



*Havemeyer Foundation*  
*Monograph Series No. 18*

*Proceedings of a Workshop*

# **INTERNATIONAL EQUINE GAMETE GROUP**

*18th – 21st September 2005*  
*Kühlungsborn, Germany*

**Editors: H. Alm, H. Torner and J. F. Wade**



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First published 2006

ISSN 1472-3158

Published by **R & W Communications**

Printed in Great Britain by Quality Print Services (Anglia) Limited

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## FOREWORD

It gave us a great pleasure to welcome delegates to Kühlungsborn/Rostock, Germany, for the 4th meeting in this series. The past 3 meetings (1999 Lopuszna, Poland; 2001 Loosdrecht, The Netherlands; 2003 Pardubice, Czech Republic) were held under the auspices of the European Equine Gamete Group, founded in 1999 by Twink Allen and Marian Tischner.

However, during the meeting in Pardubice, Mr Gene Pranzo, President of the Dorothy Russell Havemeyer Foundation, called on the scientists from Europe and North America to come together and participate jointly in this forum. In both these continents there are various groups working in the field of equine assisted reproduction and, therefore, the name of the group has been changed to the International Equine Gamete Group, and we were pleased to welcome all delegates, both from Europe and from North America. Also in future, the venue for our biennial meeting will alternate between North America and Europe.

Since our last meeting several remarkable achievements have occurred. The first cloned horse by Cesare Galli and his laboratory in Italy was reported in 2003 and, in 2005, cloned foals were announced both in Italy and in Texas, USA.

Other results in the field of reproductive biology have been obtained, providing new data in the determination of cytoplasmic maturation of horse oocytes for a better understanding of the complex regulatory system in the horse oocyte during maturation.

The number of scientists working in the area of assisted reproduction in horses is quite limited. Furthermore, the limited availability of horse gametes and embryos makes it necessary for scientists to work together. And so we hope that the 4th meeting stimulated new experimental protocols and activated collaboration between European and American scientists and groups.

We would like to express our heartfelt thanks to Gene Pranzo and the Dorothy Russell Havemeyer Foundation for their continuing support of this meeting, which provides a unique forum for discussion and collaboration.

We hope you had a pleasant and informative time at this workshop and on the Baltic sea coast of Germany.

We are looking forward to the next International Equine Gamete Group meeting, which will be held in Texas, USA, in 2007.

*Hannelore Alm, Helmut Torner,*

## HAVEMEYER SCIENTIFIC WORKSHOPS

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- 1981            **First International Workshop on Lymphocyte Alloantigens of the Horse**  
October - New York City, USA  
*Organiser: Dr D. F. Antczak*
- 1982            **Second International Workshop on Lymphocyte Alloantigens of the Horse**  
October - Cornell University, Ithaca, New York, USA  
*Organiser: Dr D. F. Antczak*
- 1983            **Third International Workshop on Lymphocyte Alloantigens of the Horse**  
April - New Bolton Center, University of Pennsylvania, USA  
*Organiser: Dr D. F. Antczak*
- 1984            **First International Symposium on Equine Embryo Transfer**  
October - Cornell University, Ithaca, New York, USA  
*Organisers: Drs D. F. Antczak and W. R. Allen*
- 1985            **Fourth International Workshop on Lymphocyte Alloantigens of the Horse**  
October - University of Kentucky, USA  
*Organisers: Drs D. F. Antczak and E. Bailey*
- 1986            **Workshop on *Corynebacterium equi* Pneumonia of Foals**  
July - University of Guelph, Canada  
*Organiser: Dr J. F. Prescott*
- 1987            **Fifth International Workshop on Lymphocyte Alloantigens of the Horse**  
October - Louisiana State University, USA  
*Organisers: Drs D. F. Antczak and J. McClure*
- 1989            **Second International Symposium on Equine Embryo Transfer**  
February - Banff, Alberta, Canada  
*Organisers: Drs D. F. Antczak and W. R. Allen*
- 1990            **International Workshop on Equine Sarcoids**  
April - Interlaken, Switzerland  
*Organisers: Dr D. F. Antczak and Professor S. Lazary*
- 1992            **Workshop on Equine Neonatal Medicine**  
January - Naples, Florida  
*Organisers: Drs D. F. Antczak and P. D. Rossdale*

**Third International Symposium on Equine Embryo Transfer**

February - Buenos Aires, Argentina

*Organisers: Drs D. F. Antczak, W. R. Allen, J. G. Oriol and R. Pashen*

1995

**Equine Perinatology**

July - Cambridge, England

*Organiser: Dr P. D. Rossdale*

**Second International Equine Leucocyte Antigen Workshop**

July - Lake Tahoe, California, USA

*Organisers: Drs D. F. Antczak, P. Lunn and M. Holmes*

**First International Workshop on Equine Gene Mapping**

October - Lexington, Kentucky, USA

*Organisers: Drs D. F. Antczak and E. Bailey*

**Erection and Ejaculation in the Human Male and Stallion: A Comparative Study**

October - Mount Joy, Pennsylvania, USA

*Organiser: Dr S. M. McDonnell*

**Bone Remodelling Workshop**

October - Corcord, Massachusetts, USA

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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

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April - Hungary

*Organisers: S. Lazary and E. Marti*

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June - Port Douglas, Australia

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**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

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**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*

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October - Fairmont Hotel, New Orleans, USA

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2002

**USDA International Plant & Animal Genome Conference**

January - San Diego, California

**Comparative Neonatology/Perinatology**

March - Palm Springs, California

*Organiser: P. Sibbons*

**Stallion Behavior IV**

June - Reykjavik, Iceland

*Organisers: S. McDonell and D. Miller*

**Rhodococcus Equi II**

July - Pullman, Washington

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**Equine Orthopaedic Infection**

August - Dublin, Ireland

*Organiser: E. Santschi*

**Inflammatory Airway Disease**

September - Boston, USA

*Organiser: Dr E. Robinson*

2003

**USDA International Plant and Animal Genome Conference**

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**Embryonic and Fetal Nutrition**

May - Ravello, Italy

*Organiser: S. Wilsher*

**Genomics and the Equine Immunity System**

June - Ithaca, New York

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**Fifth International Gene Mapping Workshop**

August - Kreuger Park, South Africa

*Organiser: E. Baily and E. Vandyke*

**Equine Recurrent Laryngeal Neuropathy**

September - Stratford-upon-Avon, UK

*Organisers: P. Dixon and E. Robinson*

**Transporting Gametes and Embryos**

October - Brewster, Massachusetts

*Organiser: E. Squires*

**Third Meeting of the European Gamete Group (EEGG)**

October - Pardubice, Czech Republic

*Organiser: J. and Z. Müller*

**Nosocomial Infections and Biosecurity in Equine Hospitals**

October - Lexington, USA

*Organiser: F. Bain and J. Taub-Dargatz*

2004

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January - San Diego, California

**Equine Viral Herpes Virus Workshop**

June/July - Tuscany, Italy

*Organiser: P. Lunn*

**Equine Embryo Transfer VI Workshop**

August - Rio de Janeiro, Brazil

*Organiser: M. Alvarenga*

**Sporting Injuries in Horses and Man: A Comparative Approach**

September - Lexington, USA

*Organiser: E. J. L. Soulsby*

**Maternal Recognition of Pregnancy in the Mare III**

November - Barbados, West Indies

*Organiser: T. A. E. Stout*

2005

**USDA International Plant and Animal Genome Conference**

January - San Diego, California

*Organiser: J. Mickelson*

**Comparative Placentology**

April - Victoria, Canada

*Organiser: P. Sibbons*

**Sixth International Gene Mapping**

July - Dublin, Ireland

*Organisers: E. Bailey and J. Flynn*

**World Equine Airway Symposium**

July - Ithaca, USA

*Organisers: D. Ainsworth, E. Robinson, N. DuCharme, B. McGorum and L. Viel*

**Genetic Relatednes Between Different Breeds of Horses using Molecular Markers**

August - Poland

*Organisers: M. Binns, G. Lothran and B. Graiak*

**International Equine Gamete Group**

September - Kühlungsborn, Germany

*Organisers: H. Alm, H. Torner, K. Hinrichs and E. Squires*

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# SESSION I:

## Semen

*Chairman:*

*E. Squires*



# SEMINAL PLASMA STUDIES IN STALLIONS

T. Katila, M. Kareskoski, E. Akcay\*, T. Reilas†, E. Koskinen\*\* and J. J. Calvete††

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## INTRODUCTION

Stallions show individual differences in seminal plasma composition (Ball *et al.* 2000; Barrier-Battut *et al.* 2002), which may be reflected in sperm survival. The exchange of seminal plasma between stallions has affected sperm motility during freezing (Aurich *et al.* 1996) and cooled storage (Katila *et al.* 2004). Also, compositions of fractions within the ejaculate differ because of the different contributions of accessory sex glands (Magistrini *et al.* 2000).

Seminal plasma has been shown to decrease sperm motility during cooled storage (Jasko *et al.* 1991) and, therefore, its proportion is commonly reduced during cooled storage by dilution with semen extenders or by centrifugation. However, in a previous experiment (Katila *et al.* 2002), where seminal plasma was removed by repeated washing and centrifugation, the centrifuged semen samples in skim milk extender exhibited lower motility after 24 h storage than the non-centrifuged samples. Skim milk may not be the best extender in the absence of seminal plasma (Rigby *et al.* 2002). In this subsequent experiment, skim milk and modified Tyrode's solution were compared with sperm-rich and sperm-poor seminal plasma, and seminal plasma was exchanged between stallions. In addition, some constituents of seminal plasma in different fractions of the ejaculate were measured.

## MATERIALS AND METHODS

In the first experiment, semen was collected from 4 stallions using an automated phantom, which fractionates semen into 5 cups (Lindeberg *et al.* 1999). Sperm-rich and sperm-poor seminal

plasmas and 2 types of extenders were compared after a cooled storage of 24 h in an Equitainer container. Centrifuged and washed spermatozoa from the 2nd cup were suspended in skim milk extender, in a modified Tyrode's medium (MT) prepared as described by Padilla and Foote (1991), in the stallion's own seminal plasma fractions and in the seminal plasma fractions of the other 3 stallions. Spermatozoa were evaluated for motion characteristics using a computerised motility analyser (Sperm Vision Minitüb, Tiefenbach b. Landshut, Germany) and for plasma membrane integrity by calcein AM/propidium iodide staining (CAM/PI). Osmolarity and the pH of the medium were measured.

In the second experiment, several components of seminal plasma were analysed and compared between stallions and ejaculate fractions. The following electrolytes were analysed using commonly applied methods: Ca, P, Mg, Cl, Na and K. Some enzymes, including alkaline phosphatase (AP), acid phosphatase (ACP),  $\beta$ -glucuronidase (B-Gase), N-acetyl- $\beta$ -glucosaminidase (NAGase), catalase (CAT) and total protein (PROT), were also measured. Seminal plasma proteins were isolated by reverse phase HPLC and identified using mass spectrometry and N-terminal sequencing.

## RESULTS

The sperm-poor part of the ejaculate showed higher motility values than the sperm-rich part, but MT maintained motility best. The lowest motility values, but the highest viability percentages, were associated with the spermatozoa in skim milk extender. The osmolarity of skim milk extender after a 24 h storage was significantly higher (374.3

mOsm) than that of MT (329.9) or seminal plasmas (sperm-poor 332.7 and sperm-rich 333.7 mOsm). The pH of skim milk extender at 24 h was significantly lower (6.77) than that of MT (7.35) or seminal plasmas (7.3 and 7.2). The seminal plasma of one stallion did not maintain sperm motility and viability as well as the seminal plasma of the other 3 stallions.

Significant differences between stallions were found in the concentrations of CAT, PROT and major horse seminal plasma proteins (HSP-1, 2 and 3). Significant differences between fractions were demonstrated in ACP, Ca, P, Mg, and Cl concentrations. The first fraction containing only pre-ejaculatory fluid showed very low CAT, PROT, Ca and Mg concentrations but high Cl and Na concentrations. In Fraction 2, ACP, AP and P levels were the highest, whereas Cl concentration was the lowest. Ca and Mg concentrations were the highest in Fraction 3. HSP-2 levels were the highest in Fraction 2, but HSP-1 and 3 showed no differences between fractions. Significant differences between stallions were found for Na in Fractions 2 and 4, for ACP in Fractions 3 and 4 and for Mg in Fraction 3.

## DISCUSSION

The previously reported differences in motility after 24 h cooled storage between centrifuged, skim milk extended sperm samples and samples containing seminal plasma can be explained by the low pH and the high osmolarity of skim milk extender. The pH and osmolarity of MT were similar to those of seminal plasmas, and spermatozoa stored in MT showed the highest motility values. On the other hand, the percentage of intact plasma membranes was the highest in skim milk, followed by MT and sperm-poor seminal plasma; sperm-rich seminal plasma had the lowest percentage. Our results are in agreement with those of Rigby *et al.* (2002). The selection of a suitable extender is dependent on the presence or absence of seminal plasma: if all seminal plasma has been removed, skim milk extender alone is not suitable, but MT needs to be added.

The sperm-rich part of the ejaculate has been demonstrated to survive better during storage than the whole ejaculate (Varner *et al.* 1987). In contrast to this, in our study, membrane integrity and all motility characteristics, except TMOT,

were significantly higher for spermatozoa in sperm-poor seminal plasma than in sperm-rich seminal plasma. In earlier reports, the differences between cups were not very significant, but Fraction 3 showed the highest numerical values for the parameters examined (Katila *et al.* 2002 and 2004). The middle seminal plasma part of the ejaculate may be the best for storage purposes, but further studies are needed to confirm this. Pre-ejaculatory fluid and the very last part of the ejaculate should probably be discarded from the inseminate, because they do not provide good storage media and their contribution to the sperm count is minimal.

It is obvious that there are differences between stallions in the quality of seminal plasma (Aurich *et al.* 1996; Katila *et al.* 2004). This was also demonstrated in this seminal plasma exchange experiment. However, seminal plasma or its specific fractions are not as good for cooled semen storage as seminal plasma removal. Conflicting reports have been published about the benefits of seminal plasma addition before freezing: Aurich *et al.* (1996) and Katila *et al.* (2002) found positive effects, but Moore *et al.* (2005) did not.

Pre-ejaculatory fluid showed the lowest concentrations and the sperm-rich fraction the highest concentrations for most of the constituents measured. The concentrations of many components of seminal plasma were the lowest in the stallion with an ejaculate of large volume and low sperm concentration. It is not known which accessory sex gland is responsible for excessive fluid production, but the seminal vesicles are the largest ones and are therefore good candidates.

## REFERENCES

- Aurich, J.E., Kühne, A., Hoppe, J. and Aurich, C. (1996) Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. *Theriogenology* **46**, 791-797.
- Ball, B.A., Gravance, C.G., Medina, V., Baumber, J. and Liu, I.K.M. (2000) Catalase activity in equine semen. *Am. J. vet. Res.* **61**, 1026-1030.
- Barrier-Battut, I., Delajarraud, H., Legrand, E., Bruyas, J.-F., Fiéni, F., Tainturier, D., Thorin, C. and Pouliquen, H. (2002) Calcium, magnesium, copper, and zinc in seminal plasma of fertile stallions, and their relationship with semen freezability. *Theriogenology* **58**, 229-232.
- Jasko, D.J., Moran, D.M., Farlin, M.E. and Squires, E.L. (1991) Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. *Theriogenology* **35**, 1059-1067.

- Katila, T., Karlsson, M., Reilas, T., Andersson, M., Kaikkonen, R. and Koskinen, E. (2002) 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.
- Katila, T., Reilas, T., Güvenc, K., Alm, K. and Andersson, M. (2004) The effect of seminal plasma on motility characteristics and viability of spermatozoa after cooled storage. 3rd Meeting of the European Equine Gametes Group. *Havemeyer Foundation Monograph Series No 13*, Eds: J. Müller, Z. Müller and J. F. Wade, R & W Publications (Newmarket) Ltd, pp 3-5.
- Koskinen, E., Karlsson, M., Reilas, T., Sankari, S., Esala, A.-L. and Katila, T. (2002) Catalase activity and total protein in fractionated stallion seminal plasma. *Theriogenology* **58**, 337-340.
- 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 artificial 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.
- Moore, A.L., Squires, E.L. and Graham, J.K. (2005) Effect of seminal plasma on cryopreservation of equine spermatozoa. *Theriogenology* **63**, 2372-2381.
- Padilla, A.W. and Foote, R.H. (1991) Extender and centrifugation effects on the motility patterns of slow-cooled stallion spermatozoa. *J. Anim. Sci.* **69**, 3308-3313.
- Rigby, S.L., Brinsko, S.P., Cochran, M., Blanchard, T.L., Love, C.C. and Varner, D.D. (2002) Advances in cooled semen technologies: seminal plasma and semen extender. *Anim. Reprod. Sci.* **68**, 171-180.
- 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.

# EFFECTS OF DURATION, FORCE OF CENTRIFUGATION AND CUSHIONED CENTRIFUGATION TECHNIQUE ON SPERM RECOVERY AND SPERM QUALITY IN STALLIONS WITH GOOD AND POOR SEMEN FREEZABILITY

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## SUMMARY

During semen processing increasing gravitational force and prolonged time of centrifugation up to 1,000 × g for 20 min achieved either by a cushioned technique (Cushion-Fluid), or use of siliconised glass tubes, resulted in a more efficient sperm recovery (83%, 92%) when compared to the routine method recommended for centrifugation of stallion semen (600 × g, 10 min; 75%) (P<0.05). After thawing, neither high speed nor prolonged time of centrifugation showed detrimental effects on motility, membrane integrity, acrosomal status and mitochondrial membrane potential of spermatozoa in ejaculates with good and poor freezability from 12 stallions (6 stallions per group × 3 ejaculates) when compared to routine centrifugation method.

In stallions with good and poor semen freezability the use of a cushioned centrifugation technique associated with clear-saline centrifugation extender (HBS) or Eqcellsire resulted in higher sperm recovery (93%, 93%) when compared to INRA-82 (86%) (P<0.05). The use of a high-speed centrifugation and clear extender without a cushion allows high sperm recovery (95%) but decreased post thaw sperm quality (P<0.05).

## INTRODUCTION

Preceding cryopreservation of stallion semen by centrifugation has become the routine method for removal of seminal plasma. For this purpose, a primary extender is used during centrifugation and a secondary extender is used after centrifugation for dilution and cryoprotection of the sperm rich fraction. Centrifugation may be used successfully,

but is not without detrimental effects on motility and morphology of spermatozoa. Despite the major role of individual composition and quality of the stallions semen the detrimental effects of centrifugation can be influenced by the time and force of centrifugation. Furthermore loss of spermatozoa after centrifugation is disadvantageous. To enhance sperm recovery after centrifugation, techniques were developed to underlay semen with a dense, liquid cushion, on which the spermatozoa float during the centrifugation process (Revell *et al.* 1997).

The work in Experiment I of this study evaluates the effects of routine centrifugation method (10 min, 600 g) vs new techniques for centrifugation (20 min, 1,000 g) either with or without the use of a cushion fluid (Cushion-Fluid, Landshut, Germany) on sperm quality post thaw in stallions with good and poor semen freezability. In Experiment II, the effects of various centrifugation extenders (Eqcellsire, INRA-82, HBS) were tested with or without cushioned centrifugation at 1,000 × g for 20 min.

## MATERIALS AND METHODS

A total of 12 breeding sires of proven normal fertility were used for the experiment. The stallions belong to the stud farm of the State of Lower Saxony at Celle, Germany, and are routinely used in an AI programme. They participated in the routine semen freezing programme, which lasts from October to February. Stallions were divided into 2 groups. Stallions with a progressive post thaw sperm motility ≤35% in more than 3 of 10 ejaculates collected before the experiment were considered to be poor freezers (n=6). Stallions above these

**TABLE 1: Comparison of a routine centrifugation method (10 min, 600 x g) vs. modified techniques for centrifugation (20 min, 1,000 x g) either with or without the use of a cushion fluid on sperm recovery and post thaw sperm quality in stallions with good and poor semen freezability**

	Centrifugation techniques					
	Control		Cushion		Glass tubes	
	INRA-82		INRA-82		INRA-82	
Extender	INRA-82		INRA-82		INRA-82	
Cushion	-		Cushion-Fluid		-	
Centrifugation	600 x g, 10 min		1,000 x g, 20 min		1,000 x g, 20 min	
	Group I	Group II	Group I	Group II	Group I	Group II
Sperm recovery	75.3 ±2.8 <sup>a</sup>	76 ±1.9 <sup>a</sup>	82.9 ±4.6 <sup>b</sup>	83 ±3.9 <sup>b</sup>	92.8 ±5 <sup>c</sup>	92 ±5.1 <sup>c</sup>
PMS	51.8 ±14.8 <sup>a</sup>	35.6 ±13.8 <sup>b</sup>	56.4 ±9.7 <sup>a</sup>	36.5 ±17.7 <sup>b</sup>	53.1 ±12.3 <sup>a</sup>	33.9 ±18.4 <sup>b</sup>
FITC/PNA-live	41.5 ±10.7 <sup>a,b</sup>	33.2 ±11.2 <sup>c</sup>	49.2 ±9.5 <sup>c</sup>	40.4 ±12.6 <sup>b</sup>	46.8 ±5.6 <sup>a</sup>	36.1 ±8.9 <sup>b,c</sup>
FITC/PNA-AR	22.1 ±8.3 <sup>a,b</sup>	27 ±10 <sup>a</sup>	18.7 ±4.8 <sup>b</sup>	24.1 ±8.9 <sup>a,b</sup>	22.1 ±6.7 <sup>a,b</sup>	21.2 ±5.5 <sup>a,b</sup>
JC-1	32.9 ±9.5	26.3 ±7.4	34.5 ±8.4	33.0 ±8.0	34.5 ±6.6	29.5 ±7.5

Group: good freezers (Group I), poor freezers (Group II) (n=6 stallions per group; 3 ejaculates/stallion).

PMS: Progressively Motile Sperm.

FITC/PNA-live: Percentage of Syto-positive stained spermatozoa stained by FITC-PNA/Syto/PI.

FITC/PNA-AR: Percentage of FITC-positive stained acrosomes stained by FITC-PNA/Syto/PI.

