.

Statistical comparison of pregnancy rates between mares that ovulated either echogenic or nonechogenic follicles was made by Chi-square analysis. Comparison of ages of mares with and without echogenic ovulatory follicles was made by Student's *t*-test. Data are presented as the mean ± sd.

RESULTS

Records for 1,694 oestrous cycles from a total of 721 mares examined during the 5 year period were reviewed. Non-echogenic and echogenic preovulatory follicles were noted in 1,589 (93.8%) and 105 (6.2%) of the 1,694 cycles, respectively. Echogenic ovulatory follicles were observed at least once during a breeding season in 16.0% of all mares. The age of mares exhibiting echogenic ovulatory follicles (15.0 ± 5.4 years) was not significantly different ($P>0.05$) than age of mares not exhibiting echogenic ovulatory follicles (11.8 ± 5.5 years). The mean interval from initial detection of echogenic particles within the follicular fluid and ovulation was 1.4 ± 0.9 days. Ovulation was induced by hCG or GnRH in 87.3% of all oestrous cycles. Treatment with hCG or GnRH preceded detection of an echogenic ovulatory follicle in 41.9% of cases. Administration of hCG or GnRH occurred following detection of echogenic ovulatory follicles in 25.7% of cases and no hCG or GnRH was administered before or after detection of an echogenic ovulatory follicle in 32.4% of cases.

Embryo recovery data were recorded for 34 mares that ovulated a single non-echogenic follicle or a single echogenic follicle during oestrous cycles within the same breeding season. Embryo recovery rate per flush was significantly higher ($P<0.05$) following ovulation of a non-echogenic preovulatory follicle vs an echogenic preovulatory follicle (Table 1). Pregnancy data was available for 1,336 oestrous cycles. The overall pregnancy rate per cycle associated with ovulation of nonechogenic follicles (42.0%) was significantly higher ($P<0.05$) than that of echogenic follicles (29.4%).

DISCUSSION

The incidence rate and pregnancy rates associated with development of echogenic ovulatory follicles have not been reported. The incidence rate of echogenic ovulatory follicles is relatively low at 6.2% of all ovulatory oestrous cycles. However, pregnancy rates following ovulation of echogenic follicles was noted to be significantly lower than that following ovulation of nonechogenic follicles. The echogenic particles may be granulosa cells that have been shed from the follicular wall or red blood cells and fibrin from preovulatory haemorrhage. Aspiration of follicular fluid from non-echogenic and echogenic follicles will be necessary to identify the echogenic particles. It is hypothesised that the decreased embryo recovery rate may have been due to degeneration of the oocyte if sloughed granulosa cells were present or entrapment of the oocyte in the follicle if preovulatory haemorrhage was present.

The echogenic ovulatory follicle phenomenon may represent a mild form of the persistent or haemorrhagic anovulatory follicle syndrome (Ginther and Pierson 1989; Pycock 2000). It is usually not possible to predict if a follicle containing echogenic particles will ovulate or not when echogenic particles are first detected. It does not appear that hCG or GnRH administration is related to the formation of echogenic ovulatory follicles. In 58.1% of cases, a decision on whether or not to administer hCG or GnRH was not made until after echogenic particles in the follicular fluid had been noted.

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COMPARISON OF EMBRYO RECOVERY RATES FROM TWO YEARS OLD AND MATURE MARES

F. Camillo, I. Vannozzi, A. Rota, S. Romagnoli and G. Aria

Dipartimento di Clinica Veterinaria, Università di Pisa,56010 San Piero a Grado, Pisa, Italy

INTRODUCTION

Embryo recovery rates in 2 year old (TY) and mature (MA) Haflinger mares were 74/85 (87%) and 22/26 (84.6%) respectively. No short or long term effects of the early use as embryo donors were observed in the TY mares. This study shows that TY old mares can be successfully used as embryo donors.

Fillies, especially if born during winter or spring, reach puberty at the age of 12–15 months (Wesson and Ginther 1981), have an ovulatory season similar to that of the mature mares at 2 years, but rarely are bred before the age of 3 years. This is due to the concern of compromising their body development and/or agonistic or show performances. To anticipate by at least one year the reproductive life of a valuable mare avoiding the possible adverse effects of an early pregnancy, 2 years old fillies could be used as embryo donors.

The use of 2 year old mares as embryo donors has been reported to result in a low embryo recovery rates by some authors (Iuliano and Squires 1985; Steiner and Jordan 1988) but not by others (Fleury *et al.* 1989; Savage *et al.* 1989.)

