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EDITORS' FOREWORD

It gave us great pleasure to welcome delegates to the third meeting of the European Equine Gamete Group. We were able to look back to the first meeting, in September 1999, and reflect on the subsequent progress which has been made in a variety of areas. These include, of course, embryo transfer techniques, improved methods of gamete handling and *in vitro* fertilisation. However, the most significant advance since the meeting in September 2001 was the creation of the first cloned mule, by Gordon Woods in Idaho, USA and the first cloned horse by Cesare Galli in Cremona, Italy. This opened a whole realm of possibility for all of us and we are, indeed, in exciting times in terms of the potential of reproductive biology. There is no doubt that the current meeting, like the previous ones, stimulated new experimental protocols and spawned a range of active collaborations.

Other significant advances are continuing to develop, as a result of increased utilisation of the equine genome map. This is an extremely powerful tool in our armoury and one from which we can all benefit.

It seemed appropriate to hold this meeting in Pardubice, to coincide with our famous Steeplechase. The city has a considerable history of horseracing and breeding and, in former times, was the home of National Stud Kladruby nad Labem, famed for the unique Kladrubian horse.

Once again we are indebted to Gene Pranzo and the Havemeyer Foundation. The generosity and altruism of this organisation have contributed in a major way to the climate of liaison in which we are all so fortunate to work. We very much look forward to the next European Equine Gamete Group meeting, in Rostock, where we will, no doubt, further strengthen the collaborative links which have arisen from this series of meetings.

Josef and Zdenek Müller

HAVEMEYER SCIENTIFIC WORKSHOPS

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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**
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Organisers: Drs D. F. Antczak and J. McClure

1989 **Second International Symposium on Equine Embryo Transfer**
February - Banff, Alberta, Canada
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1990 **International Workshop on Equine Sarcoids**
April - Interlaken, Switzerland
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1992 **Workshop on Equine Neonatal Medicine**
January - Naples, Florida
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July - Cambridge, England
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Second International Equine Leucocyte Antigen Workshop
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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
July - Saari, Finland
Organiser: Dr T. Katila

2001

USDA International Plant & Animal Genome Conference
January - San Diego, California

Equine Immunology in 2001
January - Santa Fe, New Mexico
Organiser: Dr D. P. Lunn

Asthma and Allergies II
April - Hungary
Organisers: S. Lazary and E. Marti

From Elephants to Aids
June - Port Douglas, Australia
Organiser: Professor W. R. Allen

International Equine Gene Mapping
July - Brisbane, Australia
Organiser: K. Bell

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

Foal Septicemia III
October - Tufts University European Center, Talloires, France
Organiser: M. R. Paradis

Infectious Disease Programme for the Equine Industry and Veterinary Practitioners
October - Marilyn duPont Scott Medical Center, Morvan Park, Virginia, USA
Organisers: Drs J. A. Mumford and F. Fregin

From Epididymis to Embryo
October - Fairmont Hotel, New Orleans, USA
Organiser: Dr L. H-A. Morris

2002

USDA International Plant & Animal Genome Conference
January - San Diego, California

Comparative Neonatology/Perinatology
January - Palm Springs, California
Organiser: P. Sibbons

Stallion Behavior IV
June - Reykjavik, Iceland
Organisers: S. McDonell and D. Miller

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

Equine Orthopaedic Infection
August - Dublin, Ireland
Organiser: E. Santschi

Inflammatory Airway Disease
September - Boston, USA
Organiser: Dr E. Robinson

2003

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

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October - Brewster, Massachusetts
Organiser: E. Squires

Third Meeting of the European Gamete Group (EEGG)
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SESSION I:

Semen

THE EFFECT OF SEMINAL PLASMA ON MOTILITY CHARACTERISTICS AND VIABILITY OF SPERMATOZOA AFTER COOLED STORAGE

T. Katila, T. Reilas*, K. Güvenc†, K. Alm and M. Andersson

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INTRODUCTION

Seminal plasma is an important component of stallion ejaculate. Secretions of the accessory sex glands are released in a different order during ejaculation, making the composition of the ejaculatory jets different from each other (Magistrini *et al.* 2000). This affects the ability of spermatozoa to survive storage. It has been shown that motility and viability of spermatozoa after 24 h of cooled storage (Katila *et al.* 2001) and motility of frozen-thawed spermatozoa (Katila *et al.* 2002) differ in ejaculate fractions. There are also differences between stallions. Addition of seminal plasma from stallions with high post thaw motility of sperm to ejaculates from stallions with low post thaw motility has been shown to increase motility and membrane integrity. The opposite effect was detected when seminal plasma from stallions with low sperm motility was added to stallion sperm with high motility (Aurich *et al.* 1995).

The purpose of the experiment was to study the effect of seminal plasma on the quality of cooled stored semen by exchanging seminal plasma between stallions.

MATERIALS AND METHODS

In 2 stallion pairs, one of the stallions was considered to be normal and the other showed reduced progressive sperm motility. One pair consisted of Stallion 1 (Finnhorse aged 16 years, testicular index (TI) 5.9, progressive motility 50%) and Stallion 2 (Finnhorse aged 17 years, TI 5.7, progressive motility 20%). The second pair consisted of Stallion 3 (Standardbred aged 12 years, TI 4.9, progressive motility 60%) and Stallion 4 (Finnhorse aged 24 years, TI 1.9, progressive motility 30%). Stallion 2 was the son of Stallion 4. Semen was collected 4 times, 2 times a week in February 2003, after a week of daily semen collections, using an automated fractionating semen collection device (Equidame® phantom, Haico Oy, Loimaa, Finland) which separates semen into 5 cups (Lindeberg *et al.* 1999). Cup 1 contained the pre-sperm fraction and Cup 2 did not. Three fractions (contents of Cup 1, Cup 2 and either the last or the 2 last cups) and one sample representing the whole ejaculate (15% of the contents of each cup) were processed for cooled storage. If Cup 1 did not contain any spermatozoa, contents from Cup 2 were added to make Fraction 1.

Only spermatozoa from the sperm-rich Cup 2 were used. The contents of Cup 2 were divided into 2 approximately equal parts. One was diluted with skim milk extender (Kenney *et al.* 1975) centrifuged (500 *g*, 15 min) and the supernatant removed. The remaining spermatozoa were first re-suspended in a small volume of skim milk extender and a Bürker counting chamber was used to determine sperm concentration. The suspension was then diluted with skim milk extender to a final concentration of 75×10^6 sperm/ml. The other half of Fraction 2, Fractions 1 and 3, and the sample representing the whole ejaculate were processed further to be used as seminal plasmas. They were centrifuged twice (3000 *g*, 20 min) and filtered through a 0.45 µm filter (Minisart, Sartorius, Göttingen, Germany). Semen was then mixed in a ratio of 2 parts diluted semen to 1 part seminal plasma with the stallion's own seminal plasmas and with the seminal plasmas of the paired stallion. After 24 h storage in an Equitainer, samples were warmed in a water bath (37°C). Motility parameters were evaluated using an automated semen analyser (Sperm Vision

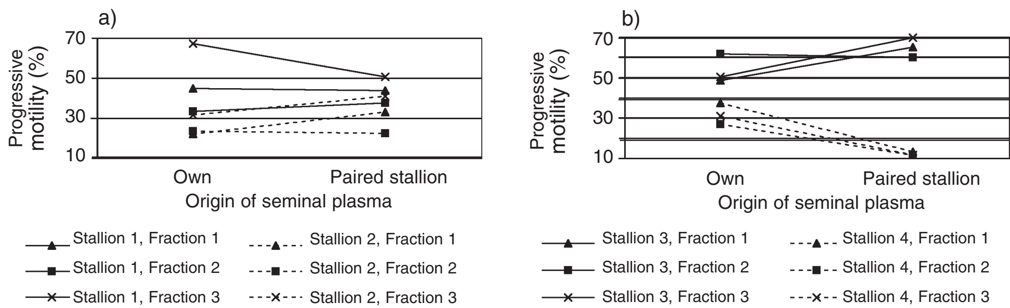

Fig 1: Dot diagram showing progressive motility of spermatozoa in stallion's own vs. stallion pair's seminal plasma after 24 h cooled storage. a) Spermatozoa from stallions 1 and 2; and b) Spermatozoa from stallions 3 and 4.

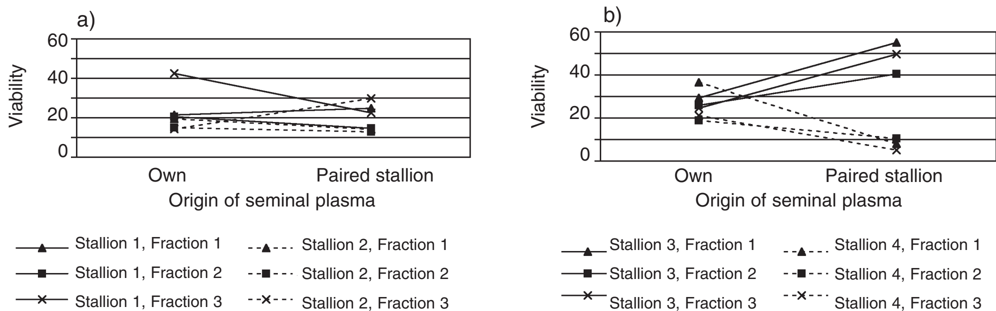

Fig 2: Dot diagram showing viability of spermatozoa in stallion's own vs. stallion pair's seminal plasma after 24 h cooled storage. a) Spermatozoa from stallions 1 and 2; and b) Spermatozoa from stallions 3 and 4.

Minitüb, Tiefenbach bei Landshut, Germany). Plasma membrane integrity was assessed using a fluorometric analyser (Fluoroscan Ascent, Labsystems, Helsinki, Finland) and the method described by Katila *et al.* (1999).

The data consist of values obtained from stallion's own seminal plasma and from the seminal plasma of the paired stallion. T-tests were used to calculate differences within the same ejaculation day and within the same ejaculate fraction between the 2 horses in each pair.

Results

The sperm concentrations of the cups differed between stallions, with Stallion 4 having particularly low concentrations in all cups. Fraction 3 showed numerically best motility values for most of the parameters measured.

Because the pairing of the stallions was based on different sperm motility, it is obvious that they differed significantly from each other in motility parameters. For the majority of parameters measured, Stallion 4 showed lowest values and Stallion 3 highest. Difference between the stallion's own seminal plasma and the paired stallion's seminal plasma for Stallions 1 and 2 was significant in Fraction 1 for straightness (STR), linearity (LIN) and wobble (WOB), in Fraction 2 for average path distance (DAP), curve line distance (DCL), average path velocity (VAP) and curve line velocity (VCL), and in Fraction 3 for viability. When Stallions 3 and 4 were compared, significant differences were discovered in Fraction 1 for PMOT, DCL, VCL and viability and in Fraction 2 for total motility (TMOT) and viability ($P<0.05$). Figure 1 depicts PMOT and Figure 2 viability of spermatozoa.

Discussion

Exchanging seminal plasma between stallions significantly changed certain motility parameters and the percentage of viable cells in some

fractions. The old stallion with obvious testicular degeneration (Stallion 4) had the lowest values numerically for almost all parameters when his spermatozoa were in his own seminal plasma. Interestingly, his seminal plasma improved the motility values of his pair, whose seminal plasma lowered the motility values of Stallion 4. For all motility parameters which differed significantly, the seminal plasma of Stallion 2 lowered the motility values of Stallion 1 and the seminal plasma of Stallion 1 increased the motility values of Stallion 2. The effect of seminal plasma exchange was consistently either positive or negative within each stallion pair for all significantly affected parameters. Thus, although seminal plasma affected sperm motility and viability during cooled storage, the effects were not consistent within fractions.

Although TMOT, PMOT and viability did not differ significantly between the fractions, other motility parameters did. Numerically, Fraction 3, which contained the last jets of ejaculation with a low sperm concentration, had the highest values for almost all parameters measured. This result was surprising, because the sperm-rich fractions have been shown to survive best during cooled storage (Varner *et al.* 1987). In our study, as spermatozoa originated only from Cup 2, the effect of the seminal plasma fractions themselves was perhaps more clearly revealed.

This study involved only 4 stallions, and the results are very preliminary. Further studies are needed to clarify the role of seminal plasma and possible differences between stallions and ejaculatory fractions in survival of spermatozoa during storage.

REFERENCES

Aurich, J.E., Kühne, A., Hoppe, H. and Aurich, C. (1995) Effects of seminal plasma on stallion semen quality after cryopreservation. *J. Reprod. Fert. Abstr. Series* **15,** 34.

Katila, T., Kuisma, P. and Andersson, M. (1999) Evaluation of frozen stallion semen. 1st Meeting of the European Equine Gamete Group. *Havemeyer Foundation Monograph Series No 1,* Eds: W.R. Allen and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 19-21.

Katila, T., Karlsson, M., Reilas, T., Andersson, M., Kaikkonen, R. and Koskinen, E. (2001) Motility and viability of fractionated stallion ejaculates after 24 h of cooled storage. 2nd Meeting of the European Equine Gamete Group. *Havemeyer Foundation Monograph Series No 5,* Eds: T.A.E. Stout and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 3-5.

Katila, T., Andersson, M., Reilas, T. and Koskinen, E. (2002) Post-thaw motility and viability of fractionated and frozen stallion ejaculates. *Theriogenology* **58,** 241-244.

Kenney, R.M., Bergman, R.V., Cooper, W.L. and Morse, G.W. (1975) Minimal contamination techniques for breeding mares: technique and preliminary findings. *Proc. Am. ass. Equine Pract.* **21,** 289-292.

Lindeberg, H., Karjalainen, H., Koskinen, E. and Katila, T. (1999) Quality of stallion semen obtained by a new semen collection phantom (Equidame) versus a Missouri artifial vagina. *Theriogenology* **51,** 1157-1173.

Magistrini, M., Lindeberg, H., Koskinen, E., Beau, P. and Seguin, F. (2000) Biophysical and 1H magnetic resonance spectroscopy characteristics of fractionated stallion ejaculates. *J. Reprod. Fert.* **56,** 101-110.

Varner, D.D., Blanchard, T.L., Love, C.L., Garcia, M.C. and Kenney, R.M. (1987) Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. *Theriogenology* **28,** 709-723.

VARIABILITY OF *IN-VITRO* SPERM ASSAYS WITHIN AND BETWEEN STALLIONS AND ITS RELATION TO FERTILITY

H. Sieme, F. Reinstorf*, M. Krienke†, D. Waberski‡, E. Klug* and H. Bollwein†

*National Stud Lower Saxony, Celle, †Department of Animal Reproduction, Veterinary College, University Munich, *Clinic for horses and ‡Institute for Reproductive Medicine, Veterinary School Hanover, Hanover, Germany*

INTRODUCTION

The predictive value of semen analysis in the evaluation of stallion fertility has been investigated in numerous studies and the results of these investigations have been reviewed (Katila 2001; Colenbrander *et al.* 2003). However, the examination of relationships between sperm quality determined by different assays and stallion fertility is often questionable because of the limited numbers of males and females examined and the use of different fertility scores. Therefore, the aim of this study was to investigate the correlation between various sperm quality parameters and different fertility parameters in a high number of stallions. Furthermore, the variability of various sperm parameters between stallions was determined.

MATERIALS AND METHODS

Stallions

From each of 69 warmblood stallions 3 ejaculates were obtained during the non-breeding (December) and breeding season (March and July). All stallions took part in a commercial AI programme of the National Studfarm Celle, Germany.

Immediately after semen collection raw ejaculates were split in 3 aliquots: One part was placed into plastic tubes and snap frozen in liquid nitrogen, another aliquot was diluted in modified skim milk extender to a final concentration of 50×10^6 spermatozoa/ml and stored at 4°C until further analysis. Furthermore, frozen semen samples were prepared following a previously described freezing procedure (Sieme *et al.* 2003).

Sperm assays

Motility analysis: The percentage of progressively motile sperm (PMS) was determined by using a computerised sperm analysing system. Cells showing more than 50 µm/s were defined as progressively motile.

Morphology: The percentage of total morphologically altered sperm (TMA) was evaluated after staining of smears of raw ejaculates using a modified Farelly procedure (Boersma *et al.* 2001).

Hypo-osmotic swelling test (HOS)

Sperm samples incubated in iso-osmotic (300 mosmol) and hypo-osmotic (150, 200, 250 mosmol/l) media were passed through a CASY 1 cell counter (Petrunkina and Töpfer-Petersen 2000). The relative volume (vol_r) was used as a measure of volume response in response to hypo-osmotic conditions ($vol_r = vol_{hypo}/vol_{iso}$).

Fluorescence staining

Aliquots containing 200×10^6 of cooled (stored for 3–6 h at 4°C) or frozen/thawed sperm were suspended in phosphate buffered saline (PBS) and washed twice (10 min at 700 x g). Sperm pellets were resuspended in PBS, used for further stainings and examined by fluorescence microscopy.

Plasma membrane integrity was evaluated by the SYBR®14/propidium iodide assay (Garner and Johnson 1995). Acrosomal status was studied using the FITC-PNA/PI assay (Blottner *et al.* 1998). Acrosome reaction was induced by incubating the sperm suspension for 20 min at 38°C with heparin. Mitochondrial membrane

TABLE 1: Inter- and intra-stallion variation of sperm attributes (%) expressed as coefficient of variation (CV) in stallion semen collected during the non-breeding season (December) and the breeding season (March, July) (Means ± SD, n=69)

Sperm-attribute (%)	Min	Max	$\bar{X}$	CV (%) inter-stallion	CV (%) intra-stallion
DFI	1.8	41.3	17.9	36.0	34.0
TMA	6.0	41.5	14.8	50.3	31.4
Cooled semen					
PMS	25.0	80.0	62.6	17.1	21.0
$Vol_{200/300}$	1.0	1.5	1.15	8.3	9.6
SYBR-L	1.0	79.0	44.9	38.8	26.8
FITC-PNA	57.0	99.0	85.9	8.3	8.0
AR	16.0	61.5	31.6	39.5	32.6
Frozen/thawed semen					
PMS	1.6	52.7	16.7	56.6	50.0
SYBR-L	3.8	59.5	21.7	41.1	41.8
FITC-PNA	12.0	70.5	39.7	25.6	20.8
AR	3.5	35.0	15.0	54.5	45.6
JC-1	7.0	61.0	28.7	37.0	38.2

CV: coefficient of variation
percentages of sperm attributes
DFI: denaturation fragmentation index of sperm DNA
TMA: total morphologically altered sperm
PMS: progressively motile sperm
$vol_{200/300}$: relative volume of sperm ($vol_r = vol_{hypo}/vol_p$)
SYBR-L: SYBR live stained sperm
FITC/PNA: acrosome intact sperm stained by FITC-PNA/PI
AR: increase of acrosome reacted sperm after incubation of sperm with heparin
JC-1: high to moderate amount of JC-1 positive stained sperm midpiece mitochondrial aggregates

TABLE 2: Relationship between sperm attributes

	DFI	TMA	PMS	$vol_{200/300}$	SYBR-L	FITC/PNA	AR
TMA	0.52						
PMS	-0.55	-0.52					
$vol_{200/300}$	-0.29	-0.29	0.36				
SYBR-L	-0.39	-0.38	0.50	0.39			
FITC/PNA	-0.24	-0.34	0.17	0.12	0.46		
AR	-0.16	-0.33	0.43	0.30	0.15	0.18	
JC-1	-0.39	-0.09	0.24	0.13	0.08	-0.01	-0.02

DFI: denaturation fragmentation index of sperm DNA
TMA: total morphologically altered sperm
PMS: progressively motile sperm
$vol_{200/300}$: relative volume of sperm ($vol_r = vol_{hypo}/vol_{iso}$)
SYBR-L: SYBR live stained sperm
FITC/PNA: acrosome intact sperm stained by FITC-PNA/PI
AR: increase of acrosome reacted sperm after incubation of sperm with heparin
JC-1: high to moderate amount of JC-1 positive stained sperm midpiece mitochondrial aggregates

TABLE 3: Correlations between various sperm attributes and different fertility parameters (pregnancy rate at the end of season (PRS), foaling rate the subsequent year (FR), pregnancy rate per cycle (PRC). Mean values of the semen samples collected in December, March and July were used (n=69 stallions)

	Pregnancy rate end of season		Foaling rate		Pregnancy rate/ cycle	
	Raw semen					
DFI	-0.51*		-0.46*		-0.51*	
TMA	-0.44*		-0.28		-0.44*	
	cooled	frozen/thawed	cooled	frozen/thawed	cooled	frozen/thawed
PMS	0.27	0.23	0.12	0.06	0.33*	0.13
$Vol_{200/300}$	-0.04		-0.14		0.34*	
SYBR-L	0.40*	0.29	0.34*	0.12	0.22	0.09
FITC-PNA	0.33*	0.26	0.23	0.20	-0.17	0.20
AR	0.21	0.19	0.27	0.23	0.03	0.18
JC-1		0.3		0.13		0.33*

* $P<0.05$
DFI: denaturation fragmentation index of sperm DNA
TMA: total morphologically altered sperm
PMS: progressively motile sperm
$vol_{200/300}$: relative volume of sperm ($vol_r = vol_{hypo}/vol_{iso}$)
SYBR-L: SYBR live stained sperm
FITC/PNA: acrosome intact sperm stained by FITC-PNA/PI
AR: increase of acrosome reacted sperm after incubation of sperm with heparin
JC-1: high to moderate amount of JC-1 positive stained sperm midpiece mitochondrial aggregates

potential was assessed by a modified JC-1 staining procedure (Garner and Thomas 1999). After thawing of the snap-frozen raw sperm samples the integrity of the DNA sperm chromatin structure was measured by using the flowcytometric sperm chromatin structure assay (SCSA™) as described by Evenson and Jost (2000). The extent of DNA denaturation was expressed in terms of DNA fragmentation index (DFI; Evenson *et al.* 2002).

Fertility estimation

For the estimation of fertility, pregnancy rate at the end of season (PRS), foaling rate in the subsequent year (FR), and pregnancy rate per cycle (PRC) were determined.

Statistical analysis

Statistical analysis was performed using SAS® (SAS Institute, North Carolina, USA). Percentage data were arcsin transformed. The sample data were checked for normal distribution to decide between parametric or nonparametric tests. Coefficient of variation (%) was used to express variation of sperm parameters among stallions. Relationships between sperm parameters and fertility were expressed by Spearman rank correlation.

RESULTS

Semen samples showed a high variability within and among stallions, whereas coefficients of variation were considerably higher in frozen/thawed semen (Table 1).

Relationships between sperm attributes were low to moderate, suggesting that sperm assays used represent different information regarding spermatozoal function (Table 2).

There were low to moderate correlations between sperm attributes and fertility (Table 3).

DISCUSSION

Although high variabilities in sperm attributes were determined in raw, cooled as well as frozen/thawed semen within and among stallions, only low to moderate relationships to fertility, also varying between different fertility indices were

detected.

Therefore, repeated ejaculates of individual stallions as well as several sperm attributes independent from each other may be required for a useful prediction of a stallion's book or breeding method (natural service or AI via fresh, cooled-stored, cooled-transported, or frozen/thawed semen).

References

Blottner, S., Wegner, I., Roelants, H. and Jewgenow, K. (1998) Durchflußzytometrische Bestimmung des akrosomalen Status von Bullen- und Hengstspermien nach Markierung mit FITC-konjugiertem PNA (peanut agglutinin). *Tierärztl. Umschau* **53,** 441-447.

Boersma, A., Rasshofer, R. and Stolla, R. (2001) Influence of sample preparation. staining procedure and analysis conditions on bull sperm head morphometry using the morphology analyser integrated visual optical system. *Reprod. Dom. Anim.* **36,** 222-229.

Colenbrander, B., Gadella, B.M. and Stout, T.A.E. (2003) The predictive value of semen analysis in the evaluation of stallion fertility. *Reprod. Dom. Anim.* **38,** 305-311.

Evenson, D.P. and Jost, L.K. (2000) Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell. Sci.* **22,** 169-189.

Evenson, D.P., Larson, K.L. and Jost, L.K. (2002) Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* **23,** 25-43.

Garner, D.L. and Johnson, L.A. (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* **53,** 276-284.

Garner, D.L. and Thomas, C. (1999) Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. *Mol. Reprod. Dev.* **53,** 222-229.

Katila, T. (2001) *In vitro* evaluation of frozen/thawed stallion semen: a review. *Acta vet. Scand.* **42,** 199-217.

Petrunkina, A.M. and Töpfer-Petersen, E. (2000) Heterogeneous osmotic behaviour in boar sperm populations and its relevance for detection of changes in plasma membrane. *Reprod. Fertil. Dev*. **12,** 297-305.

Sieme, H., Schäfer, T., Stout, T.A.E., Kulg, E., and Waberski, D. (2003) The effects of different insemination regimes on fertility in mares. *Theriogenology* **60,** 1153-1164.

SESSION II:

Oocytes

MORPHOLOGY OF OVARIES AND *IN VITRO* MATURATION OF FILLY OOCYTE–CUMULUS COMPLEXES

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Oocytes collected from ovaries of pre-pubertal females of domestic animals in IVM/IVF programmes could be used to increase the annual genetic gain by decreasing the generation interval, especially in species with a long pregnancy and a single offspring. Offspring from calves, lambs and piglets have been produced in this way (Armstrong *et al.* 1992; O'Brien *et al.* 1997; Marchal *et al.* 2001). Up to now this kind of experiment has not been performed in horses. The aim of the present study was to examine the morphology of filly ovaries and competence for *in vitro* maturation of their oocyte–cumulus complexes.

MATERIALS AND METHODS

The study was performed on ovaries of 8–15 month old slaughtered fillies (n=37) and mares (n=16; the control group) during February to July.

Immediately after collection, ovaries of each female were wrapped individually in sterile gauze and within 2–3 h transported to the laboratory in a thermos container. Before oocyte collection the ovaries were weighed and flushed 3 times in warm (37°C) physiological saline (Polfa, Lublin, Poland), supplemented with a decreasing dose of penicillin and streptomycin as described earlier (Mlodawska *et al.* 2000). Subsequently, the follicles visible and palpable on the ovaries were counted and classified into 3 groups according to size: small (≤10 mm), medium (11–20 mm and 21–30) and large (>30 mm).

Cumulus-oocyte complexes were collected either by scraping or isolation of ovarian follicles. After collection, morphology of cumulus-oocyte complexes was estimated. Oocytes surrounded with compact cumulus cells (CM), compact corona radiata (CR) or expanded cumulus (EX) were cultured in 0.5 ml TCM 199* supplemented with L-glutamine* (0.1 g/l), Na pyruvate* (1 mM), PVA* (25 mg/l) myoinositol* (5 mg/l), ascorbic acid* (75 mg/l), LH* (0.1 iu/ml), FSH* (5 µg/ml), E2*(1 µg/ml), 20% FCS (Pro Animali Ltd, Wroclaw, Poland) and antibiotics (100 iu/ml penicillin; 100 µg/ml streptomycin, 0.25 µg/ml amphotericin; Gibco) for 26–30 h. CM and CR oocytes were cultured together as CMP group. Chromatin configuration was estimated just after oocyte collection or after *in vitro* maturation using Hoechst 33342* and fluorescent microscopy.

RESULTS AND DISCUSSION

The mean weight of 8–15 month old filly ovary was 35.7 ± 19.5 g ranging from 5.9 to 104.8 g and that of mare ovary was 93.5 ± 42.6 g ranging from 20.7 to 202.7 g (P<0.01; Table 1). A distinctive feature of the filly ovary is dark brown colouration of the ovarian tissue in the peripheral area of the greater curvature, distinctly visible on the longitudinal section of the ovary and pale yellowish-pink colouration of the tissue inside the

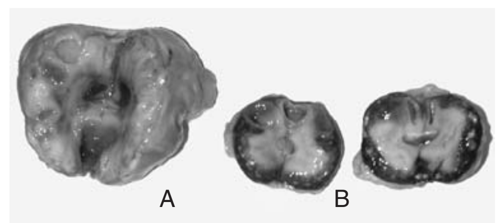

Fig 1: Mare (A) and filly (B) ovaries after sectioning. In the longitudinal section of filly ovaries there is visible dark brown colouration of the ovarian tissue in the peripheral area of the greater curvature and pale yellowish-pink colouration of the tissue inside the ovary and at depressed border. All cut surface of the mare ovary is yellowish-pink.

