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AND EMBRYOS**

*2nd – 5th October 2003  
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**Editors: E. Squires and J. F. Wade**

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## EDITORS' FOREWORD

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The economic value of horses has increased tremendously in the last several decades. This has prompted the development of reproductive techniques that can be used to improve reproductive performance of both mares and stallions. Furthermore, breeders have demanded that some of the techniques currently in use for human reproduction be developed and applied to the horse. Because of the increased economic and sentimental value of mares and stallions, techniques are needed to preserve genetic material (oocytes, embryos, sperm). The number of scientists working in the area of assisted reproduction is quite limited. In addition, the limited availability of oocytes, embryos and sperm requires that researchers must work together. The ability to transport gametes between research laboratories would facilitate development of assisted reproductive techniques. These same procedures could also be

used to transport gametes for commercial purposes.

The goal of this symposium was to share knowledge regarding transport of ovaries and oocytes; *in vitro* oocyte maturation and fertilisation; and oocyte, embryo and sperm freezing. We also learned from those working in other species. The most important outcome would be to facilitate the development of joint projects among researchers to solve basic problems of assisted reproduction.

A workshop of this nature would have been very difficult to fund and organise without the financial assistance of the Havemeyer Foundation. We would like to express thanks to Mr Gene Pranzo for his willingness to support our endeavours.

*Jan Wade*

*Ed Squires*

## 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

*Organiser: Dr H. Seeherman*

1997

**Second International Workshop on Equine Gene Mapping**

October - San Diego, California, USA

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

**Maternal Recognition of Pregnancy in the Mare**

January - Dominican Republic

*Organisers: Drs W. R. Allen and T. A. E. Stout*

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March - Gainesville, Florida, USA

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1998

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

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**Horse Genomics and the Genetic Factors Affecting Race Horse Performance**

March - Banbury Center, Cold Spring Harbor, New York, USA

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April - Lipica, Slovenia

*Organisers: Drs D. F. Antczak, S. Lazary and E. Marti*

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*Organiser: Dr M. R. Paradis*

1999

**Equine Genome Project**

January - San Diego, California, USA

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

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*Organisers: Drs D. F. Antczak, E. Bailey and K. Sandberg*

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August - Miami, Florida, USA

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**European Equine Gamete Workshop**

September - Lopuszna, Poland

*Organisers: Drs W. R. Allen and M. Tischner*

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November - Barbados, West Indies

*Organisers: Drs T. Stout and W. R. Allen*

2000

**Equine Genome Project**

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

**Uterine Infections in Mares and Women: A Comparative Study**

March - Naples, Florida, USA

*Organiser: Dr M. M. LeBlanc*



**5th International Symposium on Equine Embryo Transfer**

July - Saari, Finland

*Organiser: Dr T. Katila*

2001

**USDA International Plant & Animal Genome Conference**

January - San Diego, California

**Equine Immunology in 2001**

January - Santa Fe, New Mexico

*Organiser: Dr D. P. Lunn*

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April - Hungary

*Organisers: S. Lazary and E. Marti*

**From Elephants to Aids**

June - Port Douglas, Australia

*Organiser: Professor W. R. Allen*

**International Equine Gene Mapping**

July - Brisbane, Australia

*Organiser: K. Bell*

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

September - Loosdrecht, The Netherlands

*Organiser: Dr T. A. E. Stout*

**Foal Septicemia III**

October - Tufts University European Center, Talloires, France

*Organiser: M. R. Paradis*

**Infectious Disease Programme for the Equine Industry and Veterinary Practitioners**

October - Marilyn duPont Scott Medical Center, Morvan Park, Virginia, USA

*Organisers: Drs J. A. Mumford and F. Fregin*

**From Epididymis to Embryo**

October - Fairmont Hotel, New Orleans, USA

*Organiser: Dr L. H-A. Morris*

2002

**USDA International Plant & Animal Genome Conference**

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**Comparative Neonatology/Perinatology**