JC-1: Percentage of JC-1 positive stained sperm midpiece mitochondrial aggregates.

<sup>a,b,c</sup>: Values with different superscript differ significantly within rows (P<0.05).

criteria were assigned to the good freezer group (n=6).

Semen was collected by artificial vagina on a dummy from each stallion 3 times per week (Monday, Wednesday and Friday). Sterile gauze filtration sets were used in the collection devices and the gel free semen was evaluated for volume, concentration of spermatozoa by hemocytometer and percentage of progressively motile spermatozoa (pms). Semen was diluted in skim milk extender (INRA 82; Ijaz and Ducharme 1995) to a final concentration of  $50 \times 10^6$  spermatozoa/ml and split samples were used to compare centrifugation methods.

In Experiment I, sterile plastic centrifugation tubes were filled with 46 ml of diluted semen ( $2.3 \times 10^9$  spermatozoa/centrifugation tube) and centrifuged at  $600 \times g$  for 10 min (control group). A second plastic tube was underlaid with 5 ml of an inert, dense, isotonic solution (Cushion-Fluid, Minitüb, Landshut, Germany) prior to centrifugation at  $1,000 \times g$  for 20 min (cushion treatment). The third part of the split sample was filled into a sterile, siliconised, conical glass tube and centrifuged at  $1,000 \times g$  for 20 min (glass tube treatment). After centrifugation, supernatant was removed by aspiration (controls and glass tube group) or the sperm rich sperm phase was layered

between the interface of cushion-fluid and extender in the supernatant. Supernatant was removed first followed by careful aspiration of the cushion (cushion group).

In Experiment II, split ejaculates were prepared in order to compare various centrifugation extenders containing either egg yolk (Eqcellsire A, IMV, L'Àigle, France in combination with Eqcellsire B serving as cushion fluid) or milk (INRA-82), and a clear-saline extender (HBS, Hank's buffered saline) by cushioned centrifugation (Cushion-Fluid) at  $1,000 \times g$  for 20 min. Centrifugation of samples diluted in HBS was carried out with and without the use of a cushion.

After re-suspending the sperm pellets with milk extender containing 2% egg yolk, sperm concentration was calculated again and freezing extender was added to obtain a final concentration of  $200 \times 10^6$  spermatozoa/ml and a final concentration of 2.5% glycerol.

Semen was equilibrated for 120 min at +5°C packaged in 0.5 ml plastic straws and frozen automatically (+5°C to -140°C in 60°C/min.) using a programmable freezer (IMV, L'Àigle, France) and were plunged in liquid nitrogen and stored prior to thawing in a waterbath at 37°C for 30 s.

**TABLE 2: Comparison of various extenders during high-speed centrifugation (20 min, 1,000 x g) either with or without the use of a cushion fluid on sperm recovery and post thaw sperm quality in stallions with good and poor semen freezability**

Extender Cushion Centrifugation	Centrifugation techniques							
	Eqcellsire		INRA		HBS+cushion		HBS	
	Eqcellsire A		INRA-82		HBS		HBS	
	Eqcellsire B		Cushion-Fluid		Cushion-Fluid		-	
	1,000 x g 20 min							
	Group I	Group II	Group I	Group II	Group I	Group II	Group I	Group II
Sperm recovery	93.4 <sup>a</sup> ±5.2	93.3 <sup>a</sup> ±5.7	85 <sup>b</sup> ±5.9	86.4 <sup>b</sup> ±5.4	94.5 <sup>a</sup> ±5.7	93.1 <sup>a</sup> ±5	95.6 <sup>a</sup> ±6.6	95.9 <sup>a</sup> ±5.4
Pms	45b <sup>c</sup> ±14.5	29.3 <sup>d</sup> ±14.6	61.1 <sup>a</sup> ±15.7	49.8 <sup>a,b,c</sup> ±12.8	58.7 <sup>a,b</sup> ±15.7	46.4 <sup>a,b,c</sup> ±15.7	45.1 <sup>b,c</sup> ±15.3	40.3 <sup>c,d</sup> ±16.5
FITC/PNA-live	40.6 <sup>a,b,c</sup> ±8.7	35.2 <sup>a,b,c</sup> ±9.3	42.3 <sup>a,b</sup> ±9.9	32.5 <sup>c,d</sup> ±8.6	43.7 <sup>a</sup> ±6.9	33 <sup>c,d</sup> ±8.6	33.4 <sup>b,c,d</sup> ±8.7	29.8 <sup>d</sup> ±9.6
FITC/PNA-AR	14.9 <sup>b</sup> ±2.7	17.2 <sup>a,b</sup> ±3.7	19.2 <sup>a,b</sup> ±3.1	20.6 <sup>a</sup> ±4.8	18.7 <sup>a,b</sup> ±3.8	20.8 <sup>a</sup> ±6.1	20.7 <sup>a</sup> ±3.6	21.7 <sup>a</sup> ±5.6
JC-1	37.3 <sup>a,b</sup> ±6.1	32.1 <sup>b,c</sup> ±7.4	39 <sup>a,b</sup> ±6.2	30.2 <sup>c</sup> ±7.1	42.0 <sup>a</sup> ±6.8	32.1 <sup>b</sup> ±7.4	32.2 <sup>b,c</sup> ±8.1	27.3 <sup>c</sup> ±5.8

Groups: good freezers (Group I); poor freezers (Group II) (n=6 stallions per group; 3 ejaculates/stallion).

Eqcellsire: package containing centrifugation extender and cushion medium for centrifugation of stallion semen (IMV, L'Áigle, France)

INRA-82: skim milk extender according to Ijaz and Ducharme (1995)

HBS: Hank's buffered saline

Cushion-Fluid: high density solution for centrifugation of stallion semen (Minitüb, Landshut, Germany)

PMS: Progressively Motile Sperm.

FITC/PNA-live: percentage of Syto-positive stained spermatozoa stained by FITC-PNA/Syto/PI.

FITC/PNA-AR: percentage of FITC-positive stained acrosomes stained by FITC-PNA/Syto/PI.

JC-1: percentage of JC-1 positive stained sperm midpiece mitochondrial aggregates.

<sup>a,b,c</sup> Values with different superscript differ significantly within rows (P<0.05).

Sperm motility, morphology and function was investigated immediately after collection, centrifugation, cooled storage for 24 h at +5°C, and after freezing and thawing. The percentage of pms was determined with a computerised sperm analysing system (Stroemberg-Mika, Montreux, CH). Fluorescence stains of spermatozoa were analysed by flowcytometry (Becton-Dickinson FacsScan). Plasma membrane integrity and acrosomal status were studied using a FITC-PNA/Syto/PI assay. Mitochondrial membrane potential was assessed by a modified JC-1 staining procedure.

## RESULTS AND DISCUSSION

When semen was evaluated after thawing, results indicated that centrifugation at high speed and prolonged time (1,000 x g, 20 min) allowed recovery of more spermatozoa (P<0.05) compared to the routine method recommended for centrifugation of stallion semen (600 x g, 10 min) (Table 1). These results are in accordance with Delhomme *et al.* (2004). In their study, based on 6

stallions, a 99% recovery rate was obtained, using the cushioned centrifugation technique (Eqcellsire) compared to 77% recovery for routine centrifugation (600 x g, 10 min). However in the present study, recovery rates were not as high when compared to the latter, which might be due to the use of milk-extender instead of a saline extender during centrifugation. Under the conditions of the present study, higher speed and prolonged centrifugation time did not affect spermatozoa detrimentally in terms of post thaw motility, membrane integrity, acrosomal status and mitochondrial membrane potential. Interestingly, similar good results were also obtained when conical, siliconised glass tubes were used without a cushion.

As expected, semen samples of the good freezer group had higher (P<0.05) PMS and FITC/PNA-live stained spermatozoa (Table 1 and 2).

The use of a cushioned centrifugation technique associated with clear-saline centrifugation extender (HBS) or Eqcellsire resulted in higher sperm recovery (93%, 93%) when compared to INRA-82 (86%) (P<0.05)

(Table 2). Samples centrifuged with INRA-82 or HBS with cushioned technique had higher PMS when compared to cushioned technique with Eqcellsire and HBS without the use of a cushion ( $P < 0.05$ ). The use of high-speed centrifugation and clear extender without a cushion allows highest sperm recovery (95%) but decreased sperm quality post thaw ( $P < 0.05$ ).

In summary, high speed cushioned centrifugation combined with Eqcellsire A or clear-saline HBS improved sperm recovery rate. However, use of a cushion may be avoidable when milk extender is used during centrifugation.

## REFERENCES

- Ijaz, A. and Ducharme, R. (1995) Effect of various extenders and Taurine on survival of stallion sperm cooled to 5°C. *Theriogenology* **44**, 1039-1050.
- Delhomme, G., Vidament, M., Ecot, P. and Decuadro-Hansen, G. (2004) Evaluation of a cushioned centrifugation technique for processing equine semen for freezing. In: *Proc. 15th Int. Congr. Anim. Reprod.* Porto Seguro, Brazil, Vol. 2, 496 (abstr).
- Revell, S.G., Pettit, M.T. and Ford, T.C. (1997) Use of centrifugation over iodixanol to reduce damage when processing stallion sperm for freezing. In: *Proc. Joint Meeting Society for the Study of Fertility*, Abstr. Series, No 92, 38 (abstr).

# THE INFLUENCE OF GENITALLY PATHOGENIC BACTERIA ON SEMEN MOTILITY IN COOLED-STORED STALLION SEMEN

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## INTRODUCTION

The surface of the penis and prepuce of the stallion is normally inhabited by a variety of commensal bacteria. However, most of these are not regarded as pathogenic (Chévalier-Clément *et al.* 1991; Varner *et al.* 1998; Aurich *et al.* 2003). Collection of semen with an artificial vagina is unlikely to avoid contamination of semen with these bacteria unless an open-ended artificial vagina is used (Clement *et al.* 1995; Lindeberg *et al.* 1999). As semen is a potential source of bacterial contamination of the mare's reproductive tract and bacteria might also compromise semen quality during storage, bacterial growth is commonly controlled by the use of semen extenders containing antibiotics (Blanchard *et al.* 1987; Jasko *et al.* 1993; Varner *et al.* 1998). However, effects of potentially pathogenic bacteria on semen characteristics during cold-storage have not yet been evaluated. We have therefore determined effects of different bacteria on spermatozoal function in stallion semen during cooled-storage. Furthermore, it was evaluated whether addition of the antibiotic gentamicin is able to diminish these effects.

## MATERIAL AND METHODS

Semen was collected from genitally healthy stallions (n=6) with an artificial vagina. Native semen was checked for bacterial contamination immediately after collection by conventional methods for isolation and differentiation of bacteria (Spergser *et al.* 2002). Samples that showed more than sparse growth of commensal microorganisms were discarded. One half of each ejaculate was diluted with EquiPro semen

extender (Minitüb, Tiefenbach, Germany), either with or without gentamicin (1 g per one), respectively, and centrifuged (10 min, 700 g). The pellet was re-suspended with the respective centrifugation extender (final concentration approx. 25 Mio spermatozoa/ml). *Pseudomonas (Ps.) aeruginosa*, *Staphylococcus (St.) aureus*, *Streptococcus (Sc.) equi* subsp. *equi (Sc. equi)*, *Sc. equi* subsp. *zooepidemicus (Sc. zooepidemicus)* or *Sc. dysgalactiae* subsp. *equisimilis (Sc. equisimilis)* in a final concentration of 25 Mio bacteria/ml extended semen or culture medium alone (control) were added. All bacteria used in this study had been tested for antibiotic susceptibility by disk diffusion assay according to NCCL standards and were susceptible to gentamicin. Immediately after addition of bacteria or culture medium and after storage at 5°C for 24, 48 und 72 h, motility of diluted semen was determined with a CASA system (SpermVision, Minitüb).

## RESULTS

Immediately after addition of bacteria, no significant differences in motility of treatment groups existed. After 24 h of cooled-storage in semen samples without gentamicin, total motility with *Ps. aeruginosa* ( $25.6 \pm 4.7\%$ ) and *Sc. equisimilis* ( $18.8 \pm 4.2\%$ ) was significantly lower ( $P < 0.05$ ) than in all other groups (control:  $33.3 \pm 4.5\%$ , *St. aureus*:  $37.3 \pm 4.4\%$ , *Sc. equi*:  $34.8 \pm 4.2\%$ , *Sc. zooepidemicus*:  $35.0 \pm 4.4\%$ ; see Fig 1a). After 48 h, a pronounced decrease in motility parameters in all groups existed when compared to time 0 h. After 72 h. The decrease in motility was most apparent in semen with *Ps. aeruginosa* ( $P < 0.05$  vs. all other groups).

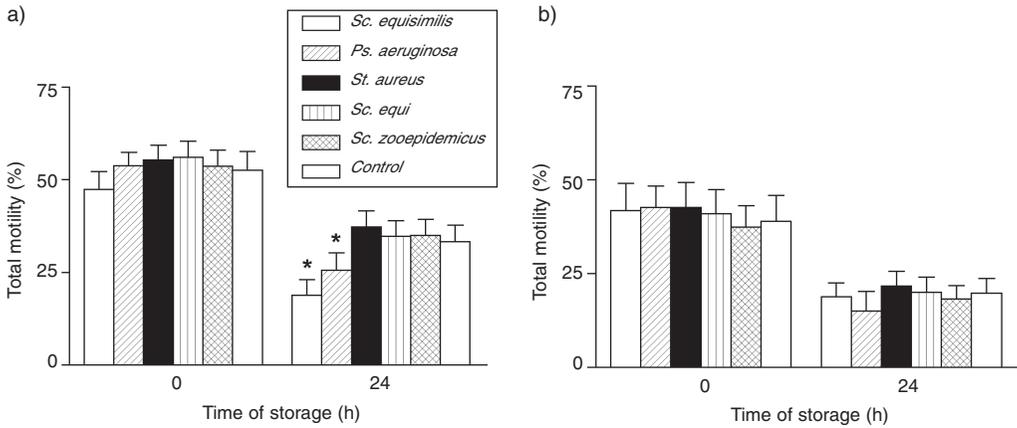


Fig 1: Motility of spermatozoa in semen diluted with EquiPro (Minitüb) extender (a) without and (b) with gentamicin (1 g/l) and incubated with *Sc. equisimilis*, *Ps. aeruginosa*, *St. aureus*, *Sc. equi*, *Sc. zooepidemicus* or control medium for 24 h. Significant differences between groups: \**Sc. equisimilis* and *Ps. aeruginosa* vs. all other groups:  $P < 0.05$ .

Addition of gentamicin to extender resulted in a significant decrease of spermatozoal motility after 24 h of storage ( $P < 0.05$  between control with and without gentamicin). Motility in semen samples stored in the presence of different bacteria was not improved when compared to respective groups without antibiotics. At all time points, no significant differences between the semen samples stored with the different bacteria and control medium could be detected (eg after 24 h: control:  $19.8 \pm 3.9\%$ , *Ps. aeruginosa*:  $15.1 \pm 5.2\%$ , *Sc. equisimilis*:  $18.8 \pm 3.7\%$ , *St. aureus*:  $21.7 \pm 3.9\%$ , *Sc. equi*:  $20.1 \pm 4.0\%$ , *Sc. zooepidemicus*:  $18.2 \pm 3.6\%$ , ns, see Fig 1b).

## DISCUSSION

All bacteria used in the present study were isolated from the genital tracts of horses showing clinically apparent alterations during breeding soundness examination. Only 2 of these bacteria, *Ps. aeruginosa* and *Sc. equisimilis* induced significantly adverse effects on semen quality during cooled-storage. Other  $\beta$ -haemolytic streptococci (*Sc. equi* and *Sc. zooepidemicus*) as well as *St. aureus* did not cause any changes in semen motility during the storage over 72 h despite the fact that these bacteria are potentially pathogenic for the genitalia of horses.

*Ps. aeruginosa* is detected on the external genitalia of healthy stallions with a frequency of 20–40% (Malmgren *et al.* 1998; Aurich *et al.*

2003). Therefore, contamination of semen with *Ps. aeruginosa* during semen collection is possible. This study demonstrates a clear detrimental effect of *Ps. aeruginosa* on spermatozoal motility during cooled storage resulting in poor semen quality after 24 h, a time at which shipped semen is usually used for insemination of mares. The lowered number of motile spermatozoa at that time point would probably contribute to a decrease in the conception rate of inseminated mares, even if they were capable of eliminating the potentially pathogenic bacteria from their uteri.

Gram negative bacteria such as *Ps. aeruginosa* may act on spermatozoal function by toxic components of their outer membranes, mainly lipopolysaccharides (endotoxins) and porins. They are released from gram-negative bacteria both during active growth and bacteriolysis, resist proteolytic enzymes and thus can persist for a long time after infection (Garten and Henning 1974). The negative effects of *Ps. aeruginosa* on equine sperm motility in the present study are in agreement with reports from man where even low levels of natural porin or lipopolysaccharides severely damage spermatozoa *in vitro* and result in reduced fertility (Paulson and Polakoski 1977). In contrast, in gram positive bacteria, specific toxic components interfering with membrane function of spermatozoa have not been identified so far. Therefore, the mechanism that induced a decrease in semen motility of spermatozoa incubated with

*Sc. equisimilis* is unknown. It can be speculated that bacteria – besides having specific mechanisms for damaging cells – might contribute to an increased production of reactive oxygen species (ROS) in stored semen (Ochsendorf 1999).

As collection of semen from stallions with a closed artificial vagina is unlikely to avoid contamination with bacteria from the external genitalia, the control of bacterial growth is routinely performed by the use of semen extenders containing antibiotics (Jasko 1993; Varner *et al.* 1998; Lindeberg *et al.* 1999). Gentamicin is frequently chosen as an antibiotic in equine semen extender because it is successful in controlling bacterial growth during cooled-storage of semen (Clement *et al.* 1995; Varner *et al.* 1998). However, in the present study, it was not able to inhibit the detrimental influence of *Ps. aeruginosa* on semen motion characteristics. This supports the suggestion that toxins derived from *Ps. aeruginosa* are responsible for spermatozoal damage and not the bacteria themselves (Garten and Henning 1974; Paulson and Polakoski 1977). In addition, antibiotics are generally effective at a temperature above 15°C, so in cooled stored semen they can only exert their effects during cooling from collection to storage temperature. After reaching the storage temperature, no further effects can be expected.

In control semen without bacteria processed with the gentamicin-containing extender, motility characteristics were negatively influenced compared to semen processed without gentamicin. Varner *et al.* (1998) did not find any adverse effect of gentamicin on semen motility parameters but Jasko *et al.* (1993) reported negative effects of gentamicin on motion characteristics of spermatozoa only when concentrations of gentamicin greater than 1 g per one were used. This concentration was not exceeded in the present study and composition of the extender EquiPro is similar to Kenney extender used by Varner *et al.* (1998) and Jasko *et al.* (1993). In EquiPro, the skim milk component is substituted by a more defined milk-derived component. This difference in the extender composition seems to influence interactions between spermatozoa and the antibiotic component gentamicin.

The present study demonstrates clearly negative effects of certain bacteria on cooled-stored stallion semen. However, the addition of antibiotics to extender can be critical for semen

motility and optimal concentrations have to be tested for the respective extender medium to avoid detrimental effects. Control of bacteria by antibiotics in extender medium has to be accompanied by optimal hygienic measures during semen collection and processing to keep bacterial contamination as low as possible.

## REFERENCES

- Aurich, C., Spergser, J., Nowotny, N., Rosengarten, R. and Aurich, J.E. (2003) Prevalence of venereal transmissible diseases and relevant potentially pathogenic bacteria in Austrian Noriker Draught horse stallions. *Wien. Tierärztl. Mschr.* **90**, 124-130.
- Blanchard, T.L., Varner, D.D., Love, C.C., Hurtgen, J.P., Cummings, M.R. and Kenney, R.M. (1987) Use of a semen extender containing antibiotic to improve the fertility of a stallion with seminal vesiculitis due to *Pseudomonas aeruginosa*. *Theriogenology* **28**, 541-546.
- Chévalier-Clément, R., Hochereau de Reviers, M.T., Perreau, C. and Magistrini, M. (1991). Alterations of the semen and genital tract of infertile stallions. *J. Reprod. Fertil. Suppl* **44**, 657-658.
- Clément, F., Vidament, M. and Guérin, B. (1995) Microbial contamination of stallion semen. *Biol. Reprod.* **1**, 779-786.
- Garten, W. and Henning, U. (1974) Cell envelope and shape of *Escherichia coli* K12, Isolation and preliminary characterisation of the major ghost membrane proteins. *Eur. J. Biochem.* **47**, 343-352.
- Jasko, D.J., Bedford, S.J., Cook, N.L., Mumfort, E.L., Squires, E.L. and Pickett, B.W. (1993) Effect of antibiotics on motion characteristics of cooled stallion spermatozoa. *Theriogenology* **40**, 885-893.
- 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 artificial vagina. *Theriogenology* **51**, 1157-1173
- Malmgren, L., Engvall, E.O., Engvall, A. and Albighn, A. (1998) Aerobic bacterial flora of semen and stallion reproductive tract and its relation to fertility under field conditions. *Acta. Vet. Scand.* **39**, 173-182.
- Ochsendorf, F.R. (1999) Infections in the male genital tract and reactive oxygen species. *Human Reprod. Update* **5**, 399-420.
- Paulson, J.D. and Polakoski, K.L. (1977) Isolation of a spermatozoal immobilization factor from *Escherichia coli*. *Fertil. Steril.* **28**, 182-185.
- Spergser, J., Aurich, C., Aurich, J.E. and Rosengarten, R. (2002) High prevalence of mycoplasma in the genital tract of asymptomatic stallions in Austria. *Vet. Microbiol.* **87**, 119-129.
- Varner, D.D., Scanlan, C.M., Thompson, J.A., Brumbaugh, G.W., Blanchard, T.L., Carlton, T.M. and Johnson, L. (1998) Bacteriology of preserved stallion semen and antibiotics in semen extender. *Theriogenology* **50**, 559-573.

# SPERM CHROMATIN ABNORMALITIES AFTER SEMEN SEXING PROCEDURE – PRELIMINARY RESULTS

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Being able to pre-select the sex of offspring at the time of insemination is one of most exciting possibilities of reproductive technology in mammals.