**Medium or chemical reagent originated from Sigma*

TABLE 1: Morphology of ovaries of 8–15 month old fillies and mares

Female	Weight of ovaries [g] x ± SD/ovary	No of observed follicles (x ± SD/ovary)		Percentage of follicles			
		Visible	Visible and palpable	Small (≤ 10 mm)	Medium (11-20 mm)	(20-30 mm)	Large (>30 mm)
Fillies (n=37)	35.7 ± 19.5[a]	4.0 ± 1.8[a]	6.2 ± 2.8[a]	60.4	32.2	4.6	2.8
Mares) (n=16	93.5 ± 42.6[b]	8.4 ± 3.6[b]	11.4 ± 3.5[b]	38.4	48.5	9.8	3.3

Values with different superscripts in columns are significantly different, [a], [b] = P<0.01

TABLE 2: Morphological assessment of cumulus-oocyte complexes collected from the ovaries of fillies and mares

Females	No of oocytes		Morphology of cumulus-oocyte complexes			
	n (100%)	x/female	CM n (%)	CR n (%)	EX n (%)	Deg n (%)
Fillies (n = 37)	238	6.4	110 (46.2)	16 (6.7)	45 (18.9)[a]	67 (28.2)[c]
Mares (n = 16)	145	9.1	62 (42.8)	15 (10.3)	52 (35.9)[b]	16 (11)[d]

Values with different superscripts in columns are significantly different, [a,b] = P<0.01 [c,d] = P<0.001
CM–oocytes surrounded with compact cumulus; CR–oocytes with compact corona radiata; EX – oocyte with expanded cumulus; Deg–degenerated oocytes

ovary and at the depressed border (Fig 1). This brown colouration, probably due to the remaining interstitial cells, debris or macrophages fades gradually over several years (Ginther 1992). All the cut surface of the ovary of the mare is yellowish-pink (Fig 1).

The present study showed almost a 2-fold lower number of ovarian follicles on the filly ovary compared to that of the mare (Table 1). Most (60.4%) of the filly follicles observed were ≤10 mm. Follicles >30 mm were found on the ovaries of 29.7% (11/37) fillies and 62.5% (10/16) mares. According to Ginther (1992), fillies generally reach puberty at about 1.5 years of age. However, fillies that are born during the first half of the year can reach puberty by spring of the next year, ie at the age of 12–15 months (Wesson and Ginther 1981; Ginther 1992). In our hemisphere mares start the reproductive season in early spring and continue up to late autumn. Thus, it can be assumed that some of the fillies used in this experiment were just about reaching puberty and first ovulation. It was confirmed by the presence of the corpus luteum which was found in about 48.7% (18/37) fillies and a large follicle (>30 mm) as mentioned above.

As shown in Table 2 a total of 238 filly oocytes (6.4/filly) were obtained, of which 46.2% had a compact multilayer cumulus and 6.7% were surrounded with compact corona radiata (Fig 2). From the ovaries of mares 145 oocytes (9.1/mare) were collected, of which 42.8 and 10.3% were CM and CR respectively. More EX oocytes were collected from mare ovaries than from the ovaries of fillies (35.9% vs. 18.9%; P<0.01). However, a higher percentage of degenerated oocytes was collected from the ovaries of fillies than from those of the mares (28.2% vs. 11%; P<0.001).

The use of fluorescent stain Hoechst 33342 for chromatin configuration assessment allows differentiation within germinal-vesicle stage of mare oocytes configuration: FN (diffusely fluorescent nucleus) and CC (chromatin condensed into one mass: TCC - tightly with smooth, oval edges or LCC - loosely with irregular outline) (Hinrichs and Schmidt 2000). Recent studies seem to suggest that chromatin configuration within the germinal vesicle indicates meiotic competence of the oocyte. The oocytes with CC configuration are meiotically competent while FN configuration represents viable oocytes, which did not yet have the ability to renew meiosis. The FN configuration is more frequent in oocytes having a compact cumulus than in those with an expanded cumulus (24% vs. 9%). Frequency of CC configuration in oocytes with a compact cumulus increases with follicular diameter and most oocytes from follicles >20 mm demonstrate CC configuration (Hinrichs 1997; Hinrichs and Schmidt 2000).

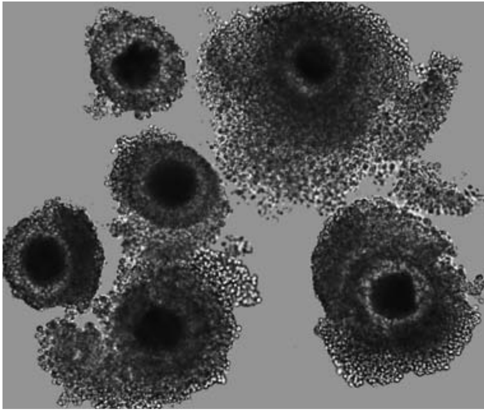

Fig 2: Filly oocytes with a compact cumulus and compact corona radiata immediately after collection (100x).

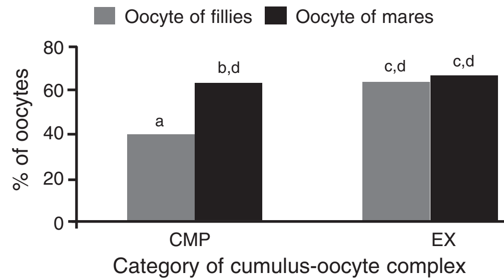

Fig 3: Percentage of filly and mare oocytes in metaphase II stage after in vitro culture. Bars with different superscripts are significantly different ^{a,b} = P<0.01 ^{c,b} P<0.05. CMP- oocytes with compact cumulus and compact corona radiata cells; EX - oocytes with expanded cumulus.

Of the filly CMP-oocytes (n=42) evaluated before culture, 6 (14.3%) showed FN configuration, 25 (59.5%) demonstrated CC (TCC/LCC) configuration and one oocyte (2.4%) was in diakinesis. Of the mare CMP oocytes (n=12) evaluated just after collection only one (8.3%) showed FN configuration and 8 (66.7%) had CC configuration. In the EX groups, FN and CC configurations were found on 0/12 and 8/12 (66.7%) oocytes of fillies and 1/19 (5.3%) and 11/19 (57.9%) oocytes of mares, respectively. The diakinesis stage was found in 2/12 (16.7%) oocytes of fillies and 4/19 (21%) oocytes of mares. The remaining filly and mare CMP and EX oocytes evaluated before culture were degenerated.

In the present study, the lowest maturation rate (40% ie 30/75) was obtained for filly CMP oocytes (Fig 3). Experiments conducted on mare oocytes showed that the proportion of mature oocytes increases with the growth of follicular diameter (Goudet *et al.* 1997; Hinrichs 1997; Hinrichs and Schmidt 2000). Goudet *et al.* (1997), obtained a lower maturation rate (20%) for oocytes with a compact cumulus originating from 5–9 mm follicles and higher (45%) when oocytes collected from 10–50 mm follicles were cultured. In our study about 60% follicles observed on filly ovaries were ≤10 mm, thus it could be assumed that most of the collected oocytes originated from these follicles. As mentioned above among the oocytes evaluated before culture, FN configuration was more frequent in filly CMP oocytes than in other oocyte groups. The above data seem to suggest that filly CMP oocytes may have lower ability to *in vitro* maturation than the remaining groups of oocytes.

Mare oocytes having initially expanded cumulus, show greater meiotic competence than oocytes with a compact cumulus (Hinrichs 1997). In our experiment, metaphase II stage attained a similar proportion of CMP (63.1% ie 41/65) and EX (66.7% ie 14/21) mare oocytes and EX (64% ie 16/25) filly oocytes (Fig 3). The lack of variation may be due to several factors. One of them may be the presence of CR oocytes (n=9) in the CMP group of mare oocytes. Recently, Torner (Torner *et al.* 2004) noted that at collection CR and EX oocytes of mares showed higher oxidative activity of mitochondria than oocytes with compact cumuli, which may indicate their greater meiotic competence. (In our study, 5 CR oocytes were also present in the filly CMP group). Maturation competence of mare CMP oocytes could have been also influenced by the size of the follicles from which oocytes were collected. As showed in Table 1, a considerable proportion of follicles observed on the mare ovaries was >10 mm, thus most of cultured oocytes could have come from medium size follicles. Likewise, additivies to culture medium could have increased the maturation rate of CMP oocytes. Further investigations are needed to determine meiotic competence and the ability for *in vitro* fertilisation of filly oocytes having both compact cumulus, corona radiata and expanded cumulus cells.

In conclusion, about 2-fold few ovarian follicles were observed on the ovaries of 8–15 month old fillies in comparison to mare ovaries. Oocytes of fillies and mares having initially

expanded cumulus show the same ability to *in vitro* maturation. In applied culture conditions filly CMP-oocytes seemed to be less competent to reach metaphase II stage than the oocytes with expanded cumuli.

REFERENCES

Armstrong, D.T., Holm, P., Irvine, B., Petersen, B.A., Stubbings, R.B., McLean, D., Stevens, G. and Seamark, R.F. (1992) Pregnancies and live birth from *in vitro* fertilisation of calf oocytes collected by laparoscopic follicular aspiration. *Theriogenology* **38,** 667-678.

Ginther, O.J. (1992) Reproductive biology of the mare. Basic and applied aspects. Equiservices, USA, Second edition.

Goudet, G., Bezard, J., Duchamp, G., Gerard, N. and Palmer, E. (1997) Equine oocyte competence for nuclear and cytolpasmic *in vitro* maturation: effect of follicle size and hormonal environment *Biol. Reprod.* **57,** 232-245.

Hinrichs, K. (1997) Cumulus expansion, chromatin configuration and meiotic competence in horse oocytes: A new hypothesis. *Equine vet. J.* **25,** 43-46.

Hinrichs, K. and Schmidt, A.L. (2000) Meiotic competence in horse oocytes: Interactions among chromatin configuration, follicle size, cumulus morphology, and season. *Biol. Reprod.* **62,** 1402-1408.

Marchal, R., Feugang, J.M., Perreau, C., Venturi, E., Terqui, M. and Mermillod, P. (2001) Meiotic and developmental competence of prepubertal and adult swine oocytes *Theriogenology* **56,** 17-29.

Mlodawska, W., Palmer, E., Duchamp, G., Okólski, A. and Bezard, J. (2000) Zona pellucida-sperm binding assay for equine oocytes. *J. Reprod. Fert.* **56,** 423-429.

O'Brien, J.K., Catt, S.L., Ireland, K.A., Maxwell, W.M.C. and Evans, G. (1997) *In vitro* and *in vivo* developmental capacity of oocytes from prepubertal and adult sheep. *Theriogenoloygy* **47,** 1433-1443.

Torner, H., Poehland, R., Kanitz, W., Goellnitz, K. and Alm, H. (2004) Parallel investigation into mitochondrial activity and chromatin configuration in horse oocytes during pre-ovulatory maturation. 3rd Meeting of the European Equine Gamete Group. *Havemeyer Foundation Monograph Series No 13,* Eds: J. Müller, Z. Müller and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 20-22.

Wesson, J.A. and Ginther, O.J. (1981) Influence of season and age on reproductive activity in pony mares on the basis of a slaughterhouse survey. *J. anim. Sci.* **52,** 119-129.

MAP KINASE AND AKT ACTIVITIES IN EQUINE OOCYTES DEPENDING ON CUMULUS MORPHOLOGY DURING MATURATION *IN VITRO*

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INTRODUCTION

At the present time, the reasons for the failure of equine oocytes to complete meiosis during *in vitro* culture are unknown, but could be linked to an alteration of the biochemical cascade of enzyme activity involved in the meiotic process. Resumption and progression of meiosis to metaphase II cannot be used as a determinant of further developmental competence of the oocyte. Extensive changes in protein synthesis and phosphorylation occur during this period and influence cytoplasmic maturation (Leibfried-Rutledge *et al.* 1989).

The meiotic cell cycle is controlled mainly by a phosphorylation-dephosphorylation regulatory cascade of protein kinases such as mitogen-activated protein kinase (MAPK, also referred to as extracellular signal-regulated protein kinase, or ERK). MAP kinase is a serine-threonine kinase activated during oocyte maturation and markedly involved in the resumption of meiosis: it is known to activate the maturation promoting factor (MPF) and induce germinal vesicle break down (GVBD) in Xenopus (Gotoh and Nashida 1995); it is a potential regulator of microtubule dynamics in the mouse and pig (Verlhac *et al.* 1994; Inoue *et al.* 1996); and it regulates events that lead to nuclear envelope assembly and disassembly (Moos *et al.* 1996). Furthermore it has been shown that in bovine oocytes MAPK is involved in regulating gene expression during meiotic maturation by phosphorylating the translation initiation factor eIF4E (Tomek *et al.* 2002). In this context it is of some interest that a specific repressor protein (4E-BP1) of eIF4E function exists which is also involved in this process (Tomek *et al.* 2002; Smiljakovic *et al.* 2003). It has been described in somatic cells that the protein kinase Akt contributes in the regulation of the biological activity of 4E-BPs (Sonenberg and Gingras, 1998).

In the several species such as pig, cattle and mouse, the 2 isoforms of MAP kinase (ERK1 and ERK2) were detected; in horse oocytes up to now only ERK2 has been observed (Goudet *et al.* 1998).

The aim of the study was to determine the presence and the activity of 2 isoforms (ERK1 and ERK2) of MAPK and Akt in equine oocytes depending on cumulus morphology and stage of meiotic development.

MATERIALS AND METHODS

Cumulus-oocyte complexes (COCs) were obtained either by follicular scraping of slaughterhouse ovaries or by follicle aspiration from living mares (Alm *et al.* 1997). According to their cumulus morphology, compact (n=144) or expanded (n=150), COCs were cultured in Hepes-buffered TCM 199 with FSH and 10% oestrus mare serum (EMS) for 0, 12, 24 (expanded) or 0, 12, 32 h (compact).

Immunoblotting with antibodies was carried out to identify the abundance and the phosphorylation state of 2 forms of MAPK, p42ERK2 and p44ERK1 as described earlier (Tomek *et al.* 2002). Akt kinase assay was performed after immunoprecipitation of total Akt in an *in vitro* kinases assay with GSK as an external substrate. The activity of Akt was determined by Western blotting using a specific phospho-GSK antibody.

For chromatin evaluation the oocytes were fixed 0, 12, 24 (expanded) or 0, 12, 32 h (compact) after culture in buffered formol saline (BFS) and stained with Hoechst 33258.

Results

Altogether, 308 COCs were recovered and 288 oocytes with compact or expanded cumulus were included into the study (3 x 10 compact and 3 x 10 expanded for the analyses of MAP kinase 0, 12, and 24 or 32 h of IVM; 108 chromatin evaluation after IVM).

Figure 1a shows the determination of activity of protein kinase Akt (PKB) with the external substrate GSK by kinase assay *in vitro*. The phosphorylation is different in compact and expanded COCs. At the time of recovery the phospohorylation was low and increased with a maximun at 12 h of IVM. In expanded COCs the phosphorylation level observed in compact COCs at 12 h after IVM was seen at the time of recovery. Looking at the chromatin we evaluated almost the same distribution of different developmental stages in compact at t0 and expanded at t12 (Fig 2). The higher level of phosphorylation correlated with the activation of chromatin.

The presence of MAPK depended on the cumulus morphology at recovery and on the duration of maturation as shown in Figure 1b. Both isoforms were detected, but in different manner.

In oocytes with expanded cumuli both isoforms were observed in non-phosphorylated form at the time of recovery. After 24 h of IVM both ERK2 and ERK1 were phosphorylated although the complete phosphorylation as described in bovine oocytes was not reached.

In oocytes from compact COCs both isoforms were present, but at a lower level than in expanded COCs. Twelve hours later one of the isoforms of MAPK – ERK2 – was partly phosphorylated. Up to the end of IVM (32 h after onset of maturation) both isoforms – ERK2, ERK1 – were phosphorylated but also not to the full extent.

In both COC-categories comparable results were obtained in the metaphase II-rate at the end of IVM (Fig 2).

Discussion and conclusion

During *in vitro* maturation, numerous cytoplasmic and nuclear events occur to prepare the oocyte for fertilisation and further development. These changes are induced by an increase in the activities of protein kinases such as mitogen-activated protein (MAP) kinases, which among others is involved in initiation and resumption of meiosis (Fissore *et al.* 1996).

ERK1 and ERK2 proteins are present in growing oocytes in slightly higher concentrations than in fully grown mouse oocytes (Harrouk and Clarke 1995). The present study shows that in horse oocytes there are differences in the phosphorylation up to the end of IVM not only depending on time but also on morphology. About 50% of ERK1 and ERK2 protein is phosphorylated at the end of IVM in oocytes of expanded COCs; in oocytes of compact, a significantly lower value is reached. In comparison to cattle oocytes, where high amounts of ERK1 and ERK2 phosphorylations were observed after 8 h of culture, and complete phosphorylation occurs after 24 h (Motlik *et al.*

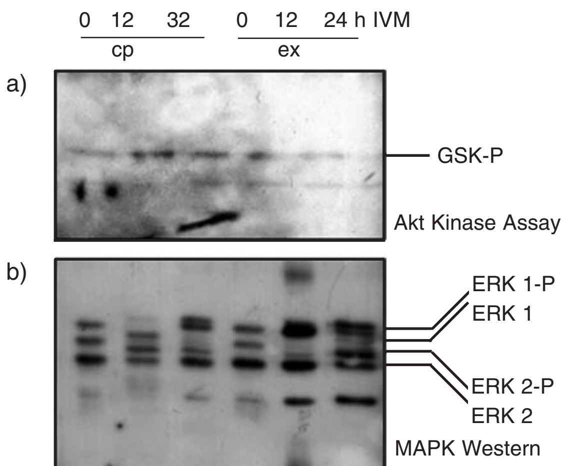

Fig 1: Analysis of activation of selected protein kinases depending on cumulus morphology and duration of IVM.

a) Determination of activity of protein kinase Akt (PKB) with the external substrate GSK by kinase assay in vitro.

b) Western Blot with anti-ERK 1 for the determination of phosphorylation of MAPK by band shift assay.

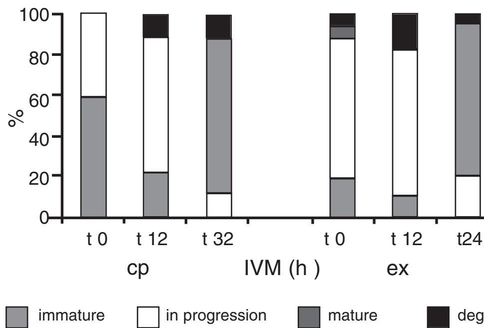

Fig 2: Distribution of chromatin configurations depending on cumulus morphology and on time of IVM (cp – compact, ex – expanded).

1998; Tomek *et al.* 2002), we observed lower levels in horse oocytes.

In conclusion, although the chromatin status in oocytes of compact and expanded COCs is similar at the end of IVM, there are differences in the abundance and in the level of phosphorylation of MAPK between different COC types during maturation *in vitro*. Reaching metaphase II configuration after IVM is not the only marker for further developmental competence of equine oocytes. Also, cumulus expansion during IVM is not necessarily related to nuclear maturation in equine oocytes as reported for mice and cattle.

These results of higher MAP kinase activity in expanded COCs correlate with previous results of higher developmental capacity of expanded COCs obtained in IVF experiments (Alm *et al.* 2001).

Acknowledgements

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References

Alm, H., Torner, H., Kanitz, W., Becker, F. and Hinrichs, K. (1997) Comparison of different methods for the recovery of horse oocytes. *Equine vet. J.* **25,** 47-50.

Alm, H., Torner, H., Blottner, S., Nürnberg G., and Kanitz, W. (2001) Effect of sperm cryopreservation and treatment with calcium ionophore or heparin on *in vitro* fertilisation of horse oocytes. *Theriogenology* **56,** 817-829.

Fissore, R.A., He, C.L. and Vande Woude, G.F. (1996) Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. *Biol. Reprod.* **55,** 1261-1270.

Gotoh, Y. and Nashida, E. (1995) Activation mechanism and function of the MAP kinase cascade. *Mol. Reord. Dev.* **42,** 486–492.

Goudet, G., Belin, F., Bezard, J. and Gerard, N. (1998) Maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK) expression in relation to oocyte competence for *in vitro* maturation in the mare. *Mol. Human Reprd.* **4,** 563-570.

Harrouk, W. and Clarke, H.J. (1995) Mitogen-activated protein (MAP) kinase during the acquisition of meiotic competence by growing oocytes in the mouse. *Mol. Reprod. Dev.* **41,** 29-36.

Inoue, M., Naito, K. and Aoki, F. (1996) Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes. *Zygote* **3,** 265-271.

Leibfried-Rutledge, M.L., Florman, H.M. and First, N.L. (1989) The molecular biology of mammalian oocyte maturation. In: Schatten H, Schatten G. (eds.): The Molecular Biology of Fertilisation, New York: Academic Press Inc., 259-275.

Moos, J., Xu, Z., Schultz, R.M. and Kopf, G.S. (1996) Regulation of nuclear envelope assembly/ disassembly by MAP kinase. *Dev. Biol.* **175,** 358-361.

Motlik, J., Pavlok, A., Kubelka, M., Kalous, J. and Kalb, P. (1998) Interplay between cdc2 kinase pathway during maturation of mammalian oocytes. *Theriogenology* **49**, 461-469.

Smiljakovic, T., Melo Sterza, F., Kubelka, M., Vohnikova, Z. and Tomek, W. (2003) Aspects of cytoplasmic maturation of bovine oocytes: Interplay between MAPK, mRNA binding complex and cytoplasmic mRNA metabolism in regulation of translation. *Biotechnology in Animal Husbandry* **19,** 1-8.

Sonenberg, N. and Gingras, A.C. (1998) The mRNA 5´cap-binding protein eIF4E and the conton of cell growth. Current opinion in cell biology **10,** 268–275.

Tomek, W., Melo Sterza, F.A., Kubelka, M., Wollenhaupt, K., Torner, H., Anger, M. and Kanitz, W. (2002) Regulation of translation during *in vitro* maturation of bovine oocytes: The role of MAP kinase, eIF4E (cap binding protein) phosphorylation and eIFeE-BP1. *Biol. Reprod.* **66,** 1274-1282.

Verlhac, M.-H., Kubiak, J.Z., Clarke, H.J. and Maro, B. (1994) Microtubule and chromatin behaviour follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development* **120,** 1017-

PARALLEL INVESTIGATION INTO MITOCHONDRIAL ACTIVITY AND CHROMATIN CONFIGURATION IN HORSE OOCYTES DURING PRE-OVULATORY MATURATION *IN VITRO*

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INTRODUCTION

Mitochondria play a vital role in the oocyte to provide ATP for fertilisation and pre-implantation embryo development. Data in human and bovine oocytes suggest that the efficiency of mitochondrial respiration in oocytes is closely correlated with the rate of embryo development after fertilisation (Stojkovic *et al.* 2001; Wilding *et al.* 2001).

Electron microscopy studies of oocytes have revealed dynamic morphological changes of mitochondria during the pre-ovulatory period. At this time, they are the most prominent organelles in the ooplasm (Motta *et al.* 2000). They form voluminous aggregates with the smooth endoplasmatic reticulum (SER), tubules and vesicles. These mitochondrial-SER aggregates (m-SER) could be involved in the production of a reservoir of energy prior to fertilisation.

However, the role of mitochondria during maturation, fertilisation and embryonic development is not fully understood (Bavister and Squirrell 2000). There appear to be large differences in the timing of changes in activity and in distribution of mitochondria during oocyte maturation among species [sheep (Smaili *et al.* 2000); human (Wilding *et al.* 2001), mouse (Calarco 1995; van Blerkom *et al.* 2002), cattle (Stojkovic *et al.* 2001), sea urchin (Sukhomlinova *et al.* 2001)]. For the most part, these studies have been done in oocytes without characterisation of their mitochondrial activity and the situation of chromatin during meiotic progression.

Therefore, the aim of the study was to examine chromatin configuration of the nucleus, mitochondrial distribution and activity in parallel studies in the same oocyte. Oocytes with defined cumulus oocyte complex (COC) morphology from follicular puncture of mares at different intervals of subsequent pre-ovulatory maturation *in vitro* (0, 18, 24 or 32 h) were used.

MATERIALS AND METHODS

COCs at different intervals *in vitro* maturation (IVM) of (n=146) were incubated for 30 min in PBS containing 3% BSA and 200 nM MitoTracker Orange CMTM Ros (Molecular Probes, Oregon, USA) under culture conditions. The cell-permeant probe MitoTracker Orange-fluorescent tetramethyl-rosamine (M-7510) is readily sequestered only by actively respiring organelles depending upon their oxidative activity. Then the cumulus cells were removed as described above, and the oocytes were washed 3 times in pre-warmed PBS without BSA. The oocytes were then fixed for 15 min at 37°C using freshly prepared 2% paraform-aldehyde in Hank's balanced salt solution. The probe M-7510 contains a thiol-reactive chloromethyl moiety and can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable fluorescent conjugate, which is well retained after cell fixation over a period of 6 weeks. After fixation the oocytes were washed 3 times in PBS and stained with Hoechst 33342 for additional chromatin observation, mounted on slides under cover slips and stored in the refrigerator prior to fluorescence microscopy evaluation.

An epifluorescence microscope (Jenalumar, Carl Zeiss, Jena, Germany) was used for all experiments. Emission wavelengths were separated by a 540 nm dichroic mirror followed by further filtering through a 570 nm long pass filter (red emission). The mitochondrial distribution pattern of horse oocytes was characterised only by observation (up to 500 x magnification) of the labelled mitochondria which were oxidative

TABLE 1: Chromatin configuration in horse oocytes depending on time of maturation *in vitro* and cumulus morphology at the time of recovery (t_0) (n=146)

Cumulus morphology t_0	Time of IVM (h)	n	Meiotic configuration (%) Immature	in progression of meiosis	Mature
Compact	0	14	57.1	42.9	0
	18	12	16.0	66.8	16.6
	24	12	0	33.3	66.7
	32	10	0	0	100
Corona radiata	0	10	30.0	70.0	0
	18	12	0	50.0	50.0
	24	14	0	35.7	64.3
	32	12	0	0	100
Expanded	0	16	12.6	81.2	6.2
	18	16	0	31.2	68.8
	24	18	0	22.3	77.7

TABLE 2: Mitochondrial distribution in horse oocytes depending on time of maturation *in vitro* and cumulus morphology at the time of recovery (t_0) (n=146)

Cumulus morphology t_0	Time of IVM (h)	n	Type of mitochondrial distribution (%)					
			homogeneous			heterogeneous		
			fine	crystalline	granulated	crystalline	granulated	cluster
Compact	0	14	78.6	0	0	21.4	0	0
	18	12	16.6	16.6	16.6	50.2	0	0
	24	12	0	0	24.9	24.9	50.2	0
	32	10	0	0	0	0	100	0
Corona radiata	0	10	0	30.0	20.0	30.0	22.0	0
	18	12	0	0	16.6	16.6	66.8	0
	24	14	0	0	0	28.6	71.4	0
	32	12	0	0	0	0	83.3	16.6
Expanded	0	16	0	25.0	25.0	0	50.0	0
	18	16	0	0	6.3	25.0	68.7	0
	24	18	0	0	0	11.1	55.5	33.3

active. The distribution patterns were mainly classified as homogeneous: distribution even throughout the cytoplasm, or heterogeneous: distribution unequal within the cytoplasm.