January - Palm Springs, California

*Organiser: P. Sibbons*

**Stallion Behavior IV**

June - Reykjavik, Iceland

*Organisers: S. McDonell and D. Miller*

**Rhodococcus Equi II**

July - Pullman, Washington

*Organiser: J. Prescott*

**Equine Orthopaedic Infection**

August - Dublin, Ireland

*Organiser: E. Santschi*

**Inflammatory Airway Disease**

September - Boston, USA

*Organiser: Dr E. Robinson*

2003

**USDA International Plant and Animal Genome Conference**

January - San Diego, California

**Embryonic and Fetal Nutrition**

May - Ravello, Italy

*Organiser: S. Wilsher*

**Genomics and the Equine Immunity System**

June - Ithaca, New York

*Organiser: D. F. Antczak*

**Fifth International Gene Mapping Workshop**

August - Kreuger Park, South Africa

*Organiser: E. Baily and E. Vandyke*

**Idiopathic Laryngeal Hemiplegia**

September - Stratford-upon-Avon, UK

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*Organiser: E. Squires*

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# SESSION I:

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## Transport of ovaries and oocytes

*Chairman:*

*Robert Godke*



# ESTABLISHMENT OF PREGNANCIES AFTER THE COLLECTION AND TRANSFER OF OOCYTES FROM TRANSPORTED OVARIES: COMMERCIAL AND EXPERIMENTAL RESULTS

E. M. Carnevale, K. A. Preis, M. A. Coutinho da Silva and E. L. Squires

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Transport of equine ovaries, while maintaining the viability of oocytes, is valuable for commercial and research endeavours. Because of the location of equine abattoirs in the United States, many laboratories cannot obtain oocytes for research unless they are transported long distances. When a valuable mare dies or is euthanised, her remaining reproductive potential lies within her ovaries. The oocytes can be collected, matured and transferred to try to produce additional foals, often requiring shipment of ovaries to a specialised facility.

Italian researchers (Galli *et al.* 2000; Lazzari *et al.* 2002) effectively transported oocytes for up to 4 h at 24–26°C in thermostatic boxes. After maturation *in vitro* for 26 h, the oocytes were fertilised by intracytoplasmic sperm injection (ICSI). Fertilised oocytes were cultured to blastocysts and transferred for pregnancies. In one study (Lazzari *et al.* 2002), 180 oocytes were obtained from 72 ovaries (2.5 oocytes/ovary) and 114 of the recovered oocytes (63%) were viable after maturation. Sperm were injected into 90 oocytes and 62 (69%) of the oocytes cleaved. Therefore, 62 of the 180 collected oocytes (34%) were capable of cleavage. Foals were also produced after fertilisation by ICSI of oocytes transported at room temperature (12–20°C) for intervals from 4–24 h (Li *et al.* 2001). The oocytes were matured *in vitro* for 28–30 h. The effect of length of transport is unknown.

In 2001, Scott *et al.* (2001) determined the potential of oocytes from different sources to develop *in vivo*. Oocytes were collected from: 1) pre-ovulatory follicles of live mares; 2) diestrus follicles of live mares; and 3) follicles from ovaries, containing a corpus luteum, that were transported from an abattoir between 6 and 8 h at 28–30°C. Immature oocytes, from live mares, were cultured for a slightly longer interval (36–38

h) than oocytes transported from the abattoir (32–36 h). Multiple oocytes were transferred per recipient. On Day 12, embryo development rates were 82, 7 and 10%, respectively, for oocytes from pre-ovulatory follicles, diestrus follicles of live mares and follicles from transported ovaries. Although significantly lower than for pre-ovulatory follicles, embryo development was not different for immature oocytes collected from mares or from transported ovaries. The transport interval was relatively short; therefore, shipment temperature was higher than could be acceptable for a longer transport. Results suggested that ovaries could be transported for short intervals while maintaining developmental potential of oocytes. Although *in vivo* conditions for embryo culture were similar for groups, reduced pregnancy rates for oocytes matured *in vitro* versus *in vivo* suggested that maturation conditions or oocyte quality were not similar.