The aim of this study was to compare embryo recovery rates from 2 year old and mature mares.

MATERIALS AND METHODS

During May–July of different breeding seasons fifty-one 24–28 months old (2-year-old = TY) and 12 4–12-year-old (mature = MA) Haflinger mares were employed as embryo donors for a total of 85 and 26 oestrus cycles, respectively.

Twenty seven 2- and 3-year-old Haflinger mares were employed as embryo recipients.

Mares were checked daily by ultrasounds for uterine status and ovarian activity; oestrus was induced by prostaglandin F2alfa ($PGF_{2\alpha}$); donor mares were naturally bred, or artificially inseminated with fresh extended semen, every second day of the oestrus from the detection of a growing follicle/s ≥34 mm until ovulation. To synchronise ovulations donors and recipients were treated with human corionic gonadotropin.

Seven to 8 days after ovulation, mares were flushed 3 times for embryo recovery (Lagneaux *et al.* 1988) either with a solution of Dulbecco's PBS containing 0.2% of bovine serum albumin or with saline, depending if the transfer of an embryo was planned or not. Recovered embryos were evaluated for morphology (McKinnon and Squires 1988).

When embryo transfer was planned, recovered embryos were washed 10 times in a new solution of DPBS, placed in a French straw and non surgically transferred in the recipients using a guarded French gun (Lagneaux *et al.* 1988).

Recipient mares, when pregnant, were treated with $PGF_{2\alpha}$ at Day 30 of gestation to induce abortion.

In the following years, foaling rates of 11 mares previously used as TY embryo donors and of 8 control mares, of the same breed, age and stud and never used as embryo donors, were compared. These animals were kept on pasture with stallions (one stallion/20 mares or less) and the production of live foals was recorded for a period of 1–8 breeding seasons.

Statistical analysis was performed using the chi square test.

RESULTS

No differences were found in embryo recovery rates and recipient pregnancy rates in the TY and

TABLE 1: Embryo recovery rates and recipients pregnancy rates at Day 14 and 30 in TY and in MA Haflinger mares

	Embryo recovery rate	Recipient pregnancy rate (Day 14)	Recipient pregnancy rate (Day 30)
Two years	74/85(87%)	8/16(50%)	8/16 (50%)
mature	22/26(84.6%)	6/11(54.5%)	5/11(45.4%)

TABLE 2: Embryo recovery rates in the different years of the study in the TY donors

1991	1992	1993	1994	1995	1996	1997	Total
10/13 (77%)	4/6 (67%)	17/18 (94%)	6/6 (100%)	17/20 (85%)	12/12 (100%)	8/10 (80%)	74/85 (87%)

TABLE 3: Outcome of consecutive embryo recovery attempts in the same mares in the TY donors

1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	6th cycle	Total
44/51 (86%)	20/24 (83%)	6/6 (100%)	2/2 -	1/1 -	1/1 -	74/85 (87%)

MA mares (Table 1). Recipients ovulated between one day before and 4 days after the donors.

Embryo quality was excellent or good in 71/74 (95.9%) and in 22/22 embryos derived from the TY and the MA mares, respectively.

Embryo recovery rates in the TY donors were similar both in the different years of the study (Table 2) and in consecutive oestrous cycles of the same year (Table 3).

TY donors and control mares, when bred, produced live foals in 42/49 (85.7%) and in 38/43 (88.4%) of the occasions, respectively.

DISCUSSION AND CONCLUSIONS

In this study the use TY Haflinger mares as embryo donors resulted in a high embryo recovery rate throughout the years and in a pregnancy rate after non surgical transfer in the normal range (Squires *et al.* 1999). Both embryo recovery rates and recipient pregnancy rates were similar in TY and in MA mares.

These results are in agreement with what found by Savage *et al.* (1989) and Fleury *et al.* (1985), which reported an embryo recovery rate of 80.6% in 2 years old Arabian fillies and of 85% in under 3 years old Mangalarga mares, respectively. On the contrary, Iuliano and Squires (1985) obtained an embryo recovery rate of only 36.3% in 2-year-old Arabian fillies and Steiner and Jordan (1988) reported a lower embryo recovery rate in three 2-year-old than in 3 mature Hanoverian mares. Breed and seasonal effects could have accounted for these differing results.