Flow cytometry is the only reliable and relatively fast method allowing separation of live X and Y spermatozoa for sex regulation. Upto 30,000 animals of different mammalian species were born after insemination with sexed semen during the past 20 years (Johnson *et al.* 2005). Nevertheless the question is still open: do the sexing procedure with its potentially mutagenic UV light and Hoechst 33342 dye which is cytotoxic in high concentrations (Durand and Olive 1982) affect probably the most important component of sperm cell - chromatin? To examine sperm chromatin abnormality, the Sperm Chromatin Structure Assay (SCSA) can be used (Evenson 1990). This is a fast and reliable flow cytometrical method which is based on metachromatic characteristics of acridine orange. Moreover, it was found that results of the SCSA are highly correlated with male fertility (Evenson and Jost 2000; Bochenek *et al.* 2001).

The aim of the work was to examine the effect of laser UV light and Hoechst 33342 fluorochrome used in the sperm sorting process on stallion sperm chromatin structure.

## MATERIALS AND METHODS

The fresh ejaculates of 6 stallions were used in the study. Each ejaculate was divided into 5 groups:

1. control, unprocessed - labelled as: 'Control'
2. sorted strictly according to XY Inc. protocols – '150/R'
3. As Group 2 except erythrosine staining (used for dead spermatozoa discrimination) – '150'

4. Group 2 but with double UV laser power (300mW) – '300/R'
5. Group 3 but with double UV laser power (300mW) – '300'

Sperm sorting was performed in MoFLoSX cytometer at speed 3,000–5,000 cells/s. Sorted fractions of X and Y spermatozoa were mixed again, centrifuged and dissolved in SSE extender (medium for liquid stallion semen storage, developed in NRIAP, Balice) and stored for 24 h in +15°C. The SCSA examination (Evenson 1990) was performed twice: immediately after sorting and after 24 h. The chromatin of control sample was examined according to the same time schedule.

## RESULTS

The complete results of sperm chromatin examination are showed in Table 1.

Mean percentage of spermatozoa with abnormal chromatin was 3.78% (SD=4.09) for the control sample. The highest level of chromatin abnormality was noted for Groups '150' and '300' – 7.68% (SD=11.77) and 9.86% (SD=15.25) respectively, both groups with no dead cell discrimination (erythrosine staining). It is worth noting that dead sperm elimination by erythrosine staining removed spermatozoa with damaged chromatin as well. This was seen, particularly, in Stallion 4 with a high level of chromatin abnormality.

After 24 h of storage, the mean level of chromatin abnormality increased to 4.92% (SD=5.52) in the control sample while in '150/R' and '300/R' groups, both with dead cell discrimination, it increased to 12.54% (SD=21.89) and 11.21% (SD=17.61) respectively. Interestingly, this level decreased slightly for groups with no erythrosin staining. Perhaps this is caused by slow

**TABLE 1: The percentage of spermatozoa with damaged chromatin after sexing procedure '0 h' and '24 h' – examination immediately after sorting and after 24 h respectively. Sign "Δ" means the increase of chromatin abnormality during 24 h of storage**

Group	Stallion						Mean	SD
	1	2	3	4	5	6		
Control/0 h	2.11	1.14	1.65	2.67	12.00	3.12	3.78	4.09
Control/24 h	2.31	2.36	1.74	2.85	16.07	4.20	4.92	5.52
Δ Control	0.20	1.22	0.09	0.18	4.07	1.08	1.14	1.52
150/R/0 h	2.02	0.70	0.71	2.16	10.54	3.76	3.32	3.72
150/R/24 h	1.87	0.87	1.48	56.48	11.54	2.99	12.54	21.89
Δ 150/R	-0.15	0.17	0.77	54.32	1.00	-0.77	9.22	22.10
150/0 h	2.46	1.06	1.10	31.32	6.85	3.29	7.68	11.77
150/24 h	1.95	0.96	1.36	19.27	7.19	2.46	5.53	7.10
Δ 150	-0.51	-0.10	0.26	-12.05	0.34	-0.83	-2.15	4.87
300/R/0 h	3.18	1.42	0.67	3.03	11.13	5.21	4.11	3.78
300/R/24 h	1.03	1.04	0.38	45.44	14.72	4.66	11.21	17.61
Δ 300/R	-2.15	-0.38	-0.29	42.41	3.59	-0.55	7.11	17.40
300/0 h	2.91	0.87	1.05	40.37	9.14	4.82	9.86	15.25
300/24 h	0.99	1.48	1.00	17.57	7.52	2.06	5.10	6.59
Δ 300	-1.92	0.61	-0.05	-22.80	-1.62	-2.76	-4.76	8.93

diffusion of DNA strand breaks from sperm cells to the medium during long storage. One of the stallions (No. 4) appeared to be exceptionally susceptible for sperm sexing procedure – its percentage of spermatozoa with abnormal chromatin increased as high as 56.48% (control, non-sexed sample: 2.67%) after the sorting procedure. In Stallion 5 a noticeably higher level of chromatin abnormality was observed (12.0% in control sample at 0 h) but the sexing procedure did not affect this level to the same extent as in Stallions 1–3 and 6.

## CONCLUSIONS

Generally, the sexing procedure did not affect sperm chromatin structure (with the exception of Stallion 4);

It seems that dead sperm elimination (erythrosine staining) helps to exclude spermatozoa with damaged (abnormal) chromatin.

The SCSA method should be obligatory when selecting stallions for sperm sexing.

## REFERENCES

- Bochenek, M., Smorag, Z. and Pilch, J. (2001) Sperm chromatin structure assay of bulls qualified for artificial insemination. *Theriogenology* **56**, 557-567.
- Durand, R.E. and Olive, P.L. (1982) Cytotoxicity, mutagenicity, and DNA damage by Hoechst 33342. *J. Histochem. Cytochem.* **30**, 111-116.
- Evenson, D.P. (1990) Flow cytometric analysis of male germ cell quality. In: *Methods In Cell Biology* Vol. 33, Academic Press, San Diego, 401-410.
- Evenson, D. and Jost, L. (2000) Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci.* **22(2-3)**, 169-89.
- Johnson, L.A., Rath, D., Vazquez, J.M., Maxwell, W.M. and Dobrinsky, J.M. (2005) Preselection of sex of offspring in swine for production: current status of the process and its application. *Theriogenology* **63(2)**, 615-624.

# EXPOSURE OF STALLION SEMEN TO TWO FREEZE-THAW CYCLES: EFFECTS ON SPERMATOZOAL VIABILITY

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The breeding careers of stallions can end abruptly because of disease, devastating injury or death. Most equine breed registries permit the use of frozen semen, even following the death of a stallion, and many owners freeze semen from their stallions to avoid absolute loss of the genetic potential of these stallions should such circumstances arise. Often, frozen semen from affected stallions is in short supply, leading to a very limited continuation of breeding when a standard insemination protocol is used. In an effort to maximise the use of limited cryopreserved semen banks of stallions that may unexpectedly be lost to breeding, a study was conducted to evaluate the effects of thawing and re-freezing of semen in smaller aliquots on sperm viability, with the intention of using the re-frozen semen for intracytoplasmic sperm injection (ICSI).

Single ejaculates from each of 10 fertile stallions were diluted at a ratio of 1:3 (semen: extender) in a milk-based extender (INRA 96, IMV International, Minnesota, USA) and subjected to centrifugation at  $400 \times g$  for 20 min in 40 ml capacity glass centrifuge tubes (Nipple tubes; Pesce Lab Sales, Pennsylvania, USA), with a 30  $\mu$ l cushion (OptiPrep; Nycomed Pharma, Oslo, Norway). Spermatozoal pellets were re-suspended in one of 2 cryopreservation extenders (a milk-based extender [EZ Freezin – ‘MFR5’; Animal Reproduction Systems, California, USA] or an egg yolk-based [EZ Freezin – ‘LE’; Animal Reproduction Systems, California, USA]). Semen was then packaged in 0.5 ml straws at a sperm concentration of 200 million/ml and straws were frozen in static nitrogen vapor at 1 cm above liquid nitrogen for 20 min. Following freezing, the straws were submerged in liquid nitrogen for storage. Semen frozen in LE or MFR5 extender was later thawed at 37°C for 30 s and prepared for

re-freezing by one of the following methods: 1) simple re-freezing of semen that remained in the same straw; 2) re-freezing of semen that had been re-packaged in another straw; or 3) re-freezing of semen that was first diluted 1:10 with the same or different extender, then loaded into 0.5 ml straws and re-frozen, as described above. This procedure was done to replicate a situation in which stored semen that had been frozen in one, perhaps unknown, extender was used for re-processing by dilution a similar or different extender type.

Straws were thawed at 37°C for 30 s for evaluation. Experimental endpoints included total spermatozoal motility (TMOT; %), progressive spermatozoal motility (PMOT; %), and curvilinear velocity (VCL;  $\mu$ m/s), as measured by computer-assisted spermatozoal analysis (CASA; IVOS Version 10; Hamilton Research, Massachusetts USA), as described by Varner *et al.* (1991). Sperm chromatin quality (COMP  $\alpha$ -t; %), spermatozoal viability (VIAB; %), and sperm acrosomal integrity (VIAB-AI; %) were measured as previously described (Graham 2001; Love *et al.* 2003). Data were analysed statistically by a general linear models method, with a least squares means procedure used to compare treatment means. Data are reported below as means  $\pm$  sd.

All experimental measures were similar ( $P > 0.05$ ) between semen frozen once in LE or MF extender, except that VCL ( $P < 0.05$ ) was higher for semen frozen in MF extender ( $167 \pm 23$ ) than that frozen in LE extender ( $141 \pm 20$ ). Mean values for MOT, PMOT, VCL, VIAB, and VIAB-AI were higher ( $P < 0.0001$ ) for semen frozen once ( $36 \pm 9$ ,  $28 \pm 8$ ,  $154 \pm 25$ ,  $42 \pm 9$  and  $46 \pm 9$ , respectively), as compared to semen frozen twice in the same package ( $15 \pm 6$ ,  $9 \pm 4$ ,  $132 \pm 21$ ,  $22 \pm 11$  and  $23 \pm 10$ , respectively). For semen frozen twice in undiluted form, no significant differences for

experimental endpoints were detected ( $P>0.05$ ) for semen re-frozen in the same package versus semen transferred to another package prior to re-freezing. For semen subjected to dilution prior to re-freezing, mean values for MOT, PMOT and VCL were slightly, but significantly, higher ( $P<0.05$ ) when the diluting extender was MF ( $17 \pm 5$ ,  $10 \pm 3$ , and  $131 \pm 20$ , respectively), as compared to LE ( $13 \pm 4$ ,  $8 \pm 2$  and  $118 \pm 21$ ), regardless in the initial extender type. Values for all experimental endpoints were not different ( $P>0.05$ ) between twice-frozen semen that was undiluted or diluted prior to re-freezing.

In summary, re-freezing of semen reduced spermatozoal quality of stallions, but the manipulations to which sperm were subjected prior to re-freezing had a minimal effect on spermatozoal quality. Diluting semen prior to re-freezing had no apparent untoward effects on spermatozoal quality, so this technique would be the logical method for re-freezing of semen to extend frozen semen banks of deceased or retired stallions for the purpose of ICSI techniques. Use of a milk-based extender may be preferable to an egg yolk-based extender for dilution of all frozen-thawed semen prior to re-freezing. A subsequent study revealed that blastocyst development after ICSI was equivalent between sperm frozen in LE extender and thawed once, as compared to sperm frozen once in LE extender, then thawed, diluted in MF extender and re-frozen (27% and 23%, respectively) when motile sperm were isolated for

ICSI (Choi *et al.* 2005). In that study, the dilution ratio prior to re-freezing was 1:100, as opposed to a 1:10 dilution ratio used in the present study. Therefore, frozen semen can be thawed and diluted considerably to achieve an abundant supply of spermatozoa suitable for ICSI procedures.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the American Quarter Horse Foundation, and by the Link Equine Research Endowment Fund, Texas A&M University.

## REFERENCES

- Choi, Y.H., Love, C.C., Varner, D.D. and Hinrichs, K. (2005) Equine blastocyst development after intracytoplasmic injection of sperm subjected to two free-thaw cycles. *Theriogenology* (in press).
- Graham, J.K. (2001) Assessment of sperm quality: a flow cytometric approach. *Anim. Reprod. Sci.* **68**, 239-247.
- Love, C.C., Thompson, J.A., Brinkso, S.P., Rigby, S.L., Blanchard, T.L., Lowry, V.K. and Varner, D.D. (2003) Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. *Theriogenology* **60**, 1127-1138.
- Varner, D.D., Vaughan, S.D. and Johnson, L. (1991) Use of a computerised system for evaluation of equine spermatozoal motility. *Am. J. Vet. Res.* **52**, 224-230.

# SESSION 2:

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## Oocytes

**Chairman:**  
***K. Hinrichs***



# EQUINE EMBRYO DEVELOPMENT AFTER INTRACYTOPLASMIC INJECTION OF TWICE FROZEN-THAWED SPERM

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Freezing of semen is most commonly performed to preserve a stallion's genetic potential. When a stallion dies or is unable to produce semen, stores of frozen semen become extremely valuable. Use of frozen semen for artificial insemination requires large numbers of sperm from multiple straws, and results in lower pregnancy rates than does insemination with fresh or cooled semen (Samper and Morris 1998). In contrast, fertilisation via intracytoplasmic sperm injection (ICSI) requires essentially only one sperm per oocyte. One standard straw of frozen semen (2 million sperm per straw) could potentially provide hundreds or thousands of doses of sperm for ICSI, if it could be thawed and aliquoted without reduction of the sperm's potential to support embryo development. Since the first successful report on ICSI by Squires *et al.* (1996), improved cleavage (>80%) and blastocyst (>30%) rates have been reported using a piezo drill for micromanipulation (Choi *et al.* 2002b; Hinrichs *et al.* 2005). If reprocessed (twice-frozen) sperm is utilised for ICSI, it may result in low embryo development because of loss of factors from the sperm cytoplasm, due to membrane damage. This would be especially likely in the case of non-motile sperm, because loss of motility is an indicator of membrane damage. Injection of sperm factor may reverse this situation, as injection of sperm factor has been associated with high rates of activation of horse oocytes both parthenogenetically and for nuclear transfer (Choi *et al.* 2002a). The aim of this study was to evaluate the blastocyst development rate after ICSI with twice frozen-thawed sperm. The effect of sperm extract on blastocyst development after ICSI with non-motile sperm was also examined.

Semen from a fertile stallion was collected and frozen in either MF (MFR5, a standard freezing

extender in our laboratory) or LE (EZ Mixin LE, an egg yolk-based extender). Motile sperm from the MF group were used for ICSI (control). Semen frozen in LE was thawed, diluted 1:100 with MF freezing extender, and re-frozen (2F treatment). Total sperm motility after thawing was evaluated using computer-assisted sperm analysis as described by Varner *et al.* (1991). Equine oocytes were collected from slaughterhouse-derived ovaries by follicular scraping and matured in M199 with 5 mU/ml FSH and 10% fetal bovine serum at 38.2°C in 5% CO<sub>2</sub> in air for 24 h. Mature oocytes were fertilised by ICSI as previously published (Choi *et al.* 2003). Four treatments were used for ICSI: 1) motile control sperm; 2) motile 2F sperm; 3) non-motile 2F sperm; or 4) non-motile 2F sperm followed by injection of sperm extract. Sperm extract was prepared by repeated freeze-thaw cycles in our laboratory (Choi *et al.* 2002a). Injected oocytes were cultured in DMEM/F-12 + 10% FBS in 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> for 8 days. Blastocyst formation was morphologically evaluated at 7 and 8 days, and embryos which did not progress to the blastocyst stage were fixed and stained with Hoechst 33258 to determine the number and status of nuclei, as previously described (Choi *et al.* 2002b).

The total motility of control sperm from a fertile stallion after thawing (once frozen in MF) was 48% and that of sperm in the 2F treatment (LE to MF) was 16%. Similar blastocyst rates were obtained in control and motile 2F sperm (27 and 23%, respectively). The rate of blastocyst development when non-motile 2F sperm were used (13%) tended to be lower than that for control sperm ( $P=0.07$ ). Injection of sperm extract after ICSI with non-motile 2F sperm significantly decreased the blastocyst development rate (2%) compared with injection of non-motile 2F sperm

alone. Semen from a subfertile stallion was similarly processed by freezing either once or twice; only motile sperm were used for ICSI. The total motility of control sperm after thawing was 22% and that of sperm in the 2F treatment was 3%. The blastocyst development rate for the control and 2F treatments was the same (9%).

These data indicate that the number of potential embryos produced from a straw of frozen stallion semen may be multiplied by dilution and re-freezing of the sperm for embryo production via ICSI. While a direct comparison was not performed, frozen motile sperm from a fertile stallion appeared to achieve a higher blastocyst rate than that from a subfertile stallion (27 vs. 9%). Reprocessing sperm did not significantly affect blastocyst production in either the fertile or the subfertile stallion. Non-motile re-processed sperm may be used for embryo production if motile sperm are not available, as blastocysts were also obtained in this treatment. It is surprising that oocytes fertilised with non-motile sperm developed to the blastocyst stage, as Lazzari *et al.* (2002) reported that non-motile sperm used for ICSI did not result in blastocyst development. We anticipated better embryo development when oocytes injected with non-motile sperm were subsequently activated with sperm extract. However, injection of sperm extract did not improve blastocyst rate after ICSI with non-motile sperm. Further study is needed to explore what factors interfere with ICSI-produced embryo development after sperm extract injection.

This work was supported by a grant from the American Quarter Horse Foundation, and by the Link Equine Research Endowment Fund, Texas A&M University.

## REFERENCES

- Choi, Y.H., Chung, Y.G., Walker, S.C., Westhusin, M.E. and Hinrichs, K. (2003) *In vitro* development of equine nuclear transfer embryos: effects of oocyte maturation media and amino acid composition during embryo culture. *Zygote* **11**, 77-86.
- Choi, Y.H., Love, C.C., Chung, Y.G., Varner, D.D., Westhusin, M.E., Burghardt, R.C. and Hinrichs, K. (2002a) 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.
- Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S. and Hinrichs, K. (2002b) Developmental competence *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilised by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. *Reproduction* **123**, 455-465.
- Hinrichs, K., Choi, Y.H., Love, L.B., Varner, D.D., Love, C.C. and Walckenaer, B.E. (2005) Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. *Biol. Reprod.* **72**, 1142-1150.
- Lazzari, G., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Barbacini, S. and Galli, C. (2002) Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of *in vitro* matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology* **58**, 709-712 (abstract).
- Samper, J.C. and Morris, C.A. (1998) Current methods for stallion semen cryopreservation: a survey. *Theriogenology* **49**, 895-899.
- Squires, E.L., Kato, H. and Blaszczyk, A. (1996) A pregnancy after intracytoplasmic sperm injection into equine oocytes matured *in vitro*. *Theriogenology* **45**, 306 (abstract).
- Varner, D.D., Vaughan, S.D. and Johnson, L. (1991) Use of a computerised system for evaluation of equine spermatozoal motility. *Am. J. Vet. Res.* **52**, 224-230.

# MEIOTIC COMPETENCE OF MARE OOCYTES SELECTED USING THE BRILLIANT CRESYL BLUE TEST

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Brilliant cresyl blue (BCB) is a vital dye used for indirect measurement of glucose-6-phosphate dehydrogenase (G6PD) activity, the enzyme synthesised by growing oocytes. The oocytes that have finished their growth phase show decreased G6PD activity and during the incubation in the BCB solution their cytoplasm turns blue. Active G6PD reduces BCB to a colourless compound, thus the cytoplasm of growing oocytes remain uncoloured (Mangia and Epstein 1975; Ericsson *et al.* 1993). Experiments on pig (Ericsson *et al.* 1993), prepubertal goat (Rodríguez-Gonzalez *et al.* 2002; 2003), heifer (Pujol *et al.* 2004) and cow (Alm *et al.* 2005) oocytes showed that the BCB test allows selection of oocytes that are more suitable for *in vitro* maturation, fertilisation (IVM/IVF) and embryo development. So far no such studies have been carried out on mare oocytes. The aim of the study was to evaluate the usefulness of the BCB test to select mare oocytes for *in vitro* maturation.

## MATERIALS AND METHODS

Oocytes were collected by scraping ovarian follicles of slaughtered mares. During collection the oocytes were divided into 2 groups according to follicle size: originating from small follicles ( $\leq 10$  mm in diameter) or from larger ones ( $> 10$  mm in diameter). Only oocytes having compact (CM;  $n=332$ ) or expanded (EX;  $n=258$ ) cumulus cells were used in the study.

Afterwards, CM and EX oocytes were exposed to  $26 \mu\text{M}$  of BCB in PBS supplemented with 0.4% BSA, 36 mg/l sodium pyruvate, 1,000 mg/l D-glucose and antibiotics (mPBS) for 90 min at  $38.5^\circ\text{C}$  in a 5%  $\text{CO}_2$  humidified air atmosphere. After exposure to BCB, oocytes were classified

according to the colour of their cytoplasm as: BCB+ (dark-blue), BCB $\pm$  (pale-blue) or BCB- (colourless). Subsequently, the EX and CM oocytes of each BCB group were incubated in TCM 199 supplemented with 20% FBS,  $5 \mu\text{g/ml}$  FSH,  $1 \mu\text{g/ml}$   $\text{E}_2$ , 36 mg/l sodium pyruvate and antibiotics for 24–26 and 29–31 h, respectively. The control groups consisted of oocytes which were cultured *in vitro* without pre-incubation in BCB. After culture, the stage of nuclear development was estimated using orcein.