The fluorescence intensity (µA) was measured by the Nikon Photometry System P 100 (Nikon, Düsseldorf, Germany). Microscope adjustments and photomultiplier settings were kept constant for all experiments. Oocytes were positioned in the plane of focus, and the area of measurement was adapted to the size of the oocyte. The data of emission intensity/oocyte were reduced by compensation for the background fluorescence. The chromatin configuration at the same object was estimated by UV-fluorescence.

RESULTS

The highest percentage of meiotically immature oocytes (in the stage of germinal vesicle [GV]) was found in compact COCs at the time of recovery (Table 1). The proportion of immature oocytes in all COC-categories decreased as the interval post IVM increased. The proportion of oocytes in progression of meiosis was highest in oocytes with expanded and corona radiata cumuli immediately after recovery. A significant proportion of oocytes in metaphase II at 18 h post IVM was observed only in the cultured expanded COCs. Oocytes from all cultured COC-categories reached at 24 h of IVM a sufficient proportion of M II (64.3% to 77.7%).

The mitochondrial distribution pattern of horses oocytes was characterised by 2 main distribution features: labelled mitochondria were distributed evenly throughout the cytoplasm – homogeneously; labelled mitochondria were distributed unequally within the cytoplasm – heterogeneously. These 2 main groups of mitochondrial distribution were divided further

depending upon the aggregation of mitochondria: small pixels of fluorescence intensity – fine; small linear spots of fluorescence intensity – crystalline; bigger areas of fluorescence with irregular shapes – granulated; and aggregates of bigger fluorescent areas – cluster. The fine aggregation type was found in homogeneously distributed oocytes only; the cluster aggregation type only in the heterogeneously distributed oocytes.

Depending on time of pre-ovulatory maturation *in vitro* the type of mitochondrial distribution changed from homogeneous distribution of fine fluorescence labelled spots to a formation of heterogeneous cluster of mitochondria at the end of IVM (Table 2). In horses the mitochondrial activity in oocytes after IVM (n=146) depended on cumulus morphology at recovery and increased in oocytes as the interval post IVM increased ($P<0.05$). Lowest oxidative activity of mitochondria was found in oocytes from compact COCs during IVM. Comparatively high levels of oxidative activity were found in oocytes with corona cadiata or expanded cumulus before IVM (468.6 ± 9.4 µA; 272.6 ± 11.4 µA, respectively) and 24 h after IVM (622.1 ± 12.1 µA, 521.7 ± 9.8, respectively).

Discussion and conclusion

The present study was conducted to monitor the changes in mitochondria and chromatin of horse oocytes during pre-ovulatory maturation *in vitro* related to COC-morphology at time of recovery. MitoTracker Orange labelling of respiring mitochondria and photometric measurement of fluorescence intensity were used to determine the respiratory activity/oocyte.

This investigation shows that the oocytes progressed to metaphase II between 24 and 32 h during IVM. The data show (Table 1) that the stage of meiotic progression is related both to the increased time interval post IVM and to the quality of the cumulus investment such as cumulus expansion at t_0.

All alterations in the oocyte nucleus and in the surrounding cumulus of the observed horse COCs were associated with dramatic changes in the respiratory activity, and the pattern of aggregation and distribution of ooplasmic mitochondria. During this short time of pre-ovulatory maturation *in vitro* a strong time-dependent mitochondrial aggregation and distribution was observed.

The mitochondrial activity in the oocytes as a parameter for intact metabolism is influenced by progression of pre-ovulatory maturation as well as cumulus expansion, meiotic configuration in the nucleus and distribution and cluster formation of mitochondria. It seems to be that the better developmental competence of expanded COCs and of oocytes with corona radiata cells is based on a higher metabolism of their energy-containing compounds in the ooplasm.

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References

Bavister, B. and Squirrell, J. M. (2000) Mitochondrial distribution and function in oocytes and early embryos. *Hum. Reprod. Suppl.* **2,** 189-198.

Calarco, P. G. (1995) Polarisation of mitochondria in the unfertilised mouse oocyte. *Dev. Genet.* **16,** 36-43.

Motta, P. M., Nottola, S. A., Makabe, S. and Heyn, R. (2000) Mitochondrial morphology in human fetal and adult female germ cells. *Hum. Reprod. Suppl.* **2,** 129-147.

Smaili, S. S., Hsu, Y. T., Youle, R. J. and Russell, J. T. (2000) Mitochondria in Ca2+ signalling and apoptosis. *Bioenerg. Biomembr.* **32,** 35-46.

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Goncalves, P. B. and Wolf, E. (2001) Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilisation and culture. *Biol. Reprod.* **64,** 904-909.

Sukhomlinova, My., Kireyev, I. I., Fais, D., Guidice, G. and Polyakov, VYu. (2001) Quantitative and ultrastructural analysis of the chondriome in ovogenesis and embryo genesis of the sea urchin Paracentrotu lividus. 2. Growth and proliferation of mitochondria in embryo genesis. *Membr. Cell. Biol.* **14,** 605-615.

van Blerkom, J., Davis, P., Mathwig, V. and Alexander, S. (2002) Domains of high-polarized mitochondria may occur in mouse and human oocytes and early embryos. *Hum. Reprod.* **17,** 393-406.

Wilding, M., Dale, B., Marino, M., diMotteo, L., Alviggi, C., Pisaturo, M. L., Lombardi, L. and De Placido, G. (2001) Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum. Reprod.* **16,** 909-917.

INVOLVEMENT OF THE INTERLEUKIN-1 SYSTEM IN FOLLICULAR AND OOCYTE MATURATION IN THE MARE

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INTRODUCTION

Interleukins (IL) are well-known for their involvement in the immune system and their role during inflammation. IL-1 is organised as a gene system that includes 2 bioactive ligands, IL-1α and IL-1β, 2 types of receptors, IL-1R1 and IL-1R2, and a natural receptor antagonist, IL-1RA, which regulates IL-1 ligand biological activity. A growing body of evidence suggests that in the ovary ovarian cells could represent sources and targets of interleukins (Gérard *et al.* 2004).

The aim of the present work was to examine the IL-1 system gene expression in various cell types of the equine ovary, and to study the role of IL-1 during follicular and oocyte maturation.

MATERIALS AND METHODS

To study the localisation of the IL-1 system components in the mare ovary, cumulus-oocyte complexes (COCs) were collected using transvaginal ultrasound-guided follicular puncture (Goudet *et al.* 1997). Oocytes and cumulus cells were isolated from pre-ovulatory and subordinate follicles and were analysed either at collection or after *in vitro* culture (30 h in TCM199 + 0.5% BSA + 50 ng/ml EGF). Granulosa cells and follicular fluids were collected from the largest follicle at the early dominance stage (diameter 24±3 mm) or during the pre-ovulatory maturation phase, 0, 6, 12, 24 and 34 h after induction of ovulation (Martoriati and Gérard 2003). Granulosa cells were also collected from subordinate follicles. RNA was extracted from oocytes, cumulus and granulosa cells and was used to study the expression of IL-1 system members in the various cell types (Martoriati *et al.* 2002; Martoriati and Gérard 2003). Follicular fluids were studied by gel electrophoresis and immunoblotting (Martoriati and Gérard 2003).

To study the *in vitro* effect of IL-1β and IL-1RA on oocyte nuclear maturation and cumulus expansion, immature COCs were collected from slaughterhouse ovaries. COCs were cultured in 8 different media for 30 h (31–50 COCs per medium). Media were as follows: medium 1 (TCM199 + 0.5% BSA), medium 2 (medium 1 + 50 ng/ml IL-1β), medium 3 (medium 1 + 5 µg/ml eLH), medium 4 (medium 3 + 50 ng/ml IL-1β), medium 5 (medium 4 + 50 ng/ml IL-1RA), medium 6 (medium 1 + 50 ng/ml EGF), medium 7 (medium 6 + 50 ng/ml IL-1β), medium 8 (medium 7 + 50 ng/ml IL-1RA), (Martoriati *et al.* 2003a). After culture, nuclear maturation of oocytes was evaluated by DNA staining.

TABLE 1: Detection of IL-1 system components mRNA by RT-PCR in various equine ovarian cell types

		IL-1α	IL-1β	IL-1RA	IL-1R1	IL-1R2
Oocytes	at collection	-	+++	-	-	+
	after culture	-	+	-	-	+
Cumulus cells	at collection	-	+	+++	++	+
	after culture	-	+	_	+/-	+
Granulosa cells		-	+	+	-	+

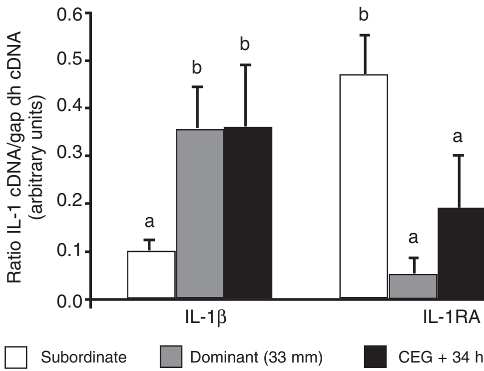

Fig 1: Relative amount of PCR products expressed in granulosa cells from subordinate, dominant and pre-ovulatory follicles. a, b are significantly different (P<0.05).

In order to study the *in vivo* effect of IL-1β and IL-1RA on follicular and oocyte maturation, IL-1β or IL-1RA were injected intra-follicularly in pre-ovulatory follicles (Martoriati *et al.* 2003b). A transvaginal ultrasound-guided injection was performed when the diameter of the dominant follicle reached 30–34 mm. The 4 experimental groups were: 1) IL-1β group: intra-follicular injection of IL-1β (1 µg/2 ml PBS) plus iv injection of physiological serum; 2) IL-1RA group: intra-follicular injection of IL-1RA (1 µg/2 ml PBS) plus iv injection of physiological serum; 3) PBS group, intra-follicular injection of 2 ml of PBS plus iv injection of saline; 4) CEG group, intra-follicular injection of PBS plus iv injection of crude equine gonadotropins (CEG, 20 mg), used to induce ovulation in the mare. 80 mares were used. In 40 mares, COCs from dominant/injected follicles were collected by transvaginal ultrasound-guided aspiration 38 h after intra-follicular injection. Oocyte nuclear stage was assessed by Hoechst staining. The ovarian activity of the other 40 mares was assessed by rectal ultrasound scanning to determine the time of ovulation after treatments.

Results

Localisation of the IL-1 system components in the mare ovary

The results indicated that equine oocytes expressed IL-1β and IL-1R2 genes, that cumulus cells expressed all the IL-1 system members but IL-1α, and that granulosa cells expressed IL-1β, IL-1RA and IL-1R2 (Table 1). In oocytes, IL-1β mRNA is significantly lower after *in vitro* culture than at collection. Similarly in cumulus cells, IL-1RA and IL-1R1 mRNA decreased during *in vitro* culture. In the dominant follicle, the levels of IL-1β and IL-1RA transcripts in granulosa cells changed during pre-ovulatory maturation (not shown). Actually, IL-1β mRNA content increased during the growth of the dominant follicle, reached a maximum 6 h after induction of ovulation, then fell and increased again from 12–34 h after induction of ovulation. IL-1RA mRNA level was very low 6 h after induction of ovulation, and progressively increased until ovulation (not shown). Moreover, the author observed that subordinate follicles display a lower level of IL-1β and a higher level of IL-1RA than dominant preovulatory follicles (Fig 1).

TABLE 2: Percentage of metaphase II oocytes after 30 h incubation in different culture media. Different superscripts indicate significant differences. See M and M for media composition

	Experiment 1	Experiment 2	Experiment 3
Medium 1	31.0%(a,b)		
Medium 2	28.6%(a)		
Medium 3	57.1%(c)	47.8 %(a)	
Medium 4	37.2%(a,b)	19.2 %(b)	
Medium 5		53.5 %(a)	
Medium 6			68.8%(a)
Medium 7			35.5%(b)
Medium 8			56.3%(a)

In vitro effect of IL-1β and IL-1 RA on oocyte maturation

As shown in Table 2, IL-1β significantly inhibits the eLH and the EGF -induced *in vitro* maturation of oocytes. The addition of IL-1RA to the culture medium inhibited the effect of IL-1β and restored the maturation rate observed in the presence of eLH or EGF alone.

In vivo effect of IL-1β and IL-1RA on ovulation and oocyte meiosis resumption

As shown in Fig 2a, the ovulation profile observed in Group 1 (IL-1β group) is similar to the ovulation profile observed in Group 4 (CEG group). In contrast, intra-follicular injection of IL-1RA, similarly to PBS, had no effect on ovulation

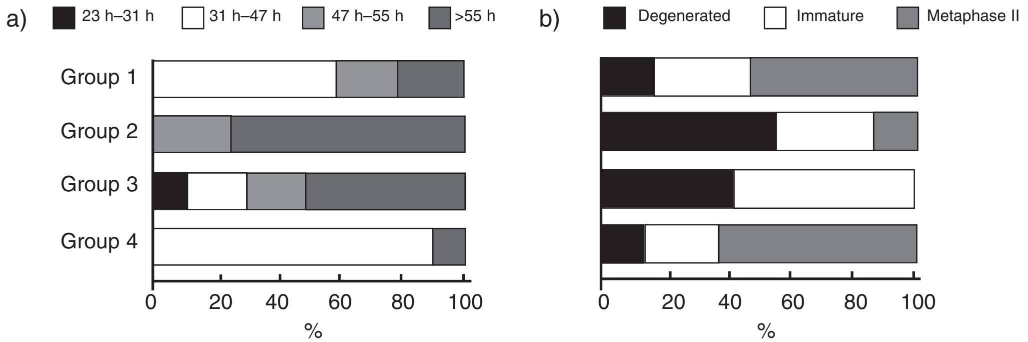

Fig 2: Ovulation profiles (a) and oocyte maturation stage (b) after intra-follicular injection of IL-1β (Group 1), IL-1RA (Group 2) or PBS (Groups 3 and 4) in the dominant follicle. Mares from the Group 4 received an ovulatory dose of CEG (iv) whereas mares from other groups received saline.

delay (Groups 2 and 3, respectively). As shown in Fig 2b, oocyte meiosis resumption was similar in Groups 1 and 4, the groups which received IL-1β intra-follicularly and CEG intravenously, respectively. In contrast, intra-follicular injection of IL-1RA or PBS did not induce nuclear oocyte maturation.

Discussion

In conclusion, we demonstrated for the first time in the mare the presence of a complete IL-1 system genes in the ovary. IL-1β and IL-1R2 transcript have been shown in oocytes and IL-1β, IL-1RA, IL-1R1 and IL-1R2 mRNA in cumulus cells. It was also demonstrated that IL-1β, IL-1R2 and IL-1RA genes are expressed in equine granulosa cells. The expression of IL-1β gene in granulosa cells and the content of IL-1β in follicular fluid seem to be regulated by gonadotropins. This suggests that IL-1β could be an intermediate paracrine factor involved in ovulation. The regulatory effect of IL-1β on cumulus-oocyte complexes during *in vitro* maturation suggests that this factor is involved in the physiology of cumulus-oocyte complexes by regulating meiosis resumption. The inhibitory effect of IL-1RA that the author observed *in vitro* demonstrated that the action of IL-1β is receptor-mediated. Finally, it was demonstrated clearly that IL-1β injection into the dominant follicle induced the *in vivo* oocyte maturation and the ovulation process whereas IL-1RA had no effect. The involvement of IL-1 in oocyte maturation and ovulation strongly suggests that IL-1 could be of crucial importance for ovarian physiology in the mare.

References

Gérard, N., Caillaud, M., Martoriati, A., Goudet, G. and Lalmanach, A. C. (2004) Interleukin-1 system and female reproduction: a review. *J. Endocr.* In press.

Goudet, G., Bézard, J., Duchamp, G., Gérard, N. and Palmer, E. (1997) Equine oocyte competence for nuclear and cytoplasmic *in vitro* maturation: effect of follicle size and hormonal environment. *Biol. Reprod.* **57,** 232-245.

Martoriati, A., Caillaud, M., Goudet, G. and Gérard, N. (2003a) Inhibition of *in vitro* maturation of equine oocytes by interleukin 1β via specific receptors. *Reprod.* **126,** 509-515.

Martoriati, A., Duchamp, G. and Gérard, N. (2003b) *In vivo* effect of epidermal growth factor, interleukin-1b, and interleukin-1RA on equine preovulatory follicles. *Biol. Reprod.* **68,** 1748-1754.

Martoriati, A. and Gérard, N. (2003) Interleukin-1 (IL-1) system gene expression in granulosa cells: kinetics during terminal preovulatory follicle maturation in the mare. *Reprod. Biol. and Endocr.* **42,** 1-10.

Martoriati, A., Lalmanach, A. C., Goudet, G. and Gérard, N. (2002) Expression of interleukin-1 (IL-1) system genes in equine cumulus-oocyte complexes and influence of IL-1β during *in vitro* maturation. *Biol. Reprod.* **67,** 630-636.

THE *IN VITRO* EFFECT OF GROWTH HORMONE (GH) ON HYALURONAN SYNTHASES, CONNEXINS 32 AND 43 EXPRESSION, AND GH RECEPTOR MRNA EXPRESSION IN EQUINE COCS.

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INTRODUCTION

During cumulus-oocyte complexes (COCS) maturation, cumulus expansion is observed in all mammalian species. Different events occur during cumulus expansion. On one hand, cumulus cells secrete hyaluronan that accumulates among cells. On the other hand, there are some modifications of gap junctions which contain proteins belonging to the connexin family.

It is well known that gonadotropins influence cumulus expansion. In the last few years, there has been evidence that other factors are involved in the maturation of cumulus-oocyte complexes (COCs).

The role of growth hormone (GH) in ovarian function, follicular growth, and steroidogenesis is well known, and some evidence shows a positive effect of GH on cumulus expansion (Hull and Harvey 2001).

The aim of this study was to determine if GH influences connexins 32 and 43 and hyaluronan synthases (Has) 1, 2 and 3 expression in equine cumulus cells. The authors also examined the expression of GH receptor mRNA in equine oocytes and cumulus cells.

MATERIALS AND METHODS

Equine COCs were collected by transvaginal ultrasound-guided aspiration on standing mares. All follicles larger than 5 mm were punctured at the end of the follicular phase, ie 35 h after induction of ovulation (Duchamp *et al.* 1987). Immature COCs were cultured individually in 20 µl of maturation medium covered with mineral oil in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 30 h. The control maturation medium was TCM 199 with Earle salts supplemented with 5 mg/ml BSA, 1 µg/ml oestradiol, and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone). It was supplemented or not with either 0.5 µg/ml equine GH (eGH) or 5 µg/ml equine luteinising hormone (eLH). After *in vitro* culture, COCs were stripped of their cumulus cells. Oocytes and cumulus cells were frozen in liquid nitrogen and stored at –80°C separately. *In vivo* matured COCs from pre-ovulatory follicle were also collected and stored in order to obtain a mature control. Some of the immature COCs from small follicles (<30 mm) were stored just after collection to obtain an immature control.

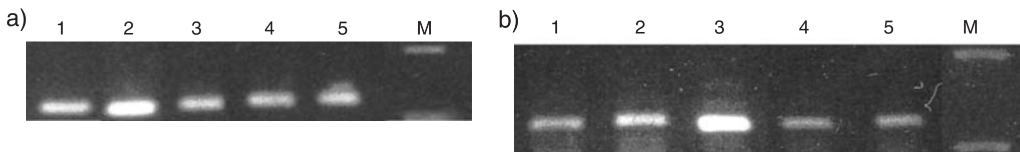

Fig 1: Expression of GH receptor mRNA in equine: a) oocytes; b) cumulus cells.
Lane 1: after in vitro maturation in the control medium; Lane 2: after in vitro maturation in the control medium supplemented with eGH; Lane 3: after in vitro maturation in the control medium supplemented with eLH; Lane 4: after in vivo maturation; Lane 5: at collection and Lane M: molecular weight markers (100 bp DNA ladder, Promega), the 100 and 200 bp markers are visualised.

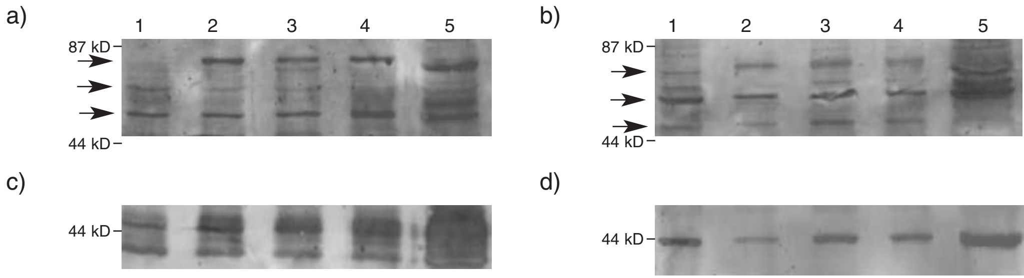

Fig 2: Representative profiles of protein expression in equine cumulus cells. a) Has 1; b) Has 3; c) connexin 43; d) actin. Lane 1: cumulus cells from COCs at collection; Lane 2: cumulus cells from COCs after in vitro maturation in the control medium; Lane 3: cumulus cells from COCs after in vitro maturation in the control medium supplemented with eGH; Lane 4: cumulus cells from COCs after in vitro maturation in the control medium supplemented with eLH; Lane 5: cumulus cells from COCs after in vivo maturation.

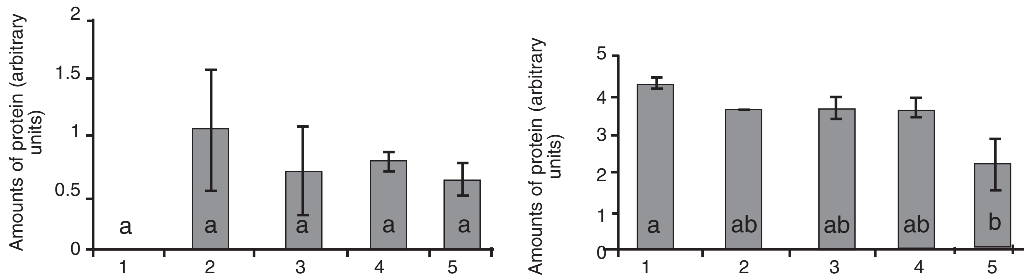

Fig 3: Semiquantitative analysis of the expression of Has 1 and connexin 43 in equine cumulus cells. Relative amount of the upper band revealed with the Has 1 antibody (A) and the connexin 43 antibody (B). Values with different superscripts differ significantly (P<0,05). 1: cumulus cells from COCs at collection; 2: cumulus cells from COCs after in vitro maturation in the control medium; 3: cumulus cells from COCs after in vitro maturation in the control medium supplemented with eGH; 4: cumulus cells from COCs after in vitro maturation in the control medium supplemented with eLH; 5: cumulus cells from COCs after in vivo maturation.

Total RNA was extracted from oocytes and cumulus cells, and RNA preparation was then reverse transcribed. Polymerase chain reaction (PCR) amplification of GH receptors cDNA was performed with specific primers.

Cumulus cells were submitted to gel electrophoresis (1D SDS-PAGE) and immunoblotting on PVDF membranes. Six successive immunoblots (Has 1, Has 2, Has 3, connexin 43, connexin 32, and actin) were performed per membrane.

Results

GH receptor mRNA expression in oocytes and cumulus cells

Amplification of cDNA with equine GH receptor-specific primers resulted in one PCR product with the expected size of 124 bp (Fig 1).

This GH receptor product was detected in all oocytes and cumulus cells whether they were analysed after collection, after *in vivo* maturation, or after *in vitro* maturation in one of the 3media used.

Effect of GH in maturation medium on hyaluronan synthases, connexin 43, and connexin 32 expression

The expression of Has1, Has2, and Has3, connexin 43 and 32, and actin were analysed in equine cumulus cells using gel electrophoresis and immunoblotting (Fig 2). The antibodies raised against Has 1 and Has 3 revealed 3 major bands between 44 and 87 kDa. The connexin 43 antibody revealed 2 major bands at 43 kDa and 45 kDa and minor bands running between 43 and 45 kDa. The actin antibody revealed one band at 44 kDa. No signal could be detected with the Has 2 and

connexin 32 antibodies in our conditions. The amounts of Has 1, Has 3 and connexin 43 were analysed as the ratio to the actin amount for each lane. It was observed that Has 1 increased during *in vitro* and *in vivo* maturation and that connexin 43 decreased during *in vivo* maturation. On the other hand, GH did not influence the expression of Has 1, Has 3, and connexin 43 in cumulus cells (Fig 3).

DISCUSSION

The aim of the present work was to determine if GH influences connexins 32 and 43 and Has 1, Has 2 and Has 3 expression in equine cumulus cells.

The authors first examined the expression of GH receptor mRNA in COCs. It was shown that GH receptor mRNA was present in oocyte and cumulus cells whatever the maturation conditions. mRNA for GH receptor has also been observed in other species such as sheep or bovine (Eckery *et al.* 1997; Kölle *et al.* 1998).

Dell'Aquila *et al.* (2001) demonstrated that GH improves cumulus expansion in equine species. This result allowed us to conclude that equine COCs have a functional GH receptor. Cumulus expansion is associated with hyaluronan accumulation among cumulus cells and modification of gap junctions, which contain connexins. In our conditions, antibodies raised against mouse Has 1 and 3 revealed 3 major bands which probably correspond to 3 equine Has in cumulus cells. Moreover, the molecular masses are consistent with masses observed for mouse Has. No signal has been detected with the anti Has 2 antibody. We could hypothesise that mouse Has 2 antibodies do not cross-react with the equine Has. Our results showed that Has 1 expression was very low in cells from immature COCs and increased during *in vitro* or *in vivo* maturation. Equine Has 1 may be the most important in the synthesis of hyaluronan by cumulus cells during expansion.

In our experimental conditions, no signal could be detected with connexin 32 antibody. Connexin 32 protein expression in equine cumulus cells may be too low to be detected with our antibody.

The data showed that equine cumulus cells express connexin 43 proteins. Similar results were obtained in other species (bovine: Sutovsky *et al.* 1993; porcine: Shimada *et al.* 2001). In our conditions, we observed two bands that could correspond to phosphorylated forms of connexin 43. The intensity of the 45 kDa band decreased during COC *in vivo* maturation. This result is consistent with data from porcine species (Shimada *et al.* 2001).

The addition of eGH or eLH to the culture medium did not alter the expression of connexin 43. eGH does not influence the connexin 43 expression in equine cumulus cells during *in vitro* maturation.

In conclusion, it was shown that the GH receptor mRNA is present in oocytes and cumulus cells whatever the maturation stage. Has 1 expression increased during *in vitro* and *in vivo* maturation and connexin 43 expression decreased during *in vivo* maturation. In our experimental conditions, GH had no effect on the expression of Has 1, Has 3, and connexin 43 in cumulus cells.

REFERENCES

Dell'Aquila, M. E., Caillaud, M., Maritato, F., Martoriati A., Gérard, N., Duchamp, G., Aiudi, G., Deals, P. F. and Goudet, G. (2001) Factors influencing nuclear maturation and cumulus expansion in the equine oocyte. European Equine Gamete Group (EEGG), *Havemeyer Foundation Monograph Series No 5.*

Duchamp, G., Bour, B., Combarnous, Y and Palmer, E. (1987) Alternative solutions to hCG induction of ovulation in the mare. *J. Reprod. Fertil. Suppl. 35,* 221-228.

Eckery, D. C., Moeller, C. L., Nett, T. M. and Sawyer, H. R. (1997) Localisation and quantification of binding sites for follicle-stimulating hormone, luteinising hormone, growyh hormone, and insulin-like growth factor I in sheep ovarian follicles. *Biol. Reprod.* **57,** 507-513.

Hull, K. L. and Harvey, S. (2001) Growth hormone: roles in female reproduction. *J. Endocr.* **168,** 1-23.

Kölle, S., Sinowatz, F., Boie, G. and Lincoln, D. (1998) Developmental changes in the expression of the growth hormone receptor messenger ribonucleic acid and protein in the bovine ovary. *Biol. reprod.* **59,** 836-842.