Although in our study most fillies were employed as embryo donors only once or twice, some mares provided embryos in 3 and 6 consecutive oestrus cycles suggesting that short time fertility of these young donors was not affected by embryo recovery procedures as previously reported for mature mares (McKinnon and Squires 1988). The foaling rates for mares previously used as embryo donors and for controls was similar, confirming that early use as donors did not affect fertility in the following years (Savage *et al.* 1989).

In conclusion, this study indicate that 2-year-old mares can be successfully used as embryo donors, as a method to anticipate foals production from valuable mares, with no negative effects on their future reproductive efficiency.

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EFFECT OF INSEMINATION TIMING ON EMBRYO RECOVERY RATE, PREGNANCY RATE AFTER TRANSFER, AND PREGNANCY LOSS RATE IN A COMMERCIAL EMBRYO TRANSFER PROGRAMME

F. L. Riera, J. E. Roldán and K. Hinrichs*

*Centro de Reproducción Equina Doña Pilar, Lincoln, Argentina; *Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas, USA*

INTRODUCTION

Scheduling insemination of donor mares is a major component of success in an embryo transfer program. Periodically, a stallion is overbooked, semen is shipped late, help needed for stallion collection is absent, or a mare ovulates earlier than anticipated, and the only chance of pregnancy on that cycle is to inseminate the mare after ovulation. We recorded data for 191 cycles in donor mares of normal fertility over one breeding season to examine the effect of insemination time on embryo recovery rate, pregnancy rate after transfer, and embryo loss rate in recipient mares.

MATERIALS AND METHODS

Thirty seven polo pony mares aging 5–20 years and weighing 450–550 kg were used as donor mares. Mares with preovulatory follicles were examined by ultrasonography and palpation per rectum twice daily, between 6 and 8 am and between 6 and 8 pm.

Eight Thoroughbred or Thoroughbred-cross fertile stallions were used to inseminate the donors. Insemination with extended fresh or cooled semen was performed before ovulation (pre) if possible; however, due to constraints as given above, some mares were first inseminated at the time ovulation was detected (within 12 h after ovulation occurred; post).

Uterine lavage for embryo recovery was performed using a standard technique with an in-line embryo filter (Riera and MacDonough 1993). Embryos were recovered on Day 7 for pre mares and at Day 8 for post mares.

Embryos were located using a dissection microscope and graded for quality on a 1–4 scale according to morphological features. Embryos were also classified according to the stage of development as morula, early blastocyst (Ebl), blastocyst (Bl), expanded blastocyst (XBl) and very expanded blastocyst (XXBl).

The embryos were transferred transcervically to the uterus of synchronised recipient mares that had ovulated from one day before to 3 days after the donor. Ultrasound examination for pregnancy was performed between Day 14 and 21 and again between Day 40 and 60.

RESULTS

Only results from single-ovulating mares were used for this analysis. There was a significant reduction in embryo recovery rate for post insemination (86/103, 83% for pre insemination vs 19/30, 63% for post, $P<0.05$). There was no significant difference in the proportion of Grade 1 embryos between pre and post groups (76/85, 89% vs. 15/19, 79% respectively). There was no difference in recipient pregnancy rates at Day 14–21 (62/85, 73% vs 13/19, 68% for pre and post embryos respectively) or for pregnancies maintained at the second examination (57/61, 93% vs 11/13, 85% respectively). The overall efficiency (pregnancies at 60 days per donor mare cycle) was 56% for pre mares and 37% for post mares.

Embryos recovered on Day 7 from pre mares were more advanced in development than were embryos recovered on Day 8 from post mares (proportions of EBl, XBl, and XXBl were 10, 70, and 20% respectively for pre mares and 31, 63 and 6% respectively for post mares).

The embryo recovery rate was similar in pre mares inseminated one or 2 times, both for single ovulating mares (52/62, 84% and 34/41, 83% respectively) and for double ovulating mares (15/11, 136% and 50/37, 135%, respectively). There was no difference in embryo recovery rate among single ovulating mares last bred 0.5, 1, 1.5, 2, or 2.5 days before ovulation (29/35, 83%; 4/6, 67%; 31/37, 84%; 6/8, 75% and 2/2, 100%, respectively).

DISCUSSION

These findings indicate that post ovulation insemination results in reduced embryo recovery, but no increase in embryo mortality after transfer. Although reduced, the recovery rate of 63% justifies using this procedure rather than abandoning the cycle. For the 8 stallions used in this programme, there was no advantage in inseminating 2 times rather than once, or for timing insemination closer than 2.5 days before ovulation.