## RESULTS AND DISCUSSION

After exposure to BCB, more BCB+ oocytes were observed among CM and EX oocytes collected from large than from small follicles (Table 1). Most BCB- oocytes (35%) were noted in the CM group from small follicles. Recent studies on cattle indicated that the G6PD was 2.5 times less active in the oocytes which exhibited a blue cytoplasm after incubation in BCB than in those which remained uncoloured (Alm *et al.* 2005). Furthermore, it was reported that ‘Blue oocytes’ are more capable of IVM than the colourless ones (cattle: Alm *et al.* 2005; pigs: Ericsson *et al.* 1993; goat: Rodríguez-Gonzalez *et al.* 2002). Thus, it may be presumed that the G6PD is less active in the mare oocytes originating from large follicles, and that these oocytes should have a better meiotic competence than those collected from the small follicles. The above presumption confirms earlier research on mare CM oocytes in which it was shown that a higher proportion of oocytes collected from the large follicles (10–50 mm in diameter) matured *in vitro*, than those derived from the smaller ones: 45% versus 20%, respectively (Goudet *et al.* 1997). Also in our

**TABLE 1: Relationship between follicular size, cumulus morphology and the intensity of the oocyte cytoplasm blue colouration after incubation in BCB**

Follicular diameter (mm)	Cumulus morphology	Classification of oocytes n (%)			Total n=100%
		BCB+	BCB+/-	BCB-	
≤ 10	CM	46 <sup>a</sup> (32.9)	45 <sup>a</sup> (32.1)	49 <sup>a</sup> (35.0)	140
	EX	89 <sup>b</sup> (69.0)	12 <sup>c</sup> (9.3)	28 <sup>d</sup> (21.7)	129
>10	CM	75 <sup>b</sup> (67.0)	15 <sup>c</sup> (13.4)	22 <sup>c</sup> (19.6)	112
	EX	54 <sup>e</sup> (84.4)	4 <sup>c</sup> (6.2)	6 <sup>c</sup> (9.4)	64

n =number of oocytes;

Values with different superscripts differ significantly a,d; c,d; b,e = P<0.05 a,b; a,c; b,c; b,d; a,e; ec = P<0.001

**TABLE 2: Chromatin configuration in mare CM oocytes after *in vitro* culture**

Groups of oocytes	Follicles with diameter (mm)									
	≤10					>10				
	Stage of nuclear development n (%)					Stage of nuclear development n (%)				
	MII	MI	GV	Deg.	Total n= 100%	MII	MI	GV	Deg.	Total n=100%
BCB+	13 <sup>a</sup> (54.2)	7 <sup>a</sup> (29.2)	1	3 <sup>a</sup> (12.5)	24	16 <sup>a</sup> (80.0)	1 (5.0)	1	2 <sup>a</sup> (10.0)	20
BCB+/-	6 <sup>a,b</sup> (33.3)	3 <sup>a</sup> (16.7)	0	9 <sup>b</sup> (50.0)*	18	4 <sup>b,d</sup> (30.8)	2 (15.4)	0	7 <sup>b,d</sup> (53.8)	13
BCB-	2 <sup>b</sup> (11.8)	1 <sup>a,c</sup> (5.8)	2	12 <sup>b</sup> (70.6)*	17	2 <sup>c,d</sup> (16.7)	0	1	9 <sup>c,d</sup> (75.0)	12
Control	11 <sup>b</sup> (22.0)	2 <sup>c</sup> (4.0)	7	30 <sup>b</sup> (60.0)	50	16 <sup>a,b</sup> (53.3)	3 (10)	0	11 <sup>b</sup> (36.7)	30

n =number of oocytes; MII – metaphase II; MI – metaphase I; GV-germinal vesicle; Deg. – degenerated oocytes; Within columns, values with different superscripts differ significantly: a,b; b,d = P<0.05; a,c = P<0.001

\* Including 1 degenerated oocyte in M I stage

**TABLE 3: Chromatin configuration in mare EX oocytes after *in vitro* culture**

Groups of oocytes	Follicles with diameter (mm)									
	≤10					>10				
	Stage of nuclear development n (%)					Stage of nuclear development n (%)				
	MII	MI	GV	Deg.	Total n=100%	MII	MI	GV	Deg.	Total n=100%
Blue	15 <sup>a</sup> (55.6)	1 (3.7)	2	9 (33.3)*	27	15 (48.4)	6 (19.4)	1	9 <sup>a</sup> (29.0)	31
BCB-	2 <sup>b</sup> (14.3)	3 (21.4)	0	9 (64.3)**	14	2 (33.3)	0	0	4 <sup>b</sup> (80.0)	6
Control	20 <sup>a</sup> (44.4)	3 (6.8)	2	20 (44.4)	45	9 (45.0)	1 (5.0)	0	10 (50.0)	20

n = number of oocytes; MII – metaphase II; MI – metaphase I; GV-germinal vesicle; Deg. – degenerated oocytes

Blue = BCB+ plus BCB+/-

\* Including 1 degenerated oocyte in MII stage;

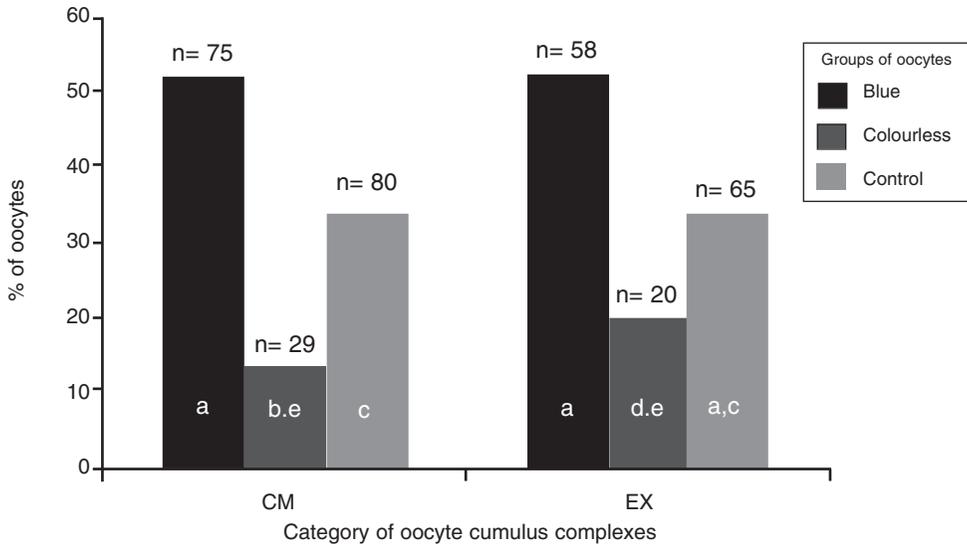
\*\* Including 4 degenerated oocytes in MII stage;

Within columns, values with different superscripts differ significantly a,b = P<0.05

study, within the CM control groups more oocytes from large than from small follicles reached metaphase II (53.3% versus 22%, respectively; P<0.01; Table 2).

After IVM, among the CM groups from small follicles, 54.2% BCB+ oocytes in Metaphase II were observed, ie significantly more than in the BCB- (11.8%) and control (22%) groups (Table

2). For all oocytes with blue stained cytoplasm (Blue-oocytes: BCB+ plus BCB±), the maturation rate was 45.2% (19/42), ie about twice as high as in the control group (P<0.05). Among the CM groups from large follicles, the percentage of oocytes reaching metaphase II was higher in the blue stained oocytes (80% ie 16/20 for BCB+ and 60.6% ie 20/33 for all of the ‘blue oocytes’ than



n = number of oocytes; Blue = BCB+ plus BCB±  
 Bars with different letters are significantly different: a,c a,d; b,c; d,c = P<0.05; a,b = P<0.001

Fig 1: Percentage of oocytes in metaphase II stage after *in vitro* culture with no respect of follicular size.

in the control group (53.3%) but the difference was not statistically significant. Whereas in the BCB- group, only 16.7% oocytes were in Metaphase II, which was fewer than in all the remaining groups (Table 2). Likewise, in other species, like pigs, prepubertal goats and cows the rate of mature oocytes was lower in BCB- than control groups or in the blue stained oocytes (Ericsson *et al.* 1993; Rodriguez-Gonzalez *et al.* 2002; Alm *et al.* 2005).

As Figure 1 shows, among the CM groups (regardless of follicular size) significantly more 'Blue-oocytes' matured to Metaphase II (52%) than in the control (33.8%; P<0.05) and BCB- (13.8%; P<0.001) groups. Thus, the foregoing results showed, that applying the BCB test for selection of mare CM oocytes before culture increases the nuclear maturation rate, especially in the oocytes originating from small follicles.

Following exposure to BCB, more oocytes with blue stained cytoplasm were found in EX than CM groups (Table 1). This finding suggests that EX oocytes should have a better meiotic competence than the CM ones. It is in agreement with the earlier studies which showed, that more equine oocytes with expanded than with compact

cumuli matured *in vitro* (for review, see Hinrichs, 1997). In our study this observation was confirmed only for the oocytes originating from small follicles (44.4% versus 22% oocytes in Metaphase II in EX and CM control groups, P<0.05, respectively, Table 2 and 3).

Table 3 presents the stage of nuclear development after IVM of EX oocytes. Because of the small number of BCB± oocytes estimated after IVM (2 and 3 from small and large follicles, respectively) these oocytes were included in the BCB+ group and presented together as Blue-one. In the EX groups from small follicles (Table 3) and in the EX groups without respect to follicular size (Fig 1), the rate of maturation to metaphase II was similar in Blue and control groups, and was significantly higher than in BCB- oocytes. However, these differences were not statistically significant between the EX groups derived from large follicles (Table 3). Also the studies on cows, did not show differences in the proportion of mature oocytes between the Blue stained oocytes and control groups (Alm *et al.* 2005). However, after IVF more embryos from the 'Blue oocytes' developed to the blastocyst stage after IVF than those from the BCB- and control ones (Pujol *et al.*

2004; Alm *et al.* 2005). Further studies are needed to find out whether the equine oocytes selected with the BCB test are suitable for *in vitro* fertilisation.

After IVM, some oocytes (n=7) having irregular Metaphase I or II plate with scattered chromosomes, large vacuoles in cytoplasm and partially broken cytoplasmic membrane were observed among the BCB groups from small follicles. Therefore, they were included in the degenerated groups. Such oocytes were found more often in EX than CM groups and in BCB-than Blue groups (Table 2 and 3). In our study, the total time of culture for EX oocytes was about 26–28 h (incubation in BCB + IVM), thus it was longer than usually used for culture of mare EX oocytes (24 h) and could have caused degeneration of the already mature oocytes. One may suppose that the exposure to BCB in mPBS itself could have negatively affected the oocytes or that these oocytes were not fully capable of normal nuclear maturation. Irrespective of follicular size and compactness of the cumulus cells, most of BCB- oocytes (64.4–80%) degenerated during *in vitro* culture (Tables 2 and 3).

To our knowledge, these are the first preliminary results of IVM of mare oocytes selected with the use of the BCB test. On the basis of these results it can be concluded that the BCB test may be useful for selection of mare oocytes more competent to mature *in vitro*, especially for oocytes with compact cumulus cells. This test enables obtaining more homogenous material suitable for *in vitro* culture. The selected oocytes with blue stained cytoplasm proved to be more capable of reaching Metaphase II than the colourless ones. However, further studies of the use of the BCB test are needed, particularly for selection of oocytes with

expanded cumulus cells.

## ACKNOWLEDGEMENTS

The authors wish to thank Barbara Tischner for her help with the English version of the abstract.

## REFERENCES

- Alm, H., Torner, H., Löhrike, B., Viergutz, T., Ghoneim, I.M. and Kanitz, W. (2005) Bovine blastocyst development rate *in vitro* is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* **63**, 2194-2205.
- Ericsson, S.A., Boice, M.L., Funahashi, H. and Day, B.N. (1993) Assessment of porcine oocytes using brilliant cresyl blue. *Theriogenology* **39**, 214 (abstract).
- Goudet, G., Bezar, 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.
- Hinrichs, K. (1997) Cumulus expansion, chromatin configuration and meiotic competence in horse oocytes: A new hypothesis. *Equine vet. J. Suppl.* **25**, 43-46.
- Mangia, F. and Epstein, C.J. (1975) Biochemical studies of growing mouse oocytes: preparation of oocytes and analysis of glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. *Dev. Biol.* **45**, 211-220.
- Pujol, M., Lopez-Bejar, M. and Paramio, M.T. (2004) Developmental competence of heifer oocytes selected using the brilliant cresyl blue (BCB) test. *Theriogenology* **61**, 735-744.
- Rodriguez-Gonzalez, E., Lopez-Bejar, M., Vellila, E. and Paramio, M.-T. (2002) Selection of prepubertal goat oocytes using the brilliant cresyl blue test. *Theriogenology* **57**, 1397-409.
- Rodriguez-Gonzalez, E., Lopez-Bejar, M., Izquierdo, D. and Paramio, M.-T. (2003) Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation. *Reprod. Nutr. Dev.* **43**,

# OOCYTE MITOCHONDRIAL DEGENERATION DURING REPRODUCTIVE AGEING IN THE MARE

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## INTRODUCTION

In mares and women, the likelihood of a normal pregnancy decreases with increasing maternal age (Morris and Allen 2002; te Velde and Pearson 2002), and it has been proposed that this may, at least in part, be a consequence of an age-induced reduction in the quantity and quality of mitochondria in the oocyte. In this respect, oocyte growth and maturation encompasses a period of considerable mitochondrial replication, with numbers increasing from tens or hundreds at primordial stages to hundreds of thousands by the time of ovulation (Jansen and de Boer 1998). Thereafter, oocyte mitochondrial replication arrests and does not re-start until the gastrulation stage of embryo development (Piko and Taylor 1987; Larsson *et al.* 1998). In addition, the few paternal mitochondria that enter the ooplasm during fertilisation are subsequently eliminated by the ubiquitin-proteasome pathway (Sutovsky *et al.* 1999). As a result, early embryonic cell divisions involve the partitioning of a finite number of oocyte-derived mitochondria over an ever-increasing number of blastomeres and energy generation during both fertilisation and early embryo development is dependent wholly on this pool of oocyte-derived mitochondria.

Maternal age is thought to negatively affect oocyte mitochondrial quality via the accumulation of damage, by reactive oxygen species (ROS) generated during oxidative phosphorylation, to both the mitochondrial DNA (mtDNA) and mitochondrial membranes. However, while an age-related increase in oocyte mtDNA mutations (Barritt *et al.* 2000) and deletions (Keefe *et al.* 1995) has been claimed in women, this remains controversial. Nevertheless, it is possible that oocyte developmental competence is

compromised by an age-dependent decrease in mitochondrial quantity and/or quality, and the aim of the current study was to determine whether the quantity or quality of mitochondria in equine oocytes was affected by maternal age and/or maturation *in vitro*.

## MATERIALS AND METHODS

### *Collection of oocytes*

Oocytes were recovered from the ovaries of slaughtered mares whose age had been estimated by examining dental wear. Animals in which the age was unclear due to dental malformation, disease or abnormal attrition were excluded from the study. Otherwise, ovaries were assigned at collection to one of 2 groups depending on the mare's estimated age, ie young ( $\leq 11$ -years-old) or aged ( $\geq 12$ -years-old), before transport to a laboratory where the cumulus-oocyte-complexes (COCs:  $n=501$ ) were collected by aspiration from follicles ranging from 5–30 mm in diameter. Approximately half of the COCs were immediately denuded of their cumulus investment and stored (GV), while the remainder were matured *in vitro* for 30 h prior to denudation and storage (IVM).

### *mtDNA quantity and quality*

For each oocyte, the mitochondrial DNA (mtDNA) copy number was quantified using triplicate samples in a real-time (quantitative) PCR detection system (MyiQ Single-color Real-Time PCR Detection System; Bio-Rad Laboratories, The Netherlands). Inter-plate variations in absolute numbers were monitored using internal controls of known high and low mtDNA copy number.

The 'quality' of the mtDNA was analysed in 40 oocytes (10 per group for the combinations of young versus aged with GV versus IVM) using conventional PCR for one DNA sequence located in the 'D-loop' and 2 sequences located elsewhere in the mitochondrial genome but containing a potential 'deletion hotspot', ie direct sequence repeats of 12 and 13 base pairs, respectively. After electrophoresis, the amplicons of the latter sequences were analysed for the presence of deletions on the basis of gross amplicon size, whereas the products of the first and one other PCR reaction were also sequenced to look for DNA heteroplasmy (ie mutations).

### **Morphology of oocyte mitochondria**

Oocyte mitochondrial morphology was examined in 20 oocytes (5 per group for young versus aged combined with GV versus IVM) by transmission electron microscopy (TEM) of COCs fixed in Karnovsky fixative and embedded in Durcupan ACM (Fluka, Switzerland). Ultrathin sections (50 nm) of the oocyte were mounted on grids and stained with lead citrate before electron microscopy to examine mitochondrial diameter and appearance.

## **RESULTS**

The mean ( $\pm$  sem) number of mtDNA copies in analysed oocytes was  $2.6 \times 10^6 \pm 8.2 \times 10^4$ . mtDNA copy number did not differ between GV oocytes from young or aged mares, or IVM oocytes from young mares. However, the mtDNA copy number in IVM oocytes from aged mares was significantly lower than in the other 3 groups ( $P < 0.001$ ). With regard to mtDNA quality, no deletions were detected and although mutations were detected, they were all single nucleotide polymorphisms (SNPs) and were neither common nor associated with either mare age or oocyte maturation. On the other hand, TEM demonstrated that mitochondria in IVM oocytes from aged mares, but not in any other group, were often swollen with extensively damaged cristae.

## **DISCUSSION**

In the current study, we used quantitative PCR and TEM to demonstrate that maternal age is associated with a reduction in mitochondrial

quantity in equine oocytes. This reduction in number manifested following maturation *in vitro* and presumably resulted from the IVM induced mitochondrial damage detected by TEM and characterised by swelling and loss of internal architecture. This resembled a previous finding in mice, in which mitochondria in the oocytes of aged females were found to be more sensitive to experimentally induced damage than those in pubertal animals (Thouas *et al.* 2005). In both cases, it is possible that in older females reactive oxygen species (ROS) generated during constitutive oxidative phosphorylation have simply had more opportunities to inflict damage on the oocyte's mitochondrial membranes, but that the damage only becomes apparent when the oocyte's energy demand increases, eg during (*in vitro*) maturation. The combination of an increased demand for energy and damaged mitochondrial membranes may then lead to either insufficient ATP production and/or increased ROS production, thereby creating a vicious circle ending in swelling and destruction of the mitochondria.

The significance of the apparent reduction in mitochondrial numbers and quality in the oocytes of aged mares relates to the likely consequences for fertilisation and embryo development. In women, low oocyte mtDNA copy numbers have been associated with reduced fertilisability (Reynier *et al.* 2001) while, in mice, extensive damage to an oocyte's mitochondria restricts the ability of that oocyte to subsequently undergo germinal vesicle breakdown, meiotic spindle formation, chromosome segregation, polar body extrusion (Takeuchi *et al.* 2005) and blastocyst formation (Thouas *et al.* 2005). Although still to be proven, it seems likely that the decreases in mitochondrial quantity and quality detected in the current study in IVM oocytes of aged mares would negatively influence oocyte developmental competence and may contribute to poor fertility in these animals.

## **REFERENCES**

- Barritt, J.A., Cohen, J. and Brenner, C.A. (2000) Mitochondrial DNA point mutation in human oocytes is associated with maternal age. *Reprod. Biomed. Online* **1**, 96-100.
- Jansen, R.P. and de Boer, K. (1998) The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. *Mol. Cell Endocrinol.* **145**, 81-88.
- Keefe, D.L., Niven-Fairchild, T., Powell, S., and

- Buradagunta, S. (1995) Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil. Steril.* **64**, 577-583.
- Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S. and Clayton, D.A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* **18**, 231-236.
- Morris, L.H. and Allen, W.R. (2002) Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. *Equine vet. J.* **34**, 51-60.
- Piko, L. and Taylor, K.D. (1987) Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev. Biol.* **123**, 364-374.
- Reynier, P., May-Panloup, P., Chretien, M.F., Morgan, C.J., Jean, M., Savagner, F., Barriere, P. and Malthiery, Y. (2001) Mitochondrial DNA content affects the fertilisability of human oocytes. *Mol. Hum. Reprod.* **7**, 425-429.
- Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C. and Schatten, G. (1999) Ubiquitin tag for sperm mitochondria. *Nature* **402**, 371-372.
- Takeuchi, T., Neri, Q.V., Katagiri, Y., Rosenwaks, Z. and Palermo, G.D. (2005). Effect of treating induced mitochondrial damage on embryonic development and epigenesis. *Biol. Reprod.* **72**, 584-592.
- te Velde, E.R. and Pearson, P.L. (2002) The variability of female reproductive ageing. *Hum. Reprod. Update* **8**, 141-154.
- Thouas, G.A., Trounson, A.O. and Jones, G.M. (2005) Effect of female age on mouse oocyte developmental competence following mitochondrial injury. *Biol. Reprod.* **73**, 366-373.

## MOUSE PLCzeta mRNA INDUCES CALCIUM OSCILLATIONS AND ACTIVATION IN MARE OOCYTES

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In all species studied so far, fertilisation induces a series of repetitive long-lasting intracytoplasmic  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) elevations or oscillations responsible for oocyte activation and for supporting embryonic development (Fissore *et al.* 1992; Kline and Kline 1992; Nakada *et al.* 1995). The pattern of  $[Ca^{2+}]_i$  transients at fertilisation is species specific, with  $[Ca^{2+}]_i$  rises occurring at of 3–10 min in the mouse (Kline and Kline 1992; Faure *et al.* 1999), 10–35 min in man (Taylor *et al.* 1993; Tesarik *et al.* 1995), and 20–50 min in the bovine oocyte (Fissore *et al.* 1992; Nakada *et al.* 1995), and lasting for several hours.

Current research supports the role of the phosphoinositide pathway and production of 1,4,5-inositol trisphosphate (IP3) for triggering the initiation of  $[Ca^{2+}]_i$  release from intracellular stores (Wu *et al.* 2001). It is believed that the sperm releases a 'factor' responsible for initiating IP3 production. It is well known that a group of phospholipase C (PLC) enzymes, with different regulatory sequences, are responsible for IP3 production in somatic cell systems (reviewed by Rhee 2001). Recently, a sperm-specific PLC, PLCzeta, was proposed as the putative 'sperm factor' (Saunders *et al.* 2002). Injection of PLCzeta mRNA into mouse oocytes, induced fertilisation-like  $[Ca^{2+}]_i$  oscillations and embryonic development to the blastocyst stage. This construct was also able to induce fertilisation-like  $[Ca^{2+}]_i$  transients in bovine eggs (Malcuit *et al.* 2005).