Shimada, M., Maeda, T. and Terada, T. (2001) Dynamic changes of connexin-43, gap junctional protein, in outer layers of cumulus cells are regulated by PKC and PI3-kinase during meiotic resumption in porcine oocyte. *Biol. Reprod.* **64,** 1255-1263.

Sutovsky, P., Fléchon, J. E., Fléchon, B., Motlik, J., Peynot, N., Chesné, P. and Heyman, Y. (1993) Dynamic changes of gap junctions and sytoskeleton during *in vitro* culture of cattle oocyte cumulus complexes. *Biol. Reprod.* **49,** 1277-1287.

THE EFFECT OF CYSTEAMINE ON *IN VITRO* NUCLEAR MATURATION AND GSH CONTENT IN EQUINE OOCYTES

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In recent years, assisted reproduction technology has improved reproductive performance in mammals. In bovine species, extensive research has been carried out in the field of *in vitro* embryo production (IVP), and satisfactory results are now achieved routinely for *in vitro* oocyte maturation (IVM), spermatozoa capacitation, and *in vitro* fertilisation (IVF) techniques. On the contrary, assisted reproduction methods have produced only limited success in equine species. A possible reason is the lack of detailed information about the physiological mechanisms that control follicle development, oocyte maturation and fertilisation in this species.

Previous studies have shown that the low IVP success rate in the equine species is mainly due to the large proportion of oocytes that fail to reach a complete cytoplasmic and nuclear maturation (metaphase II) during *in vitro* culture (Goudet *et al.* 1997; Squires *et al.* 1999; Hinrichs and Schmidt 2000)

Recent studies indicate that oxidative stress could be an important factor affecting oocyte viability during *in vitro* culture (Guerin *et al.* 2001). Glutathione (GSH) is a major intracellular free thiol known to play an important role in protecting the oocyte from oxidative damage and in male pronucleus formation after fertilisation. GSH precursors such as cysteamine, added during *in vitro* culture of bovine, porcine and ovine oocytes, have been shown to increase intracellular glutathione synthesis and consequently to improve *in vitro* maturation (IVM) and embryo development (de Matos *et al.* 1995; Abeydeera *et al.* 1999; de Matos *et al.* 2002).

The aim of our study was to determine whether equine oocytes benefit from the addition of cysteamine during IVM and to compare the GSH content of horse oocytes after *in vivo* and *in vitro* maturation.

MATERIALS AND METHODS

Oocyte collection and in vitro maturation

In vivo matured cumulus-oocyte complexes (COCs) were collected by transvaginal ultrasound-guided aspiration from ovulatory follicles and intra-oocyte GSH concentration was determined after cumulus cell removal and MII stage assessment. Immature COCs were collected by *in vivo* aspiration or from ovaries isolated from slaughtered animals. They were matured either in 500 µl of control medium (TCM199 + 20% fetal calf serum + 50 ng/ml EGF), or control medium supplemented with 100 µM cysteamine, or in defined medium (TCM199 + 0.4% of BSA), or defined medium supplemented with 50 ng/ml EGF with and without 100 µM cysteamine, for 30 h at 38.5°C and 5% CO_2 in air. After culture, COCs

TABLE 1: Percentage of matured oocytes after IVM of COCs collected *in vivo* and from ovaries of slaughtered animals

Maturation conditions	n	%MII
Collected in vivo		
M199 + Serum + EGF	51	76.5[a]
M199 + Serum + EGF + Cyst	43	79.0[a]
Slaughterhouse		
M199 + Serum + EGF	34	73.5[a]
M199 + Serum + EGF + Cyst	40	70.1[a]
M199 + BSA 0.4% + EGF	48	70.8[a]
M199 + BSA 0.4% + EGF + Cyst	46	69.6[a]
M199 + BSA 0.4%	37	51.3[b]

a, b: $P<0.05$

were denuded and stained with 1 µg/ml bisbenzimide fluorescent dye (Hoechst 33342) and observed in a drop on a slide with an epifluorescence microscope in order to determine the nuclear stage of meiotic progression. Oocytes that reached the MII stage were analysed for GSH intra-oocyte concentration.

Intracellular GSH determination

The oocyte GSH content was determined by 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB)-GSH reductase recycling micro-GSH assay according to Baker *et al.* (Baker *et al.* 1990) with slight modifications. Briefly, oocytes were suspended in 50 µl of water and frozen and thawed on melting ice 3 times. Standards containing from 0.19 to 200 pmol in 50 µl were prepared in water, simultaneously with the samples. A volume of 50 µl of each sample and standards was added in a 96 well microtitre plate. Reaction mixture was prepared with 0.15 mm of DTNB, 0.2 mm of NADPH and 1.0 U GSH reductase/ml (final concentrations) in 0.1 M phosphate buffer supplemented with 1 mm EDTA, pH 7.8. Samples and standards were analysed at 405 nm in a microtitre plate reader (SpectraCount, Packard, USA) with one initial mixing and repeated-reads functions at 2 min intervals for 30 min.

Statistical analysis

Oocyte maturation rates were compared using Chi-square test. The non-parametric Kruskal-Wallis test was performed using StatXact 4 software (CYTEL, Massachusetts, USA) in order to compare means of intra-oocyte GSH between groups.

RESULTS AND DISCUSSION

As shown in Table 1, the nuclear maturation rate after IVM of *in vivo* collected immature oocytes was similar to those isolated from ovaries of slaughtered animals. However, oocytes matured in M199 supplemented only with BSA exhibited a maturation rate significantly lower than other treatments (P<0.05). The addition of cysteamine did not influence the maturation rate in all treatments used in both *in vivo* collected immature oocytes and those isolated from slaughtered ovaries. GSH content is shown in Table 2. After IVM in presence of serum, oocytes collected *in vivo* or from ovaries of slaughtered animals had a similar GSH content. The addition of cysteamine did not influence the GSH concentration at the end of maturation period. *In vivo* matured MII oocytes displayed a similar GSH content to oocytes matured *in vitro* in the presence of serum and EGF. In the absence of serum and in the presence of BSA as protein source in IVM medium, the GSH content was significantly higher than all other maturation conditions tested. GV oocytes analysed at collection displayed the significantly lowest GSH content.

The results of this study demonstrate that *in vitro* maturation conditions are able to stimulate a significant increase in intra-oocyte GSH content, similarly to *in vivo* matured oocytes. Moreover, the addition of cysteamine affects neither the GSH

TABLE 2: GSH content (mean±se) in immature and metaphase II oocyte after IVM of COCs collected *in vivo* and from ovaries of slaughtered animals

Treatments	Cysteamine	n	GSH content (pmol/oocyte)
In vivo collected			
MII at collection	-	11	5.89 ± 0.48[b]
GV at collection	-	10	1.25 ± 0.37[a]
M199 + serum + EGF	-	13	4.74 ± 0.46[b]
M199 + serum + EGF	+	15	5.06 ± 0.43[b]
Slaughterhouse			
GV at collection	-	11	1.16 ± 0.29[a]
M199 + serum + EGF	-	13	4.84 ± 0.33[b]
M199 +serum + EGF	+	14	5.09 ± 0.37[b]
M199 + BSA 0.4%	-	19	7.18 ± 0.43[c]
M199 + BSA0.4% + EGF	-	15	7.87 ± 0.70[c]
M199 + BSA0.4% + EGF	+	18	9.89 ± 0.60[c]

[a, b]: P<0.05

content in MII oocytes nor the maturation rate of equine oocytes. We hypothesised that the higher GSH content in media containing BSA instead of serum may be due to unknown factor(s) present in the serum that can inhibit GSH synthesis. Analogously to other species, intra-oocyte GSH concentration increased during *in vivo* and *in vitro* maturation. However, *in vitro* fertilisation of equine oocyte after IVM still remains unsuccessful both in the presence or absence of cysteamine. Based on our data we conclude that the low *in vitro* developmental competence of equine oocyte is not due to the lack or defective GSH synthesis but to other factor(s) that could be involved in cytoplasmic maturation, oocyte-sperm interaction and early development.

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REFERENCES

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Prather, R.S. and Day, B.N. (1999) Glutathione content and embryo development after *in vitro* fertilisation of pig oocytes matured in the presence of a thiol compound and various concentrations of cysteine. *Zygote* **7,** 203-210.

Baker, M.A., Cerniglia, G.J. and Zaman, A. (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal. Biochem.* **190,** 360-365.

de Matos, D.G., Furnus, C.C., Moses, D.F. and Baldassarre, H. (1995) Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. *Mol. Reprod. Dev.* **42,** 432-436.

de Matos, D.G., Gasparrini, B., Pasqualini, S.R. and Thompson, J.G. (2002) Effect of glutathione synthesis stimulation during *in vitro* maturation of ovine oocytes on embryo development and intracellular peroxide content. *Theriogenology* **57,** 1443-1451.

Goudet, G., Bezard, J., Duchamp, G., Gerard, N. and Palmer, E. (1997) Equine oocyte competence for nuclear and cytoplasmic *in vitro* maturation: effect of follicle size and hormonal environment. *Biol. Reprod.* **57,** 232-245.

Guerin, P., El Mouatassim, S. and Menezo, Y. (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod.* **7,** 175-189.

Hinrichs, K. and Schmidt, A.L. (2000) Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. *Biol. Reprod.* **62,** 1402-1408.

Squires, E.L., McCue, P.M. and Vanderwall, D. (1999) The current status of equine embryo transfer. *Theriogenology* **51,** 91-104.

TRANSVAGINAL FOLLICULAR ASPIRATIONS IN MARES – EFFECT ON HEART RATE AND BEHAVIOUR

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INTRODUCTION

Application of transvaginal ultrasound-guided follicular aspiration, for research purposes or in the valuable mare, requires careful consideration of the biological consequences for the animal. Possible acute effects, like pain, risk of injury or infection must be assessed and the long-term effects on ovarian function and fertility have to be considered.

It was shown recently that repeated ovarian punctures of mares induce reparative fibrosis of the ovarian stroma and may, in rare cases, cause chronic oophoritis (Bøgh *et al.* 2003). Increased amount of connective tissue in the ovarian stroma and the tunica albuginea has also been described in cattle after multiple aspirations (Kruip *et al.* 1994; Kurykin and Majas 1996; Petyim *et al.* 2001; McEvoy *et al.* 2002; Chastant-Maillard *et al.* 2003). In spite of these morphological changes to the ovarian stroma, folliculogenesis, and the ability to ovulate pre-ovulatory follicles regularly, to develop corpora lutea and to establish pregnancies is unaffected in mares which have undergone repeated follicular punctures (Kanitz *et al.* 1995; Bøgh *et al.* 2003).

In mares, transvaginal ultrasound-guided follicular aspirations are generally conducted under sedation and central analgesia. Several reports conclude that the procedure is well tolerated, with signs of minor discomfort which may be attributed to traction on the ovarian ligaments (Brück *et al.* 1992, 2000; Cook *et al.* 1992, 1993; Bracher *et al.* 1993). The objective of the present study was to assess change in heart rate and behavioural signs of discomfort in mares during the procedures involved in transvaginal ultrasound-guided aspiration.

MATERIALS AND METHODS

During the procedures involved in the preparation for and conduction of transvaginal ultrasound-guided follicular aspiration, behaviour and heart rates were recorded in 5 Standardbred mares. The heart rate was measured using a heart rate monitor (Polar Pacer, Polar Electro, OY, Kempele, Finland). The degree of discomfort was retrospectively characterised into 4 categories (minor = 1, mild = 2, moderate = 3, severe = 4) according to the intensity and combination of the following behavioural parameters: sighing, shifting bodyweight, shifting legs, looking back, arching back, muscle tremor, lifting of hind leg, scraping with front leg, kicking with hind leg, groaning and grinding teeth on a bar.

Experiment I

In an initial experiment, measurements were taken during the following procedures: resting in the box, resting in the crush (considered as baseline value), emptying of the rectum, transrectal ultrasound scanning, perineal wash, injection of sedativum and analgesicum, insertion of transducer and needle guide into the vagina, additional insertion of hand into the rectum, traction of the ovary towards the transducer, holding of the ovary under traction (fixation), follicular puncture and flushing of the follicular cavity. Sedation was achieved with detomidine hydrochloride (10 g/kg bwt, Domosedan^R, Smith Kline Beecham, Ballerup, Denmark). After 5 min, central analgesia was induced with butorphanol tartrate (30 g/kg bwt, Torbugesic^R, Fort Dodge Laboratories Inc., Iowa, USA).

Experiment II

TABLE 1: Pain assessment (minor = 1, mild = 2, moderate = 3, severe = 4) in 5 mares during various procedures involved in follicular aspiration under sedation (Experiment I)

Treatment	Mare 1	Mare 2	Mare 3	Mare 4	Mare 5
Resting in box	-	-	-	-	-
Resting in crush	-	-	-	-	-
Emptying rectum	1	-	-	1	1
Ultrasound scanning	-	-	-	1	1
Perineal wash	-	-	-	-	1
No manipulation 1-3 min	-	-	-	-	-
Injection of sedative	-	-	-	1	-
Injection analgesic	-	-	-	1	-
Insertion of needle guide	-	1	-	-	1
Additionally hand resting in rectum	-	-	-	-	-
Traction on ovary1	2	2	1	2	1
Puncture/flushing of follicle 1	1	2	1	3	1
Puncture/flushing of follicle 2	1	1	1	3	1
Puncture/flushing of follicle 3	1	na	2	3	na
Traction on ovary 2	2	2	1	na	-
Puncture/flushing of follicle 1	2	1	-	na	-
Puncture/flushing of follicle 2	3	na	1	na	-
No manipulation 1-3 min	-	-	-	-	-

na = not applicable

TABLE 2: Pain assessment (minor = 1, mild = 2, moderate = 3, severe = 4) in 5 mares during various procedures during transvaginal follicular aspiration before and after sedation (Experiment II)

Treatment	Mare 1	Mare 2	Mare 3	Mare 4	Mare 5
Resting in box	-	-	-	-	-
Resting in crush	-	-	-	-	-
Emptying rectum	1	-	-	2	1
Ultrasound scanning	-	-	-	-	1
Perineal wash	-	-	-	-	1
No manipulation 1-3 min	-	-	-	-	-
Insertion of needle guide	2	-	1	1	2
Additionally hand resting in rectum	-	-	-	2	-
Traction on ovary 1	3	3	3	4	2
Fixation of ovary 1	1	1	1	4	-
Traction on ovary 2	3	3	2	4	2
Fixation of ovary 2	3	3	1	2	3
Follicular puncture	1	2	2	2	3
No manipulation 1-3 min	-	-	-	-	-
Injection of sedative	-	-	-	-	-
Injection analgesic	-	-	-	-	-
Perineal wash	-	-	-	-	-
Insertion of needle guide	1	-	1	1	-
Additionally hand resting in rectum	1	-	-	1	-
Traction on ovary 1	2	2	1	3	1
Fixation of ovary 1	1	-	-	-	-
Traction on ovary 2	2	1	1	3	1
Fixation of ovary 2	-	-	-	3	-
No manipulation 1-3 min	-	1	-	-	-
Sum of pain scores	21	16	13	32	17

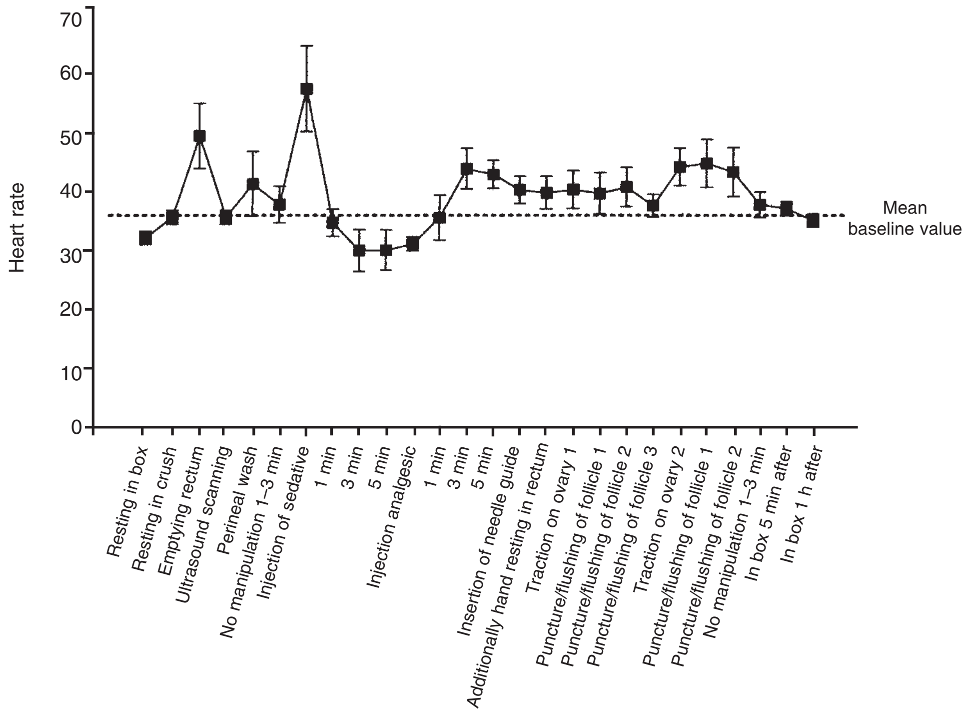

Fig 1: Heart rate (mean ± SEM) in 5 mares during various procedures involved in follicular aspiration under sedation (Experiment 1).

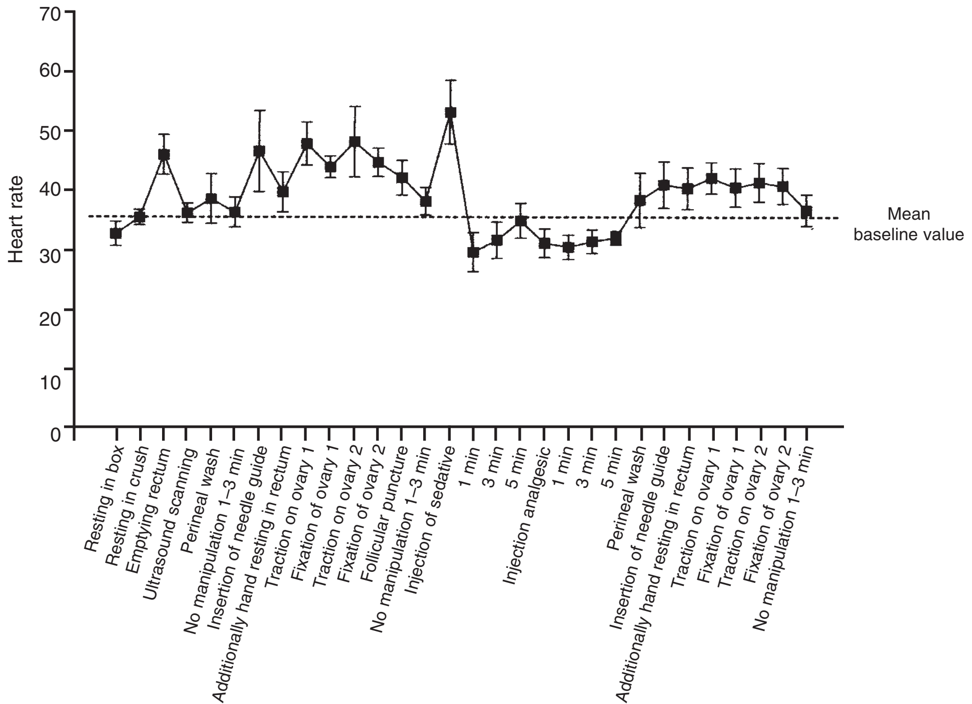

Fig 2: Heart rate (± SEM) in 5 mares during various procedures involved in follicular aspiration before and after sedation (Experiment 2).

In a second experiment, measurements were taken during the same procedures before and after sedation and analgesia.

RESULTS

Experiment I

In all mares, baseline values of the heart rate were below 40/min. Without sedation, emptying the rectum induced an elevation of the heart rate in all mares. The first intravenous injection resulted in all mares in a short, pronounced increase of the heart rate. After sedation and analgesia, the heart rate was maintained at a slightly elevated level without any distinct deviations during the procedures of ovarian traction, follicular aspiration and flushing (Fig 1).

Minor signs of discomfort were observed in individual mares during emptying of the rectum, rectal ultrasound scanning, perineal washing, intravenous injection and insertion of transducer and needle guide into the vagina. After sedation, traction on the ovarian ligaments induced minor to mild reactions in all 5 mares. Reactions in response to follicular puncture and flushing varied between minor to moderate. Signs of discomfort and pain were expressed more often and more pronounced by Mare 4 compared to the other 4 animals (Table 1).

Experiment II

Similarly to the first experiment, heart rates increased briefly in response to emptying of the rectum in the unsedated mare and to the first intravenous injection. Elevations of the heart rate were also induced by insertion of transducer and needle guide into the vagina and by traction on the ovarian ligaments. The heart rate was slightly lower while the ovaries were held under traction or when a needle was inserted into the ovary of the unsedated mare. After the mares were sedated and analgesia induced, the increase in heart rate was less distinct for any of these procedures (Fig 2).

Similar to Experiment I, the degree of pain reactions differed distinctly between the individual mares as visualised by the sum of the pain scores (Table 2). In unsedated mares, none to mild signs of discomfort were observed during emptying of the rectum, ultrasound scanning, perineal wash, insertion of transducer and needle guide into the vagina and the hand resting in the rectum. Minor to severe reactions were noticable in almost all mares during traction on the ovarian ligaments, fixation of the ovary towards the transducer and follicular puncture. Severe reaction such as a combination of groaning, looking back, shifting legs, lifting hind leg and arching back, were only obsered in Mare 4 during ovarian traction and fixation. After sedation and analgesia, signs of discomfort were less pronounced.

CONCLUSIONS

The results of the present study indicate that routine procedures of gynaecological examination such as emptying of the rectum, rectal ultrasound scanning or perineal washing, as well as intravenous injections may cause elevations of heart rate and/or slight behavioural reactions. Individual mares may experience these procedures as minor discomfort, but distinct reactions of avoidance were not observed.

In the sedated mare, the procedure of transvaginal ultrasound-guided follicular aspiration conducted under well controlled conditions was generally well tolerated with only minor to mild signs of discomfort. As indicated by heart rate elevations and behavioural reactions, this was clearly attributable to the traction on the ovarian ligaments and to a lesser extent due to follicular puncture. However, distinct signs of pain may be observed in individual mares, in which short ovarian ligaments impede a close contact between the ovary and transducer. In these mares, transvaginal follicular aspiration may have to be refrained from.

ACKNOWLEDGEMENTS

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REFERENCES

Bøgh, I.B., Brink, P., Jensen, H.E., Lehn-Jensen, H. and Greve, T. (2003) Ovarian function and morphology in the mare after multiple follicular punctures. *Equine vet. J.* **35,** 575-579.

Bracher, V., Parleviet, J., Fazeli, A.R., Pieterse, M.C., Vos, P.L.A.M., Dieleman, S.J., Taverne, M.A.M. and Colenbrander, B. (1993) Repeated transvaginal ultrasound-guided follicle aspiration in the mare. *Equine vet. J.* **15,** 75-78.

Brück, I.B., Raun, K., Synnestvedt, B. and Greve, T.

(1992) Follicle aspiration in the mare using a transvaginal ultrasound-guided technique. *Equine vet. J.* **24,** 58-59.

Brück, I.B., Høier, R., Synnestvedt, B. and Greve, T. (2000) Steroid concentrations in follicular fluid aspirated repeatedly from transitional and cyclic mares. *Theriogenology* **54,** 877-888.

Chastant-Maillard, S., Quinton, H., Lauffenburger, J., Cordonnier-Lefort, N., Richard, C., Marchal, J., Mormede, P. and Renard, J.P. (2003) Consequences of transvaginal follicular puncture on well-being in cows. *Reprod.* **125,** 555-563.

Cook, N.L., Squires, E.L., Ray, B.S., Cook, B.S. and Jasko, D. J. (1992) Transvaginal ultrasonically guided follicular aspiration of equine oocytes: preliminary results. *J. equine vet. Sci.* **12,** 204-207.

Cook, N.L., Squires, E.L., Ray, B.S. and Jasko, D.J. (1993) Transvaginal ultrasound-guided follicular aspiration of equine oocytes. *Equine vet. J. Suppl.* **15,** 71-74.

Kanitz, W., Becker, F., Alm, H. and Torner, H. (1995) Ultrasound-guided follicular aspiration in mares. *Biol. Reprod. Mono.* **1,** 225-231.

Kruip, T.H.A.M., Boni, R., Wurth, Y.A., Roelofsen, M.W.M. and Pieterse, M.C. (1994) Potential use of ovum pick-up for embryo production and breeding in cattle. *Theriogenology* **42,** 675-684.

Kurykin, J. and Majas, L. (1996) Ultrasound-guided puncture of follicles in heifers. *Acta vet. Balt. Estonian Veterinary Review, Suppl.* 30-33.

McEvoy, T.G., Thompson, H., Dolman, D.F., Watt, R.G., Reis, A. and Staines, M.E. (2002) Effects of epidural injections and transvaginal aspiration of ovarian follicles in heifers used repeatedly for ultrasound-guided retrieval of ova and embryo production. *Vet. Rec.* **151,** 653-658.

Petyim, S., Båge, R., Forsberg, M., Rodríguez-Martínez, H. and Larsson, B. (2001) Effects of repeated follicular punctures on ovarian morphology and endocrine parameters in dairy heifers. *J. vet. Med.* **48,** 449-463.

SESSION III:

Embryos

ABNORMALITIES OF PLOIDY IN EQUINE EMBRYOS PRODUCED *IN VIVO* OR *IN VITRO*

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INTRODUCTION

Chromosomally abnormal cells are a fairly common finding in morphologically normal mammalian conceptuses (Hare *et al.* 1980; Long and Williams 1982; Murray *et al.* 1986; Viuff *et al.* 1999; Bielanska *et al.* 2002), and have been proposed as a major cause of early embryonic loss in a number of species, including the horse (*Equus caballus:* Blue 1981; Ball 1988). In cattle (Hare *et al.* 1980), sheep (Murray *et al.* 1986) and pig conceptuses (Long and Williams 1982), conventional cytogenetic analysis of cells in metaphase has suggested that most spontaneous chromosomal aberrations are various types and degrees of mixoploidy, ie the abnormal embryos are predominantly diploid-polyploid mosaics. Recently, the analysis of chromosomal abnormalities in embryos has been revolutionised by the application of fluorescent in situ hybridisation (FISH), in which DNA probes are used to selectively label specific chromosomes. Most significantly, because FISH can be carried out on interphase nuclei, a large proportion of an embryo's cells can be analysed without the need to force the cells into metaphase during a preparatory period of cell culture. FISH has thus made it possible to demonstrate that a large proportion of *in vitro* produced human (Bielanska *et al.* 2002) and bovine (Viuff *et al.* 1999) blastocysts are mixoploid, and that polyploid cells are a more common finding in *in vitro* than *in vivo* produced bovine blastocysts (Viuff *et al.* 1999).

Although chromosomal abnormalities have also been proposed as a significant contributor to early embryonic loss in the horse (Blue 1981; Ball 1988), their occurrence has yet to be proven. Indeed, the few studies that have been published have used conventional karyotypic analysis and were unable to demonstrate any chromosomal abnormalities in equine conceptuses (Blue 1981; Romagnano *et al.* 1987), and only a low incidence of aberrations in spermatozoa (Scott and Long 1980) or oocytes (King 1990; Lechniak *et al.* 2002). Not surprisingly then, the effect of *in vitro* production on the incidence of chromosomal abnormalities in equine embryos has yet to be reported. The aim of the current study was to validate FISH probes for the identification of equine chromosomes in interphase nuclei, and to use these probes to examine the incidence of abnormal ploidy in *in vivo* and *in vitro* produced embryos.