There have been conflicting reports in the literature regarding the fertility of post ovulation breeding in the mare. Huhtinen *et al.* (1996) found that embryo recovery rate decreased as time from ovulation to insemination increased, but mares inseminated within 16 h of ovulation had similar recovery rates to mares inseminated before ovulation. Woods *et al.* (1990) reported that insemination within 12 h of ovulation did not significantly decrease pregnancy rates. However, these studies involved few mares (14–20 per group) which may have limited the power to detect differences between treatments. In the study of Woods *et al.* (1990), pregnancy rates achieved in mares inseminated 0–6 h post ovulation and 6–12 h post ovulation (79% and 65% respectively) were similar to our embryo recovery rates for pre and post breedings (83% and 63%, respectively), suggesting that most fertilisation failure/embryo loss occurs in mares inseminated 6–12 h after ovulation.

The delay from ovulation to fertilisation in post breedings incorporates both the time needed for the sperm to arrive at the site of fertilisation and time needed for capacitation. Oocyte aging may result in lack of fertilisation or in reduced viability of the fertilised eggs. It is also possible that some of the embryos from post ovulation breeding lack the ability to signal the oviduct for descent into the uterus, and thus perish in the oviduct. The embryos recovered from post breedings, even though they were recovered one day later, were at an earlier stage of development than were embryos from pre breedings. However, there was no significant difference in quality. These findings agree with those of Huhtinen *et al.* (1996), and also with the ultrasonographic findings of Woods *et al.* (1990), who reported that post embryos were more than one day smaller in diameter than pre embryos at Days 11–15 of pregnancy. Since insemination was performed within 12 h of ovulation, the >1 day developmental delay in post embryos may reflect the time needed for the sperm to capacitate before fertilisation, or possibly retardation of embryo growth because of compromised oocyte viability. The time required for the sperm to capacitate is not known in the stallion. When mares were bred before ovulation, anaphase was recognised by 6 h after ovulation (Bezard *et al.* 1989). However in mares bred post ovulation, telophase was not seen until 10 h post coitum (Enders *et al.* 1987). Although the data are scant, this suggests about a 4 h delay relating to transport and capacitation of sperm after insemination. The contrast between our results and these previous data suggest that more work is needed on the physiology of *in vivo* sperm transport and capacitation in the horse, and on the effects of oocyte aging on embryo development.

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FACTORS AFFECTING PREGNANCY RATES AND EARLY EMBRYONIC DEATH AFTER EQUINE EMBRYO TRANSFER

E. M. Carnevale, R. J. Ramirez, E. L. Squires, M. A. Alvarenga and P. M. McCue

Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523, USA

Success of a commercial embryo transfer program is dependent upon identifying factors that affect pregnancy and embryonic death. Previous studies have examined the effect of method of transfer, technician, size, age of embryo, embryo morphology, season of the year, synchrony between donor and recipient, and culture and storage of embryos (Squires *et al.* 1982; Iuliano and Squires 1985; McKinnon and Squires 1988; Cook *et al.* 1989; Carney *et al.* 1991; Squires *et al.* 1992; Squires and Seidel 1995; Fluery and Alvarenga 1999; McCue *et al.* 1999; Squires *et al.* 1999). This is a retrospective study involving 638 embryos transferred into recipients at Colorado State University Equine Reproduction Laboratory during 1996, 1997 and 1998. The objective of this retrospective study was to determine which factors (recipient or embryo) had significant effects on pregnancy and embryonic loss rates. Donor mares were either mares housed at Colorado State University or those housed at various breeding farms throughout the United States. Embryos were collected 7 or 8 days after ovulation either at CSU or collected on the farm, cooled and transported within 24 h to Colorado State University. Recipients were light horse mares between 2 and 18 years of age and 400 to 600 kg. Cycling recipients were examined by palpation and ultrasound at regular intervals and daily during oestrus. Reproductive tracts were examined for the detection of follicular activity, day of ovulation, presence of the corpus luteum, uterine oedema, and fluid in the uterine lumen. Recipients were classified as 'acceptable' if a well-defined corpus luteum and a good to excellent cervical and uterine tone were present. Recipients were classified as marginally acceptable if they had a small or poorly imaged corpus luteum and/or poor to fair cervical and uterine tone. Embryos were measured and graded based on morphology ((McKinnon and Squires 1988). Embryos shipped from another location for subsequent transfer were placed in a plastic culture tube containing transport medium (Ham's F10 with 10% fetal calf serum plus 1% penicillin/streptomycin), which was gassed with 5% CO_2, 5% O_2 and 90% N_2. Embryo were packaged in Equitainers as previously described and transported via a commercial airline or parcel delivery service (Squires *et al.* 1992). Transport time varied from 6 to 32 h. Pregnancy rates on Day 50 were significantly higher for recipients that had excellent to good uterine tone and were graded as acceptable recipients vs recipients that had fair to poor uterine tone and graded marginally acceptable (60% vs 45%). Embryonic factors that affected pregnancy rates significantly were morphology grade, diameter and stage of development. Embryos 100 to 200 µm resulted in lower pregnancy rates. As expected, those embryos given a quality grade of 1 (excellent) resulted in higher pregnancy rates that those graded 2, 3 or 4. Fewer ($P<0.05$) pregnancies resulted after non-surgical vs surgical transfers, but embryos transferred by non-surgical techniques had significantly lower mean quality scores. Pregnancy rates were lowest during July and August.