The success of assisted reproduction techniques in the horse depends, at least partially, on methods that can reliably induce  $[Ca^{2+}]_i$  oscillations and thus embryonic development to the blastocyst stage. In this regard, Bedford *et al.* (2003) showed, for the first time, that injection of stallion sperm extracts (eSF; 5 mg/ml) induces

$[Ca^{2+}]_i$  oscillations and activation in mare oocytes. Transients triggered by eSF in our study occurred every 20–45 min and lasted for 60–120 min. Because the pattern of fertilisation (IVF) induced  $[Ca^{2+}]_i$  oscillations is unknown for the horse, sperm were injected (ICSI) into *in vitro* and *in vivo* matured mare oocytes and  $[Ca^{2+}]_i$  transients were monitored (Bedford *et al.* 2003, 2004). Interestingly, only 30–40% oocytes displayed  $[Ca^{2+}]_i$  oscillations after ICSI, thus partially explaining the difficulty in achieving consistent activation and embryonic developmental rates in the horse via this technique. These results emphasise the need to find activation protocols that will enhance long-lasting  $[Ca^{2+}]_i$  oscillations in mare oocytes for both nuclear transfer and ICSI technology.

The objectives of this study were therefore to ascertain the ability of PLCzeta to induce long-lasting  $[Ca^{2+}]_i$  oscillations and activation in mare oocytes. For this purpose a c-RNA construct from mouse PLCzeta was microinjected at a concentration of 1  $\mu\text{g}/\mu\text{l}$  ( $\approx$ 20–40 pl) into mare oocytes matured *in vitro* for 36 h. This concentration was based on previous results in bovine oocytes (Malcuit *et al.* 2005).

Preliminary results are encouraging in that 10/12 (83%) microinjected oocytes displayed  $[Ca^{2+}]_i$  oscillations (Fig 1) that lasted for at least 3 h, but less than 5 h. This is longer than that reported with eSF (Bedford *et al.* 2003). Such oscillations started within 30 min of PLCzeta injection, occurring every 25–60 min with amplitude decreasing and frequency increasing over time. Moreover, 25/31 (81%) of treated oocytes had one or 2 pronuclei or had cleaved (2-cell) by 24 h after injection of PLCzeta, whereas none of the oocytes (0/3) injected with vehicle medium only were activated.

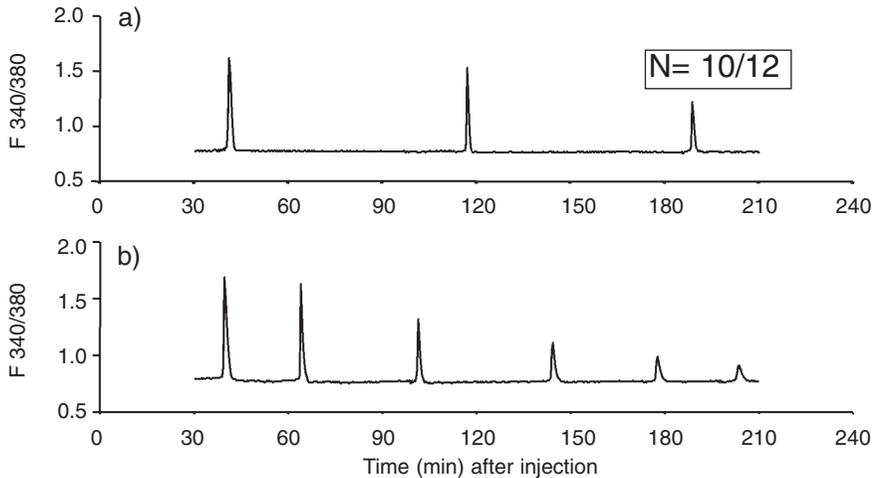


Fig 1: Representative  $[Ca^{2+}]_i$  oscillation profiles observed in *in vitro* matured mare oocytes microinjected with  $1 \mu\text{g}/\mu\text{l}$  of mouse PLCzeta mRNA. From the 10 oocytes that showed  $[Ca^{2+}]_i$  responses, 6 and 4 oocytes displayed a pattern consistent with Panels A and B, respectively.

In conclusion, mouse PLCzeta mRNA could consistently trigger  $[Ca^{2+}]_i$  oscillations and activation in mare oocytes. More work is needed to titrate the optimal concentration; it is suspected that appropriate calibration of the PLCzeta concentration will result in induction of long-lasting  $[Ca^{2+}]_i$  oscillations and thus optimise the chances of obtaining high rates of parthenogenetic embryonic development. Therefore this research may provide a better avenue than is currently available for activation of mare oocytes for assisted reproduction technology.

## REFERENCES

- Bedford, S.J., Kurokawa, M., Hinrichs, K. and Fissore, R.A. (2003) Intracellular calcium oscillations and activation in horse oocytes injected with equine sperm extracts or sperm. *Reproduction* **126**, 489-499.
- Bedford, S.J., Kurokawa, M., Hinrichs, K. and Fissore, R.A. (2004) Patterns of intracellular calcium oscillations in horse oocytes fertilised by intracytoplasmic sperm injection: Possible explanations for the low success of this assisted reproduction technique in the horse. *Biol. Reprod.* **70**, 936-944.
- Faure, J.E., Myles, D.G. and Primakoff, P. (1999) The frequency of calcium oscillations in mouse eggs at fertilisation is modulated by the number of fused sperm. *Dev. Biol.* **213**, 370-377.
- Fissore, R.A., Dobrinsky, J.R., Balise, J.J., Duby, R.T. and Robl, J.M. (1992) Patterns of intracellular  $Ca^{2+}$  concentration in fertilised bovine oocytes. *Biol. Reprod.* **47**, 960-969.
- Kline, D. and Kline, J.T. (1992) Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80-89.
- Malcuit, C., Knott, J.G., He, C., Wainwright, T., Parys, J.B., Robl, J.M. and Fissore, R.A. (2005) Fertilisation and inositol 1,4,5-trisphosphate (IP3)-induced calcium release in type-1 inositol 1,4,5-trisphosphate receptor down-regulated bovine eggs. *Biol. Reprod.* **73**, 2-13.
- Nakada, K., Mizuno, J., Shiraishi, K., Endo, K. and Miyazaki, S. (1995) Initiation, persistence, and cessation of the series of intracellular  $Ca^{2+}$  responses during fertilisation of bovine oocytes. *J. Reprod. Dev.* **41**, 77-84.
- Rhee, S.G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Ann. Rev. Biochem.* **70**, 281-312.
- Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K. and Lai, F.A. (2002) PLCz: A sperm-specific trigger of  $Ca^{2+}$  oscillations in eggs and embryo development. *Dev.* **129**, 3533-3544.
- Taylor, C.T., Lawrence, Y.M., Kingsland, C.R., Biljan, M.M. and Cuthbertson, K.S.R. (1993) Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilisation. *Hum. Reprod.* **8**, 2174-2179.
- Tesarik, J., Sousa, M. and Mendoza, C. (1995) Sperm-induced calcium oscillations of human oocytes show distinct features in oocyte center and periphery. *Mol. Reprod. Dev.* **41**, 257-263.
- Wu, H., Smyth, J., Luzzi, V., Fukami, K., Takenawa, T., Black, S.L., Allbritton, N.L. and Fissore, R.A. (2001) Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod.* **64**, 1338-1349.

# CHANGES IN MITOCHONDRIAL ACTIVITY IN HORSE OOCYTES AND ZYGOTES DURING MATURATION AND FERTILISATION *IN VITRO*

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## INTRODUCTION

The technology for *in vitro* embryo production from equine oocytes after IVM and IVF needs a better understanding for all *in vitro* methods which are included. Nevertheless, biochemical and morphological information concerning oocyte maturation and fertilisation in mares are quite rare.

Immature equine oocytes are capable of complete meiosis *in vitro*. However, the subsequent fertilisation and embryonic development are not efficient, possibly due to an impaired cytoplasmic maturation. The mammalian 'oocyte quality' after final maturation is strongly influenced by cytoplasmic maturation before and during IVM (Hytel *et al.* 1997; Dieleman *et al.* 2002). A prominent marker for cytoplasmic maturation are the mitochondria. They play an essential role in the oocyte to provide ATP for fertilisation and pre-implantation embryo development. Morphological information concerning mitochondria in horse oocytes or zygotes is quite limited (Grondahl *et al.* 1995; Hochi *et al.* 1996) and not directed at the respiratory activity of mitochondria in horse oocytes and zygotes.

Because of limited availability all equine oocytes with complete cumulus not less than 3 cumulus layers were used for the technique of IVM, but they differ in cumulus morphology and further developmental competence (Alm *et al.* 2001; Choi *et al.* 2004). Electron microscopic studies (Alm *et al.* 2000) had shown differences of mitochondrial distribution in horse oocytes with different initial cumulus morphology (compact or expanded).

Based on the morphological data concerning mitochondria in horse oocytes and the published difference in developmental competence of horse

oocytes with different initial cumulus morphology, the aim of the present work focused on the respiratory activity of mitochondria as a functional parameter of cytoplasmic quality in horse oocytes and zygotes. Therefore, we examined the mitochondrial activity in horse oocytes related to the meiotic stage and initial cumulus morphology during IVM (0–32 h) and investigated this parameter after IVF (0–32 h) in zygotes.

## MATERIALS AND METHODS

### *Analysis of changes in mitochondrial activity in horse oocytes during IVM*

Cumulus-oocyte-complexes (COC's) were recovered by ultrasound-guided follicle aspiration *in vivo*. The morphology of freshly recovered COC's was evaluated under an inverted microscope at x 60 magnification. The recovered oocytes were classified as having compact, expanded or few layers of cumulus cells (Torner and Alm 1995) and stored in a holding medium (modified TCM 199, Sigma) at room temperature for a maximum of 2 h. Oocytes without cumulus or with corona radiata only were not used in the experiments. According to their cumulus morphology COC's were cultured in Hepes-buffered TCM 199 (Sigma) containing FSH and 10% (v/v) heat-treated horse serum (Sigma) for 0, 18, 24 or 32 h. COC's according to their cumulus investment at the time of recovery and to their time of culture were processed to evaluate mitochondrial activity and chromatin configuration in parallel studies in the same oocyte.

As described for porcine oocytes (Torner *et al.* 2004) the defined COC's were incubated for 30 minutes in phosphate-buffered saline (PBS)

containing 3% (w/v) BSA and 200 nM MitoTracker Orange CMTM Ros (Molecular Probes, Oregon, USA) under culture conditions. The cumulus cells were mechanically removed from the oocytes by repeated pipetting and subsequent treatment with 3% sodium citrate. The denuded 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% (v/v) paraformaldehyde in Hank's balanced salt solution. After fixation the oocytes were washed 3 times in PBS and mounted between slide and cover slide in a mixture of Moviol V4-88 (133 mg/ml, Hoechst) and n-propyl gallate (5 mg/ml, Sigma) containing 2.5 µg/ml bis-benzimide (Hoechst 33342, Sigma) to detect chromatin configuration. The slides were kept at 4°C in darkness until evaluation.

An epifluorescence microscope was used for all experiments. At first, in each oocyte the chromatin configuration was estimated by UV-fluorescence at 410 nm. The chromatin configuration was classified according to the onset of meiotic stages into diplotene (Dipl), diakinesis (Dia), Metaphase 1 (M 1), Anaphase 1 (A 1), Telophase 1 (T 1), Metaphase 2 (M 2) and activated oocyte chromatin – pronuclei (PN).

For the subsequent evaluation of mitochondrial activity in the oocyte the emission wavelengths were separated by a 540 nm dichroic mirror followed by further filtering through a 570 nm long pass filter (red emission). The fluorescence intensity per oocyte (µA) was measured by the Nikon Photometry System P 100. Microscope adjustments and photo multiplier settings were kept constant for all experiments. The same measurement procedure was used as described in pig oocytes (Torner *et al.* 2004).

#### *Analysis of mitochondrial activity in horse zygotes during IVF*

COC's were recovered by scraping follicles from ovaries of slaughtered mares of unknown reproductive history. Only oocytes with compact cumulus (n=222) were selected from all recovered COC's and matured for 32 h in TCM 199 containing FSH and 10% estrus mare serum. For *in vitro* capacitation we used cryopreserved semen from one ejaculate of one stallion; a motile sample of thawed spermatozoa for IVF was obtained by swim-up separation and subsequent treatment with

200 µg/ml heparin (Torner *et al.* 2002).

After IVM 5 oocytes were placed in a 50 µl droplet of TALP-medium and approximately 5 µl of the final sperm suspension were added to each droplet to give a final concentration of  $1 \times 10^6$  motile sperm/ml in the fertilisation droplet. Fertilisation was carried out for 0, 2, 4, 8, 16 and 32 h. Oocytes or zygotes were processed for investigation of chromatin and mitochondrial activity as described above. Only 50 eggs with representative stages of penetration/fertilisation were evaluated out of 222.

## RESULTS AND DISCUSSION

The respiratory activity in the oocytes measured by fluorescence intensity for 570 nm emission/oocyte is associated both with the type of cumulus at the time of recovery and with the time of IVM. The lowest fluorescence intensity/oocyte was found in oocytes with compact cumulus investment before IVM (0 h), and the highest intensity was measured in oocytes originating from COC-group with few cumulus layers at the end of IVM (32 h). Oocyte with different cumulus investment at the beginning of IVM showed significant differences in the dynamic of respiratory activity.

The data in Figure 1 demonstrate that the mitochondrial activity in the oocyte is also associated with the chromatin configuration of the oocyte nucleus.

After IVF of *in vitro* matured oocytes with initial compact cumulus investment the following events during IVF (0 to 32 h p.insem.) in the fertilised eggs were representative: Metaphase 2 (M 2; 0–2 h), decondensed sperm head (DSH; 4

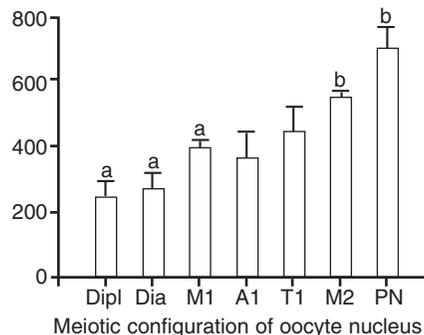


Fig 1: Mitochondrial activity in horse oocytes related to the meiotic stage of nucleus. a,b Values with different superscripts differ significantly ( $P < 0.05$ ).

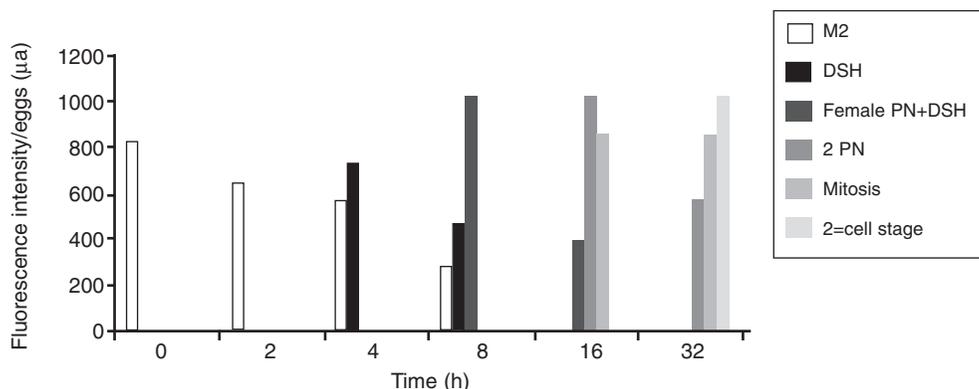


Fig 2: Mitochondrial activity in horse zygotes during IVF depending on stage of fertilisation.

h), female pronucleus and DSH (PN+DSH; 8 h), two pronuclei (2 PN; 16 h), mitosis (16–32 h), and 2-cell stage (32 h).

The data in Figure 2 demonstrate the level of mitochondrial activity depending on the progress of penetration and fertilisation in horse zygotes during 0–32 h post IVF.

In conclusion, the results of the present study demonstrate the increasing mitochondrial activity in horse oocytes during IVM and the close correlation of the different meiotic stages of nucleus chromatin. It seems that the higher respiratory activity of mitochondria from oocytes with initial expanded or few layers of cumulus could be an explanation for their higher developmental competence. After fertilisation *in vitro* the level of mitochondrial activity in horse zygotes depends on the correct first onset of different penetration and fertilisation stages.

## REFERENCES

- Alm, H., Neumann, H., Torner, H., Tomek, W., Wollenhaupt, K., Kanitz, W. and Becker, F. (2000) Ultrastructure, protein phosphorylation and mRNA status of equine oocytes matured *in vivo* and *in vitro*. *J. Reprod. Fert.* **56**, 473-482.
- 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.
- Choi, Y.H., Love, C.B., Varner, D.D. and Hinrichs, K. (2004) Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127**, 187-194.
- Dielemann, S.J., Hendriksen, P.J., Viuff, D., Thomsen, P.D., Hyttel, P., Knijn, H.M., Wrenzycki, C., Kruij, T.A., Niemann, H., Gadella, B.M., Bevers, M.M. and Vos, P.L. (2002) Effects of *in vivo* pre-maturation and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology* **57**, 5-20.
- Grondahl, C., Hyttel, P., Grondahl, M.L., Eriksen, T., Gotfredsen, P. and Greve, T. (1995) Structural and endocrine aspects of equine oocyte maturation *in vivo*. *Mol. Reprod. Dev.* **42**, 94-105.
- Hochi, S., Kozawa, M., Fujimoto, T., Hondo, E., Yamada, J. and Oguri, N. (1996) *In vitro* maturation and transmission electron microscopic observation of horse oocytes after vitrification. *Cryobiology* **33**, 300-310.
- Hyttel, P., Fair, T., Callesen, H. and Greve, T. (1997) Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* **47**, 23-32.
- Torner, H. and Alm, H. (1995) Meiotic configuration of horse oocytes in relation to the morphology of the cumulus-oocyte-complex. Eds: D.C. Sharp and F.W. Bazer In: *Biology of Reproduction Monograph Series 1, Equine reproduction VI*. Ann Arbor, MI: Edwards Brothers, Inc. pp 253-259.
- Torner, H., Alm, H., Mlodawska, W., Warnke, C., Göllnitz, K., Blottner, S. and Okolski, A. (2002) Determination of development in horse zygotes and spermatozoa during fertilisation *in vitro*. *Theriogenology* **58**, 693-696.
- Torner, H., Brüßow, K.-P., Alm, H., Ratky, J., Pöhland, R., Tuchscherer, A. and Kanitz, W. (2004) Mitochondrial aggregation patterns and activity in porcine oocytes and apoptosis in surrounding cumulus cells depends on the stage of preovulatory maturation. *Theriogenology* **61**, 1675-1689.

# THE INFLUENCE OF CUMULUS CELLS DURING THE VITRIFICATION OF IMMATURE EQUINE OOCYTES

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## INTRODUCTION

Oocyte cryopreservation is a potentially valuable technique for storing female germ cells for subsequent use in assisted reproduction. However, oocytes are very susceptible to freezing-induced damage and the developmental competence of frozen-thawed oocytes is currently poor. While few studies have examined the cryopreservability of equine oocytes, freezing at the germinal vesicle (GV) stage dramatically reduces their ability to resume meiosis; Hochi *et al.* (1994) reported that <16% of oocytes frozen using a controlled rate technique reached Metaphase II (MII) during post thaw *in vitro* maturation (IVM). And while Hurtt *et al.* (2000) achieved a more promising 30% MII with vitrified oocytes, this still compares unfavourably to the 50–80% typical for fresh oocytes.

Although the precise mechanisms of freezing-induced oocyte damage are not clear, disruption of the cytoskeleton and meiotic spindle are common findings (Park *et al.* 1997). Freezing and thawing has also been reported to cause cumulus cell death (Ruppert-Lingham *et al.* 2003) and damage the gap-junctions between the oocyte and the surrounding cumulus cells (Hochi *et al.* 1996), where oocyte-cumulus interaction is critical to successful maturation *in vitro*. Additionally, in horses, cumulus morphology at oocyte collection (compact versus expanded) is related to developmental competence, with an expanded cumulus associated with a higher likelihood of blastocyst formation (Choi *et al.* 2004). The effect of cumulus morphology on the ability of oocytes to survive cryopreservation has not been reported.

The aims of the current study were to investigate the effect of vitrification on the integrity of immature equine cumulus oocyte complexes (COCs) in terms of cumulus cell

viability, oocyte-cumulus gap junction function and MII spindle morphology, and to examine the relationship between initial cumulus morphology and post warming developmental competence.

## MATERIALS AND METHODS

### *Vitrification*

COCs were vitrified using a modified open pulled straw (OPS) technique. First, the oocytes were immersed for 30 s in 'pre-vitrification' medium; M199 containing 0.014% (w/v) bovine serum albumin (BSA), 10% (v/v) ethylene glycol (EG) and 10% (v/v) dimethyl sulphoxide (DMSO). Next, they were immersed for 15 s in vitrification medium (VM: M199 containing 20% EG, 20% DMSO and 0.5M sucrose), transferred to fresh VM and then loaded into pulled straws and plunged into liquid nitrogen. 'Warming' was performed in M199 supplemented with 0.014% BSA and 0.3M sucrose. When required, oocytes were matured *in vitro* by culture in M199 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 10 mM cysteamine, 0.1 units/ml porcine FSH and 0.1 units/ml equine LH, at 38.7°C in humidified 5% CO<sub>2</sub>-in-air. In all experiments, both VM-exposed (but not frozen) and untreated COCs were used as controls.

### *Experiment 1*

After vitrifying and warming, 48 immature COCs were incubated for 10 min in a 2 µM solution of the cell membrane impermeant DNA marker, Ethidium homodimer-1 (Ethd-1: Molecular Probes), to label dead cells. The COCs were then fixed in 4% (w/v) paraformaldehyde in PBS and stained further with

DAPI to label previously live cells, and thereby allow live and dead cells to be counted using an epifluorescence microscope. To localise dead cells within the cumulus mass, a further 46 vitrified-warmed COCs were stained with Ethd-1, fixed, stained with another DNA marker (ToPro3: Molecular Probes) and examined using a confocal microscope. To investigate the significance of freezing-induced cumulus cell death on the ability of oocytes to resume meiosis *in vitro*, 108 vitrified-warmed COCs were stained with Ethd-1, examined with an inverted epifluorescence microscope and divided into classes based on the approximate proportion of dead cumulus cells (+1, +2 or +3), before IVM for 30 h.