MATERIALS AND METHODS

Embryo production and spread preparations

Embryos were flushed from the uterus of mares on Day 6 or 7 after ovulation (*in vivo*) or produced by *in vitro* maturation of oocytes from slaughtered mares, intracytoplasmic sperm injection and subsequent culture for 6 days in the oviduct of a progesterone-treated ewe (*in vitro*). The *in vitro* embryos were frozen using a 4-step glycerol protocol, to allow their transport to the laboratory, and thawed immediately prior to the preparation of the cell spreads. The cell spreads were prepared by lysing an embryo with a 0.1% Tween/HCl solution and letting the cells disperse onto a glass microscope slide. The dispersed cells were then fixed with a 3:1(v:v) mixture of methanol:glacial acetic acid, dried and stored at –80°C.

Production and validation of FISH probes

FISH probes were prepared from bacterial artificial chromosomes specific for equine chromosomes 2 and 4 (BACs: a gift from the

Biological Resources Centre, INRA, France). After culture of the bacteria and isolation of the equine DNA sequences, the probes were labelled with either biotin-16-dUTP or digoxigenin-11-dUTP (both from Roche Diagnostics, Penzberg, Germany) so that they could be distinguished using an epifluorescence microscope as red (avidin-CY3) or green (sheep-antiDIG-FITC), respectively. The remaining nuclear DNA was counterstained with 4',6'-diamino-2-phenyl-indole (DAPI; Vector Laboratories, Burlingame, CA, USA). Binding of the FISH probes to the appropriate regions of the appropriate chromosomes was verified by labelling equine lymphocytes arrested in metaphase. The utility of the probes for labelling interphase nuclei was also validated using lymphocytes before, finally, the embryos were stained.

Scoring system

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

RESULTS

Cell numbers

In total, 44 embryos (22 *in vivo* and 22 *in vitro* produced) were examined. However, 2 of the *in vitro* produced embryos were excluded from further analysis because of an abnormally low cell number (<30 cells/embryo). The remaining *in vivo* and *in vitro* produced embryos had averages of approximately 1600 and 150 nuclei per embryo, respectively.

Abnormal ploidy

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

DISCUSSION

Chromosomal abnormalities have often been advanced as a cause of early embryonic loss in the mare (Blue 1981; Ball 1988) despite the absence of any concrete proof for their occurrence. In the current study, we used FISH with chromosome specific probes to demonstrate that chromosomally abnormal cells can be found in macroscopically normal equine embryos. However, the majority of the 'abnormal' embryos detected were mixoploids (2n/3n, 2n/4n) that contained only a small proportion of polyploid nuclei, and it is unclear whether such low degrees of mixoploidy would actually compromise embryo viability. For example, if the majority of the abnormal cells were located in the trophectoderm rather than the inner cell mass, as appears to be the case in porcine (Long and Williams 1982) and bovine mixoploid embryos (Viuff *et al.* 2002), those embryos with only a few polyploid cells would probably have been viable. On the other hand, it is very unlikely that either of the 2 embryos with more than 50% polyploid nuclei would have given rise to a live foal at term. Interestingly, as in other species (Viuff *et al.* 1999), *in vitro* production (IVP) of embryos tended to increase the incidence of chromosomally abnormal cells (40% vs 18% for *in vivo* embryos); a phenomenon that may well lead to a reduced viability of IVP embryos.

Of course, the current study examined only very gross chromosome abnormalities of chromosome number (of ploidy) whereas, in human embryos, autosomal trisomy is a much more common finding in spontaneously aborted pregnancies (Eiben *et al.* 1990). It is therefore probable that, by not examining all 32 chromosome pairs, the current study underestimated the true incidence of chromosomal aberrations in horse embryos. Future studies could usefully examine the effects of maternal age and/or the precise manner of *in vitro* embryo production on the incidence of chromosomal

abnormalities, and investigate whether the abnormal cells tend to be annexed to the trophectoderm or not.

REFERENCES

Ball, B.A. (1988) Embryonic loss in mares. Incidence, possible causes, and diagnostic considerations. In: *The veterinary clinics of North America. Equine practice. Reproduction*, Ed: S.D. Van Camp, W.B. Saunders company, Philadelphia. pp 263-290.

Bielanska, M., Tan, S.L. and Ao, A. (2002) High rate of mixoploidy among human blastocysts cultured *in vitro*. *Fert. Steril.* **78,** 1248-1253.

Blue, M.G. (1981) A cytogenetical study of prenatal loss in the mare. *Theriogenology* **15,** 295-309.

Eiben, B., Bartels, I., Bahr-Porsch, S., Borgmann, S., Gatz, G., Gellert, G., Goebel, R., Hammans, W., Hentemann, M., Osmers, R., Rauskolb, R. and Hausmann, I. (1990) Cytogenetic analysis of 750 spontaneous abortions with the direct-preparation method of chorionic villi and its implications for studying genetic causes of pregnancy wastage. *Am. J. hum. genet.* **47,** 656-663.

Hare, W.C., Singh, E.L., Betteridge, K.J., Eaglesome, M.D., Randall, G.C., Mitchell, D., Bilton, R.J. and Trounson, A.O. (1980) Chromosomal analysis of 159 bovine embryos collected 12 to 18 days after estrus. *Can. J. Genet. Cytol.* **22,** 615-626.

King, W.A. (1990) Chromosome analysis of horse oocytes cultured *in vitro*. *Genet. Sel. Evol.* **22,** 151-160.

Lechniak, D., Wieczorek, M. and Sosnowski, J. (2002) Low incidence of diploidy among equine oocytes matured *in vitro*. *Equine vet. J.* **34,** 738-740.

Long, S.E. and Williams, C.V. (1982) A comparison of the chromosome complement of inner cell mass and trophoblast cells in day-10 pig embryos. *J. Reprod. Fert.* **66,** 645-648.

Murray, J.D., Moran, C., Boland, M.P., Nancarrow, C.D., Sutton, R., Hoskinson, R.M. and Scaramuzzi, R.J. (1986) Polyploid cells in blastocysts and early fetuses from Australian Merino sheep. *J. Reprod. Fert.* **78,** 439-446.

Romagnano, A., Richer, C.L., King, W.A. and Betteridge, K.J. (1987) Analysis of X-chromosome inactivation in horse embryos. *J. Reprod. Fert. Suppl* **35,** 353-361.

Scott, I.S. and Long, S.E. (1980) An examination of chromosomes in the stallion (Equus caballus) during meiosis. *Cytogenet. Cell Genet.* **26,** 7-13.

Viuff, D., Palsgaard, A., Rickords, L., Lawson, L.G., Greve, T., Schmidt, M., Avery, B., Hyttel, P. and Thomsen, P.D. (2002) Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. *Mol. Reprod. Dev.* **62,** 483-488.

Viuff, D., Rickords, L., Offenberg, H., Hyttel, P., Avery, B., Greve, T., Olsaker, I., Williams, J.L., Callesen, H. and Thomsen, P.D. (1999) A high proportion of bovine blastocysts produced *in vitro* are mixoploid. *Biol. Reprod.* **60,** 1273-1278.

A NEW METHOD FOR NON-SURGICAL EMBRYO TRANSFER AND DEEP UTERINE INSEMINATION IN THE MARE

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Embryo transfer is undoubtedly the way forward for breeding Sporthorses and the related technology can also provide a therapeutic alternative for genetically valuable mares that are either incapable of maintaining a pregnancy or produce inferior foals due to endometrial and/or placental insufficiency. Their embryos can be transferred surgically, via flank or midline laparotomy incisions, or non-surgically by transcervical deposition of the embryo into the uterine lumen. Laparoscopic and transvaginal ultrasound-guided intra-uterine embryo transfer techniques have also been described (Gastal *et al.* 2002) but transcervical transfer still remains the preferred method for its speed, simplicity, reduced costs and minimal post transfer complications.

Pregnancy rates after non-surgical transfer are usually lower than those achieved by surgical transfer, particularly in the hands of less experienced operators. For example, Iuliano *et al.* (1985) reported a surgical transfer pregnancy rate of 72% compared to 45% for non-surgical transfer. These lower non-surgical pregnancy rates have been attributed variously to release of $PGF_{2\alpha}$ (Kask *et al.* 1997) and/or oxytocin (Handler *et al.* 2001) induced by manipulation and/or penetration of the cervix, or uterine infection or other localised inflammatory responses coupled with reflux of embryos back through the cervix (Squires *et al.* 1999).

Argentina is renowned internationally for its utilisation of embryo transfer techniques in the polo pony breeding industry and has possibly the highest rate of equine embryo transfer in the world. For example, Riera and McDonough (1993) reported 353 pregnancies from the non-surgical transfer of 580 embryos (61%). More recently, Losinno *et al.* (2000) achieved an 81% pregnancy rate following non-surgical transfer of 993 embryos. It is suggested that technicians who perform large numbers of non-surgical transfers become experienced and adept at passing the transfer gun or pipette through the cervix and depositing the embryo in the right part of the uterus. This level of experience is not possible for the authors of the present paper and they therefore determined to modify the non-surgical transfer method to aid passage of the transfer pipette through the recipient mare's cervix while still maintaining high levels of sterility and manipulative gentleness. Accordingly, the following method of transfer was designed and tested. This included conveying the embryo in a larger volume of medium (2.5 ml) than is used traditionally (0.5 ml).

The uteri of donor mares were flushed conventionally on Day 7 after ovulation and when an embryo was recovered it was washed 4 times in increasing concentrations of ovum culture medium (OCM; ICN Biomedicals, Ohio, USA) and graded according to McKinnon and Squires (1988). While waiting the 10–15 min prior to transfer, the embryo was maintained in the dark at 37°C in 100% OCM. A warmed plastic disposable insemination pipette, guarded within a plastic sleeve, was used to transfer embryos to recipient mares that had ovulated 0–3 days after the donors. To load the embryo, a 5 ml syringe was attached to the pipette and a 1.5 ml air dam drawn into the syringe before aspirating approximately 0.5 ml of OCM, a further air dam and then a further 1.5 ml OCM containing the embryo, a second air dam and finally a further 0.5 ml of OCM. For transfer the recipient mare was lightly sedated with 1 ml of 10 mg/ml acepromazine maleate (ACP; C-Vet, Lancashire, UK) and her rectum was evacuated of faeces. The perineum was cleaned thoroughly with separate washes of povidine-iodine (Pevidine; C-Vet,

Lancashire, UK) in warm water, rinsed with running tap water and dried with paper towelling. She was then transferred to stocks in a heated room where her perineum was sprayed with 70% methanol and again dried with paper towels. A duck-billed Polanskys speculum was inserted into the vagina and the external os of the cervix visualised using a focused pencil torch. A modified Velsellum forceps was then inserted into the vagina and, under visual control, the ventral portion of the cervical os was grasped with the forceps. Pulling backwards on the forceps straightened the cervix and elevated the uterus in the abdomen. The transfer operator was then able to pass the sleeved pipette into the vagina, withdraw the sleeve to expose the pipette and pass the latter into the os of the cervix, straight through the cervix and well up one of the uterine horns in one easy movement, with no other handling of the cervix *per vaginum* or manipulation of the uterus *per rectum*. The embryo, in the large volume of medium (2.5 ml) was fired into the uterine lumen by swiftly depressing the plunger of the syringe and the pipette was then immediately and swiftly withdrawn from the uterus and cervix, again in one precise movement and simultaneously with release of the forceps from the cervix. The recipient mares all then received 10 mega benzylpenicillin (Crystapen; Schering-Plough Animal Health, Welwyn Garden City, UK)) intravenously immediately after the transfer and procaine penicillin (Duphapen; Fort Dodge Animal Health, Southampton, UK) once daily intramuscularly for 2 further days. Transfer of 20 Grade I embryos by this method, by inexperienced operators, gave 17 established pregnancies (85%).

This new method of non-surgical embryo transfer has several novel features that make it manipulatively straightforward to deposit the embryo in a sizeable volume of medium deep into the recipient mare's uterus without the need to place the operator's hand in the mare's vagina or rectum. It is simple to perform and does not rely upon vast expertise and experience to achieve high pregnancy rates.

References

Gastal, M.O., Gastal, E.L., Rivera, A.P. and Silva, L.A. (2002) Evaluation of the transvaginal ultrasound-guided intrauterine injection technique for embryo transfer in mares. *Theriogenology* **58,** 725-728 (abstract).

Handler, J., Konigshofer, M., Schams, D and Aurich, C (2001) Secretion of oxytocin caused by cervical dilation in the horse mare. *J. Reprod. Fert.* **27,** 11 (abstract).

Iuliano, M.F., Squires, E.L. and Cook, V.M. (1985) Effect of age of equine embryos and method of transfer on pregnancy rate. *J. anim. Sci.* **60,** 258-263.

Kask, K., Odensvik, K and Kindahl, H (1997) Prostaglandin F release associated with an embryo transfer procedure in the mare. *Equine vet. J.* **29,** 286-289.

Losinno, L., Aquilar, J.J and Lisa, H. (2000) Impact of multiple ovulations in a commercial equine embryo transfer programme. Proceedings of the 5th International Symposium on Equine Embryo Transfer. *Havemeyer Foundation Monograph Series No 3*, Eds: T. Katila and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 81-83.

McKinnon, A.O. and Squires, E.L. (1988) Morphological assessment of the equine embryo. *J. Am. vet. med. Ass.* **179,** 767-790.

Riera, F.L. and McDonough, J. (1993) Commercial embryo transfer in polo ponies in Argentina. *Equine vet. J. Suppl.* **15,** 115.

Squires, E.L., McCue, P.M. and Vanderwall, D. (1999) The current status of equine embryo transfer. *Theriogenology* **51,** 91-104.

IN VIVO FERTILISATION AFTER INTRAFALLOPIAN TRANSFER OF *IN VITRO* MATURED EQUINE OOCYTES

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INTRODUCTION

In vitro production of embryos is common in human and in livestock species. However, in the horse, the results are still disappointing. Only 2 foals have been born using standard IVF procedure. The 2 major problems to successful IVF are oocyte maturation and sperm capacitation. Oxidative stress appears to be an important factor impairing *in vitro* embryo development. The failure of *in vitro* maturation of equine oocytes could also be due to an oxidative stress. Glutathione (GSH) is a major non-protein sulphydryl compound in mammalian cells which plays a critical role in protecting the cell from oxidative damages (Lafleur *et al.* 1994). GSH is synthesised during oocyte maturation *in vitro* in mouse (Calvin *et al.* 1986), hamster (Perreault *et al.* 1988), pig (Yoshida *et al.* 1993), and cattle (Miyamura *et al.* 1995). The addition of cysteamine to *in vitro* culture media stimulates the intracellular GSH synthesis (Takahashi *et al.* 1993; de Matos *et al.* 1995).

In this study, the objective was to evaluate, by intrafallopian transfer and *in vivo* fertilisation, the effect of cysteamine on the ability of equine oocytes to be fertilised after *in vitro* maturation. The evaluation of nuclear maturation is not sufficient to assure the ability of oocytes to be fertilised after *in vitro* culture. Therefore, the ultimate test to appreciate the quality of a gamete following *in vitro* culture is by obtaining an embryo. The oocytes were collected *in vivo* by transvaginal ultrasound-guided follicular aspiration, matured *in vitro* with or without cysteamine and transferred in the oviduct of mares.

MATERIALS AND METHODS

Animals

Adult, cycling welsh pony mares weighing between 250 and 350 kg bwt were used as donor mares and recipient mares. Animals were kept inside and fed concentrates and straw. The follicular and uterine status was assessed daily by ultrasonography with a 5 Mhz linear probe.

Donor and recipient mares were used while in oestrus stage. When a pre-ovulatory follicle >33 mm was present, the mares received CEG (crude equine gonadotropin, 15 mg iv; Duchamp *et al.* 1987) in order to increase the LH levels which have a beneficial effect on oocyte quality (Goudet *et al.* 1997).

Oocyte collection

Oocyte collection took place 24 h after the CEG administration to the donor mares. All follicles with a diameter ≥10 mm were aspirated by transvaginal ultrasound-guided procedure. Animals were restrained in stocks and were sedated with 1 mg detomedine iv (Domosedan® Pfizer, France) for preparation (emptying the rectum and perineal cleaning). Immediately before starting the procedure, mares were sedated again with 1.5 mg iv and also received 25 mg propantheline bromide (ref P-8891 Sigma, France) to relax the rectum. The oocyte collections were performed using an Aloka SSD 900 coupled with a 7.5 Mhz convex probe (Ecotron, France). Follicular aspiration was performed with a single lumen needle for follicles ≥25 mm, or a double lumen needle for follicles <25 mm using the method previously described by Duchamp *et al.* (1995). Antibiotics

(dihydrostreptomycin 4000,000 IU/animal and benzylpenicillin 4 g/animal) were given once intramuscularly at the end of the procedure.

In vitro maturation of oocytes

Before culture each COC (cumulusoocyte complex) was classified according the gross morphology of the cumulus (compact; moderate expansion and full expansion) as described by Goudet *et al.* (1998). The collected oocytes from the pre-ovulatory follicles were not used in this experiment. The oocytes from immature follicles were rinsed in 500 µl of PBS (phosphate buffered saline, Dulbecco A, BR14, Oxoid, France) and each oocyte was individually cultured in 20 µl droplet media under mineral oil. (ref M8410, Sigma, France) In this experiment, 2 media were compared: 1) the control medium containing TCM 199 (ref M4530, Sigma, France) with 50 ng/ml EGF (epidermal growth factor, ref E4127, Sigma, France) and 20% fetal calf serum (ref F4135, Sigma, France); 2) the cysteamine medium which was the control medium supplemented with 100 µM cysteamine (ref M9768, Sigma, France). The oocytes were incubated for 30 h at 38.5°C in humidified atmosphere of 5% CO_2 in air. After incubation, the oocytes were either transferred in the oviduct of a recipient mare, or stained for nuclear evaluation.

Oocyte transfer

The transferred oocytes were rinsed 3 times in PBS and kept at 38.5°C. The transfer was performed via standing flank laparotomy with a fire-polished glass pipette connected to a syringe. Seven to 10 were transferred in the oviduct contralateral to the dominant follicle according to procedure described by Carnevale *et al.* (1995). The non-transferred oocytes were first rinsed in PBS and their cumulus was assessed. Then, the oocytes were rinsed again and the cumulus cells were mechanically removed. After the third rinsing oocytes were stained with Hoechst (ref 33342, Sigma France); and classified as previously described by Goudet *et al.* (1998) (germinal vesicle, dense chromatin, metaphase I, metaphase II and degenerated).

Recipient mares

Food was withdrawn the day before surgery. The oocyte transfer took place about 30 h after CEG administration to the recipient mare. Antibiotics were given 5–6 h before surgery and for another 4 days. The recipients were inseminated 5 h before transfer with fresh semen (400×10^6 total spermatozoa in 20 ml INRA96® extender (IMV technology, France). Using semen from the same ejaculate kept at 15°C, recipient mares were inseminated again immediately after surgery with 200×10^6 total spermatozoa in 10 ml extender.

Fertility assessment

The embryos were collected by flushing the uterus on Day 10 post ovulation of the recipient mares. Embryos resulting from the transferred oocytes were differentiated from the potential embryo produced by the recipient mare by a DNA test parentage (Guerand *et al.* 1997).

RESULTS AND DISCUSSION

A total of 120 oocytes were obtained from 245 immature follicles resulting in a recovery rate of 49%. The oocytes were assigned to different treatment groups as given in Table 1.

Although slightly in favour of the cysteamine group the nuclear maturation was not statistically different between the 2 groups. The proportion of oocytes reaching the metaphase II stage (Table 2) at the end of the culture period in our study is rather low (36% in the control group and 47% in the cysteamine group). However, our global maturation rate of 43% is similar to the figures generally reported in the literature, which vary from 40% to 70% in the equine species (Squires 1996; Goudet *et al.* 1997; Bøgh *et al.* 2002). We obtained 2 embryos from 27 transferred oocytes (7%) in the cysteamine group and 6 embryos from 27 transferred oocytes (22%) in the control group (Table 3). The production of embryos has not been increased by the cysteamine under our conditions as opposed to what has been observed in other

TABLE 1: Number and distribution of the oocytes at end of the incubation period

	No. cultured oocytes	No. transferred oocytes	No.stained oocytes
Control Group	52	27	25
Cysteamine Group	65	27	38
Total	117	54	63

TABLE 2: Percentage of nuclear maturation of the non-transferred oocytes and valuation of nuclear maturation after culture

Medium	Nuclear stage				
	Metaphase II	Metaphase I	Dense chromatine	Germinal vesicle	Degenerated
Control	36% (9/25)	8% (2/25)	8% (2/25)	8% (2/25)	40% (10/25)
Cysteamine	47% (18/38)	11% (4/38)	8% (3/38)	3% (1/38)	32% (12/38)
Total	43% (27/63)	10% (6/63)	8% (5/63)	5% (3/63)	35% (22/63)

TABLE 3: Fertilisation rates according to the treatment groups, the number of collected embryos and result of parentage test

Recipient	Media	No. transferred oocytes	No. embryos total	Embryos from the recipient	Embryos from the donors
1	Control	10	2	1	1
2	Control	10	5	0	5
3	Control	7	1	1	0
4	Cysteamine	10	2	1	1
5	Cysteamine	10	2	1	1
6	Cysteamine	7	0	0	0
Total		54	12	4	8

species such as bovine (De Matos *et al.* 1995, De Matos *et al.* 1996); ovine (De Matos *et al.* 1999); porcine (Grupen *et al.* 1995; Bing *et al.* 2001); buffalo (Gasparrini *et al.* 2003). These results are in agreement with Luciano *et al.* (2004) who demonstrated recently that the addition of 100 µM of cysteamine during *in vitro* maturation of equine oocytes does not influence GSH synthesis.

The global results, 8 embryos from 54 transferred oocytes giving a 15% recovery rate are similar to those reported in other studies. From 29 oocytes aspirated during dioestrus stage *in vivo* Scott *et al.* (2001) obtained 2 pregnancies on Day 16 (7%) following i*n vitro* maturation and *in vivo* fertilisation. Zhang *et al.* (1989) obtained 7 embryos from 29 oocytes from slaughterhouse matured *in vitro* and fertilised *in vivo*. Hinrichs *et al.* (2002) obtained a high fertilisation rate of 77% after *in vitro* maturation. The recipient mares were euthanased 40–44 h after transfer and oocytes were recovered from the oviduct. When comparing the percentage of embryos per transferred oocytes, 18% of the oocytes recovered from the oviduct had undergone cleavage (2 cells or more). The discrepancy between the fertilisation rate and the relatively low embryo recovery rate in all studies combined may suggest that the percentage of embryos able to reach the blastocyst stage is probably lower. This may be due to a blockage of embryonic development or to an intrafallopian embryonic reduction and gives a new light to overall low results obtained following uterine collection. This confirms that the ultimate test to appreciate the quality of a gamete following *in vitro* culture is by obtaining at least a blastocyst.

Conclusions

In our study the addition of 100 µM of cysteamine to a classic culture medium did not improve equine oocyte maturation and embryonic development. However this study confirms that it is possible to produce embryos after *in vitro* maturation of equine oocytes. Intrafallopian transfer of *in vitro* matured oocytes can be an option in commercial programs for mares with delayed ovulations, lack of pre-ovulatory maturation, or chronic lesions of the reproductive tract.

References

Bing, Y.Z., Naga, T. and Rodriguez-Martinez, H. (2001) Effects of cysteamine, FSH, estradiol-17 beta. on *in vitro* maturation of porcine oocytes. *Theriogenology* **55,** 867-876.

Bøgh, I.B., Bezard, J., Duchamp, G., Batilsen, M., Gerard, N., Daels, P. and Greve, T. (2002) Pure preovulatory follicular fluid promotes *in vitro* maturation of *in vivo* aspirated equine oocytes. *Theriogenology* **57,** 1765-1779.

Calvin, H.I., Grooshans, K. and Blake, E.J. (1986) Estimation and glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus

transformation in the fertilised egg. *Gamete res.* **14,** 265-275.

Carnevale, E.M. and Ginther, O.J. (1995) Defective oocytes as cause of subfertility in old mares. *Biol. Reprod.* **1,** 209-214.

De Matos, D.G., Furnus, C.C., Moses, D.F. and Baldassarre, H. (1995) Effect of cysteamine on glutathione level and developmental capacity of bovine oocytes matured *in vitro*. *Mol. Reprod. Dev.* **42,** 432-436.

De Matos, D.G., Furnus, C.C., Moses, D.F., Martinez, A.G. and Matkovic, M. (1996) Stimulation of gluthation synthesis of *in vitro* matured bovine oocytes and its effect on embryo development and freezability. *Mol. Reprod. Dev.* **45,** 451-457.

De Matos, D.G., Gasparrini, B., Furnus, C.C. and Thomos, J.G. (1999) Glutathione synthesis during *in vitr*o maturation of ovine oocytes: Effects of cysteamine and ß-mercaptoethanol. *Theriogenology* **53,** 368.

Duchamp, G., Bezard, J. and Palmer, E. (1995) Oocyte yield and the consequences of puncture of all follicles larger than 8 mm. *Biol. Reprod.* **1,** 223-241.

Duchamp, G., Bour, B., Combarnous, Y. and Palmer, E. (1987) Alternative solutions to hCG induction of ovulation in the mare. *J. Reprod. Fert.* **35,** 221-228.

Gasparrini, B., Sayoud, H., Neglia, G., De Matos, D.G., Donnay, I. and Zicarelli, L. (2003) Glutathione synthesis during *in vitro* maturation of buffalo (Bubalus bubalis) oocytes: Effects of cysteamine on embryo development. *Therigenology* **60,** 943-952.

Goudet, G., Bezard, J., Belin, F., Duchamp, G., Palmer, E. and Gerard, N. (1998) Oocytes competence for *in vitro* maturation is associated with histone H1 kinase and is influenced by estrous stage in the mare. *Biol. Reprod.* **59,** 456-462.

Goudet, G., Bezard, J., Gerard, N. and Palmer, E. (1997) Equine oocyte competence for nuclear and cytoplasmique *in vitro* maturation: effect of the size and hormonal environment. *Biol. Reprod.* **57,** 232-245.

Grupen, C.G., Nagashima, H. and Nottle, M.B. (1995) Cysteamine enhances *in vitro* development of porcine oocytes matured and fertilised *in vitro*. *Biol. Reprod.* **53,** 173-178.

Guerand, M., Mahla, R., Lagneaux, D., Amigues, Y., Palmer, E. and Bezard, J. (1997) Parentage testing of Day 10 equine embryos by amplified PCR analysis of micro satellites. *Equine vet. J.* **25,** 69-71.

Hinrichs, K., Love, C.C., Brinsko, S.P., Choi, Y.H. and Varner, D.D. (2002) *In vitro* fertilisation of *in vivo*-matured equine oocytes: Effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilisation *in vivo* after oviductal transfer. *Biol. Reprod.* **67,** 256-262.

Lafleur, M.V.M., Hoorweg, J.J., Joenje, H. and Westmijze, E.J. (1994) The ambivalent role of glutathione in the protection fo DNA against singlet oxygen. *Free Radic Res.* **21,** 9-17.

Luciano, A.M., Goudet, G. and Gérard, N. (2004) The effect of cysteamine on *in vitro* nuclear maturation and GSH content in equine oocytes. 3rd Meeting of the European Equine Gamete Group. *Havemeyer Foundation Monograph Series No 13,* Eds: J. Müller, Z. Müller and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 29-31.

Miyamura, M., Yoshida, M., Hamano, S. and Kuwayama, M. (1995) Glutathione concentration during maturation and fertilisation of bovine oocytes. *Theriogenology* **43,** 282.

Perreault, S.D., Barbee, R.R. and Slott, V.L. (1988) Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. *Dev. Biol.* **125,** 181-186.