The incidence of early embryonic death was 16% (65 of 419 from Days 12–50). Embryonic loss rate was significantly higher in recipients used 7 or 9 days vs 5 or 6 days after ovulation. For those mares that experienced early embryonic loss, the highest incidences of embryonic death occurred during the interval from Day 17–25. Embryonic

vesicles that were imaged with ultrasound during the first pregnancy examination (5 days after transfer) resulted in significantly fewer embryonic deaths than vesicles imaged on subsequent examination.

Reduced uterine tone and quality scores for recipients were associated with reduced pregnancy rates and tended to be associated with increased embryonic loss rates. Reduced uterine tone in recipients may indicate a uterine environment that is not maximally compatible for embryonic growth and development. In a previous study (McCue *et al.* 1999), lower circulating concentrations of progesterone appeared to be correlated with reduced uterine and/or cervical tone. In the present study, the incidence of embryonic loss was not different for fresh vs cooled embryos. Thus, when properly done, cooling and transporting an embryo is an effective way of utilising recipients without a decrease in pregnancy rates or an increase in early embryonic loss. The results of this study suggest that recipients should be critically evaluated on Day 5 after ovulation and used as recipients for embryos within 7 days of ovulation. The morphology of the embryo was predictive of the potential of the embryo to result in a pregnancy or to undergo embryo losses.

Embryos that were delayed in development were associated with lower pregnancy rates. Recipient quality and selection appears to be the major factor that could be successfully manipulated to increase pregnancy rates after embryo transfer.

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ADMINISTERED OESTROGENS ARE LUTEOLYTIC DURING EARLY PREGNANCY IN MARES

T. A. E Stout and W. R. Allen*

*Department of Equine Sciences, Utrecht University, Section of Reproduction, Yalelaan 12, 3584 CM Utrecht, The Netherlands; *Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK*

INTRODUCTION

Recent surveys have reported early pregnancy loss (EPL) rates of between 5 and 24% in young, healthy mares and up to 70% in aged, subfertile mares (see Ball 1993 for review), thereby confirming that EPL is a significant cause of economic loss to the horsebreeding industry. In the present monograph, Carnevale *et al.* report similar rates of EPL for pregnancies established by embryo transfer (12–25%) and they discuss some of the factors that may have contributed to these losses. In general, while it is clear that the majority of EPL occurs within the first 35 days of gestation, when maintenance of the primary corpus luteum is essential to conceptus survival, the conditions under which the CL might undergo luteolysis in the presence of a normal conceptus are not well understood. On the other hand, it has been widely speculated that oestrogens secreted by the developing horse conceptus may effect maternal recognition of pregnancy and luteostasis, as they do in pigs (Bazer and Thatcher 1977). However, this hypothesis has remained unproven, primarily because administration of exogenous oestrogens does not reliably prolong luteal activity in cycling mares. Indeed, Goff *et al.* (1993) reported that the administration of oestrogens to mares in late dioestrus stimulates, rather than inhibits, luteolysis, thereby mirroring the situation in the domestic ruminants in which oestrogens promote luteolysis by enhancing endometrial oxytocin receptor development and, thereby, oxytocin-induced $PGF_{2\alpha}$ release (McCracken *et al.* 1984). Moreover, in cattle, hormonal treatments given to protect against failure of maternal recognition of pregnancy and loss of the primary CL, namely, exogenous progesterone or a mid-dioestrous injection of a GnRH analogue, have been proposed to do so by reducing circulating oestradiol concentrations and thereby weakening the luteolytic drive (Mann and Lamming 1995). Similar treatments are being used increasingly in mares in an attempt to minimise early pregnancy loss, despite a lack of evidence to support their efficacy. Furthermore, there is no rationale for such treatments unless it can be established that oestrogens are pro-luteolytic during early pregnancy in equids; such an effect is both unproven and contrary to the existing dogma. The aim of this study was to determine if administered oestrogens might be luteolytic during early pregnancy in the mare and, if so, whether they act by stimulating $PGF_{2\alpha}$ release directly or by enhancing endometrial oxytocin-sensitivity.