### Experiment 2

The presence of gap junctions in vitrified-warmed COCs was examined immunohistochemically using a mouse monoclonal antibody against connexin 43. Gap junction function was then investigated in 36 vitrified-warmed and 53 control COCs by microinjecting a 3% solution of the membrane impermeant dye Lucifer yellow (LY) into the ooplasm. Ten min post injection the extent of LY diffusion into the cumulus complex was examined using an epifluorescence microscope. Gap junctions were classified as 'open' if at least 80% of cumulus cells fluoresced, 'partially open' if fewer cells were stained and 'closed' if LY was confined to the oocyte.

### Experiment 3

To examine the influence of initial cumulus morphology on vitrification-induced cytoskeleton damage, COCs classified as having a compact (Cp: n=63) or an expanded (Ex: n=59) cumulus were vitrified. After warming, the oocytes were matured *in vitro* for 30 h and fixed to allow examination of maturation stage and MII spindle quality using confocal microscopy and fluorescent labels for microtubules (anti  $\alpha$ -tubulin antibody), microfilaments (phalloidin) and DNA (ToPro3). To examine the influence of culmulus morphology on post-warming developmental competence, 229 Cp and 153 Ex vitrified COCs were matured *in vitro* for 28 h. Oocytes that reached MII were fertilised by ICSI, and cleavage and blastocyst-formation rates were recorded on Days 2 and 9 post-ICSI, respectively.

## RESULTS

Vitrifying COCs significantly increased the proportion of dead cumulus cells (14% versus 3% for controls;  $P<0.05$ ). However, the dead cells were almost always located at the periphery of the cumulus mass and rarely in the 2–3 layers closest to the oocyte. Furthermore, the proportion of dead cumulus cells (+1, +2 or +3) did not affect the ability of oocytes to reach MII during IVM (approx. 30% in all groups).

In both control and vitrified COCs, the gap-junction protein connexin-43 was present at the interface between neighbouring cumulus cells. Moreover, gap junction function, as determined by LY diffusion, did not differ between vitrified and control COCs; approximately 50% in each group had 'open' gap junctions.

Finally, cumulus morphology at collection did not affect the proportion of vitrified oocytes that developed to MII during IVM (40–45%), but MII spindle quality was better for Cp than Ex COCs (63% versus 33% normal spindles: compared to >95% in controls). Cleavage rates were higher in control (56%) than vitrified oocytes, but there was no difference between vitrified Cp and Ex COCs (34% versus 27% cleavage). Only one of 156 vitrified, sperm-injected oocytes (from a Cp COC) developed into a blastocyst (versus 13% of injected control oocytes), and it was not therefore possible to determine whether cumulus morphology affected post vitrification developmental competence.

## CONCLUSIONS

The cumulus complex has been proposed to protect the oocyte during freezing and thawing (Ruppert-Lingham *et al.* 2003). In the current study, vitrifying COCs resulted in the death of an appreciable proportion of cumulus cells together with a reduction in oocyte meiotic competence; there was, however, no apparent relationship between the extent of cumulus damage and meiotic competence. This is presumably because cell death hardly ever affected the innermost cumulus cells most likely to 'communicate' with the oocyte, while the gap junctions critical to oocyte-cumulus communication also survived the vitrification process. This suggests that cumulus cell death is a minor contributor to the vitrification-induced reduction in oocyte quality.

Overall, the percentage of oocytes that reached MII following vitrification (40-45%) was promising. However, vitrification frequently resulted in disruption of the meiotic spindle sufficient to render normal chromosome segregation impossible. Since spindle quality was best preserved in oocytes with a Cp cumulus at recovery, this group should have the best chance of yielding a chromosomally normal embryo. Unfortunately, because only one blastocyst was produced from a vitrified oocyte, it was possible only to conclude that vitrification dramatically reduces oocyte developmental competence, probably for reasons other than spindle disruption alone.

## REFERENCES

- Choi, Y.H., Love, L.B., Varner, D.D. and Hinrichs, K. (2004) Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127**, 187-194.
- Hochi, S., Fujimoto, T., Choi, Y.H., Braun, J. and Oguri, N. (1994) Cryopreservation of equine oocytes by 2-step freezing. *Theriogenology* **42**, 1085-1094.
- Hochi, S., Kozawa, M., Fujimoto, T., Hondo, E., Yamada, J. and Oguri, N. (1996) *In vitro* maturation and transmission electron microscopic observation of horse oocytes after vitrification. *Cryobiology* **33**, 300-310.
- Park, S.E., Son, W.Y., Lee, S.H., Lee, K.A., Ko, J.J. and Cha, K.Y. (1997) Chromosome and spindle configurations of human oocytes matured *in vitro* after cryopreservation at the germinal vesicle stage. *Fertil. Steril.* **68**, 920-926.
- Ruppert-Lingham, C.J., Paynter, S.J., Godfrey, J., Fuller, B.J. and Shaw, R.W. (2003) Developmental potential of murine germinal vesicle stage cumulus-oocyte complexes following exposure to dimethylsulphoxide or cryopreservation: loss of membrane integrity of cumulus cells after thawing. *Hum. Reprod.* **18**, 392-398.



# SESSION 3:

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## Embryos

**Chairman:**  
**T. Stout**



## EMBRYO FREEZING

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The number of equine embryos frozen is relatively small compared to the number of fresh embryos transferred. This is likely to change once the breed registries accept the use of this technology and allow foals to be registered from embryo recipients receiving frozen-thawed equine embryos. The largest equine breed, the American Quarter Horse Association, is in the process of deciding whether to accept foals born from frozen-thawed embryos. There appear to be 3 major reasons for freezing equine embryos: 1) import-export; 2) embryo banking; and 3) to reduce recipient herds. With the recent availability of equine FSH (eFSH), superovulation and collection of multiple embryos from the mare provides extra embryos that can be frozen. It is likely that breed registries will change their attitudes about registering foals from frozen embryos and, with the simplified new procedures for vitrification, embryo freezing is likely to become more of a standard procedure used in equine embryo transfer.

One major drawback to freezing equine embryos is that the embryo must be of a certain size and developmental stage to survive the freezing and thawing process. Based on numerous studies, only embryos <300  $\mu\text{m}$  (morula or early blastocysts), with a zona pellucida, survive the freezing-thawing process. Whether one uses slow-cooling or vitrification procedures, embryos >300  $\mu\text{m}$  result in very poor pregnancy rates after freezing, thawing and transfer (Slade *et al.* 1985; Squires *et al.* 1989; Maclellan *et al.* 2002). This means that the mare's uterus must be flushed for an embryo approximately 6.5 days after ovulation. Mares flushed later than this time provide embryos that have an acellular capsule formed underneath the zona. Apparently, this acellular capsule may, in fact, impede the penetration of

cryoprotectants into the inner cell mass and trophoblastic cells and explains why larger embryos freeze very poorly.

There are 2 approaches to timing the flushing of the mare's uterus in order to obtain small embryos. One is to examine the mare by ultrasonography either once or several times per day in order to determine the exact time of ovulation and then flush the mare 6.5 days after ovulation. The other approach is to flush the mare's uterus 8 days after administration of hCG. Studies conducted in our laboratory have shown that the majority of mares flushed 8 days after hCG provide embryos that are <300  $\mu\text{m}$  in size (Eldridge-Panuska *et al.* 2005). Generally, mares ovulate approximately 36 h after hCG. Thus, when flushing the mare's uterus 8 days post hCG, the embryo is approximately 6.5 days old.

There are 2 different procedures used for freezing of equine embryos. One is a slow-cooling method similar to the protocol used for freezing bovine embryos. Several studies (Slade *et al.* 1985; Lascombes 2002; Maclellan 2002) have reported excellent fertility of embryos slow-cooled, thawed and transferred into recipients. Typically, embryos are packaged in 0.5 ml plastic straws, cooled to 6°C at 4°C/min, seeded and held for 15 min, then cooled at 0.3°C/min to -35°C or 38°C and then plunged into liquid nitrogen. The majority of studies have been done with the cryoprotectant glycerol, although ethylene glycol appears to be a good alternative to glycerol for equine embryos. The disadvantage of the slow-cool method is the time involved in freezing, as well as the need for a programmable cell freezer.

The alternative is a vitrification procedure which is much simpler and does not require a cell freezer. Several studies have been conducted in our laboratory in the last 2 years to evaluate the

fertility of embryos that have been vitrified and thawed. In the initial study by Eldridge-Panuska *et al.* (2005), 39 lactating light horse mares from 3–15 years of age were used. Embryo collections were made 6–6.5 days after detection of ovulation or 8 days after administration of hCG. Upon identification, embryos were washed through 3–6 drops of holding medium (Vigro Holding Plus, AB Technology, Inc., Washington, USA). Embryos were classified for developmental stage and measured. The vitrification solutions were: VS1 – 1.4 M glycerol for 5 min, then VS2 – 1.4 M glycerol + 3.6 M ethylene glycol for 5 min, then VS3 – 3.4 M glycerol + 4.6 M ethylene glycol for <1 min. The embryos were loaded into a straw and the straw was heat-sealed and placed into a cooled plastic goblet surrounded by liquid nitrogen for 1 min. The entire goblet containing the straws was then plunged into liquid nitrogen. Embryos were transferred nonsurgically into the uteri of recipients. Twenty-six of 48 embryos transferred resulted in viable pregnancies (54%).

In 2004 (Hudson *et al.* 2005), a study was done to determine if cooling embryos for 12–19 h prior to vitrification would result in similar pregnancy rates to embryos vitrified immediately upon collection and to determine the viability of vitrified embryos from superovulated mares. Mares were administered 12.5 mg of eFSH (Bioniche Animal Health, Georgia, USA) twice daily for 5–7 days. Embryos were flushed from the uterus 6.5 days after ovulation, if ovulations were asynchronous, or 8 days post hCG for synchronous ovulations. Upon identification, each embryo was measured using an eyepiece micrometer and graded. After sizing and grading, each embryo was rinsed 4 times in Vigro Holding Solution (AB Technology, Inc., Washington, USA). Embryos were either assigned to be vitrified immediately or to be cooled in a passive cooling device (Equitainer, Hamilton Thorne Biosciences, Massachusetts, USA) for 12–19 h prior to vitrification. All embryos were vitrified as described by Eldridge-Panuska *et al.* (2005). Embryos were transferred during the month of August 2004 into synchronised recipients. Embryos were transferred into recipients that had ovulated 4–6 days previously to the transfer. Embryos were warmed by removing the straw from the liquid nitrogen tank and holding the straw in air at room temperature for 10 s prior to plunging into a bath of 20–22°C water for an

additional 10 s. The straws were removed from the water bath and flicked like a clinical thermometer 4–5 times to ensure mixing of the solutions. For transfer, the straws were loaded into a special Cassou gun designed for cut straws. Each embryo was transferred into a recipient within 8 min of removal of the straw from the liquid nitrogen tank. There were no differences in pregnancy rates between embryos vitrified immediately after collection (15 of 20, 75%) and embryos cooled for 12–19 h prior to vitrification (13 of 20, 65%). This provides the flexibility of shipping an embryo from a farm or veterinary clinic to a centralised facility that has the expertise for vitrification. Alternatively, the embryos can be collected and vitrified immediately.

In summary, pregnancy rates from transfer of vitrified equine embryos have been quite acceptable (50–70%). These rates are similar to those obtained from transfer of fresh embryos. Furthermore, the viability of embryos obtained from superovulated mares is similar to that from single-ovulating, nonsuperovulated mares.

## REFERENCES

- Eldridge-Panuska, W.D., Caracciolo di Brienza, V., Seidel, G.E. Jr., Squires, E.L. and Carnevale, E.M. (2005) Establishment of pregnancies after serial dilution or direct transfer by vitrified equine embryos. *Theriogenology* **63**, 1308-1319.
- Hudson, J., McCue, P.M., Carnevale, E.M., Welch S. and Squires, E.L. (2005) The effects of cooling and vitrification of embryos from eFSH-treated mares on pregnancy rates after nonsurgical transfer. *J. Equine vet. Sci.* (In press)
- Lascombes, F.A. and Pashen, R.L. (2000) Results from embryo freezing and post-ovulation Cryopreservation of equine embryos using glycerol and 1,2 propanediol as cryoprotectants. *Equine vet. J.* **15**, 64-66.
- Maclellan, L.J., Carnevale, E.M., Coutinho da Silva, M.A., McCue, P.M., Seidel, G.E. Jr. and Squires, E.L. (2002) Cryopreservation of small and large equine embryos pre-treated with cytochalasin-B and/or trypsin. *Theriogenology* **58**, 717-720.
- Slade, N.P., Takeda, T., Squires, E.L., Elsdon, R.P. and Seidel, G.E. Jr. (1985) A new procedure for the cryopreservation of equine embryos. *Theriogenology* **24**, 45-58.
- Squires, E.L., Seidel, G.E. Jr. and McKinnon, A.O. (1989) Transfer of cryopreserved equine embryos to progestin-treated ovariectomized mares. *Equine vet. J.* **8**, 89-91.

# EXPRESSION AND LOCALISATION OF BLASTOCOEL FORMING AND MAINTAINING PROTEINS IN EQUINE BLASTOCYSTS

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Mammalian blastocyst formation requires establishment of the trans-trophectodermal ion gradient established by the action of sodium potassium ATPases. Prerequisite is formation of trophoblast epithelium sealed by tight junctions and connected by e-cadherin. Recently, we showed that Aquaporins (AQPs) (Budik *et al.* 2004) are present in equine blastocyst mediating fluid movement across the cell membranes. The expression pattern of aquaporins during early equine embryonic development could influence blastocyst expansion and freezing abilities since the ability to facilitate water movements through the trophoctoderm is an important factor for both processes.

So far it has been shown that  $\alpha 1/\beta 1$  ATPase isoenzyme is present in equine blastocysts, but nothing is known about expression of aquaporins in equine embryos.

In the present study, equine cumulus oocyte complexes (n=5, pooled) and different stages of pre-implantative equine embryos between Day 8 and 14 of development (n=4; Day 8, 10, 12 and 14) were investigated for abundance of sodium potassium and aquaporin transcripts by means of RT-PCR. After positive detection by RT-PCR, immunohistochemistry was performed on embryonic sections using specific antibodies. The same antibodies were tested in western blotting using protein preparations of the corresponding embryos.

It was shown that transcripts for  $\alpha 1/\beta 1$  subunits of sodium potassium ATPase were present in all embryos examined. Immunohistochemistry of  $\alpha 1$ - subunit showed a basolateral distribution of immunoreactive protein in the trophoctodermal layer and a strong, diffuse abundance in the endodermal cell layer.

It could be demonstrated that equine AQP 3,

known to be involved in transport of either water or glycerol, is expressed in cumulus oocytes complexes and all embryonic stages investigated, respectively. Immunohistochemistry of AQP 3 showed a diffuse cytoplasmatic abundance in Day 8 equine embryonic sections changing into a more basolateral pattern on Day 12. Expression of AQP 5, responsible for transport of water, but not of glycerol, seems to coincide with the expansion of the blastocyst, detected not earlier than Day 8 of embryonic development. Immunohistochemistry demonstrated abundance of aquaporin 5 on the apical side of the trophoctoderm only.

Our results confirmed the presence of  $\alpha 1/\beta 1$  sodium potassium ATPase transcripts and demonstrated the existence of aquaporin 3 and 5 gene products for the first time in equine blastocysts. A positive correlation of AQP 5 expression with embryo size and abundance of the  $\alpha 1$  subunit of sodium potassium ATPase could be demonstrated by real time PCR.

Changes of water permeability in the apical membrane of the trophoctodermal cells might be mediated by different amounts of AQP5 present in this membrane. Apparently the speed of blastocyst expansion is regulated by modulation of water flow through the apical membrane of the trophoctoderm. Presence of a target sequence for protein kinase A (PKA) in the deduced amino acid sequence of the amplified equine AQP5 cDNA sequence indicate a regulation via cAMP may be caused by hormonal stimulus similar to the regulation of AQP2 in kidney collecting ducts.

## REFERENCES

- Budik, S., Walter, I., Aurich, C. and Pittner, F. (2004) Aquaporin expression in equinen embryonen. Jahrestagung Physiologie und Pathologie der Fortpflanzung 37, 19-20.

# EFFECT OF PROTEIN KINASE C ACTIVATION FOLLOWED BY KINASE INHIBITION ON EMBRYONIC DEVELOPMENT OF *IN VIVO*-DERIVED EQUINE OOCYTES AFTER ICSI

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## INTRODUCTION

During fertilisation in mammals, the penetration of a spermatozoon into ooplasm triggers repetitive rises in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ). This increase activates protein kinase C (PKC), a downstream regulator of egg activation events. Another calcium-independent intracellular signal via diacylglycerol (DAG) also activates PKC. PKC activity increases dramatically as a result of sperm penetration as it does when parthenogenetic agents are employed to activate eggs (Gallicano *et al.* 1997). Inactivation of MAP kinase by PKC was suggested to be required for pronucleus formation in mice, rats, and pigs (Sun *et al.* 2001; Lu *et al.* 2002; Quan *et al.* 2003). With rat IVF, the degree of MAP kinase inactivation by phorbol 12 myristate, 13-acetate (PMA), a PKC activator, was consistent with the proportion of oocytes with pronuclei (Lu *et al.* 2002). PKC activation after intracytoplasmic sperm injection (ICSI) of bovine oocytes improved embryonic development (Suh and Seidel 2005). However, no similar study has been reported in horses.

This study evaluated the effect of the PKC activator, PMA, and subsequent treatment with the protein serine/threonine kinase inhibitor, 6-dimethylaminopurine (6-DMAP), on embryonic development of *in vivo*-derived equine oocytes after ICSI.

## MATERIALS AND METHODS

### *Oocyte recovery and maturation*

For recovery of *in vivo* oocytes, 2 groups of follicles (10 to 20 and >20 mm) were aspirated

using a 12-gauge, double-lumen needle under ultrasound guidance during March to July. Follicular oocytes of 14 mares were aspirated 10 times at 10 day intervals, without use of hCG. Each follicle was aspirated repeatedly using 20–30 ml of PBS containing heparin to detach the oocytes from the follicle wall. Recovered oocytes were rinsed 3 times in PBS, and then matured in 1 ml of EMM1, a defined medium similar to SOF, in a 4-well dish for  $30 \pm 1$  h under 5%  $CO_2$  in air at 38.5°C. EMM1 contained 10% FCS and hormones (15 ng/ml FSH, 1  $\mu$ g/ml LH, 1  $\mu$ g/ml E2, 500 ng/ml progesterone, 100 ng/ml EGF, and 10 ng/ml IGF-1).

### ICSI

Oocytes were denuded of cumulus cells by repeated pipetting in GMOPS (Vitrolife, Sweden) with 200 iu/ml of hyaluronidase. Oocytes with a 1st polar body were selected and kept in EMMI until ICSI. For motile sperm recovery, a piece of a cut straw of frozen stallion semen (approximately 50  $\mu$ l) was thawed and transferred into the bottom of a 15 ml conical tube containing 2 ml of F-CDM, washed at 300 g for 5 min, and supernatant was removed leaving approximately 50  $\mu$ l of sperm pellet. A Piezo driven injection system was used for sperm injection. Immediately before injection, 0.5  $\mu$ l of sperm suspension was placed in 5  $\mu$ l GMOPS containing 5% (w/v) polyvinylpyrrolidone (PVP, Sigma) under oil. Injection of sperm was carried out in a 50  $\mu$ l drop of GMOPS+0.5% FAF-BSA. Each sperm was immobilised by applying a few pulses to score the tail with the Piezo drill (PMM Inc, Japan). The scored sperm was washed once before injection. All manipulations were performed at 30°C.

**TABLE 1: Cleavage and blastocyst development of *in vivo*-derived equine oocytes after ICSI**

Follicle size (mm)/treatment	No. ICSI/IVC	No. Cleaved (%)	No. blastocyst (%)
10 – 20 / Control	50	20 (40.0)	7 (14.0)
≥20 /Control	12	5 (41.7)	1 (8.3)
Average Control	62	25 (40.3) <sup>a</sup>	8 (12.9)
10 – 20 / PMA	50	31 (62.0)	9 (18.0)
≥20 / PMA	12	10 (83.3)	5 (41.7)
Average PMA	62	41 (66.1) <sup>b</sup>	14 (22.6)

<sup>a,b</sup> Averaged means differ ( $P < 0.01$ )

### *In vitro* culture and evaluation of embryo development

ICSI zygotes were cultured 9 days in DMEM/F12+10% FCS, either directly after ICSI, or after activation in G1.3 (Vitrolife, Sweden) containing 0.02  $\mu$ M PMA for 2 h, and then 2.0 mM 6-DMAP for 3 h. *In vitro* culture was in 50  $\mu$ l drops of medium covered with oil (up to 10 embryos) at 38.5°C under 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> and medium was replaced with fresh medium every 3 days. Cleavage rates and number of cells were recorded at 24, 48 and 72 h. Morula/blastocyst rates were recorded at Days 7, 8 and 9. Embryos were stained with 5  $\mu$ g/ml of Hoechst 33342 for 20 min at the end of culture and classified as blastocysts if they contain more than 64 cells (Hinrichs *et al.* 2005). Data were analysed by Chi-square.

## RESULTS

A total of 161 and 30 oocytes were recovered from 10–20, and >20 mm follicles, respectively. Average maturation rates to MII in respective groups were 71.0 and 80.0%, respectively ( $P > 0.05$ ). Average cleavage rates in 10–20 and >20 mm follicle groups were 51.0 and 62.5%, respectively ( $P > 0.05$ ) and those of control and activated groups averaged over follicle sizes were 40.3 and 66.1%, respectively ( $P < 0.01$ , Table 1). Blastocyst rates per oocyte averaged 16.0 and 25.0% for small and large follicles, and 12.9 and 22.6% for control and PMA, respectively ( $P > 0.05$ ).