Scott, T.J., Carnevale, E.M., Maclellan, L.J., Scoggin, C.F. and Squires, E.L. (2001) Embryo development rates after transfer of oocytes matured *in vivo*, *in vitro*, or within oviducts of mares *Theriogenology* **55,** 705-715.

Squires, E.L. (1996) Maturation and fertilisation of equine oocytes. *Vet. Clin. N. Am.* **12,** 31-45.

Takahashi, M., Nagai, T., Hamano, S., Kuwayama, M., Okamura, N. and Okanao, A. (1993) Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryos. *Biol. Reprod.* **49,** 228-232.

Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M. and Pursel, V.G. (1993) Glutathione concentration during maturation and after fertilisation in pig oocytes: relevance the abitlity of oocytes to form male pronucleus. *Biol. Reprod.* **49,** 89-94.

Zhang, J.J., Boyle, M.S. and Allen, W.R. (1989) Recent studies on in fertilisation of equine oocytes. *Equine vet. J.* **21,** 101-104.

LOCALISATION OF ZP_3 PROTEINS AND N-ACETYLGLUCOSAMINE RESIDUES ON EQUINE AND PORCINE ZONA PELLUCIDA

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INTRODUCTION

In equine species, *in vitro* fertilisation rates are below 30% (Palmer *et al.* 1991; Dell'Aquila *et al.* 1996; Alm *et al.* 2001). The major barrier to fertilisation is the *zona pellucida* (ZP) as authors observed binding of the sperm to the ZP but no penetration. Moreover, when the ZP is opened by zona drilling or partial zona dissection, *in vitro* fertilisation rates range from 30–80% (Choi *et al.* 1994; Azuma *et al.* 1995; Li *et al.* 1995). In pigs, the spermatozoa penetrate the ZP easily in high numbers (Abeydeera 2002). Therefore, our aim was to use porcine ZP as a model to correlate special features of the composition and structure of equine ZP to the inability of sperm penetration.

Few studies have been performed on the equine ZP. However, 3 glycoproteins of 60, 80 and 100 kDa were identified on the ZP (Miller *et al.* 1992). In porcine, 5 glycoproteins belonging to the ZPA, ZPB and ZPC gene families have been identified : ZP_1, ZP_2, $ZP_3\alpha$, $ZP_3\beta$ and ZP_4 (Harris *et al.* 1994). During fertilisation, spermatozoa are bound by a complex formed with the $ZP_3\alpha$ and $ZP_3\beta$ proteins (Yurewicz *et al.* 1998). In the mouse, the ZP contains 3 major glycoproteins: ZP_1, ZP_2 and ZP_3. Gamete binding is mediated by the sperm surface enzyme galactosyltransferase that recognises N-acetylglucosamine residues on the ZP glycoprotein ZP_3.

In the equine, the mechanism of gamete binding is not known, but a galactosyltransferase is present on the surface of equine spermatozoa (Fayrer-Hosken *et al.* 1991). We hypothesised that $ZP_3\alpha$ and $ZP_3\beta$ proteins and N-acetylglucosamine residues may be involved in this process. Therefore, we compared the localisation of $ZP_3\alpha$ and $ZP_3\beta$ proteins and N-acetylglucosamine residues on equine and porcine oocytes.

MATERIALS AND METHODS

Immature oocytes and oocytes after *in vitro* and *in vivo* maturation were used. Immature equine oocytes were collected from slaughtered mares. *In vitro* matured equine oocytes were obtained after 30 h of culture in TCM199 supplemented with 20% fetal calf serum and 50 ng/ml epidermal growth factor (EGF). *In vivo* matured equine oocytes were punctured *in vivo* from pre-ovulatory follicles. Immature and *in vivo* matured porcine oocytes were collected from slaughtered sows. *In vitro* matured porcine oocytes were obtained after 44 h of culture in TCM199 supplemented with 570 µM cysteamine, 10 ng/ml EGF and 400 ng/ml FSH.

Oocytes were denuded and fixed in 2.5% paraformaldehyde in PBS. They were first incubated with a chicken anti-porcine $ZP_3\alpha$ or $ZP_3\beta$ antibody (provided by Dr. S. Koelle, University of Munich, Germany), and then with a tetramethylrhodamine isothiocyanate-conjugated anti-chicken antibody. In the same oocytes, the N-acetylglucosamine residues were detected with a fluorescein isothiocyanate conjugated wheat germ agglutinin (WGA, Sigma). Fluorescence detection was performed with an inverted confocal microscope (Olympus IX 81).

RESULTS

Equine oocytes

Equine immature oocytes and oocytes after *in vivo* and *in vitro* maturation were stained with the anti-$ZP_3\alpha$ antibody. For all of them, the staining was homogeneous in the ZP and the oocyte (Fig 1). With the anti-$ZP_3\beta$ antibody the staining was homogeneous only for the *in vitro* matured oocytes. For the immature and *in vivo* matured

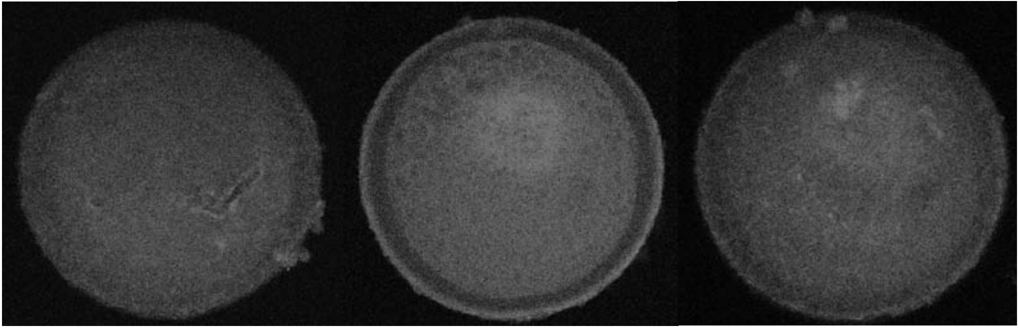

Fig 1: Three optical sections in one immature equine oocyte labelled with the anti $ZP_3\alpha$ antibody.

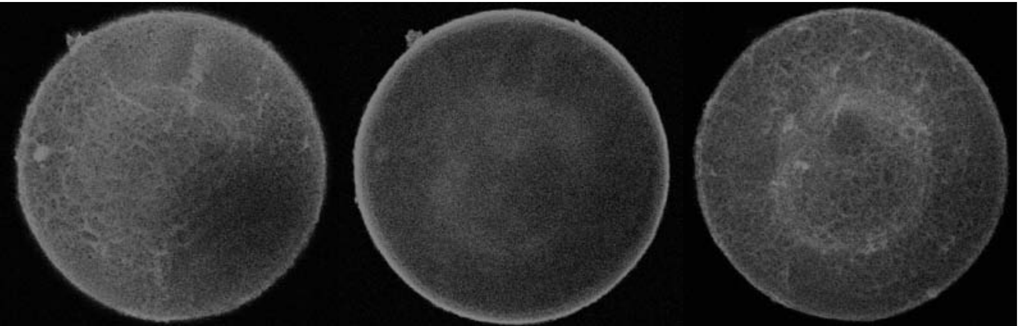

Fig 2: Three optical sections in one immature equine oocyte labelled with the anti $ZP_3\beta$ antibody.

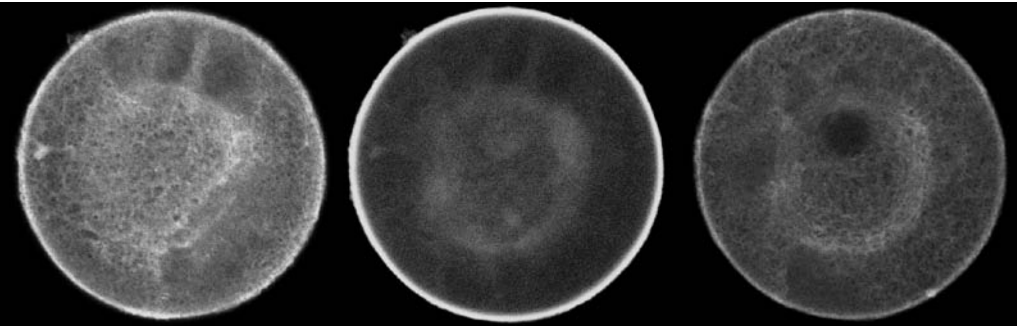

Fig 3: Three optical sections in one immature equine oocyte labelled with the WGA lectine.

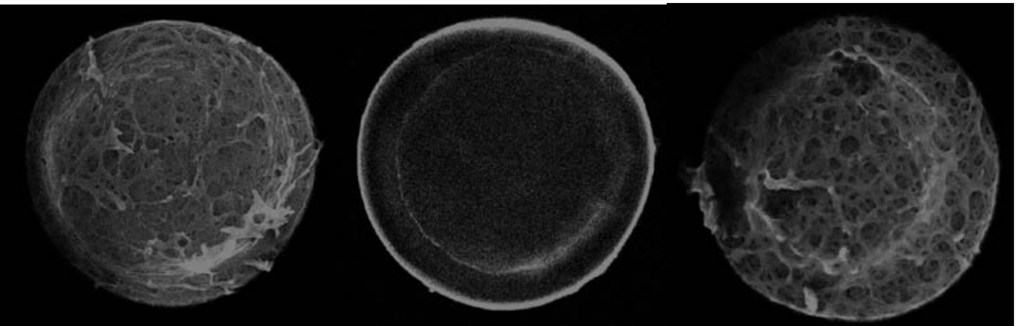

Fig 4: Three optical sections in one immature porcine oocyte labelled with the anti $ZP_3\beta$ antibody.

oocytes the staining was faint in the oocyte and more intense at the periphery of the ZP (Fig 2). For all the oocytes, the N-acetylglucosamine residues were localised in the oocyte and the ZP, but it was more intense at the periphery of the ZP (Fig 3).

Porcine oocytes

Porcine immature oocytes and oocytes after *in vivo* and *in vitro* maturation were stained with the anti-$ZP_3\alpha$ antibody. For the immature and *in vitro* matured oocytes the staining was observed in the ZP. For the *in vivo* matured oocytes, the staining was either homogeneous or more intense in the ZP. With the anti-$ZP_3\beta$ antibody a strong labelling was observed in the ZP for all the oocytes. Moreover, $ZP_3\alpha$ proteins were organised in a network in immature porcine oocytes and $ZP_3\beta$ proteins were organised in a network in all porcine oocytes. Fig 4 shows the spongy appearance of the ZP with strongly stained processes from cumulus cells. For all porcine oocytes, the N-acetylglucosamine residues were localised in the ZP and the staining was more intense at the periphery of the ZP.

Discussion

This work showed for the first time, the presence of 2 different ZP_3 proteins on equine ZP. The $ZP_3\alpha$ protein was localised in the oocyte and in the ZP whereas the $ZP_3\beta$ protein was localised mainly in the ZP and in the processes of the cumulus cells.

According to the stage of maturation, some modifications of the localisation of the ZP_3 proteins were observed in equine and porcine oocytes. These modifications could be necessary for the penetration of spermatozoa across the zona pellucida after oocyte maturation. The localisation of ZP_3 proteins was different between *in vitro* and *in vivo* matured oocytes. Thus, the oocytes obtained by *in vitro* maturation were different to *in vivo* matured oocytes.

The localisation of ZP_3 proteins was different between equine and porcine oocytes. Moreover, in the latter, proteins were organised in a network, but no network was observed in equine oocytes. It is likely that ZP_3 proteins are not the major component of the equine ZP and that the proportions of ZP proteins differ between porcine and equine. Finally, in equine, the N-acetylglucosamine residues co-localised with the $ZP_3\beta$ protein, whereas in porcine, they co-localised neither with the $ZP_3\alpha$ protein nor with the $ZP_3\beta$ protein. This residue may be associated with another protein of the porcine ZP.

In conclusion, during IVF, the penetration rate is very low in equine whereas polyspermy is frequent in porcine. Different localisations and proportions of ZP glycoproteins may contribute to the decreased permeability of the equine ZP.

References

Abeydeera, L.R. (2002) *In vitro* production of embryos in swine. *Theriogenology* **57,** 257-273.

Alm, H., Torner, H., Blottner, S., Nürnberg, G. and Kanitz, W. (2001) Effect of sperm cryopreservation and treatment with calcium ionophore or heparin on *in vitro* fertilisation of horse oocytes. *Theriogenology* **56,** 817-829.

Azuma, T., Choi, Y.H., Hochi, S. and Oguri, N. (1995) Effect of glucose in the culture medium on development of horse oocytes matured and microfertilised *in vitro*. *Reprod. Fert. Dev.* **7,** 1067-1071.

Choi, Y.H., Okada, Y., Hochi, S., Braun, J., Sato, K. and Oguri, N. (1994) *In vitro* fertilisation rate of horse oocytes with partially removed zonae. *Theriogenology* **42,** 795-802.

Dell'Aquila, M.E., Fusco, S., Lacalandra, G.M. and Maritato, F. (1996) *In vitro* maturation and fertilisation of equine oocytes recovered during the breeding season. *Theriogenology* **45,** 547-560.

Fayrer-Hosken R.A., Caudle A.B. and Shur B.D. (1991) Galactosyltransferase activity is restricted to the plasma membranes of equine and bovine sperm. *Mol. Reprod. Dev.* **28,** 74-78.

Harris J.D., Hibler D.W., Fontenot G.K., Hsu K.T., Yurewicz E.C. and Sacco A.G. (1994) Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. *DNA sequence* **4,** 361-393.

Li L.Y., Meintjes M., Graff K.J., Paul J.B., Denniston R.S. and Godke R.A. (1995) *In vitro* fertilisation and development of *in vitro* matured oocytes aspirated from pregnant mares. *Biol. Reprod. Mono.* **1,** 309-317.

Miller C.C., Fayrer-Hosken R.A., Timmons T.M., Lee V.H., Caudle A.B. and Dunbar B.S. (1992) Characterization of equine zona pellucida glycoproteins by polyacrylamide gel electrophoresis and immunological techniques. *J. Reprod. Fert.* **96,** 815-825.

Palmer E., Bézard J., Magistrini M. and Duchamp G. (1991) *In vitro* fertilisation in the horse. A retrospective study. *J. Reprod. Fert. Suppl.* **44,** 375-384.

Yurewicz E.C., Sacco A.G., Gupta S.K., Xu N. and Gage D.A. (1998) Hetero-oligomerization dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. *J. Biol. Chem.* **273,** 7488-7494.

EFFECT OF FREEZE-THAWING ON THE CELLULAR INTEGRITY OF EQUINE EMBRYOS

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INTRODUCTION

The commercial expansion of equine embryo transfer has been hindered by various factors, including the failure to develop a cheap and reliable protocol for superovulation. In addition, horse embryos are difficult to cryopreserve and, because embryos are in short supply, the emphasis has remained on minimising wastage by transferring embryos fresh or after a short period (<24 h) of storage at 5°C (Squires *et al.* 1999). In fact, small horse embryos (<250 µm) can be cryopreserved with reasonable success (pregnancy rates around 60%: Lascombes and Pashen 2000), although this is still some 10–20% lower than pregnancy rates obtained with fresh or chilled embryos in comparable circumstances. The recovery of freezable embryos is labour intensive with mares needing to be checked for ovulation at least twice daily to achieve the dual aim of recovering an embryo as soon as possible after its entry into the uterus on approximately Day 6 after ovulation, but before it expands beyond the critical 250 µm soon after. This challenge is complicated further by variations in the rate of embryo development between mares (slower in older animals: Meadows *et al.* 1999) and semen preservation technique (slower after frozen semen insemination: P. Jou, personal communication). These problems could be largely circumvented by the development of a technique for successfully cryopreserving larger expanded blastocysts. With regard to the impediments to this goal, Legrand *et al.* (2001) postulated that the blastocyst capsule, which forms around the embryo during expansion, impedes cryoprotectant entry. This hypothesis was supported by their finding that the rate of cell death during freezing correlated with capsule thickness and that partial capsule digestion with trypsin prior to freezing allowed pregnancy to be established with a high proportion of expanded blastocysts (5 out of 6). Subsequent experiments to verify the beneficial effect of partial capsule digestion have produced much less impressive pregnancy rates (2/11, Legrand *et al.* 2002: 0/14, Maclellan *et al.* 2002). A second possible source of reduced embryo viability is cytoarchitectural damage resulting from intracellular ice-crystal formation during freezing (Dobrinsky *et al.* 2000). Disruption of the actin cytoskeleton appears to be an important contributor to the poor cryopreservability of porcine blastocysts, and stabilising the cytoskeleton with the actin polymerisation inhibitor cytochalasin-B markedly improves their post thaw viability (Dobrinsky *et al.* 2000). The aim of the current study was to determine whether freezing and thawing equine embryos leads to significant cytoskeletal damage and, if so, to determine whether partial capsule digestion with trypsin, or cytoskeleton stabilisation with cytochalasin-B, reduced either the rate of cell death or the degree of cytoskeleton disruption.

MATERIALS AND METHODS

Embryos were recovered on Day 7 after ovulation by uterine lavage with Dulbecco's phosphate buffered saline (PBS) supplemented with 0.5% fetal calf serum. The embryos were examined microscopically and measured with the help of an eyepiece micrometer. Only good quality embryos (Grade 1–2), were selected for freezing, and these were allotted randomly to one of 4 experimental groups:

1) 'Fresh' embryos were stained with the membrane impermeable DNA-stain 4,6-diamino-2-phenyl-indole (DAPI) to identify

dead cells, and then fixed in 4% paraformaldehyde.

2) 'Conventionally' frozen embryos (n=14) were first exposed for 10 min to each of 2.5, 5, 7.5 and 10% glycerol in PBS at room temperature. Thereafter, they were loaded into 0.5 ml straws and frozen using a programmable machine (Planar Series II). Initial cooling was at 1°C/min down to –6°C, where the embryos were held for 10 min and seeded. Subsequent cooling was at 0.3°C/min down to –33°C, after which the straws were plunged into liquid nitrogen.

3) 'Cyto-B' embryos (n=11) were frozen as in (2) except that 7.5 µg/ml cytochalasin-B was included in the glycerol solutions.

4) 'Trypsin' embryos (n=11) were pre-incubated in 0.2% w/v trypsin for 15 mins before freezing as in (2).

After thawing, the embryos were stained with DAPI, to allow counting of dead cells by fluorescent microscopy, before being fixed overnight in 4% paraformaldehyde and stored in PBS at 4°C. Prior to further examination, the embryos were permeabilised with 0.1% Triton X-100 and then restained with DAPI, to enable counting of total cell numbers, and with Alexafluor 488®, Phalloidin (Molecular Probes Europe BV, Leiden, The Netherlands) to allow visualisation of the actin cytoskeleton using a multiphoton excitation microscopy system (Bio-Rad Radiance 2100MP) mounted on a Nikon TE300-inverted microscope. To quantify the effect of cryopreservation on the actin cytoskeleton, embryos were graded on a scale of 1-3 for cytoskeleton integrity, where:

- A Grade 1 cytoskeleton was characterised by sharp actin staining along the contiguous cell borders, indicating intact cell plasma membranes.
- A Grade 2 cytoskeleton showed less sharply demarcated cell borders with some 'clumping' of the actin.
- A Grade 3 cytoskeleton had relatively large areas lacking distinguishable cell borders, with the actin instead accumulated in large, randomly distributed clumps.

RESULTS

Embryo diameter did not differ between experimental groups (391 ± 28 µm; range 160-1,000 µm) and did not, therefore, confound the effect of treatment. In general, freezing and thawing led to a marked increase in the number of dead cells (11.6 ± 1.5% in frozen-thawed versus 0.15 ± 0.6% in fresh embryos: P<0.001) and visible disruption of the actin cytoskeleton (average cytoskeleton score 2.1 ± 0.1 for frozen-thawed versus 1.2 ± 0.1 for fresh embryos). On the other hand, there was no significant effect of freezing technique (conventional versus trypsin versus cyto-B) on the number of dead cells post thaw or on average cytoskeletal quality. It was, however, noted that only trypsin treated embryos (4/11; 36%) had a Grade 1 cytoskeleton after freezing and thawing while all cyto-B embryos were recorded as Grade 2. On a grosser level, 3 of 11 trypsin, 2 of 14 conventional but none of 11 cyto-B embryos fractured during thawing, and would have been considered unfit for transfer to a recipient.

The expected deleterious effect of increased embryo size on freezability was present, but only statistically significant when embryos were frozen conventionally, after which those >300 µm in diameter had suffered higher rates of cell death than smaller embryos (19.0 ± 2.9 vs 8.4 ± 2.1%: P=0.023). The effect of embryo size was not statistically significant in the trypsin (13.8 ± 4.9% vs 5.4 ± 0.7%; P=0.141) or cyto-B (12.4 ± 5.5% vs 8.1 ± 2.3%: P=0.420) groups, primarily because of lower rates of cell death in larger embryos.

CONCLUSIONS

Clearly, freezing and thawing expanded equine blastocysts leads to cytoskeletal disruption and cell death. This is more marked in larger embryos (>300 µm) but may be ameliorated by pre-freeze treatment with either trypsin or cytochalasin B, where the former may enhance the access of cryoprotectant and the latter may increase the pliability, and thus decrease the fragility, of the cell membranes. Further investigation revealed that the Grade 2 cytoskeleton pattern observed in all frozen-thawed cyto-B embryos was an effect of cyto-B *per se* rather than of cryopreservation. Moreover, in non-frozen embryos, a normal cytoskeletal pattern was re-established after a subsequent 4 h incubation in cyto-B free medium;

it remains to be seen to what extent cryopreservation compromises this repolymerisation. Nevertheless, cytochalsin-B treatment shows promise as a means of minimising cell membrane disruption during freezing or, indeed, manipulation of equine embryos. Trypsin treatment also appeared to reduce freezing induced damage of equine blastocysts. However, one notable side effect was increased 'stickiness' of the capsule that made handling more difficult. Given that the capsule is vital to embryonic survival *in vivo* (Stout *et al.* 1997), it is postulated that the disappointing pregnancy rates reported after transfer of trypsin-treated embryos may arise from loss of the weakened, sticky coat during transfer to a recipient mare.

REFERENCES

Dobrinsky, J.R., Pursel, V.G., Long, C.R. and Johnson L.A. (2000) Birth of piglets after transfer of embryos cryopreserved by cytoskeleton stabilisation and vitrification. *Biol. Reprod.* **62,** 564-570.

Lascombes, F.A. and Pashen, R.L. (2000) Results from embryo freezing and post ovulation breeding in a commercial embryo transfer programme. 5th International Symposium on Equine Embryo Transfer. *Havemeyer Foundation Monograph Series 3*, Eds: T. Katila and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 95-96.

Legrand, E., Krawiecki, J.M., Tainturier, D., Corniere, P., Delajarraud, H. and Bruyas, J.F. (2001) Does the embryonic capsule impede the freezing of equine embryos? 5th International Symposium on Equine Embryo Transfer. *Havemeyer Foundation Monograph Series 3*, Eds: T. Katila and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 62-65.

Legrand, E., Bencharif, D., Barrier-Battut, I., Delajarraud, H., Corniere, P., Fieni, F., Tainturier, D. and Bruyas, J.F. (2002) Comparison of pregnancy rates for Days 7–8 equine embryos frozen in glycerol with or without previous enzymatic treatment of their capsule. *Theriogenology* **58,** 721-723.

Maclellan, L.J., Carnevale, E.M., Coutinho da Silva, M.A., McCue, P.M., Seidel Jr, G.E. and Squires, E.L. (2002) Cryopreservation of small and large equine embryos pre-treated with cytochalasin-B and/or trypsin. *Theriogenology* **58,** 717-720.

Meadows, S., Lisa, H. and Welsh, C. (1999) Factors affecting embryo recovery, embryo development and pregnancy rate in a commercial embryo transfer programme. First European Equine Gamete Group Meeting. *Havemeyer Foundation Monograph Series 1,* Eds: W.R. Allen and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 61-62.

Squires, E.L., McCue, P.M. and Vanderwall, D. (1999) The current status of equine embryo transfer. *Theriogenology* **51,** 91-104.

Stout, T.A.E., Meadows, S. and Allen, W.R. (1997) The stage specific formation of equine blastocyst capsule is essential for the establishment of pregnancy. In: Havemeyer Foundation Workshop on Maternal Recognition of Pregnancy in the mare. Peurto Plata, Dominican Republic, Abstract.

RECOVERY AND FREEZING OF EMBRYOS FOR USE IN COMMERCIAL EQUINE EMBRYO TRANSFER PROGRAMME

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INTRODUCTION

Animal Embryo Centre is a centre specialised in embryo transfer with cows and horses. We try to do embryo transfer in a commercial way and flush about 300 horses and 150 cows per year.

The cows we flush are dairy cows and Wagyus. Wagyu is a Japanese breed and is complete new in Europe. The Wagyu meat is famous in Japan and sold as Kobe-beef.

We flush Wagyus in Wales Belgium and Holland.

From the cow ET development we learned a lot, which we can use in horse embryo transfer. Embryo transfer (ET) with cows became popular after superovulation and freezing of embryos was possible. Before that it was commercial not possible. One time there were 15 recipients ready for transferring and you had no embryos, and the other time you had 15 embryos and no recipients, which is very frustrating.

Now with cows we only use the normal heat and transfer seven days later. In fact we freeze all the embryos. Also the worldwide trading in cow embryos is normal.

For us was clear that if ET with horses can get commercial it is necessary that you can freeze the embryos and use the normal heat of the recipient mare.

We are working together with Dr Pedro You in Canada, who is already successful in Canada for seven years with freezing horse embryos. Last year his pregnancy rate from frozen embryos was 72%.

This year is our 3rd year with horse ET. We have several vets and an ET/OPU specialist working at our centre. We are the first Centre in Europe which is EU certificated for Horse embryos.

EMBRYO TRANSFER AND FREEZING

We all know what ET is and we all know the advantages.

Donor mares

Which mares are being used for ET. The most important is that there are genetically superior above the breed average. They must belong to the at least 5% best horses on the index list. The mares we flushed the last 3 years were:

1. *Young 2-year-old mares*
   They had very good result in de second half of the breeding season.
2. *Sport mares*
   Between several contests we flushed mares. The results are very individual. But average the result were not as good as with mares who are not in sport.
3. *Very old mares*
   With all the problems in the world. No pregnancies in the last 10 years. Internal infection for 3 years, or longer. Resistant bacteria against all antibiotic
4. *Mares used for breeding.*
5. *Arabs, Friesian, lumping horses, dressage horses, Quarter horses etc.*

Semen

The quality of semen is, as you know very important for having a good recovery rate.

Because we use semen from a lot of different stallions and stations we notice that there is a significant difference between:

- Stallions individual
- Stallion stations (producing station)
- Frozen and fresh
- Stallion's age
- Several breeds: Arabs, Friesian jumping etc

We do have stallions with recovery rate of 70%, but also with 0%. There is also a difference in recovery rate between stallion stations: between 55% and 5%.

There is a lot of work for us to do before we can trust on a perfect semen quality.

Recipient mares

A good management of all the recipient mares is a must. Important is the daily check with the stallion, ultrasound for the ovulation moment and ultrasound before the transferring.

ET for the freezing programme is much more difficult. The embryo for freezing successfully must be in the morula or in the early blastocyst stage. To get such an embryo you need a lot of information.

1. Exact moment of ovulation, that means at least 2 times ultrasound a day.
2. There is a difference in the development of the embryo when you use fresh or frozen semen.
3. Embryo development in the different seasons.
4. Donor mare influence on the embryos

Results

Our results are very divers. We do work in a practical situation, sometimes we breed the mares and sometimes the mares are bred at home. As you can conclude from the chapter above there are so many factors which influent the result, that it is very hard to give an average percentage of what we are doing. I think it is good to sum up the factor who do have an influence on the results:

- Is the mare bred at our centre or not? When it is we have a higher recovery rate
- In which season we are is also important. We flush from February until December. To lowest recovery and pregnancy rates we have in spring and autumn, and the highest rates from June till September.
- From June till September we also have a higher pregnancy rate.
- The semen influence is extremely important for the recovery rate.
- What mares do we use as donor mares., young healthy mares with good semen have a higher recovery- and pregnancy rate.
- Quality of the recipient mare.