MATERIALS AND METHODS

On Days 14 and 22 of pregnancy and Day 14 of the oestrous cycle, Welsh pony mares (n = 3 in each of 6 groups) were given either an iv injection of 0.01 mg/kg oestradiol-ß alone, or an im injection of 0.03 mg/kg oestradiol-ß followed 6 h later by an iv injection of 20 iu/500 kg oxytocin. Jugular vein plasma samples were collected at 10 min intervals from 1 h before to 1 h after oestradiol-ß or oxytocin injection and assayed for their concentrations of 13,14-dihydro,15-keto $PGF_{2\alpha}$ (PGFM). Additional blood samples were collected twice daily to monitor post treatment peripheral progesterone concentrations and pregnancy was monitored by palpation and ultrasound scanning of the reproductive tract on alternate days.

RESULTS

Oestradiol benzoate followed by oxytocin, but not oestradiol-ß alone, caused a rapid decline in

peripheral serum progesterone concentrations. In the pregnant mares, however, progesterone concentrations plateaued and stabilised at around 2–3 ng/ml so that the pregnancies were maintained. Circulating PGFM concentrations did not rise within the first h after oestradiol-ß injection in any of the animals whereas oxytocin challenge 6h after oestradiol-benzoate resulted in a significant rise in plasma PGFM concentrations in one Day 14 dioestrous mare (of one analysed thus far) and two of three Day 22 pregnant mares, but not in either of 2 Day 14 pregnant mares.

CONCLUSIONS

These results indicate that a single oestrogen challenge, given as an iv injection of oestradiol-ß, does not stimulate uterine $PGF_{2\alpha}$ release or reduce luteal activity in the mare when administered either late in dioestrus or early in pregnancy. On the other hand, more prolonged oestrogen therapy (im oestradiol benzoate) followed by oxytocin challenge caused partial luteolysis in all pregnant mares treated on Day 14 or 22 after ovulation; although there was no discernable rise in PGFM concentrations in those treated on Day 14. This argues against the effect of oestradiol benzoate being mediated primarily by enhancement of the oxytocin-$PGF_{2\alpha}$ pathway and, since oxytocin injection alone does not influence peripheral serum progesterone concentrations in Day 22 pregnant mares (authors, unpublished observations), the luteolysis seen in the present study was unlikely to have been caused by the administered oxytocin *per se.* On the other hand, an earlier experiment recorded a similar fall in circulating progesterone concentrations in Day 23 pregnant mares to which oestradiol benzoate was administered once daily for 10 days (Gerstenberg 1998), which also supports the proposal that oestradiol benzoate has a luteolytic action which is independent of exogenous oxytocin and which rarely results in complete CL lysis.

In summary, we present preliminary evidence that the administration of longer-acting oestrogens to mares during early pregnancy may compromise luteal survival. Although these findings need to be confirmed and extended, they nonetheless lend support to the hypothesis that high systemic oestrogen concentrations may be detrimental to pregnancy in the mare.

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RESULTS FROM EMBRYO FREEZING AND POST OVULATION BREEDING IN A COMMERCIAL EMBRYO TRANSFER PROGRAMME

F. A. Lascombes and R. L. Pashen*

*Laboratorio Estancia 'El Boyero', C.C. 101 Casbas 6417, Provincia de Buenos Aires, Argentina; *Australian Animal Genetics Pty. Ltd., 54 Kalimna Drive, Mornington, Victoria, 3931, Australia*

EMBRYO FREEZING

The uteri of 20 donor mares were flushed repeatedly during a 70 day period with the intention of freezing any embryos collected. A previous unpublished study had indicated that large embryos (≥ 220 µm) failed to develop when frozen and thawed but that those <220 µm could survive and develop normally *in utero*. To increase the chance of obtaining small embryos, an intensive palpation regime was undertaken whereby mares were palpated 4 times daily (4.00 am, 10.00 am, 4.00 pm and 10.00 pm) until ovulation was detected. The mares were inseminated when appropriate and embryos collected at 156 ± 6 h after ovulation. Of 75 flushes, 60 (80%) were successful and yielded a total of 74 embryos. Of these, 64 (86%) were classified as 'small' and were frozen. The remaining embryos were transferred directly to recipient mares.