## DISCUSSION

Degeneration rates of immature *in vivo* oocytes recovered from small (10–20 mm) or large

follicles (>20 mm) did not differ after IVM, 10.7 and 0%, respectively ( $P > 0.05$ ). This corresponds to similar cleavage and blastocyst rates between oocytes from these 2 follicle groups, implying equivalent developmental competence *in vitro*.

PKC is involved in many egg activation events, such as cortical granule exocytosis (Bement 1992), second polar body initiation (Gallicano *et al.* 1993; Moses and Kline 1995), restructuring internal cytoskeletal components, and pronuclear formation (Bement 1992; Gallicano *et al.* 1993; Moses and Kline 1995). In this study, activation of PKC with PMA and subsequent inhibition of protein phosphorylation with 6-DMAP in equine ICSI zygotes improved the cleavage rate. Other activation protocols employing ionomycin, ethanol, and thimerosal also improved fertilisation of equine oocytes, with no further evaluation of embryo development to blastocysts *in vitro* (Li *et al.* 2000). Injection of a single sperm may not induce consistent [Ca<sup>2+</sup>]<sub>i</sub> oscillations in horse oocytes (Bedford *et al.* 2003), but the sperm also may play a role in fertilisation in an unknown way. In this respect, it may be more logical to activate PKC of sperm-injected oocytes and subsequently inactivate protein phosphorylation. In a previous study with bovine ICSI oocytes, inactivating MAP kinase after PKC stimulation with PMA markedly improved cleavage, although PMA alone was not effective (Suh and Seidel 2005).

In conclusion, activation of protein kinase C and subsequent inactivation of protein phosphorylation clearly improved cleavage rates of *in vivo*-derived equine oocytes after ICSI.

## ACKNOWLEDGEMENTS

This project was sponsored by benefactors of the Preservation of Equine Genetics Program (PEG).

## REFERENCES

- Bedford, S.J., Kurokawa, M., Hinrichs, K. and Fissore, R.A. (2003) Intracellular calcium oscillations and activation in horse oocytes injected with stallion sperm extracts or spermatozoa. *Reproduction* **126**, 489-499.
- Bement, W.M. (1992) Signal transduction by calcium and protein kinase C during egg activation. *J. Exp. Zoology* **263**, 382-397.
- Gallicano, G.I., Schwarz, S.M., McGaughey, R.W. and Capco, D.G. (1993) Protein kinase C, a pivotal regulator of hamster egg activation, functions after elevation of intracellular free calcium. *Dev. Biol.* **156**, 94-106.
- Gallicano, G.I., McGaughey, R.W. and Capco, D.G. (1997) Activation of protein kinase C after fertilisation is required for remodeling the mouse oocyte into the zygote. *Mol. Reprod. Dev.* **46**, 587-601.
- Hinrichs, K., Choi, Y.H., Love, L.B., Varner, D.D., Love, C.C. and Walckenaer, B.E. (2005) Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. *Biol. Reprod.* **72**, 1142-1150.
- Li, X., Morris, L.H. and Allen, W.R. (2000) Effects of different activation treatments on fertilisation of horse oocytes by intracytoplasmic sperm injection. *J. Reprod. Fert.* **119**, 253-260.
- Lu, Q., Smith, G.D., Chen, D.Y., Han, Z.M. and Sun, Q.Y. (2002) Activation of protein kinase C induces mitogen-activated protein kinase dephosphorylation and pronucleus formation in rat oocytes. *Biol. Reprod.* **67**, 64-69.
- Moses, R.M. and Kline, D. (1995) Calcium-independent, meiotic spindle dependent metaphase-to-interphase transition in phorbol ester treated mouse eggs. *Dev. Biol.* **171**, 111-122.
- Quan, H.M., Fan, H.Y., Meng, X.Q., Huo, L.J., Chen, D.Y., Schatten, H., Yang, P.M. and Sun, Q.Y. (2003) Effects of PKC activation on the meiotic maturation, fertilisation and early embryonic development of mouse oocytes. *Zygote* **11**, 329-337.
- Sun, Q.Y., Lai, L., Park, K.W., K\_hholzer, B., Prather, R.S. and Schatten, H. (2001) Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilisation *in vitro*. *Biol. Reprod.* **64**, 879-889.
- Suh, T.K. and Seidel Jr., G.E. (2005) Activation of protein kinase C and subsequent inhibition of protein phosphorylation improve embryonic development of bovine oocytes after ICSI. *Biol. Reprod.* **73**, 156-157.

# BLASTOCYSTS, PREGNANCIES AND FOALS FROM ICSI AND NUCLEAR TRANSFER

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Major advances have been made in assisted reproduction in the horse in the last 5 years. Procedures for intracytoplasmic sperm injection (ICSI), nuclear transfer (NT) and embryo culture have allowed production of both fertilised and cloned equine blastocysts *in vitro* (Lazzari *et al.* 2002; Choi *et al.* 2002b; Galli *et al.* 2003; Choi *et al.* 2004). However, few reports are available on pregnancies and foals resulting from *in vitro*-produced equine embryos. Li *et al.* (2001, 2003) reported the birth of 2 live foals produced by ICSI, and Galli and coworkers (C. Galli, personal communication, 2004) have also produced 2 live ICSI foals. Galli and co-workers (2004; Lagutina *et al.* 2005) also produced 2 foals after transcervical transfer of blastocysts produced by NT and *in vitro* culture. To our knowledge, these represent the only foals resulting from *in vitro*-produced (*in vitro* maturation/ *in vitro* fertilisation or NT/ *in vitro* culture to the blastocyst stage) blastocysts. We report here pregnancies and foals resulting from transfer of *in vitro*-produced blastocysts in our laboratory.

Horse oocytes were recovered from slaughterhouse-derived ovaries by follicular scraping, and were matured *in vitro* in M199 with 10% FBS and 5 mU/ml FSH for 24–30 h in a humidified atmosphere of 5% CO<sub>2</sub> at 38.2°C. For ICSI embryos, oocytes in metaphase II were subjected to ICSI with frozen-thawed sperm, without additional activation stimulus, as described by Choi *et al.* (2002b). Injected oocytes were cultured in DMEM/F-12 medium with 10% fetal bovine serum, with or without oviductal cell co-culture, for 6.5–7 days before transcervical transfer to the uteri of recipient mares. A portion of the embryos were transported in 1 ml capped vials in this medium at 35–38°C for 4–6 h before transfer. For NT embryos, oocytes in Metaphase II

were subjected to NT as described by Choi *et al.* (2002a). This was performed by enucleation of the oocytes using a Piezo drill, followed by direct injection into the cytoplasm of cultured fibroblasts synchronised in G1 by growth to confluence or treatment with roscovitine, 15 µg/ml for 24 h. Recombined oocytes were activated by injection of sperm extract, with or without further treatment with ionomycin, and were then treated for 4 h with 2 mM 6-dimethylaminopurine, with or without addition of 5 µg/ml cytochalasin B. Recombined, activated oocytes were then cultured in DMEM/F-12 medium with 10% fetal bovine serum under 5%CO<sub>2</sub>, 5%O<sub>2</sub>, and 90% N<sub>2</sub> for 7–8 days, at which time development to blastocyst was assessed. Blastocysts were transferred transcervically to recipient mares according to mare availability.

Blastocyst development rates for ICSI embryos in the above systems ranged from 15–38%. Transfer of 10 ICSI blastocysts cultured under these conditions resulted in 5 pregnancies, of which 3 were carried to term with live foals. Two of these foals were included in a previous report by Hinrichs *et al.* (2005). The remaining 2 pregnancies developed trophoblast only and were lost before 35 days. In 2004, NT blastocyst development rates ranged from 0–10%. Eleven NT blastocysts were transferred, resulting in 4 pregnancies (36% pregnancy rate), of which 2 were carried to term with live foals. The first foal was carried for 389 days and was born after a normal parturition with minimal assistance. The foal was small at birth, weighing 27 kg, and was slow to stand and nurse. After the second day post partum, all parameters were normal and the foal has developed normally. The second foal was born without assistance at 340 days and weighed 45 kg at birth. This foal stood and nursed within normal

time limits, and has developed normally. The remaining 2 NT pregnancies were lost at 4 months and 9 months gestation. In 2005, NT blastocyst development rates ranged from 1–13%. Transfer of 26 NT embryos of 3 different genotypes resulted in 16 pregnancies (62% pregnancy rate), of which 2 were lost before 30 days, and one was lost at 110 days in association with neurological disease necessitating euthanasia of the mare. The remaining 13 pregnancies are ongoing at the time of writing (from 2.5–6 months gestation).

These data show that *in vitro* embryo production in the horse can result in efficient blastocyst development; however, blastocyst development after ICSI is higher than that seen after NT (~30% vs. ~6%). Embryos produced from *in vitro*-matured oocytes and cultured to the blastocyst stage *in vitro* are capable of establishing viable pregnancies after transcervical embryo transfer. Strangely, trophoblast-only pregnancies appeared to be more common in ICSI embryos than in NT embryos. This may be attributable, in part, to the fact that the work on ICSI embryo transfer described here was undertaken prior to that on NT embryo transfer, and changes in culture environment (gas atmosphere, drop size, day of media change) may have positively influenced embryo health in later studies. Further evaluations of the pregnancy rate and incidence of trophoblast-only pregnancy, after transcervical transfer of *in vitro* cultured ICSI blastocysts, are currently being performed.

This work was supported by the Link Equine Research Endowment Fund, Texas A&M University and the Smart Little Lena Co-Managers.

## REFERENCES

Choi, Y.H., Love, C.C., Chung, Y.G., Varner, D.D., Westhusin, M.E., Burghardt, R.C. and Hinrichs, K. (2002a) 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.

Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S. and Hinrichs, K. (2002b) Developmental competence *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilised by intracytoplasmic sperm injection with fresh or frozen-thawed sperm. *Reproduction* **123**, 455-465

Choi, Y.H., Roasa, L.M., Love, C.C., Varner, D.D., Brinsko, S.P. and Hinrichs, K. (2004) Blastocyst formation rates *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilised by intracytoplasmic sperm injection. *Biol. Reprod.* **70**, 1231-1238.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G. (2003) A cloned horse born to its dam twin. *Nature* **424** 635.

Hinrichs, K., Choi, Y.H., Love, L.B. and Varner, D.D. (2005) Transfer of *in vitro*-produced equine embryos. 6th International Symposium on Equine Embryo Transfer. *Havemeyer Foundation Monograph Series No 14* Eds: M. Alvareng and J.F. Wade, R&W Communications, pp 38-39.

Lagutina, I., Lazzari, G., Duchi, R., Colleoni, S., Ponderato, N., Turini, P., Crotti, G. and Galli, C. (2005) Somatic cell nuclear transfer in horses: effect of oocyte morphology, embryo reconstruction method and donor cell type *Reproduction* **130**, 559-567.

Lazzari, G., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Barbacini, S. and Galli, C. (2002) Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of *in vitro* matured oocytes with frozen-thawed spermatozoa from semen of different fertilities *Theriogenology* **58**, 709-712.

Li, X., Morris, L.H.A. and Allen, W.R. (2001) Influence of co-culture during maturation on the developmental potential of equine oocytes fertilised by intracytoplasmic sperm injection (ICSI). *Reproduction* **121**, 925-932.

Li, X., Morris, L.H.A., Wilsher, S. and Allen, W.R. (2003) Fetal development after intracytoplasmic sperm injection (ICSI) in the horse. Workshop on 'From Epididymis to Embryo'. *Havemeyer Foundation Monograph Series No 6* Eds: L.H.A. Morris and J.F. Wade, R&W Publications (Newmarket) Ltd, pp 75-76.

# PRODUCTION OF EMBRYONIC STEM CELL LINES

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The cells of pre-implantation mammalian embryos undergo their first differentiation at the blastocyst stage of development to produce 2 distinct cell types: i) trophoblast layer that will eventually become the trophoblast layer of the definitive placenta; and ii) inner cell mass (ICM) cells that constitute the putative embryo proper. Lines of pluripotent embryonic stem (ES) cells can be established by mechanically or immunosurgically separating the ICM cleanly from the trophoblast and repeatedly culturing and sub-culturing the former for 20 or more passages without loss of the basic undifferentiated phenotype of the cells and with retention of their developmental potential to form derivatives of all 3 embryonic germ layers, ectoderm, endoderm and mesoderm.

We have created 3 lines of ES cells by isolating ICM cells from Day 8 horse blastocysts and culturing them for 15–28 passages on a feeder layer of horse (2 lines) or mouse (1 line) embryonic fibroblasts, in a medium supplemented with human derived leukaemia inhibitory factor

(LIF) to suppress differentiation. The cells of each line grew as small spherical clumps (embryoid bodies) and they continued to express on their surface an internationally recognised panel of ES markers that included alkaline phosphatase, SSEA-1, TRA-1-60, TRA-1-81 and the key embryonic gene, OCT-4 and which, together, confirmed the pluripotency and undifferentiated ES lineage of the cells. However, none of the lines formed teratomas when they were injected into the testes of immunologically incompetent NUDE mice, thereby illustrating a fundamental difference between human ES cells and those of most animal species.

Experiments are in progress to determine the genetic stability and the differentiation potentials and requirements of the ES cells, and their levels of expression of inherited major histocompatibility complex (MHC) antigens, before and after *in vitro* or *in vivo* transformation into potentially therapeutic end stage cells like chondrocytes, tenocytes, osteocytes and epidermal cells.



# SESSION 4:

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## General

**Chairman:**  
**W. R. Allen**



# APOPTOSIS IN EQUINE GRANULOSA CELLS ASPIRATED AT TWO DIFFERENT SIZES OF THE DOMINANT FOLLICLE

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## INTRODUCTION

Investigation of histological atresia at different stages of the equine cycle showed that there were most atretic follicles on Day 6, primarily growing follicles on Day 14 and, between Day 14 and Day 17, there was a significant increase in number of follicles undergoing early atresia. Just before ovulation all follicles larger than 10 mm apart from the pre-ovulatory follicle were atretic (Driancourt *et al.* 1982). In a study using mares ovariectomised on Days 6, 11, 15 and during oestrus, surprisingly there were fewer atretic follicles as the cycle progressed (Pedersen *et al.* 2000). The proportion of follicles with apoptotic granulosa cells decreased during the cycle with 65.5%, 58.1%, 40.4% and 34.9% on Days 6, 11, 15 and during oestrus, respectively. In this study we had expected to find that more follicles became atretic as the cycle progressed as a result of selection of the dominant follicle taking place. The high percentage of apoptosis on Day 6 suggested that, at this point, there was a mixed population of growing as well as degenerating follicles, presumably from the previous cycle (Pedersen *et al.* 2000). Tracking growing follicles and detecting the point at which the subordinate follicles reach a plateau in growth and start to become atretic is difficult when regressing follicles from a previous wave are present. Removal of growing and regressing follicles would result in a pool of growing follicles only, thus making it easier to identify the point at which deviation and selection of the dominant follicle takes place. Gastal *et al.* (1997) found that deviation in growth rates took place when the largest follicle reached 23 mm in mares. A further understanding of how selection and dominance happen in the mare could be obtained by knowing when subordinate follicles

are selected against. The aim of the present study was to establish whether there is a specific point during follicle development when atresia of the subordinate follicles is initiated.

## MATERIALS AND METHODS

In sedated Standardbred mares ( $n=5$ ) follicles  $\geq 10$  mm were removed by transvaginal ultrasound-guided aspiration. Aspirated follicles were monitored to establish whether they re-filled and continued to grow, or luteinised. If the aspirated follicles re-filled and kept growing, they were re-aspirated. Subsequent to follicle aspiration, the growth of follicles larger than 1 mm was monitored daily by ultrasonography until the largest follicle reached 21–25 mm (Experiment 21–25 mm) or 31–35 mm (Experiment 31–35 mm). All follicles  $\geq 10$  mm were then aspirated transvaginally. Each follicle was flushed 10 times with embryo flush medium in order to retrieve the granulosa cells. The recovered granulosa cells were kept at 35°C for a maximum of 1 h after retrieval. The granulosa cells were centrifuged at 1,000 rpm for 6 min at room temperature and then fixed in 4% paraformaldehyde in PBS. Granulosa cells were stained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) after cytospinning. Cell nucleus morphology was evaluated in order to detect morphological apoptosis (Wyllie *et al.* 1980; Kerr *et al.* 1994; Nakahara *et al.* 1997). Apoptotic cells were identified as cells showing condensed chromatin masses and/or fragmented nuclei. Small groups of apoptotic bodies were counted as remnants of one apoptotic cell. One hundred GCs per follicle were counted randomly at a magnification of X40 and scored as healthy or apoptotic. The experiment

was conducted 3 times in each mare for each of the 2 groups.

## RESULTS

A total of 52 (Experiment 21–25 mm) and 49 (Experiment 31–35 mm) follicles were examined. In Experiment 21–25 mm, mean percent apoptotic granulosa cells in follicles was 27%, 7.7%, 5.4% and 6.9% in the 10 mm, 11–15 mm, 16–20 mm, 21–25 mm groups, respectively (Fig 1). In Experiment 31–35 mm, mean percent apoptotic granulosa cells was 21.3%, 22.9%, 49.8%, 43%, 25% and 13.2% in the 10 mm, 11–15 mm, 16–20 mm, 21–25 mm, 26–30 mm and 31–35 groups, respectively.

## DISCUSSION

Preliminary results of this study indicate that a suppression of follicles smaller than 31 mm, was ongoing once the largest follicle had reached 31–35 mm. Follicles of 16–20 mm had the highest frequency of apoptotic granulosa cells, and follicles larger than 20 mm appeared less sensitive to this suppression the larger they were. According to Gastal *et al.* (1997) the destiny of the dominant and the subordinate follicles is established within one day and with a size difference of only 4 mm (23 versus 19 mm). Apparently the future dominant follicles start suppressing the

subordinate follicles soon after, as the subordinate follicle maintain adequate viability for one day after deviation, so that it can convert to dominant status if the dominant follicle fails or is ablated (Gastal *et al.* 1997). This is in agreement with the present study showing increased frequency of apoptosis in subordinate follicles once the largest follicle has reached 31–35 mm. The time to grow from 21–25 mm to 31–35 mm was 2–4 days in the present study.

Similar to our findings, Bezard *et al.* (1997) reported that when a follicle >28 mm was present, the percentage of atretic follicles was positively correlated with follicle size, apart from the pre-ovulatory follicle: 17%, 41%, 72% and 12% of follicles <10 mm, 10–19 mm, 20–27 mm and >28 mm, respectively, were classified as atretic after using Feulgen staining of aspirated granulosa cells.

Apoptotic granulosa cells in the dominant follicles appear to be a normal phenomenon. In pre-ovulatory ovine follicles, as ovulation approached, there was a progressive increase in apoptotic cells in the area of the granulosa cells where the follicle was expected to rupture (Murdoch 1995). Avian granulosa cells from slow growing, small (atresia-prone) follicles were found to undergo rapid apoptosis after incubation for 6–24 h, whereas cells from the largest pre-ovulatory follicle showed less apoptosis (Johnson *et al.* 1996). The reason for pre-ovulatory follicles not undergoing apoptosis to the same extent as

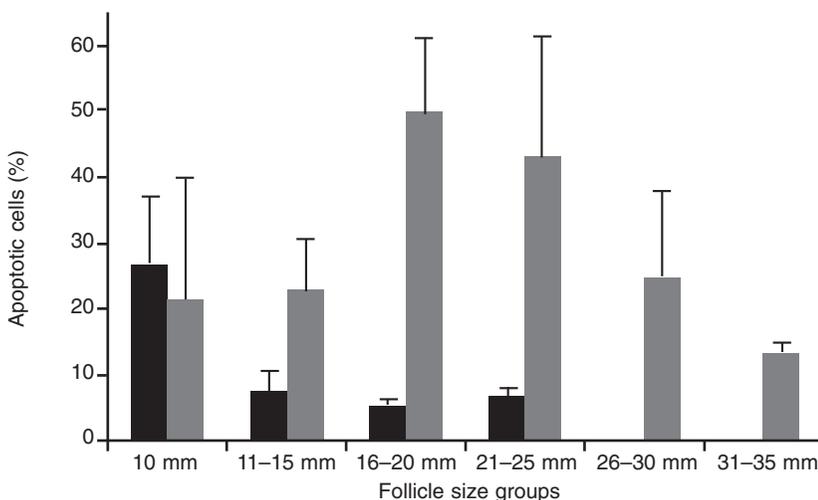


Fig 1: Mean percent apoptotic granulosa cells in different follicle size groups in Experiment 21–25 mm (black) and Experiment 31–35 mm (grey).

smaller follicles may be that IGF-I and oestradiol promote granulosa cell survival by increasing resistance to apoptosis (Quirk *et al.* 2004) and that expression of death-suppressing genes are capable of making cells resistant to apoptosis (Johnson *et al.* 1996).

A nuclear stain only was used in the present study, as TUNEL stain applied concomitantly, only in a few cases stained cells that were not already morphologically apoptotic. Collins *et al.* (1997) showed that nuclear morphological changes occurred before DNA fragmentation could be detected. When aspirating granulosa cells from ovaries *in vivo*, blood contamination may cause cells other than granulosa cells to be present. Neutrophils and monocytes are easily distinguished from granulosa cells, but lymphocytes not easily so.

In conclusion, atresia of the subordinate follicles is initiated when the future dominant follicle grows beyond 21–25 mm.

## ACKNOWLEDGEMENTS

The research project was funded by the Danish Research Agency, project no. 23-02-0133.

## REFERENCES

Bezard, J., Mekarska, A., Goudet, G., Duchamp, G. and Palmer, E. (1997) Meiotic stage of the preovulatory equine oocyte at collection and competence of immature oocytes for *in vitro* maturation: effect of interval from induction of ovulation to follicle puncture. *Theriogenology* **47**, 386.

Collins, J.A., Schandl, C.A., Young, K.K., Vesely, J. and Willingham, M.C. (1997) Major DNA fragmentation is a late event in apoptosis. *J. Histochem. Cytochem.* **45**, 923-934.