When you breed an old problem mare with a not fertile stallion and also frozen semen, you have a chance on an embryo but it is about 10% or lower. And if you would have an embryo the pregnancy rate is also low.

When we use a young fertile mare and a young fertile stallion with fresh semen the chance on an embryo is about 70%. When we transfer the embryo we have a pregnancy chance from 70% even if you transfer fresh or frozen.

The breeders are interested in foals and they decide. Sometimes the donor mares are so worthfull and mostly the last and only chance on a foal is ET. They are not really interested in numbers.

EU certification

Our centre is the first and at this moment the only who has an EU certificate for producing horse embryos. To get this certificate was a serious problem because there were at that moment no regulations and rules.

After the rules were made our first step was writing a protocol, and describe every single detail we do, and how we do it.

Weekly being checked by the veterinary authorities is also a part of the EU certificate. Some examples what we also need to do:

- Visitor registration
- Registration of all the medicines we buy, and use on the horses. Which horse gets what kind of medicine, how much on what day etc.
- The same as above also for semen. The semen also has to be produced at a EU certified semen station from a EU certificated stallion
- The donormare must be CEM free, not been bred natural within the last 60 days etc.
- In the laboratory every process has to be written down, even the cleaning and the clothes we wear.

- All the recipient mares must be chipped for a closed registration system. The system must be waterproof.

Disadvantages from the EU producing is a lot of paperwork and registration.

The advantage is that it is possible to start a trading network in embryos, and that's the reason why we thought it is necessary for us and our clients to be EU certified.

Trading international means certification

Start trading in the EU with EU certification means that it's important that more centres besides us become EU certified. A perfect situation would be that every country who would like to do something with horse genetics and embryo transfer has at least one EU centre.

The second step is to build a global network for frozen embryo. There is already a demand for frozen embryos from several countries all over de world who want to import them. The problem is that there are no import regulations, and if there are it is impossible to confirm to those regulations.

Another problem that should be solved is that there are not enough people who know how to freeze embryos. But this is actually the smallest problem.

The perfect situation is that we found a global network with people who are specialised in equine fertilisation, embryo transfer and export and import regulations In that way we have a backup knowledge group for every member of the group.

COMMERCIAL POSSIBILITIES

It is important that you reach the horse owners/breeders with the best horses in each country. Then you have to convince them that the genetical improvement goes faster with ET in the first place and second that they can sell their embryos. Also it is a perfect way of making a profit.

At our centre we have several buyers for horse genetics.

I would like to mention some of the methods we use at the moment:

1. Lease a donormare for one year. All the costs are for the person who leases the mare and he decides the stallion which is being used. Mostly they don't pay very much for leasing the mare but pay a reasonable surplus per pregnancy.
2. Lease one ore two heats from a donormare.
3. Just buy an embryo
4. Buy an embryo in three times. First pay for the embryo, than a surplus when the recipient is pregnant(40 days), en last when the foal is born healthy
5. Buy a pregnancy including the recipient mare.

Last year frozen embryos were sold in an auction for the first time in Europe. Nine embryos have been sold, 6 went to Canada. This year we sold on the same auction a pregnancy and two embryos from the same donor mare. Also, this year some of the embryos were sold to the USA.

Most of the buyers are interested in jumping bloodlines at this moment we do have demand from Spain, Australia, South-Africa Argentina en Chile for jumping bloodlines genetics.

SUMMARY

ET and the future for a commercial equine embryo transfer programme.

At the moment there is already a commercial equine embryo programme and it works. But there is still a lot of work to do, to get it accepted in Europe and the rest of the world. Lots off improvements still have to be done:

- Get better quality of semen. Especially for the breeder but also for us.
- More commercial focused organisation, with EU certification
- More global organisation with exports and import facilities.
- Convince breeder that it is worth it to do ET
- Find the best horses in the world
- Convince the investors in horse genetics that there are new possibilities to fasten the genetical improvement.
- Make everyone clear that it is a reliable organisation which they can rely on.

Important is that it is possible to create a situation were a profit for breeder, investor and ET station can be managed. Then we can build on a long-term organisation with unlimited possibilities.

SESSION IV:

Stem cells

GENE EXPRESSION IN THE EARLY EQUINE EMBRYO - A SEARCH FOR MARKERS OF EMBRYONIC VIABILITY

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Introduction

The accumulation of fluid during blastocyst formation and the ability to produce signalling substances interacting with the uterus are key factors for viability of the equine embryo and successful establishment of pregnancy. It has been suggested that the developmental capacity of embryos derived from insemination with frozen-thawed semen might be reduced compared to embryos conceived after insemination with fresh semen. In veterinary practice, progesterone treatment is often given to mares during early gestation in order to improve uterine function. However, clear evidence for such an effect still has to be provided. It was therefore the aim of the present study to investigate expression of genes specifically involved in the regulation of early embryonic development and to analyse effects of semen cryopreservation and of progesterone supplementation on embryonic gene expression the horse.

Materials and methods

As blastocyst formation is considered a critical step during early pregnancy, genes suggested to be involved in the formation of the blastocoel were chosen. These are mainly Na-K-ATPase and members of the aquaporin family. Expression of the following genes was investigated in Day 10 equine embryos: $\alpha 1/\beta 1$ Na-K-ATPase isoenzyme, aquaporin 5, cyclooxygenase 1 and 2 (COX 1 and 2), prostaglandin synthase (PGES) and cytochrome P450. Embryos were obtained by repeated flushing with 1 litre of phosphate buffered saline without supplements and subsequent filtering of the recovered fluid. During oestrus, mares had been inseminated at 48 h intervals with fresh semen and were not treated with progesterone (Group 1), at 24 h intervals with frozen-thawed semen and no progesterone treatment (Group 2) or were inseminated at 48 h intervals with fresh semen and received a progesterone releasing intravaginal device (PRID, 1.55 mg progesterone) from the day of ovulation until Day 10 of the cycle (Group 3). Selected embryos of good morphological quality, taken at random from Groups 1–3, were washed and frozen at –80°C until thawing, total RNA preparation and analysis by qualitative reverse transcriptase PCR (RT-PCR). Equine, rat or murine primers were used. After qualitative detection by sequencing of the RT-PCR fragments, quantitative analysis by real-time PCR was performed. A total of 14 embryos (Group 1: n=6, Group 2: n=4, Group 3: n=4) were submitted to quantitative real-time PCR. Relative gene expression of α_1 and β_1 Na-K-ATPase, aquaporin 5, COX 1, COX 2 and PGES in comparison to 2 housekeeping genes known to be constantly expressed in embryonic tissue (ß-actin and GAPDH) was analysed. The authors could show that both housekeeping genes can be used in equine embryos.

Results and discussion

Mean diameter of embryos after insemination with fresh semen were 4.1 ± 1.0 mm in untreated mares (n=25) and 4.9 ± 0.7 mm in progesterone-treated mares (n=8). Embryo diameter after insemination with frozen-thawed semen was 3.9 ± 1.6 mm (n=15; n.s. between groups).

All enzymes investigated were expressed in equine embryos 10 days after ovulation. Blastocyst growth is achieved mainly by water influx which is mediated by Na-K-ATPase, an

enzyme composed of an α_1 and a β_1 subunit in the horse. The $\alpha 1$ catalytic subunit is responsible for pumping 3 Na+-ions out of the cell in exchange to 2 K+-ions into the cell, thus rising the osmotic pressure into the blastocoel and leading to water influx. For the first time, the authors could demonstrate the presence of aquaporin 5 in equine embryos. In mice, these transmembrane channels have been shown to facilitate water influx. Aquaporin 5, in the equine embryo is located strictly apical which is different from Na-K-ATPase is present in the basal membranes of the trophectodermal cells. Quantitative analysis resulted in a positive correlation between aquaporin 5 expression and embryo size ($r=0.71$, $P<0.01$). Aquaporin 5 therefore appears to be a potential marker for assessment of embryo development and possibly viability.

PGES which is involved in the synthesis of prostaglandin E, the substance responsible for oviductal transport of the equine embryo, was significantly lower in embryos from progesterone-treated mares compared to embryos from mares not treated with progesterone and either inseminated with fresh or with frozen-thawed semen ($P<0.05$). This indicates an effect of exogenous progesterone on the embryonic genome. To what extent such a treatment may influence the maternal recognition of pregnancy in the horse requires further studies. For none of the other enzymes except PGES, was there a significant difference in quantitative gene expression between groups. Both COX 1 and COX 2, the 2 main enzyme complexes leading to the synthesis of prostaglandins, were also expressed in equine embryos. This implicates the existence of an external stimulus turning on the inducible COX 2 pathway. Oestrogen production of the equine embryo is considered one important factor for maternal recognition of pregnancy. Cytochrome P450 which converts progesterone to oestrogens was expressed by the equine embryo, however its relative abundance was not different between embryos from different groups of mares.

In conclusion, the authors could demonstrate for the first time expression of a member of the aquaporin family in equine embryos. The correlation of aquaporin 5 relative gene expression with embryo size suggests an involvement in embryonic blastocoel formation. Maternal and external factors that influence plasma progesterone concentration might affect the expression of genes important for early embryonic life and thus influence embryonic viability and maternal recognition of pregnancy.

WHAT IS DIFFERENT ABOUT THE HORSE EMBRYO?

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INTRODUCTION

Blastulation is the first occurrence of cell differentiation in the embryo when it separates into the inner cell mass (ICM) and trophectoderm (TE). The ICM gives rise to the embryo and some of the extra-embryonic membranes; and the TE forms the extra-embryonic ectoderm and trophoblast cells, which collectively form the trophoblast lineage and constitute most of the fetal placenta (Rossant and Croy 1986). This study examined the characteristics of horse TE and compared the ability of TE cells to support embryonic development following nuclear transfer (NT) with somatic cells.

MATERIALS AND METHODS

TE cells were dissected from Day 7–8 blastocysts. The characteristics of fresh TE cells were examined immunocytochemically using antibodies specific for the embryonic stem (ES) cell markers, alkaline phosphates (AKP), SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 and Oct-4, all of which have been successful in human and mouse embryos. NT was performed as described by Li *et al.* (2002) using enucleated MII stage oocytes as the recipient cytoplasm and adult skin fibroblast cells (AFC) or TE cells that had been passaged 1–3 times. Oocytes reconstructed from either TE or adult AFC were cultured for 3–7 days until they reached the 16–32 cell stage of development or were allowed to continue to the morula-blastocyst stage.

RESULTS AND DISCUSSION

Expression of AKP activity, and 4 of 5 stem cell markers (excluding SSEA-4) was demonstrated in ICM and TE cell colonies or vesicles. The rates of early embryonic development *in vitro* of the 2 types of reconstructed oocytes were the same for the 2 kinds of donor cells used for NT (Table 1).

TABLE 1: Embryonic development of cloned horse embryo *in vitro*

Donor cell	% of reconstructed embryos reaching	
	2-cell stage	morula-blastocyst
AFC	31%	10%
TE	28%	7%

First cell differentiation of ICM and TE from fertilised oocytes showed genetic interaction between the 2 cell types to support continuation of embryonic development. ICM cells separated from blastocyst stage embryos were used to create ES cell lines because these were considered most likely to have the potential to differentiate into the 3 embryonic layers: ectoderm, mesoderm and endoderm (Rossant and Croy 1986; Jones and Thomson 2000). However, we showed that horse TE cells also express the same ES cell surface receptors as cells derived from the ICM, highlighting major differences between the essential characteristics of horse trophectoderm cells and those of mice and other species. TE cells also showed similar potential to somatic cells to support embryonic development *in vitro* of reconstructed oocytes following nuclear transfer.

REFERENCES

Rossant, J. and Croy, B.A. (1986) In Immunoregulation and fetal survival. Eds: Gill, T.G. and Wegmann, T.G. *Oxford University Press,* pp. 156-168.

Li, X., Morris, L.H.A. and Allen, W.R. (2002) *In vitro* development of horse oocytes reconstructed wit the nuclei of fetal and adult cells *Biol. Reprod.* **66,** 1288-1292.

Jones, J.M. and Thomson, J.A. (2000) Human embryonic stem cell technology *Semin. Reprod. Med.* **18,** 219-223.

INFLUENCE OF IGF-I ON CYTOPLASMIC MATURATION OF HORSE OOCYTES *IN VITRO* AND ORGANISATION OF THE FIRST CELL CYCLE FOLLOWING NUCLEAR TRANSFER AND PARTHENOGENESIS

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INTRODUCTION

Studies of nuclear transfer in mammals have shown that nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC) and nuclear swelling resulting in nuclear reprogramming, are all prerequisites for first cell cycle organisation and further development of embryos created by nuclear transfer (Campbell *et al.* 1996; Prather 2000; Shin *et al.* 2002). In the horse, 2-cell cleavage and blastocyst development is very inefficient, whether the oocytes are reconstructed using fetal or adult fibroblasts as donor nuclei (Choi *et al.* 2002; Li *et al.* 2002, 2003). There have been only 2 reports to date of successfully cloned equids and these were both associated with very low rates of embryonic and fetal development compared to other species (Galli *et al.* 2003; Woods *et al.* 2003).

There is only limited information available about the first cell cycle organisation of horse oocytes reconstructed by nuclear transfer and the influence of culture conditions on nuclear remodelling of such reconstructed oocytes. This study was undertaken to investigate the influence of IGF-I on nuclear and cytoplasmic maturation of horse oocytes during culture *in vitro*. In addition, the organisation of chromatin and microtubules during the first cell cycle was compared in enucleated horse oocytes reconstructed by nuclear transfer and oocytes stimulated to divide parthenogenetically.

MATERIALS AND METHODS

Horse ovaries were obtained from a commercial abattoir and cumulus-oocyte complexes (COCs) were matured *in vitro* for 28–30 h at 38°C in 5% CO_2-in-air in TCM199 supplemented with 10% v:v heat inactivated fetal bovine serum, with or without 200 ng/ml added IGF-I. The same medium was used to culture the reconstructed oocytes after nuclear transfer.

The influence of IGF-I on cytoplasmic maturation was assessed by analysing the changes in MPF (cdc2 and cyclin B1) and MAPK (ERK1 and ERK2). Four groups of oocytes were prepared to be at different stages of the meiotic cycle. Namely, they were used directly after collection of the COCs, or they were cultured for 12, 24 or 28-30 h, with and without 200 ng/ml added IGF-I. Fifteen denuded oocytes were used for each treatment after which their proteins were extracted with SDS electrophoresis sample buffer then heated to 100°C for 5 min, before being frozen at -80°C. Western blot analysis was then performed as reported previously by Dai *et al.* (2000).

The nuclear transfer procedure was performed as described by Li *et al.* (2002, 2003). Fibroblast-cytoplasm couplets that had fused successfully were then activated chemically by immersing them in PBS medium containing 5 µM ionomycin for 5 min followed by culture for 4 h in TCM199 medium containing 5 µg/ml CCB and 10 µg/ml cycloheximide. For parthenogenesis, MII oocytes were subjected to the same conditions applied to activate the reconstructed oocytes. The rates of 2-cell and blastocyst stage development were counted 28–30 h after activation and at Day 7–8, respectively, in both the nuclear transfer and parthenogenesis groups. Oocytes submitted to either nuclear transfer or parthenogenesis were used to analyse the degree of organisation in the first cell cycle at 12–15 h after cell fusion or parthenogenic treatment, as described previously (Tremoleda *et al.* 2001; Li *et al.* 2003). The microtubules were labelled by incubating the fixed

TABLE 1: Influence of IGF-1 on nuclear maturation and 2-cell cleavage of horse oocytes following nuclear transfer or induction of parthenogenesis

IGF-1	Maturation total no./MII (%)	No. oocytes used	No. cleaved and developed further (%)*		
			2-cell stage	Blastocyst	Others#
Nuclear transfer		Reconstructed			
+	262/123 (47)	76	20 (26)[a]	6 (11)[a]	46
-	202/91 (45)	62	14 (23)[a]	2 (3)[a]	48
Parthenogenesis		Metaphase II			
+	47	24 (51)[b]	0[b]	23	
-	46	16 (35)[ab]	0[b]	30	

* [a] vs [b]: Values in the same column with different superscripts are significantly different when evaluated by z-test (P<0.05)
# Others include the total number of degenerate and embryos developmentally blocked before 2-cell stage

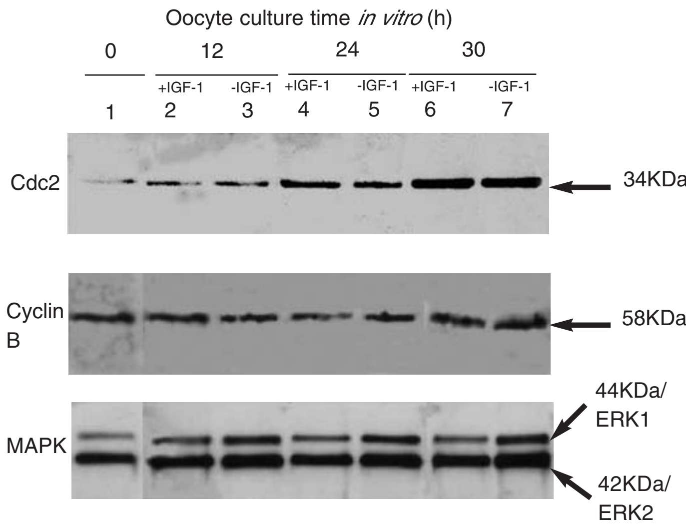

Fig 1: Changes in MPF (cdc2 and cyclin B1) and MAPK (ERK1 and ERK2) in horse oocytes after different times in culture (0, 12, 24 or 28–30 h) and with or without IGF-I added to the medium. Western blot analysis was performed using 3 monoclonal antibodies against, respectively, cdc2, cyclin B and ERK1/ERK2. Each sample was made from 15 oocytes.

oocytes for 90 min at 38°C in a 1:250 dilution of a monoclonal anti-α-tubulin antibody, followed by incubation for 1 h in a blocking solution. Next, the oocytes were exposed for 1 h at 38°C to a goat anti-mouse gamma globulin conjugated to fluorosaneisothiocyanate (FITC) and diluted 1:250 in PBS containing 0.5% Triton X-100 and 0.5% BSA. DNA was detected fluorescently by placing the oocytes for 10 min in PBS containing 5 µg/ml propidium iodide.

Results and discussion

The results are summarised in Table 1 and Figure 1. *In vitro* maturation of horse oocytes cultured with or without IGI-I supplementation, and their first cell cycle organisation, were studied in reconstructed horse oocytes made by somatic cell nuclear transfer, versus intact oocytes stimulated parthenogenetically. The rates of maturation of metaphase II oocytes (47% and 45%) and of reconstructed oocytes that developed to the 2-cell (27% and 25%) and blastocyst stages (11% and 3%), were not different between the media, with or without IGF-I, respectively. However, significantly more parthenogenic embryos exhibited 2-cell development with IGF-I (P<0.05). The results also demonstrated that the first cell cycle organisation in the reconstructed oocytes involved 2 different ways of nuclear remodelling. The donor nucleus in the Type I embryo showed normal nuclear remodelling which resulted in

normal embryonic development. In the Type II embryos, however, the donor nucleus formed a polyploid nucleus or the embryo fragmented. Addition of IGF-I to the maturation medium significantly increased the rate of normal Type I embryonic development from the reconstructed oocytes (45% vs 28%, $P<0.05$). Maturation-promoting factor (MPF, including cdc2 and cyclin B) and mitogen-activated protein kinase (MAPK, including ERK1 and ERK2) were present at the beginning of culture, just after the oocytes had been harvested from the ovaries. The quantities of cyclin B remained stable no matter how long a period of *in vitro* culture the oocytes underwent, whereas cdc2 showed a tendency to accumulate in the oocytes towards the end of the 30 h culture period. Addition of IGF-I to the medium may induce a bigger accumulation of MAPK in the cytoplasm of the horse oocyte, especially in the ERK2 component which might, in turn, increase the chance of the reconstructed oocyte undergoing nuclear remodelling to form a Type I embryo following nuclear transfer.

References

Campbell, K.H.S., Loi, P., Cappai, P. and Wilmut, I. (1996) Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev. Reprod.* **1,** 40-46.

Choi, Y.H., Love, C.C., Chung, Y.G., Varner, D.D., Westhusin, M.E., Burghardt, R.C. and Hinrichs, K. (2002) Production of nuclear transfer horse embryos by Piezo-driven injection of somatic cell nuclei and activation with stallion sperm cytosolic extract. *Biol. Reprod.* **67,** 561-567.

Dai, Y., Lee, C., Hutchings, A., Sun, Y. and Moor, R. (2000) Selective requirement for Cdc25C protein synthesis during meiotic progression in porcine oocytes. *Biol. Reprod.* **62,** 519-532.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G. (2003) A birth announcement calls for a rethink on the immunological demands of pregnancy. *Nature* **424**, 635.

Li, X., Morris, L.H.A. and Allen, W.R. (2002) *In vitro* development of horse oocytes reconstructed with the nuclei of fetal and adult cells. *Biol. Reprod.* **66,** 1288-1292.

Li, X., Tremoleda, J.L. and Allen, W.R. (2003) Effect of the number of passages of fetal and adult fibroblasts on nuclear remodelling and first embryonic division in reconstructed horse oocytes after nuclear transfer. *Reproduction* **125,** 535-542.

Prather, R.S. (2000) Pigs is pigs. *Science* **289,** 1886-1887.

Shin, M.R., Park, S.W., Shin, H. and Kim, N.H. (2002) Nuclear and microtubule reorganization in nuclear-transferred bovine embryos. *Mol. Reprod. Dev.* **62,** 74-82.

Tremoleda, J.L., Schoevers, E.J., Stout, T.A.E., Colenbrander, B., Bevers, M.M. (2001) Organisation of the cytoskeleton during *in vitro* maturation of horse oocytes. *Mol. Reprod. Dev.* **60,** 260-269.

Woods, G.L., Vanderwall, D.K., Li, G.P., Aston, K.I., Bunch, T.D., Meerdo, L.N., Pate, B.J. (2003) A mule cloned from fetal cells by nuclear transfer. *Science* www.sciencexpress.org /1086743.

HORSE CLONING, SCIENTIFIC, TECHNICAL, ECONOMICAL AND GENETIC ASPECTS

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Recent success at equine cloning has been achieved by Woods *et al.* (2003) who produced a mule from fetal cells and *Galli et al.* (2003) who produced the first foal from adult fibroblasts. K. Hinrichs (personal communication) lost a cloned pregnancy at 9 months and, at the time of this workshop, had 3 pregnancies at 8, 4 and 3 months.

The most interesting scientific finding is the unexpected possibility of a mare carrying its own copy, as it was thought that recognition of a different animal was key to endometrial cup reaction.

Technically, the approaches are different and success can be attributed to different factors. Woods *et al.* avoided the *in vitro* procedures, using pre-ovulatory oocytes from ultrasonographically guided ovum pick-up (OPU) and transferring the embryos immediately after fusion, whereas both Galli and Hinrichs used oocytes obtained from slaughtered mares, *in vitro* maturation and cultured the embryos up to the blastocyst stage.

For Woods *et al.* (2003) the key is activation by a high calcium concentration whereas for Choi *et al.* (2004) it is obtained by a sperm extract. In the work of Galli *et al.* (2003), the improvement comes from denuding oocytes before fusion.

Several companies have started to store cells, expecting a market for cloning either for copying pets or for true genetic and economic reasons.

There is little chance of making a perfect copy; in other species, altered growth *in utero*, abnormalities and over-weight neonates have occurred. Also, variability between clones is higher than that between twins, in which geneticists predict performance differences, as the heritability is only 15 and 35% for most disciplines, meaning that 65 to 85% of the variability is not genetic. Theoretically, clones could increase the genetic information on one genotype.

Even the colour markings differ and the old methods of identification will remain efficient to differentiate 2 clones whereas DNA tests will not.

In terms of using a copy of an outstanding individual to increase its progeny, the most favourable markets are disciplines which use geldings. When their ability is realised, it is often too late to breed from them. The use of clones widens the basis of selection and increases its intensity. However, the time for production of a pubertal clone increases the interval between generation. The overall benefit is a compromise between increased selection intensity and increased interval between generations. In racing, most performers are stallions and cloning provides little profit. In show jumping, about half the males are castrated and the genetic progress is accelerated by 10%. In eventing, dressage and endurance, almost all the performers are gelded and the genetic progress can be increased by 50%.

Older stallions with genetic superiority over younger animals might be the first market, as the income they produce each year is higher than the expected cost of cloning. Only when efficiency of cloning improves will multiplying females to increase numbers of their progeny become economic. However, this will raise a question about the role of mitochondrial DNA in the heredity transmitted by females.

REFERENCES

Choi, Y.H., Love, L.B., Westhusin, M.E. and Hinrichs, K. (2004) Activation of equine nuclear transfer oocytes: methods and timing of treatment in relation to nuclear remodeling *Biol. Reprod.* **70**, 46-53.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G. (2003) Pregnancy: a cloned horse born to its dam twin. *Nature* **7**, 635. Erratum: *Nature*. **425,** 680.

Woods, G.L., White, K.L., Vanderwall, D.K., Li, G.P., Aston, K.I., Bunch, T.D., Meerdo, L.N. and Pate, B.J. (2003) A mule cloned from fetal cells by nuclear transfer. *Science* 22, 1063. Epub 2003 May 29.

SESSION V:

General

SOMATIC CELL AMPLIFICATION OF EARLY PREGNANCY FACTORS IN THE FALLOPIAN TUBE

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This abstract concerns a potential function for ovarian somatic cells that are shed with the oocyte from a collapsing Graafian follicle at the time of ovulation, in particular those of the cumulus oophorus. Such cells have received little experimental attention once dissociated from the surface of the oocyte. However, their destiny within the Fallopian tubes should not be seen as one of redundancy. On the contrary, their ability to continue molecular conversations with the oocyte – and then zygote – needs serious consideration, as do interactions with the enveloping epithelium and mucosal layers of the Fallopian tube. Relevant literature is limited, but useful reviews include those of Motta *et al.* (1995, 1997), Familiari *et al.* (1998), Hunter (2003) and Hunter and Einer-Jensen (2003).

At the outset, it should be emphasised that large numbers of follicular cells (~10^5) accompany each oocyte into the ampulla of the Fallopian tube at ovulation, and that a suspension of such cells remains in the vicinity of the oocyte or embryo and is physiologically active. Although their synthetic activity is doubtless modified after ovulation and once more after fertilisation, these 'granulosa' cells can secrete steroid hormones, prostaglandins, diverse peptide molecules and glycoproteins. Paracrine actions should thus be anticipated, for the preceding molecules would be presented to the epithelium at relatively high concentrations and in the presence of cytokines. Accordingly, ovarian follicular cells in suspension in the Fallopian tube could act to transmit and amplify signals originating in the zygote, and might exert a trophic influence on ovarian activity either directly or via the endosalpinx. Such a putative effect would be primarily on secretion of progesterone as seen in ovarian venous blood, but it might also modify patterns of maturation in the follicular hierarchy. Not to be overlooked, there could also be a positive feedback influence on embryonic development and gene expression.

Considerations of molecular concentration strongly suggest that any influence of the follicular cell suspension and/or embryo on the ipsilateral ovary would require local counter-current systems of transfer rather than a systemic route. An appropriate blood vasculature exists and likewise a prominent lymphatic bed in the mesosalpinx adjoining the Fallopian tube (Andersen 1926). There is already clear evidence for a local transfer of ovarian hormones to the tissues of the Fallopian tube via the ovarian vein and tubal branch of the ovarian artery (Hunter *et al.* 1983). This is over and above the passage of ovarian hormones in the systemic circulation which necessarily leads to a massive dilution. In the present context, a counter-current transfer into the ovarian artery from the tubal venous arcade after confluence with the ovarian vein would be envisaged.