The embryo freezing and thawing protocols employed 10% v:v glycerol in Dulbecco's modified phosphate buffered saline (DPBS) as the cryoprotectant, added in 2 steps of 5% glycerol for 10 min followed by 10% glycerol for 20 min at room temperature. The embryos were frozen in a programmable embryo-freezing machine set at 20°C, cooled initially at 1°C /min to -6°C, held for 10 min, seeded after 5 min then cooled at 0.3°C/min to -32°C, 0.1°C to -35°C and then plunged into liquid nitrogen. The embryos were thawed in air for 15 s followed by immersion in a water bath at 35°C for 20–30 s. Glycerol was removed in 3 steps going from 5% glycerol + 0.5M sucrose for 5 min to 0.5M sucrose for 5 min and then DPBS for 5 min. A total of 44 embryos were thawed and transferred to synchronised recipient mares. These recipients ovulated within 24 h of the donors and, where possible, those which ovulated after the donors were used. Pregnancy was determined by ultrasound examination 7–10 days post transfer and confirmed at around Days 42 and 60 of gestation.

Embryos were transferred on 2 farms with the first (Farm 1) achieving 14 pregnancies from 25 embryos transferred (56%) and the second (Farm 2) achieving 10 pregnancies from 19 transfers (52%). This demonstrated that equine embryos <220 µm can be frozen and thawed in a commercial environment with acceptable results.

The intensive palpation regime and accurate detection of ovulation helped to achieve an acceptable embryo recovery rate from donor mares.

Other presentations at this workshop confirm our finding that expanded embryos do not freeze well (O'Donovan *et a*l. 2000) whereas small embryos can result in pregnancies following freezing (Huhtinen *et al.* 2000). The reasons for this are not clear but it is thought that a combination of factors including impedance of penetration of the cryoprotectant into the inner cell mass (ICM) by the capsule, and the small cell size of the ICM, makes equine embryos more susceptible to the toxic effects of the glycerol.

POST OVULATION BREEDING

A retrospective analysis of the results of post ovulation breeding was undertaken. This was performed when inseminating semen from a subfertile stallion and when asynchronous twin ovulations were predicted. Mares to be mated were palpated up to 4 times daily and inseminated after ovulation was detected. When twin follicles were

detected on ultrasound, and the operator thought they would ovulate >24 h apart, mating was delayed until the first follicle had ovulated and this was followed by an iv injection of hCG (2,500 iu). Mares were re-mated prior to ovulation of the second follicle which usually occurred 24–36 h after the first ovulation.

In mares with a single ovulation, mating took place 2–14 h post ovulation. The following results were obtained: of 13 recovery attempts performed following matings 2–5 h post ovulation, 11 (85%) yielded embryos. When mating occurred 6–8 h post ovulation, 15 of 16 flushes (94%) produced embryos. Following matings at 9–10 h post ovulation, embryos were recovered from2 of 3 attempts (67%). Ten mares were mated >10 h post ovulation and only one (10%) flush produced an embryo. These results show that acceptable rates of fertilisation can be achieved when mares are mated up to 10 h post ovulation.

In mares with asynchronous twin ovulations, comparisons were made between embryo collection rates when mating occurred before ovulation and those when mares were mated pre- and post ovulation. The following results were obtained:

For pre-ovulation mating in mares with ovulations 24 h apart, 9 flushes (21%) produced no embryo, 15 (37%) produced one and 18 (42%) produced 2. In mares with ovulations 36 h apart, 3 flushes (17%) produced nothing, 8 (44%) produced one embryo and 7 (39%) produced 2 embryos. In mares with ovulations 48 h apart, 4 flushes (31%) produced nothing, 5 (38%) produced one embryo and 4 (31%) produced 2 embryos. When mating occurred once before and once after ovulation, and ovulations occurred 24 h apart, 3 flushes (15%) produced nothing, 7 (35%) produced one embryo and 10 (50%) produced 2 embryos. When ovulations were 36 h apart, 2 flushes (22%) produced nothing, 5 (56%) produced one embryo and 2 (22%) produced 2 embryos. When ovulations were 48 h apart, 3 flushes (27%) produced nothing, 2 (18%) produced one embryo and 6 (55%) produced 2 embryos. In all cases embryo recovery did not differ between the groups.