Transportation of ovaries at 28–30°C for 6–8 h resulted in pregnancies. However, for longer periods of transportation, cooler temperatures are desirable to reduce tissue degeneration. Effect of shipment temperature was examined by Preis *et al.* (2004). Ovaries were shipped, between 19 and 24 h, from an abattoir at 2 temperatures (approximately 12 or 22°C, ranges of 9–16 and 19–25°C). One ovary, from an individual mare, was shipped at each temperature. Upon arrival, oocytes were collected and matured for 24 h before transfer into inseminated recipients. Multiple oocytes (9–15) were transferred per recipient. On a given day, oocytes (up to 15) were rapidly selected from the group with fewer oocytes, and a similar number of the second group identified for transfer. Recipients ovulated at the approximate time of transfer. Developing embryonic vesicles were collected 16 days after

transfer and submitted for parentage identification. Rates of embryo development were not different for oocytes collected from ovaries transported at 12 or 22°C (11/73 and 13/73). Embryo development rates were lower ( $P < 0.05$ ) for transferred versus ovulated oocytes (24/146, 16% and 8/12, 67%). On average, 8 oocytes were collected per donor, and approximately half of the collected oocytes were transferred into recipients. With a 16% rate of embryo development per oocyte, one pregnancy potentially would have been obtained per donor.

In the study by Preis *et al.* (2004) maturation rates could not be determined as oocytes were transferred with a full cumulus complex. In the subsequent year, oocytes were transported from the same abattoir at 22°C. Polar bodies were observed in 86 of 193 (45%) oocytes. During the same period, pre-ovulatory oocytes were collected and cultured and 39 of 41 (93%) had a polar body. Although not directly comparable, of the 73 oocytes transported at 22°C and transferred into recipients by Preis, potentially, only 33 (73 x 45%) had polar bodies. Therefore, the development rate of oocytes with polar bodies would be 39% (13/33).

Ovaries have been transported for commercial oocyte transfers after the death or euthanasia of valuable mares (Carnevale *et al.* 2003). During the past 3 years, ovaries from 21 mares have been obtained for collection and transfer of oocytes. From these ovaries, 237 oocytes (range, 1–28; mean, 11 oocytes per donor) were collected; and 197 oocytes were transferred into 40 recipients (1–14 oocytes per transfer). At least one embryonic vesicle was observed in 16 recipients (40%), but embryo loss rates were high (9/16, 56%). To date, 3 healthy foals have been produced from transfer of oocytes collected from transported ovaries, and 4 pregnancies (>120 days) are ongoing. Two donors had 2 pregnancies while other transfers resulted in one pregnancy per donor. Seven late-term pregnancies/foals were obtained for the 21 donors (7/21, 33%). This represents one or more pregnancy/foal for 24% (5/21) of donors and from 18% (7/40) of transfers. Number of transfers per donor was dependent on clients and number and quality of oocytes and sperm. Primarily, cooled semen obtained from various stallions was used. Age, health status, reproductive status and time of year varied for transfers. In some cases, ovaries were removed

directly after euthanasia of the donor; some donors were found dead, and time of death was unknown. Because of variability between cases and low overall success, it is difficult to determine the extent that variable factors affect this procedure. Successful transfers often appeared to be associated with: 1) ovary recovery during natural breeding season; 2) donors <20 years; 3) acute illness or accident; and 4) abundant follicular activity. Small follicles, prolonged illness, undetermined time of death, necrotic odour, and failure of good cumulus expansion in culture were often associated with unsuccessful attempts. Results of commercial transfers demonstrate that viable oocytes can be obtained from transported ovaries and that pregnancies can be obtained after death of a mare. Additional research is needed to determine factors that affect oocyte viability during transport to maximise success of this procedure.

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# INTERACTIONS BETWEEN TIME OF OOCYTE RECOVERY (IMMEDIATE VS. DELAYED) AND DURATION OF *IN VITRO* MATURATION ON DEVELOPMENTAL COMPETENCE OF HORSE OOCYTES

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## INTRODUCTION

Horse ovaries may have to be transported long distances before oocyte recovery is performed, yet there is little information on the effect of time of oocyte recovery on meiotic and developmental competence. Previous studies have evaluated oocyte meiotic competence after prolonged storage, but have not compared it with that of oocytes collected immediately after slaughter (Del Campo *et al.* 1995; Guignot *et al.* 1999). Franz *et al.* (2002) recently reported that maturation of oocytes collected immediately after slaughter was equivalent to (for oocytes with expanded cumuli; Ex) or higher than (for oocytes with compact cumuli; Cp) that for oocytes transported for 5–9 h at 27–29°C. It was found recently that oocytes recovered immediately after slaughter had a higher rate of resumption of meiosis than did oocytes recovered after holding ovaries for 5–9 h at slowly decreasing temperature (30–25°C) before recovery (Hinrichs *et al.* 2002); however, in contrast to the report of Franz *et al.* (2002) it was found that a large proportion of the oocytes recovered immediately after slaughter were still in metaphase I (MI) after 24 h maturation. It was not clear whether these oocytes were arrested in MI, or were slower in reaching MII than were oocytes collected after a delay. In that study, in which all oocytes were matured for 24 h, there were no differences in cleavage rates or embryo development between immediate-collected and delay-collected Ex oocytes. In Cp oocytes, there was a tendency for oocytes in the delay-collected group to have higher rates of normal cleavage (to > 2 nuclei; 32% vs. 9% for immediate-collected); however, this difference was not significant.