Driancourt, M.A., Mariana, J.C. and Palmer, E. (1982) Effect of the stage of the estrous-cycle on the follicular population in pony mares. *Reprod. Nutr. Dev.* **22**, 803-812.

Gastal, E.L., Gastal, M.O., Bergfelt, D.R. and Ginther, O.J. (1997) Role of diameter differences among follicles in selection of a future dominant follicle in mares. *Biol. Reprod.* **57**, 1320-1327.

Johnson, A.L., Bridgham, J.T., Witty, J.P. and Tilly, J.L. (1996) Susceptibility of avian ovarian granulosa-cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene-expression. *Endocrinology* **137**, 2059-2066.

Kerr, J.F.R., Winterford, C.M. and Harmon, B.V. (1994) Morphological criteria for identifying apoptosis. In *Cell biology: a laboratory handbook*. pp 319-329 (Academic Press, Inc)

Murdoch, W.J. (1995) Programmed cell death in preovulatory ovine follicles. *Biol.Reprod.* **53**, 8-12.

Nakahara, K., Saito, H., Saito, T., Ito, M., Ohta, N., Sakai, N., Tezuka, N., Hiroi, M. and Watanabe, H. (1997) Incidence of apoptotic bodies in membrana granulosa of the patients participating in an *in vitro* fertilisation program. *Fertil. Steril.* **67**, 302-308.

Pedersen, H.G., Telfer, E. and Watson, E.D. (2000) Follicular granulosa cell apoptosis through the equine oestrous cycle. 14th International Congress on Animal Reproduction, Stockholm: 1, 61

Quirk, S.M., Cowan, R.G., Harman, R.M., Hu, C.L. and Porter, D.A. (2004) Ovarian follicular growth and atresia: the relationship between cell proliferation and survival. *J. Anim. Sci.* **82**, 40-52.

Wyllie, A.H., Kerr, J.F. and Currie, A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**, 251-306.

# OVULATION INDUCTION FOR EMBRYO TRANSFER: hCG VERSUS GnRH ANALOGUE

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## INTRODUCTION

During an individual mare's physiological breeding season, she may vary in length of oestrus, interval from onset of oestrus to ovulation and size of the follicle(s) at ovulation. Modern breeding technologies, such as embryo transfer and artificial insemination (AI) with fresh, chilled and frozen semen, require precise prediction of the time of ovulation. The use of ovulation inducing agents can improve breeding efficiency by reducing the duration of oestrus, increasing the number of ovulations occurring within 48 h after administration, decreasing the number of inseminations or matings per oestrus and synchronising ovulation and insemination. A wide range of products is available to hasten ovulation in cycling mares.

## MATERIALS AND METHODS

Data on embryo recovery rates were collected during 3 physiological breeding seasons (March–September, 2003–2005) from 63 Thoroughbred and 7 Pony experimental mares aged 2–20 ( $\bar{x}$  = 8.9) years. The mares were treated once during a total of 443 oestrous cycles with one of 4 ovulation inducing agents when they exhibited a dominant ovarian follicle of >35 mm diameter in association with adequate endometrial oedema. Semen was collected by artificial vagina from 2 identical twin Pony stallions of known high fertility. The mares were inseminated once with  $500 \times 10^6$  progressively motile spermatozoa in a skim milk-glucose extender. The following ovulation inducing agents were administered coincidentally with the insemination: i) crude equine pituitary extract (CPE, INRA, Nouzilly, France, 20 mg iv); ii) human Chorionic

Gonadotrophin (hCG; Chorulon, Intervet, Milton Keynes, Buckinghamshire, 3,000 iu iv); iii) gonadotrophin-releasing hormone (GnRH) analogue, deslorelin acetate (Ovuplant, Peptech Animal Health, Christchurch, New Zealand, 2.1 mg sc in vulval lips); and iv) GnRH analogue, BioRelease Deslorelin (BET Pharm, Lexington, Kentucky, 0.75 or 1.5 mg, im). Ovulation was diagnosed by daily ultrasonography combined with daily blood sampling for serum progesterone measurements. On Day 7 after ovulation, the uterus of each mare was flushed twice with 1–2 l of flushing medium (Emcare; ICP Ltd, Auckland, New Zealand) and recovered embryos were evaluated microscopically and graded on the basis of their morphology.

## RESULTS

Grouping all 4 drugs together, ovulation occurred within 24 h after treatment in 17.5% of cases, 24–48 h in 80.4% and >48h in 2.1% of cases. Embryo recovery rates were 15 embryos from 40 attempts (37.5%) in mares treated with CPE; 26 from 49 flushes (53%) in mares treated with Ovuplant; 44 from 80 flushes (55%) in mares treated with Chorulon; 63 from 92 flushes (68.5%) in mares treated with 0.75 mg BioRelease Deslorelin; and 126 from 182 flushes (69%) in mares treated with 1.5 mg BioRelease Deslorelin (Table 1).

For experimental purposes, when being used as embryo donors or recipients, 47 mares were treated with 1.5 mg of BioRelease Deslorelin in 2004/2005 in 1–7 consecutive oestrous cycles for a total of 249 cycles; and 55 mares were treated with 0.75 mg BioRelease Deslorelin in 2005 during 1–7 consecutive cycles for a total of 139 cycles.

**TABLE 1: Total embryo recovery**

	Flushes	Embryos	% embryo recovery
CPE	40	15	37.5
Ovuplant	49	26	53
hCG	80	44	55
0.75 mg BioRelease Deslorelin	92	63	68.5
1.5 mg BioRelease Deslorelin	182	126	69.2

## DISCUSSION

Ovulation inducing agents are necessary for modern breeding programmes. Unfortunately, the drug most often used, human Chorionic Gonadotrophin (hCG; Chorulon), can stimulate the formation of anti-hCG antibodies after repeated injections (Duchamp *et al.* 1987). The half-life of such antibodies can range from 30 days to several months after 2–4 injections (Roser *et al.* 1979) and their cross-reaction with endogenous equine LH can reduce the efficiency of treatment and significantly delay or even completely suppress ovulation, especially in older mares. McCue (2004) recommended that hCG should be used once or twice only during a breeding season and that an alternative gonadotrophin, like CPE, should be used instead; it induced ovulation in 78% of mares previously immunised against hCG. Johnson *et al.* (2002) investigated the effects of multiple subcutaneous implants of deslorelin acetate (Ovuplant) in cycling mares and noted a prolongation of the interovulatory interval and suppression of follicular size. A single implant caused a consistent suppression of serum LH and FSH concentrations for 10–14 days, indicating a degree of ‘down-regulation’ of pituitary response to endogenous GnRH, occasionally leading to a complete suppression of ovarian activity for some weeks.

Data from the present study show clearly that the lower dose of BioRelease Deslorelin (0.75 mg) is adequate to consistently induce ovulation in Thoroughbred-size mares (500–580 kg). Fleury *et al.* (2004) reduced the dose of BioRelease Deslorelin even further to 0.5 mg and noted that mares treated with this amount, when they

exhibited follicle diameters of only 30–35 mm, took about 5 h longer to ovulate than mares given same dose of drug when the dominant follicle had reached  $\geq 35$  mm in diameter. It was surprising to observe a definite reduction in the diameter of the dominant follicle 24 h after administration of BioRelease Deslorelin in the present study but this did not prevent normal ovulation within the 24–48 h interval. Overall, in the mares treated with 0.75 mg BioRelease Deslorelin, ovulation occurred within 24 h of treatment in 27.3%, 24–48 h in 71.9% and >48 h in 0.75% of cases. And in the mares treated with 1.5 mg BioRelease Deslorelin, ovulation occurred within 24 h of treatment in 18.9%, between 24–48 h in 79.1% and >48 h in 2.0% of cases.

One day after flushing their uteri, the donor mares were injected with a prostaglandin analogue to induce luteolysis and hasten their return to oestrus. During the past 2 seasons, no adverse effects of maturation on follicular growth were noted and there was no prolongation of the interovulatory interval in mares treated in several consecutive oestrous cycles with the GnRH analogue, BioRelease Deslorelin. Further, embryo recovery rates suggest that this drug is a possible alternative to hCG, CPE and Ovuplant for induction of ovulation in mares.

## REFERENCES

- Duchamp, G., Bour, B., Combarous, Y. and Palmer, E. (1987) Alternative solutions to hCG induction of ovulation in the mare. *J. Reprod. Fert.* **35**, 221–228.
- Roser, J.F., Kiefer, B.L., Evans, J.W., Neely, D.P. and Pacheco, C.A. (1979) The development of antibodies to human chorionic gonadotrophin following its repeated injection in the cyclic mare. *J. Reprod. Fert.* **27**, 173–179.
- McCue, P.M., Hudson, J.J., Bruemmer, J.E. and Squires, E.L. (2004) Efficacy of hCG at inducing ovulation: a new look at an old issue. *Proc. Am. Assc. Equ. Pract.* Vol. **50**, 510–513.
- Johnson, C.A., McMeenm S.L. and Thompson Jr. D.L. (2002) Effects of multiple GnRH analogue (deslorelin acetate) implants on cyclic mares. *Theriogenology* **58**, 469–471
- Fleury, P.D.C., Alonso, M.A., Alvarenga, M.A. and Douglas, R.H. (2004) Intervals to ovulation after treatment with oestradiol cypionate (ECP) or BioRelease Deslorelin (BRT-DES). 6th International Symposium of Equine Embryo Transfer. *Havemeyer Foundation Monograph Series No 14*, Eds: M. Alvarenga and J.F. Wade, R&W Communications pp 89.

## MECLOFENAMIC ACID EXTENDS DONOR-RECIPIENT ASYNCHRONY IN EQUINE EMBRYO TRANSFER

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Synchrony between the embryo and the uterine environment is essential for the establishment of pregnancy. The uterine environment changes markedly under the influence of progesterone so that an embryo exposed to an asynchronous uterus may be subjected to 'out-of-phase' growth factors and hormone levels. This may result in changes in growth rates or the death of the embryo (Barnes 2000). In addition, asynchrony may preclude the embryo from conveying its maternal recognition of pregnancy signal and suppressing the cyclical luteolytic response in the mare.

Despite the dangers of an asynchronous uterine environment for the developing embryo, successful embryo transfer in large domestic animal species is not limited to strict donor-recipient synchrony. Equids permit a wider degree of asynchrony than cattle and other ruminants and it is generally accepted that, to achieve optimal pregnancy rates when carrying out embryo transfer in equids, ovulation in the recipient mare should occur +1 to -2 days with respect to that in the donor (Douglas 1982; Squires *et al.* 1982). Previous attempts to extend this window have proved unsuccessful, probably because of the necessity for the equine blastocyst to transmit its antiluteolytic maternal recognition of pregnancy signal on or before Day 10 after ovulation to prevent the cyclical upregulation of endometrial oxytocin receptors that would trigger the prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ )-driven luteolytic cascade. For example, recipient mares that ovulated 2 or 3 days before the donor mare failed to maintain pregnancy even though treated with progestagens (Clark *et al.* 1987; Pool *et al.* 1987). The administration of prostaglandin synthetase inhibitors, such as flunixin meglumine or phenylbutazone, to mares also failed to prevent luteolysis and extend the luteal phase of the cycle

(Daels and Hughes 1993). However, recent studies in the dromedary camel demonstrated an ability of the prostaglandin synthetase inhibitor, meclofenamic acid, to prolong the lifespan of the cyclical corpus luteum (CL) and thereby allow asynchronous recipient camels to be used for embryo transfer (Skidmore and Billah 2004). In this study we investigated the effect of treating asynchronous recipient mares with meclofenamic acid prior to and after embryo transfer.

The uteri of artificially inseminated donor mares were flushed on Day 7 after ovulation (Day 0) and the embryos recovered were transferred non-surgically to either meclofenamic acid-treated or untreated control mares that had ovulated either 2 or 3, or 4 or 5 days, before the donor ( $n = 20$  and 16, respectively). Treated recipient mares received 1g/day of meclofenamic acid (Arquel, Parke Davis and Co.), orally beginning on Day 9 after ovulation and continuing until Day 7 after embryo transfer. Both groups of meclofenamic treated

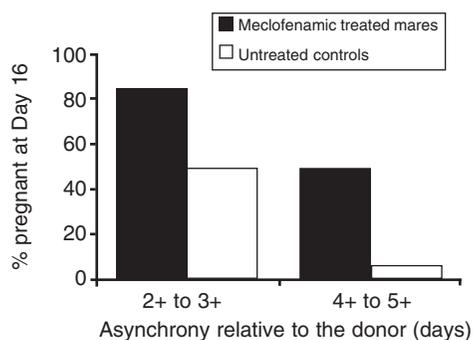


Fig 1: Both groups of meclofenamic treated mares showed significantly higher pregnancy rates at Day 16 (+2 or + 3; 17/20, 85% and + 4 or + 5; 8/16, 50%) compared to the untreated control mares with the same degree of asynchrony (+2 or + 3; 8/16, 50% and + 4 or + 5; 1/16, 6%;  $P=0.04$  and 0.02, respectively).

mares showed significantly higher pregnancy rates at Day 16 (+2 or + 3; 17/20, 85% and + 4 or + 5; 8/16, 50%) compared to the untreated control mares with the same degree of asynchrony (+2 or + 3; 8/16, 50% and + 4 or + 5; 1/16, 6%: P=0.04 and 0.02, respectively) (Fig 1).

Cyclic luteolysis was not prevented in the mares treated with meclofenamic acid that failed to become pregnant. Thus, meclofenamic acid must act in a manner other than by the suppression of luteolysis to permit the widening of the window of acceptable asynchrony for embryo transfer. The non-steroidal anti-inflammatory (NSAID) fenamates, of which meclofenamic acid is a member, have been shown to inhibit the 5-lipoxygenase arm of the arachidonic acid pathway, in addition to inhibiting the synthesis of cyclooxygenase (Civelli *et al.* 1991). Furthermore, meclofenamic acid can modulate a range of ion channels and block both gap junction intercellular communications (Harks *et al.* 2001) and the binding of PGE<sub>2</sub> to its receptors (Rees *et al.* 1988). But how such pharmacological actions might assist the embryo to interact with the maternal endometrium in such a way that it can survive in such an asynchronous environment remains to be elucidated.

## REFERENCES

Barnes, F.L. (2000) The effects of the early uterine

- environment on the subsequent development of embryo and fetus. *Theriogenology* **53**, 649-658.
- Civelli, M., Viagano, H., Acerbi, D., Caruso, P., Bongrani, A. and Folco, G.C. (1991) Modulation of arachidonic acid metabolism by orally administered morniflumate in man. *Agents Actions* **33**, 233-239.
- Daels, P.F. and Hughes, J.P. (1993) *The Abnormal Oestrous Cycle*. In: Equine Reproduction Eds: A.O. McKinnon and J.L. Voss pp 144-160.
- Douglas, R.H. (1982) Some aspects of equine embryo transfer. *J. Reprod. Fert.* **32**, 405-408
- Harks, E.G.A., De Roos, A.G., Peters, P.H., De Haan, L.H., Brouwer, A., Ypey, D.L., Van Zoelen, E.L. and Theuvsnet, A.P. (2001) Fenamates: A novel class of reversible gap junction blockers. *J. Pharm. exp. Therap.* **298**, 1033-1041.
- Pool, K.F., Wilson, J.M., Webb, G.W., Kraemer, D.C., Potter, G.D., and Evans, J.W. (1987) Exogenous hormone regimes to utilise successfully mares in dioestrus (Days 2 to 14 after ovulation) as embryo transfer recipients. *J. Reprod. Fert.* **35**, 429-432.
- Rees, M.C.P., Canete-Soler, R., Bernal, A.L. and Turnbull, A.C (1988) Effect of fenamates on prostaglandin E receptor binding. *The Lancet* Sep 3, 541-542.
- Skidmore, J.A. and Billah, M (2004) Embryo transfer in the Dromedary camel (*Camelus dromedarius*) using asynchronous, meclofenamic treated camels as recipients. 6th International Symposium on Equine Embryo Transfer. *Havemeyer Foundation Monograph Series No 14*, Eds: M. Alvarenga and J.F. Wade, R&W Communications, pp 97-98.
- Squires, E.L., Imel, K.L., Iuliano, M.F., and Shidler, R.K. (1982) Factors affecting reproductive efficiency in an embryo transfer programme. *J.*

# MANAGEMENT OF EARLY TWIN PREGNANCIES IN MARES

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## INTRODUCTION

Twin pregnancies in mares cause economic losses as they result in high rates of abortion, stillbirth or the delivery of dead or weak deformed foals. Early transrectal ultrasonographic detection of twins and manual crushing of one conceptus is the method of choice for managing equine twins before Day 24 of gestation. Transvaginal ultrasound-guided twin reduction techniques are described for twin pregnancies that advance beyond Day 25 of gestation. Reported success rates for the ultrasound-guided procedures varied between 20% and 50% (Mari *et al.* 2004; Macpherson and Reimer 2000). Surgical and transcutaneous methods (Pascoe and Stover 1989a,b) or ultrasound guided application of intracardial KCL-injections (McKinnon and Foss 1993) during later pregnancy are carried out with changeable success.

The present study aimed to prove the influence of day of gestation when performing the reduction, and the influence of treatment (over 5 days) with Flunexine. Flunexine belongs to the group of non-steroidal anti-inflammatory drugs (NSAID) which seem to have an inhibitory effect on prostaglandin synthesis influencing the arachidonic acid pathway. Former studies using NSAID focused only on short term effects, aimed at inhibiting prostaglandin synthesis evoked by the aspiration procedure itself.

## MATERIALS AND METHODS

In our experiments, 28 twin pregnancies were managed by ultrasound-guided aspiration of allantoic fluid for 4 years. Mares with twin pregnancies from Day 23 to Day 52 after breeding



Fig 1: Unilateral equine twins on Day 32 of gestation.

were directed to the ultrasound-guided procedure (Fig 1). Sixteen mares received a therapeutic dose of Flunexine (2,500–3,000 mg Finadyne, for iv injection or for oral application by paste, Essex, München) by local veterinarians 6–12 h before aspiration and for 5 days after this procedure. Pregnancy after aspiration for the survival of one embryo was controlled after 10–12 days.

A transvaginal ultrasound-guided aspiration system (VETEC GmbH Rostock) with a 6.5 MHz sector probe (HITACHI computer sonograph CS 9000) and an aspiration needle shaft was used. The replaceable needle had an outer diameter of 1.1 mm; an inner diameter of 0.8 mm and a length of 80 mm. During aspiration vaginal roof, uterus and the conceptus was penetrated by the needle guided via guide-line. Allantoic fluid was removed as far as possible but repeated penetration of the conceptus to recover the last residue was avoided. Special attention was given to protecting the endometrium during aspiration. Puncture of the embryo was not intended. The decision, what embryo was aspirated, was based on several criteria (Allen and Stewart 2001). The size of the



Fig 2: Sequence of photos during aspiration of one equine conceptus.

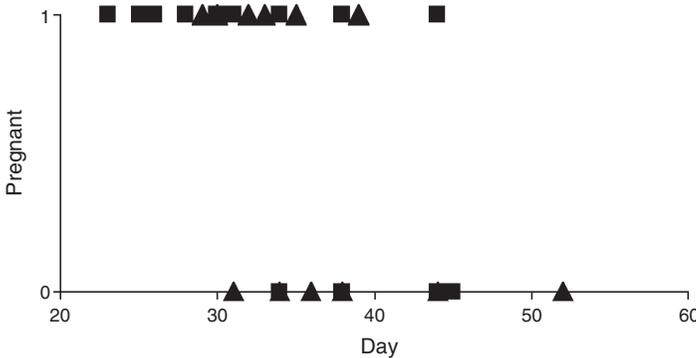


Fig 3: (g treatment with NSAID without treatment; 1= remaining pregnancy of one embryo; 0= loss of both twins). Embryonic losses and remaining pregnancies in relation to the day of gestation and according to a treatment.

conceptus, its heart beat and the possibility to manipulate the uterine horn with the embryo during the reduction process were important in the case of bilateral embryos. In unilateral twins, caudally positioned embryos were aspirated. Collection of fluid from unilateral embryos requires special attention to protect the embryonic membranes of the remaining embryo.

**RESULTS AND CONCLUSIONS**

All in all, survival of the remaining single embryo was obtained in 16 mares (success rate 59.3%). In the group of treated mares, a success rate of 71.4% was achieved. In untreated mares only 28.6% of the embryos survived. A significant influence of the day of aspiration was confirmed in treated mares. Ultrasound guided aspiration beyond Day 36 shows lower survival rates of remaining embryos. In untreated animals more mares lost their pregnancy (53.8%). An influence of the day is distinguishable beyond Day 36. Results of twin reduction in relation to day of pregnancy and a Flunixin treatment is pictured in Figure 2.

We conclude that, in management of twin pregnancies: 1) All mares should be bred regardless of the number of pre-ovulatory follicles. Withholding mares with multiple pre-

ovulatory follicles from breeding decreases the pregnancy rate per time and is followed by a loss of breeding time; 2) Check all mares for twins, regardless of the number of detected ovulations; 3) Ultrasound guided reduction of twins should be done before Day 36 of gestation; and 4) The additional application of NSAID proved to be useful.

**REFERENCES**

Allen, W.R. and Stewart, F. (2001) Equine placentation. *Reprod. Fertil. Dev.* **13**, 623-634.

Mari, G., Iacono, E., Merlo, B. and Castagnetti, C. (2004) Reduction of twin pregnancy in the mare by transvaginal ultrasound-guided aspiration. *Reprod. Domest. Anim.* **39**, 437-477.

Macpherson ML, Reimer JM. (2000) Twin reduction in the mare: current options. *Anim. Reprod. Sci.* **2**, 233-244.

McKinnon, A.O. and Voss, J.L. (1993) Equine Reproduction. Lea and Febiger, Philadelphia: 274-277.

Pascoe, D.R. and Stover, S.M. (1989a) Effect of surgical manipulation, placental fluid, and flunixin meglumine on fetal viability and prostaglandin F2 alpha release in the gravid uterus of mares. *Am. J. vet. Res.* **50**, 1505-1511.

Pascoe, D.R. and Stover, S.M. (1989b) Surgical removal of one conceptus from fifteen mares with twin concepti. *Vet. Surg.* **18**, 141-145.



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