In practice, when mares are mated post ovulation, flushing must be delayed for 24–36 h. Otherwise, embryo recovery is poor suggesting that fertilisation occurs up to 24 h after mating.

LIST OF PARTICIPANTS

Twink Allen
United Kingdom

Giulio Aiudi
Italy

Javier Aguilar
Argentina

Maria Albrizio
Italy

Marco Alvarenga
Brazil

Sergei Amstislavsky
Finalnd

Mari Aoki
Japan

Kaija Arpiainen
Finland

Isabelle Battut
France

Keith Betteridge
Canada

Ingrid Boegh
Denmark

Heinrich Bollwein
Germany

Sonia Boyazoglu
United States

Jean-Francois Bruyas
France

Dominik Burger
Switzerland

Michele Caira
Italy

Francesco Camillo
Italy

Elaine Carnevale
United States

Celina Checura
Argentina

Raffaele Cherchi
Italy

Mario Cinone
Italy

Juan Manuel Cortez
Argentina

Peter Daels
France

Maria Dell'Aquila
Italy

Vito De Nigris
Italy

Jerry Dilsaver
United States

Reynald Dupras
Canada

Bruce Eilts
United States

Lilian Franz
United States

Eduardo Gastal
Brazil

Melba Gastal
Brazil

David Gerber
South Africa

Fahrad Ghasemi
Iran

Hamid Ghasemzadeh-Nava
Iran

Paola Ghergo
Italy

Ollie Ginther
United States

Robert Godke
United States

Ken Greaney
New Zealand

Johannes Handler
Brazil

Marc Henry
Brazil

Katrin Hinrichs
United States

Mirja Huhtinen
Finland

John Hyland
Australia

Judit Juhaz
Sultanate of Oman

Tertti Katila
Finland

Erkki Koskinen
Finland

Hanna Kukkola
Finland

Giovanni Lacalandra
Italy

Fernanda Landim Alvarenga
Brazil

Francisco Lascombes
Argentina

Suzanne Laurence
Canada

Henrik Lehn-Jensen
Denmark

Stan Leibo
United States

Ktlin Leisson
Estonia

Xihe Li
United Kingdom

Heli Lindeberg
Finland

Allison Lindsey
United States

Heraldo Lisa
Northern Ireland

Luis Losinno
Argentina

Charles Love
United States

Jorge MacDonough
Argentina

Lisa Maclellan
United States

Margo MacPherson
United States

Maarit Maki
Finland

Gaetano Mari
Italy

Patrick McCue
United States

Sally Meadows
Northern Ireland

Paolo Minoia
Italy

Lee Morris
United Kingdom

Peter Nagy
Sultanate of Oman

Yasuo Nambo
Japan

Ann Nheim
Sweden

Amir Niasari-Naslaji
Iran

Jesper Nielsen
Denmark

Leila Nurmikivi
Finland

Nicole Oberstein
Germany

Mary O' Donovan
United States

Julio Oriol
Dominican Republic

Richard Owen
United Kingdom

Dale Paccamonti
United States

Eric Palmer
France

Pascale Palmer
France

Robert Pashen
Australia

Hanne Pedersen
United Kingdom

Morten Petersen
Denmark

Katja Piltti
Finland

Gene Pranzo
United States

Tiina Reilas
Finland

Martina Reims
Finland

Luigi Ricci
Italy

Fernando Riera
Argentina

Simon Robinson
Australia

Mikko Rvinen
Finland

Marie Saint-Dizier
France

Kirsi Sojakka
Finland

Dan Sharp
United States

Dan Shaw
New Zealand

Harald Sieme
Germany

Cristi Sporleder
Argentina

Ed Squires
United States

Tom Stout
The Netherlands

Julian Skidmore
United Kingdom

Bruce Taylor
New Zealand

Ragnar Thomassen
Norway

Merja-Liisa Toivonen
Finland

Jordi Tremoleda
The Netherlands

Luisa Valentini
Italy

Dickson Varner
United States

Jan Wade
United Kingdom

Elaine Watson
United Kingdom

Sandra Wilsher
United Kingdom

AUTHOR INDEX