The present study was conducted to: 1) determine if immediate-collected oocytes are

capable of reaching MII given an adequate duration of maturation; 2) to determine if time of oocyte recovery after slaughter affects oocyte developmental competence; 3) to determine which duration of oocyte maturation is associated with highest oocyte developmental competence; and 4) to determine the effect of initial cumulus morphology on the above parameters.

## MATERIALS AND METHODS

Oocytes were collected from ovaries either at the abattoir immediately after slaughter, or in the laboratory after transporting ovaries at room temperature for 4–8 h. Oocytes in the Immediate group were placed in maturation culture within 1.5 h of slaughter, after being classified as Ex or Cp according to cumulus morphology. Oocytes in the Delay group were recovered from ovaries 4–8 h after slaughter and similarly classified and cultured. Oocytes were cultured in M199 with 5 µU/ml FSH and 10% FCS for 24, 30 or 36 h, then MII oocytes were subjected to intracytoplasmic sperm injection (ICSI) with frozen-thawed sperm using a piezo drill. Presumptive zygotes were cultured for 7.5 days in DMEM/F-12 with 10% FCS.

## RESULTS

Oocytes in the Immediate group had a significantly higher rate of resumption of meiosis (combined MI and MII) than did oocytes in the Delay group (74% vs. 84% for Ex; 34% vs. 65% for Cp;  $P < 0.01$ ). However, oocytes in the Immediate group progressed to MII significantly more slowly in culture, having a higher proportion at MI at 24 h ( $P < 0.01$ ). Duration of maturation did not significantly affect blastocyst development within

either Immediate or Delay Ex groups. Overall, blastocyst development for Delay Ex oocytes was significantly higher than for Immediate Ex oocytes (75/261, 29% vs. 40/251, 16%;  $P < 0.001$ ). Duration of maturation significantly affected blastocyst development within Delay Cp oocytes: blastocyst development for the 24 h maturation group (3/32, 9%) was significantly lower than that for 30 or 36 h maturation (11/32, 35% and 14/37, 38%, respectively;  $P < 0.05$ ). There was a similar trend among maturation durations in Immediate Cp oocytes (9%, 18% and 23% for 24 h, 30 h and 36 h, respectively) but these differences were not significant. While Delay Cp oocytes matured for 30 or 36 h tended to have a higher blastocyst development rate (35% and 38%, respectively) than did similarly matured Immediate Cp oocytes (18% and 23%, respectively), these differences were not significant.

When Ex and Cp oocytes were compared at each maturation duration, in the Delay group blastocyst development was significantly higher for Ex than for Cp at 24 h ( $P < 0.05$ ) but there was no difference between cumulus morphologies when oocytes were matured for 30 h and 36 h. There were no significant differences between Ex and Cp oocytes among maturation durations in the Immediate group. The highest rates of blastocyst formation obtained for Ex oocytes (26/81, 32%; Delay/30 h) and for Cp oocytes (14/37; 38%; Delay/36 h) were not significantly different.

## CONCLUSIONS

These data support previous findings (Hinrichs *et al.* 2002) that oocytes collected immediately after slaughter are slower to mature to MII than are oocytes collected after a delay. The reason for the discrepancy between these studies and that of Franz *et al.* (2002), who reported high rates of maturation to MII in oocytes collected within 1 h of slaughter and matured for 22–26 h (63% for expanded oocytes and 59% for compact oocytes), is not immediately apparent. Our data also support the apparent trend seen previously (Hinrichs *et al.* 2002) for an increase in cleavage and development in oocytes recovered after a delay. A similar increase in developmental competence has been reported in cattle after holding ovaries at 30°C for 4 h before oocyte collection (Blondin *et al.* 1997). In our previous study, we also looked at the development of oocytes that had been recovered immediately after slaughter, but held in medium at

room temperature for 5–9 h before the onset of maturation culture. This group had a high proportion of oocytes that resumed meiosis, similar to immediately cultured oocytes, but the oocytes were able to reach MII within 24 h of maturation culture. Embryo development to 96 h in this group was similar to that for the immediately cultured or delayed group for Ex oocytes, but in Cp oocytes was intermediate between the immediate and delayed culture groups (9%, 18% and 32% for immediate, held and delayed oocytes, respectively). As all oocytes in that study were matured for 24 h, more work is needed to determine the effect of immediate recovery but delayed culture on developmental competence of horse oocytes.