A series of experimental models has been devised for testing these proposals in pigs. Differences in the number of ovulations on the 2 ovaries and hence in the number of eggs and cumulus cells in the 2 Fallopian tubes have been exploited. As an end point, a molecular approach has been adopted based upon regional sampling of the endosalpinx and examination of corresponding gene activity, proceeding from mRNA. In order to demonstrate unilateral differences in gene activity in selected regions of the endosalpinx, we have model (a) involving unilateral ovariectomy and compensatory hypertrophy and (b) with unilaterally obstructed Fallopian tubes (ie one tube double-ligated and transected in the caudal isthmus to prevent passage of spermatozoa and fertilisation). In an attempt to highlight the

amplification role of follicular cells liberated from the oocyte surface, we also have a model (c) in which the number of granulosa cells in the vicinity of the embryos has been supplemented by transplantation directly into the Fallopian tube. Also, hopefully most dramatic of all, there is the model (d) in which embryo-activated cumulus cells are transplanted into the Fallopian tubes of unmated, cyclic animals in an attempt to generate an early pregnancy influence in the absence of embryos. Microarray procedures are being used to screen for the presence of novel genes.

Despite these systematic proposals, one cannot overlook the seemingly contrary evidence from procedures of embryo transplantation and related techniques. An impressive body of evidence exists from rodents, domestic farm animals and primates – especially man – that embryos generated by *in vitro* fertilisation can be transplanted directly into the uterus and give rise to full-term pregnancies (Edwards 1980; Biggers, 1981). Indeed, in our own species, spermatozoa and oocytes instilled into the uterus can generate a full-term pregnancy, and transplantation of an ovary into the uterus in a patient with blocked tubes (Estes' operation) can lead to a low incidence of pregnancy (review: Hunter 1988 and 1998). Such a body of observations places a substantial question mark over precisely how important the Fallopian tubes are for maturation of the gametes and early development of the embryo. However, the exceptional situation in primates may be explained largely by the morphology of the female tract, especially in the region of the utero-tubal junction, and substantial overlap in the composition of tubal and uterine fluids (Hunter 1998). As to the results of embryo transplantation in animals, there are 2 points of relevance. Firstly, that there is invariably a strong morphological selection of embryos to be transplanted which doubtless acts to improve the results over a spontaneous event. Secondly, that if reproductive success could be examined in a large population of breeding animals rather than in limited experiments, exposure of embryos to the Fallopian tube before entry into the uterus would be noted to improve fecundity over that in animals in which embryos are denied such exposure.

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REFERENCES

Andersen, D.H. (1926) Lymphatics and blood vessels of the ovary of the sow. *Contrib. Embryol. Carneg. Instn Wash.* **17,** 107-123.

Biggers, J.D. (1981) *In vitro* fertilisation and embryo transfer in human beings. *N. Engl. J. Med.* **304,** 336-342.

Edwards, R.G. (1980) *Conception in the Human Female.* Academic Press, London pp 413-415.

Familiari, G., Verlengia, C., Nottola, S.A. Tripodi, A., Hyttel, P., Macchiarelli, G. and Motta, P.M. (1998) Ultrastructural features of bovine cumulus-corona cells surrounding oocytes, zygotes and early embryos. *Reprod. Fertil. Develop.* **10,** 315-326.

Hunter, R.H.F. (1988) *The Fallopian Tubes: Their Role in Fertility and Infertility.* Springer-Verlag, Berlin & Heidelberg.

Hunter, R.H.F. (1998) Have the Fallopian tubes a vital role in promoting fertility? *Acta Obstet. Gynec. Scand.* **77,** 475-486.

Hunter, R.H.F. (2003) *Physiology of the Graafian Follicle and Ovulation.* Cambridge University Press, Cambridge pp 301-304.

Hunter, R.H.F., Cook, B. and Poyser, N.L. (1983) Regulation of oviduct function in pigs by local transfer of ovarian steroids and prostaglandins: a mechanism to influence sperm transport. *Europ. J. Obstet. Gynaec. Rep. Biol.* **14,** 225-232.

Hunter, R.H.F. and Einer-Jensen, N. (2003) Potential amplification of early pregnancy signals by ovarian follicular cells in suspension within the Fallopian tube. *Zygote* **11,** 237-243.

Motta, P.M., Nottola, S.A., Familiari, G., Macchiarelli, G., Vizza, E. and Correr, S. (1995) Ultrastructure of human reproduction from folliculogenesis to early embryo development. A review. *Ital. J. Anat. Embryol.* **100,** 9-72.

Motta, P.M., Nottola, S.A. and Familiari, G. (1997) *Cumulus corona cells at fertilisation and segmentation. A paracrine organ.* In: Microscopy of Reproduction and Development. *A Dynamic Approach* Ed: P.M. Motta, Antonio Delfino Editore, Rome, pp. 177-186.

PREGNANCY RATES AND ENDOCRINE EVENTS IN MARES TREATED WITH A GNRH AGONIST

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INTRODUCTION

Early embryonic losses are one essential reason for reduced fertility performances in mares. The frequency of such events, that means the loss of confirmed pregnancies, is indicated between 6.5% and 15% (Allen 2001; Rickets 2003). Although there is no evidence that a deficiency of progesterone production is a cause of pregnancy loss in mares, many mares are treated with progestagens to prevent embryonic losses (Allen 2001). Moreover, observations made by Pycock and Newcomb (1996) and by Newcomb *et al.* (2001) indicate that pregnancy rates in horses and pony mares were significantly higher after GnRH analogue application than in control animals. Nevertheless, the mechanisms for this increase in pregnancy rates are up to now unclear. Moreover, it is not clear if this effect depends on fertility status of the mares. Therefore the aim of this study was to prove the effect of a GnRH analogue on pregnancy rate of mares in a stud under considering the following factors: reproductive status of the mares, type of sperm preservation and the number of oestrus for insemination. In addition to this the authors investigated the influence of the applied GnRH analogue on progesterone and LH concentrations.

MATERIALS AND METHODS

Experiment 1

The field study was performed during the breeding season in the year 2000. Altogether, 205 warmblood mares were daily checked for signs of oestrus. After showing heat symptoms or after induction of luteolysis with $PGF_{2\alpha}$ (Estrumate®, ESSEX, Germany) follicular development was monitored by ultrasound technique. Prior to the expected time of ovulation ultrasound scanning of the ovaries was done in 6 h intervals to predict the right time for insemination. Fresh or frozen/thawed semen from 32 stallions was used for AI. Buserelin (Receptal®, Hoechst, Germany, 40 µg/animal) was applied in 136 cycles 10 days after AI. Control animals received 10 ml 0.9% NaCL (placebo). Placebo was also given in n= 136 cycles. That means, that n= 67 mares were used in 2 successive oestrous cycles. On Day 16 after AI pregnancy diagnosis was performed by ultrasound scanning of the uterus.

Experiment 2

Altogether the blood of 20 mares with foals at foot was used for hormone determination. Ten days after the first ovulation after parturition all mares received $PGF_{2\alpha}$ (Estrumate®, ESSEX, Germany) for induction of luteolysis. Blood samples were taken on Day 0, 2, 4 and 8 after ovulation for measurement of the circulating progesterone concentrations. On Day 9 and 11 after ovulation blood samples were collected at 1 h intervals over a period of 6 h for measurement of circulatiug progesterone and LH concentrations. On Day 10 blood samples were taken every 20 min for a period of 6 h for LH determination. A single intramuscular injection of 40 mg Buserelin (Receptal®, Hoechst, Germany, n= 10) or 10 ml 0.9% NaCl (placebo, n=10) was applied immediately after the third blood sample had been taken on Day 10. Determination of progesterone was done by 3H-RIA (B/F separation by charcoal). LH determination was done by a homologous 125I-RIA (double-antibody-technique).

For statistical analysis data were analysed by a

TABLE 1: Mean values for pregnancy rate depending on treatment and type of spermatozoa

Treatment	Type of spermatozoa	Number of treatments (n)	Pregnancy rate (%)
Placebo	Fresh	43	41.8
	Frozen/thawed	93	31.2
Buserelin	Fresh	64	48.4
	Frozen/thawed	72	41.6

TABLE 2: Mean values for pregnancy rate depending on treatment after first or second AI

Treatment	Treatment after first or second AI	Number of treatments (n)	Pregnancy rate (%)
Placebo	1st	89	32.6
	2nd	47	38.3
Buserelin	1st	64	42.2
	2nd	72	51.4

mixed model, with 4 fixed factors (treatment, type of spermatozoa, heat, reproductive status of the mare) and a random factor (stallion) nested in the factor type of spermatozoa using the procedure MIXED of SAS© (SAS Systems, Release 8.2, SAS Institute Inc., Cary, NC 1999). Significance was set at P=0.05.

Results

In Experiment 1 altogether 272 pregnancy diagnoses were performed on Day 16 post ovulation. Independent of treatment and other factors 108 mares (39%) were pregnant. After the first AI 37.7% of the mares (71/188) were pregnant, whereas the same parameter was 44.0% (37/84) after the second insemination. The mean pregnancy rate among treated mares was 44.8% (61/136), whereas the same parameter was 34.5% (47/136) in the control group. Tables 1 and 2 contain mean values for pregnancy rates depending on treatment and type of spermatozoa (fresh or frozen/thawed) and depending on treatment with GnRH analogue or placebo after first or second AI.

Mean values for pregnancy rates in Table 1 and 2 show the tendency to be higher after treatment with GnRH analogue, but it has to be considered that the means are not adjusted for the factors cited under materials and methods. Data in Table 3 show adjusted means and confidence intervals for the means after the use of the described mixed model.

TABLE 3: Adjusted means for pregnancy rate and confidence intervals for the means

Treatment	Pregnancy rate (%)	Confidence intervals
Placebo	36.4[a]	< 25.8; 46.8 >
Buserelin	48.4[b]	< 38.4; 58.4 >

[a,b] P= 0.08

In Experiment 2 the mean progesterone concentration increased continuously from Day 0 to Day 8 after ovulation (treated group: from 0.81 0.48 ng/ml to 5.47 ± 0.48 ng/ml, control group: from 0.63 ± 0.68 ng/ml to 5.83 ± 0.68 ng/ml). From Day 9 to 11 of oestrous cycle mean progesterone concentrations from 5.29 ± 0.65 ng/ml to 5.71 ± 0.65 ng/ml were measured in treated mares, whereas the same parameter was 5.15 ± 0.96 ng/ml to 6.01 ± 0.99 ng/ml in control mares. The progesterone concentrations from Day 9 to Day 11 showed an opposite development in

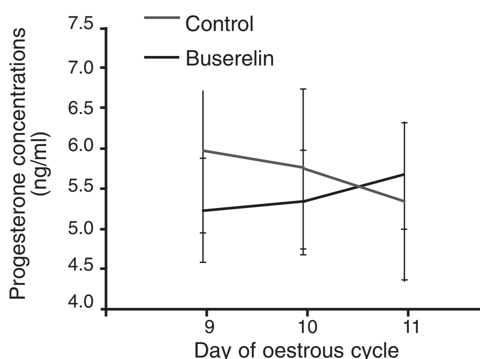

Fig 1: Progesterone concentrations on Day 9 to 11 depending on treatment.

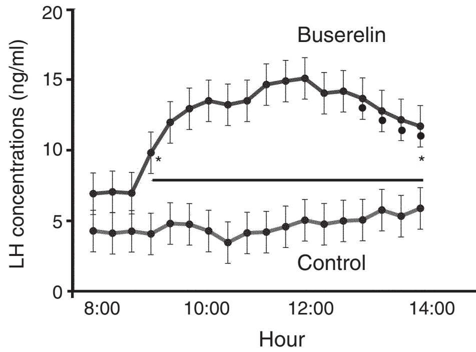

Fig 2: LH concentrations on Day 10 depending on treatment.

the treated group and the control group but these differences were not significant (Fig 1). On Day 10 of the oestrus cycle treated mares showed a significant increase of mean LH concentration after injection of Buserelin. In these mares the mean LH concentration for this day was 12.15 ± 1.36 ng/ml, which was 7.51 ng/ml higher than in control mares. This difference was significant (Fig 2).

Discussion

Newcomb *et al.* (2001) showed on the basis of the data obtained, that pregnancy rate in mares was significantly higher after application of the GnRH analogue Buserelin in comparison to control animals. But up to now there is no satisfactory explanation about the underlying mechanisms for this effect. We studied the effect of Buserelin under different conditions to Newcomb *et al.* (2001). One main difference was the integration of AI. with fresh or frozen/thawed semen into the experimental schedule. The other difference was the mean pregnancy rate in the herd used. Independent of these differences we found a positive effect of the GnRH analogue Buserelin on pregnancy rate. This positive effect was independent of the reproductive status of the mare and type of spermatozoa. Moreover, it was seen after first and second AI. The analysis of the data obtained shows clearly that the factors stallion and type of spermatozoa have a strong influence on pregnancy rates independent of treatment. As expected, LH concentrations were significantly influenced by treatment, but there was no significant effect on progesterone concentrations. So, the mechanism(s) for this effect of treatment with GnRH analogue on pregnancy rate remain unclear. Further investigations will be directed to a better understanding of the action of GnRH analogue on corpus luteum function.

References

Allen, W.R. (2001) Luteal deficiency in and embryo mortality in the mare. *Reprod. Dom. Anim.* **63,** 121-131.

Newcomb, J.R., Martinez, T.A. and Peters, A.R. (2001) The effect of the gonadotropin-releasing hormone analog, Buserelin, on pregnancy rates in horses and pony mares. *Theriogenology* **55,** 1619-1631.

Pycock, J.F. and Newcomb, J.R. (1996) The effect of the gonadotrophin-releasing hormone analog, Buserelin, administered in diestrus, on pregnancy rates and pregnancy failure in mares. *Theriogenology* **46,** 1097-1101.

Ricketts, S.W. (2003) Early pregnancy failure as seen during the course of equine stud farm practice in Newmarket, 1996-2003. *Pferdeheilkunde* **19,** 633-638.

NON-PHARMACOLOGICAL SUPPRESSION AND PHARMACOLOGICAL SYNCHRONISATION OF OESTRUS AND OVULATION IN THE MARE

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Unwanted cyclical oestrous behaviour can occasionally disturb training and competition schedules in racing Thoroughbred fillies and female high class eventers, showjumpers, dressage horses and other types of Sporthorse to the extent the affected individuals must be retired from active competition and sent to stud prematurely. Administration of progestagens or other compounds to suppress such troublesome oestrous cyclicity is internationally banned as 'the administration of a non-normal nutrient' and there therefore exists a pressing need for the development of an alternative non-pharmacological method to achieve the same desired end point; namely, suppression of oestrous signs and ovulation for extended periods with a minimum of handling, treatments and costs. Conversely, there is an equally urgent need for therapeutic protocols that will reliably and efficaciously synchronise ovulation in individuals or groups of mares for the purposes of timed insemination using cooled or deep frozen semen, or for the establishment of synchronised recipient mares in embryo transfer programmes. Might the same therapy be applied usefully in the two apparently diametrically opposed situations?

Recent experiments suggests it could be. All 11 of 11 Pony mares in which the conceptus was ruptured by manual pressure exerted *per rectum* between 16 and 22 days after conception passed into a prolonged period of pseudopregnancy (mean 82 ± 13 days; range 64 to 228 days) during which oestrous signs were absent, waves of follicles came and went in the ovaries and serum progesterone concentrations remained persistently >4 ng/ml (Fig 1: Lefranc and Allen 2004). Thus, in return for the initially very few and essentially straightforward manipulations it was possible to

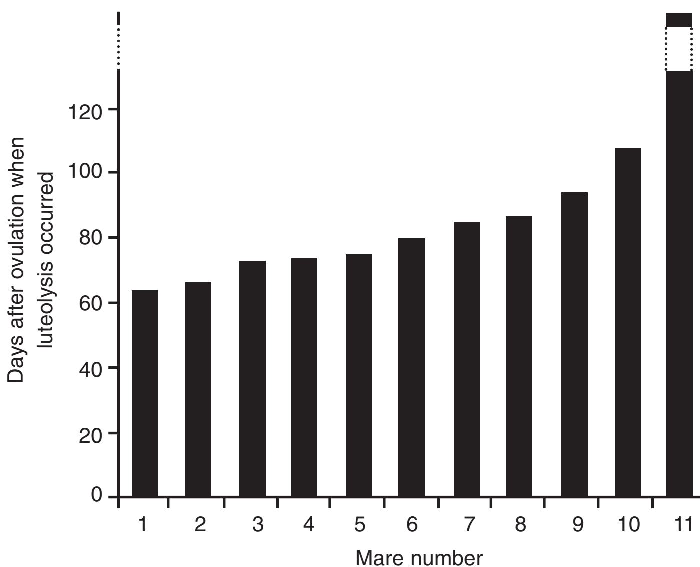

Fig 1: Length of pregnancy/pseudopregnancy in 2 pony mares following manual rupture of the conceptus per rectum between Days 16 and 22 ofter ovulation (Reproduced from Lefranc and Allen 2004).

suppress cyclicity completely for around 3 months in all 11 treated mares. Furthermore, it is clear that it would be possible to give a single intramuscular injection of a PGF analogue (eg Estrumate; Schering-Plough Animal Health, Middlesex) to any of these mares in pseudopregnancy to initiate luteolysis and a return to oestrus in 2–3 days. Moreover, the interval from that prostaglandin treatment to the subsequent ovulation could be guessed reasonably accurately by ultrasonographically assessing the level of follicular development at the time of prostaglandin administration. Of course, the precise timing of the ovulation could be greatly narrowed by treatment with any one of the many ovulation-inducing hormone preparations currently available when the dominant follicle reached ± 35 mm in diameter.

In summary, luteostasis induced 'naturally' by the antiluteolytic actions of the young conceptus could be the lynch pin in a range of subsequent options to either suppress, or induce and synchronise, oestrus and ovulation in individuals or groups of cycling mares.

Reference

Lefranc, A.-C. and Allen, W. R. (2004) Non-pharmacological suppression of oestrous in the mare. *Equine vet. J.* **36,** 183-185.

WORKSHOP SUMMARY

The third biennial meeting of the European Equine Gamete Group (EEGG) provided concrete evidence of the continuing progress being made in the field of equine gamete biology in Europe, a process in which the opportunity for discussion provided by, and collaborations initiated during, the EEGG meetings have played a valuable part. For this, we are indebted to Twink Allen and Marian Tischner, who initiated the group, and to the Dorothy Russell Havemeyer Foundation which, steered by Mr Gene Pranzo, has generously sponsored all of our meetings. The latest meeting remained true to the founding principles of EEGG, with a good balance between fundamental scientific research and the application of gamete technology to practice, including discussion of the ways in which these practical applications can be hampered by logistical or bureaucratic complications.

STALLION FERTILITY

There were only 2 presentations on stallion fertility or semen quality. This is surprising when it is considered that both papers emphasised how little we actually know about the factors that influence semen 'quality' and in-field fertility. Indeed, it is clear that we have no more than a rudimentary understanding of the role or effect of the various accessory sex gland secretions that contribute to an ejaculate, on semen quality and preservability, and there is still no reliable *in vitro* test for predicting in-field fertility of a stallion. The failure to develop a simple test for 'fertilising capacity' is undoubtedly due to the complex array of properties that a sperm must possess if it is to fertilise an oocyte *in vivo*. On the other hand, flow cytometric analysis of semen samples for parameters such as viability, membrane status (eg capacitation and/or the acrosome reaction), mitochondrial activity and DNA integrity (sperm chromatin structure assay) does appear to offer a rapid and objective way of analysing large numbers of sperm. It must be hoped that a range of flow-cytometrically assessable parameters can be identified that, in combination, provide a good prospective indication of fertilising capacity. The importance of research in this area was highlighted by the frustration of the clinicians at embryo recovery or pregnancy rates that fluctuate dramatically as a function of the source of the semen (stallion and/or stud). While there is much more to be done before we can accurately predict the fertility of a stallion, flow-cytometry may also be able to simplify and accelerate this process. For example, the importance of a given sperm characteristic could be established more rapidly by separating out sperm populations that do or do not express that characteristic and using the separated samples for low dose insemination. In this way, many fewer mares would be needed to examine the influence of a sperm property on fertility, and the probability that the selected characteristic was the source of any difference in pregnancy rates would be enhanced.

OOCYTE MATURATION

A number of papers were presented on aspects of equine oocyte maturation *in vitro* (IVM). Of course, the low maturation rates (compared to other species) and the poor quality of the oocytes that do reach the metaphase II stage are significant limiting factors in developing a practical system for *in vitro* production (IVP) of horse embryos. Encouragingly, rapid progress is being made in characterising a number of the developmental processes (eg mitochondrial redistribution, glutathione content, cumulus expansion and MAP kinase activity) that occur during oocyte maturation *in vivo* and whose inadequate replication *in vitro* may contribute to reduced oocyte survival or quality. Indeed, while the production of live, normal offspring is the only wholly reliable index of oocyte developmental competence, it must be hoped that reference to *in vivo* oocyte development patterns will simplify the development of better IVM media. In this respect,

however, it was surprising that very few of the reported experiments employed truly defined culture media. Most researchers preferred to include serum and, while it is accepted that IVM rates are better in serum-containing media, its variable and imprecise composition means that repetition of results cannot be guaranteed and, therefore, interpretation must be more cautious.

IVF

The major aim of perfecting IVM is, of course, to provide raw material for *in vitro* fertilisation (IVF). Given the number of papers on IVM, it was surprising that there was only one presentation on IVF *per se*. Presumably this is primarily because intra-cytoplasmic sperm injection (ICSI) has been relatively successful, whereas conventional IVF has not and, thus, is extremely frustrating. However, while ICSI is the current method of choice for equine IVP, there are undoubted advantages of perfecting conventional IVF in the longer term. In a research setting, IVF would allow lower-cost, less time-consuming production of larger numbers of embryos for studies on early development. More importantly, while doubts remain as to the possible effects on the development of embryo, fetus and eventual adult of introducing the whole sperm instead of just the sperm nucleus into the ooplasm, conventional IVF must be considered the most innocuous and desirable means of fertilising an oocyte *ex vivo*.

We heard how the structure of the zona pellucida and the distribution of its important surface proteins is altered by IVM. Such alterations may in part explain the poor penetrability of IVM oocytes, but not why *in vivo* matured horse oocytes are also penetrated very poorly *in vitro*. We still need to find out why it is so difficult to successfully stimulate a stallion sperm to penetrate an oocyte outside the mare.

Embryo quality

One relatively new and exciting area of research introduced at this meeting was the analysis of embryo quality using molecular biological techniques. It is now possible to use DNA-specific probes and fluorescent *in situ* hybridisation (FISH) to analyse the chromosomal constitution of most of the cells in an embryo. It was demonstrated that abnormalities of ploidy occur in equine embryos and may lead to early embryonic death, as has long been postulated but never proven. The prospect of more probes, and ‘paints’ for entire chromosomes, means that we may soon be able to detect monosomies and even fairly subtle translocations, either of which may be lethal to a developing embryo. While it is difficult to see pre-implantation genetic screening becoming an important clinical tool in the horse, these techniques may be useful for analysing the cause of failed embryonic development and for studying the effects of advanced maternal age, IVP or cloning on the chromosomal integrity of embryos. In this respect, we also learned that cloning by nuclear transfer quite often leads to aberrant early cell division, such as a round of DNA replication in the absence of cytoplasmic division to yield a tetraploid and, therefore, non-viable embryo.

Studies of embryonic gene expression are similarly in their infancy in the horse, but may be of use in establishing markers for embryonic normality, and certainly will be invaluable for investigating processes critical to early embryonic development. Of course, such markers will never rival the birth of a live foal as a means of verifying embryonic quality, but they may provide useful and user-friendly tools for examining the effect of factors such as maternal age, in vitro culture or cloning on embryonic development and normality.

Cloning

It was unfortunate that Cesare Galli was unable to join us in Pardubice, but pleasing that this was because of the interest generated by the birth of his healthy cloned foal. Fascinatingly, the surrogate mother was also the nuclear donor and she, thus, gestated her own clone, a feat that raises a whole spectrum of questions about the immunology of pregnancy. We heard reports of the extremely low blastocyst formation rate after somatic cell nuclear transfer (2–10%), the low pregnancy rate after transfer of these blastocysts to recipient mares, and the even lower number of pregnancies that progress to term. Promisingly, however, all 4 of the foals born so far appear to be clinically normal, and it may be that, like the goat,but in contrast to the sheep and cow, abnormal conceptuses are eliminated during development so that only ‘normal’ foals are born. If so, this should negate many of the anticipated objections to cloning based on welfare of the cloned offspring. A sensible approach to the practical use of cloning was outlined by Eric Palmer who plans to use cloning not as a means of recreating equine athletes but as a way of producing ‘gamete donors’. A large

proportion of the horses competing at the highest level in many disciplines are geldings, and cloning appears to be a potentially valuable way of rescuing 'lost' germ-lines. The biggest advantage of this approach is that any epigenetic abnormalities in the cloned horse *per se* should be wiped out during re-programming of the gametes and not, therefore, passed to the offspring. In fact, the contribution of a male clone to its offspring should be identical to its progenitor since, during normal fertilisation, the sperm essentially contributes only its nuclear DNA to the zygote. The situation in the female is of course complicated by introduction of mitochondria from the oocyte donor and it is uncertain what role mitochondrial DNA plays in the development or function of an individual. In any case, the anticipated rapid progress of equine cloning should be fascinating to follow.

Embryo transfer

It is good to be able to report that embryo transfer at last appears to be attracting commercial interest in Europe. We are already a long way behind the USA and South America with regard to its use in practice. It remains to be seen if this interest will be maintained and if the logistical and legal barriers to the transport of embryos within Europe and, in particular, between continents can be resolved quickly. If so, the commercial demand in countries where ET is widely accepted may well stimulate development of an export market in countries where little ET is currently done, but interesting bloodlines are found. With regard to embryo transportation, pregnancy rates with frozen-thawed embryos appear to be almost matching those with fresh embryos. While the use of ethylene glycol as a cryoprotectant may be part of this improvement, the most important factor is probably the precise determination of the time of ovulation to ensure flushing at the morula-early blastocyst stages. However, given the increasing evidence that factors such as semen type (frozen versus fresh), mare age and time of year significantly influence the rate of embryo development, the chilled transport of embryos to a few large recipient farms within Europe still seems a more realistic solution for the problems of recipient provision if the uptake of ET continues to increase. Certainly, the cryopreservation of expanded blastocysts remains a problem, notwithstanding the use of treatments to thin the capsule or stabilise the cytoskeleton. The reluctance to use cryopreservation on a large scale is likely to remain until reliable techniques are developed to accelerate oviductal transport of embryos or cryopreserve expanded blastocysts.

Acknowledgments

The enthusiastic discussion sessions and earnest but light-hearted atmosphere in which the sessions were conducted were ample proof of the success of this meeting, both scientifically and as a social 'event'. For this, we owe a vote of thanks to a number of people: Twink Allen, who organised the scientific programme; R & W Publications who were responsible for the smooth running of the meeting; Gene Pranzo and the Dorothy Russell Havemeyer Foundation who again provided generous financial support and who deserve our thanks and congratulations for continuing to insist on the small workshop format that, as ever, ensured lively, interesting and informative discussions; and finally Zdenek Muller and his family who did an exemplary job of hosting us and organising a number of extremely enjoyable and exclusive VIP visits and social events, the most memorable of which was the day out at the world famous Pardubice Steeplechase ending in a trip around the course in a horse and carriage.

In the light of the understandable and undoubtedly sensible decision by the Havemeyer Foundation to broaden subsequent equine gamete meetings to include the 'rest of the world', and prevent the development of 'rival' groups in different continents, it must be hoped that we can keep the traditionally smaller and less affluent nations, such as the Czech Republic, 'on board'. The way in which we were welcomed and hosted in Pardubice was evidence, if any were needed, that we will all be the poorer for it if we do not.

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