In summary, we conclude that blastocyst development after ICSI of horse oocytes is dependent on the time of oocyte recovery and on an interaction between cumulus morphology and the duration of *in vitro* maturation. The highest rate of blastocyst development was achieved in both cumulus groups when oocytes were collected after a delay rather than immediately following slaughter; and after 30 h maturation for Ex oocytes but 36 h for Cp oocytes.

## ACKNOWLEDGEMENTS

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# SESSION 2:

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## **Comparison of developmental competence of *in vitro* and *in vivo* matured oocytes**

**Chairman:**

***Elaine Carnevale***



# OOCYTE MATURATION AND EFFECT ON SPERM OR SPERM EXTRACT INDUCED INTRACELLULAR CALCIUM OSCILLATIONS

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## INTRODUCTION

The molecular determinants of oocyte developmental competency are largely unknown in mammals. In the horse, due to the difficulties linked to obtaining embryos for *in vitro* development, either through *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) techniques, the characterisation of these factors is complicated further.

In all mammalian species studied, fertilisation induces a specific pattern of  $[Ca^{2+}]_i$  oscillations that are responsible for oocyte activation (Fissore *et al.* 1992; Kline and Kline 1992). Studies in laboratory species show that the pattern of these oscillations may be altered in immature and aged oocytes (Gordo *et al.* 2002), so the ability of the oocyte to respond with a normal pattern of oscillations may be correlated with developmental capacity. It has also been shown that aged mouse and human oocytes die by apoptosis upon fertilisation, rather than following the expected developmental pattern (Morita and Tilly 1999; Gordo *et al.* 2002).

Although oscillations resulting from IVF have not been studied in the horse, equine oocytes display  $[Ca^{2+}]_i$  transients upon ICSI or stallion sperm extract (eSF) microinjection (Bedford *et al.* 2004). Oocytes matured *in vitro* for 24 or 42 h displayed a similar pattern of  $[Ca^{2+}]_i$  transients upon eSF microinjection. ICSI induced responses were similar in *in vivo* matured mare oocytes injected around 45 h post hCG, or those matured *in vitro* for 40 h; both groups of oocytes were

activated at similar rates. Even though the pattern of  $[Ca^{2+}]_i$  responses was similar in oocytes under different maturation conditions, it is not known whether those oscillations were capable of initiating normal development. Therefore, the long-term objectives are to investigate  $[Ca^{2+}]_i$  responses in *in vivo* or *in vitro* matured oocytes and relate them to their capacity to undergo normal development or apoptosis.

## MATERIALS AND METHODS

### Oocytes

Oocytes with expanded cumuli for *in vitro* maturation were obtained by scraping the follicular walls of abattoir-collected ovaries (Texas A&M, Texas, USA) and placed into equilibrated maturation medium (TCM 199 with Earle's salts; Gibco Life Technologies, Inc., New York, USA) supplemented with 5  $\mu$ U/ml FSH (Sioux Biochemical Inc.; Iowa, USA), 10% fetal bovine serum, and 25  $\mu$ g/ml gentamycin. The vial containing the oocytes was sealed and packaged into a commercial incubator (Minitube of America, Inc.; Wisconsin, USA) for overnight shipment at 38°C. Upon arrival, maturation continued in an incubator up to 24 or 48 h. Oocytes having intact cytoplasmic membranes with no signs of degeneration or fragmentation and with a visible perivitelline space, were selected for micromanipulation. For these experiments, oocytes were injected with 5  $\mu$ g/ $\mu$ l eSF, prepared as previously described (Bedford *et al.* 2004), for  $[Ca^{2+}]_i$  monitoring. Following these experiments, horse oocytes were incubated in culture medium but without FSH at 38°C in 5% CO<sub>2</sub> in humidified air for an additional 24 h prior to evaluation of activation status and caspase activity.

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### Microinjection procedure

Microinjection procedures were performed as previously described (Wu *et al.* 1998). Denuded MII horse oocytes were microinjected using a Nikon Diaphot microscope (Nikon Inc.; New York, USA) and Narishige manipulators (Medical Systems Corp.; New York, USA). Injection pipettes containing 0.5 mM fura-2 dextran (fura-2D; Molecular Probes; Oregon, USA) or 5 µg/µl eSF were advanced into the cytoplasm of each individual oocyte and reagent was injected by pneumatic pressure (PLI-100 Picoinjector, Harvard Apparatus; Massachusetts, USA). The injection volume was 15–20 pl.

### [Ca<sup>2+</sup>]<sub>i</sub> monitoring

Fluorescence of fura-2D loaded oocytes subjected to microinjection or ICSI was monitored as previously described (Wu *et al.* 1998). Illumination was by a 75-W xenon arc lamp on a Nikon Diaphot microscope equipped with a 40X UV oil immersion objective (Nikon Inc.; New York, USA). Excitation wavelengths were of 340 and 380 nm, and the corresponding emitted light (attenuated 32-fold by neutral density filters) was quantified by a photomultiplier tube, which averaged the fluorescence signal over the whole oocyte. The 340/380 fluorescence ratio correlates with [Ca<sup>2+</sup>]<sub>i</sub>.

### Assessment caspase activity and oocyte activation

Assays for caspase activity were performed as described by Gordo *et al.* (2002) using the PhosphorFluorimetry kit (Oncoimmunin, Inc.; Maryland, USA), a fluorescein-conjugated DEVD (Asp-Glu-Val-Asp)-specific caspase substrate.

## PRELIMINARY RESULTS AND OBSERVATIONS

Fewer oocytes matured for 48 h mounted normal [Ca<sup>2+</sup>]<sub>i</sub> responses (3/6 vs. 3/3; P<0.05) and the interval between spikes averaged 17.6 and 6.8 min for oocytes matured *in vitro* for 24 and 48 h respectively (P=0.07). In all instances, [Ca<sup>2+</sup>]<sub>i</sub> responses lasted as long as these were monitored (ie 360 min). Hoechst staining of oocytes at 24 h post injection revealed that 2/3 and 3/6 of the 24 and 48 h matured oocytes were activated,

respectively. One oocyte of the 24 h group was still in MII, whereas non-activated 48 h matured oocytes had no chromatin (2) or fragmented DNA (1), both of which are signs of developmental incompetence.

Control non-injected oocytes did not display caspase activity. Additionally, 3 oocytes that were fragmented prior to injection were not denuded completely, and when stained, only granulosa cells appeared to display caspase activity. Only one of the 24 h matured oocytes was submitted to the caspase assay and no caspase activity was observed. All 3 oocytes from the 48 h group submitted to this assay showed widespread activity throughout the cytoplasm. One of these oocytes had been incubated for 48 h and had been observed to cleave to the 2-cell stage. At the time of staining there was widespread cytoplasmic fragmentation and caspase activity.

## CONCLUSIONS

The results suggest that *in vitro* aged mare oocytes display an altered pattern of [Ca<sup>2+</sup>]<sub>i</sub> oscillations and decreased embryonic developmental potential, as evidenced by activation of caspases within their cytoplasm upon eSF microinjection. The findings may allow optimisation of the time of maturation of mare oocytes whereby fertilisation or activation may result in maximal developmental competency. The results may also provide new insights in the mechanism(s) that underlies decreased developmental potential of aged oocytes.

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# USE OF OOCYTE TRANSFER TO DETERMINE DEVELOPMENTAL COMPETENCE OF *IN VITRO* AND *IN VIVO* MATURED OOCYTES

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Various methods have been used to assess oocyte viability in the mare. Assessment of viability *in vitro* and *in vivo* may not be comparable. One method to determine viability of the oocyte is oocyte transfer (OT). During OT, oocytes are placed into the oviducts of inseminated recipients; and fertilisation and embryo development occur within the recipients. Because capacitation, fertilisation and embryo development occur within the equine reproductive tract, effects of an *in vitro* environment are minimised. Potential methods to determine development include recovery of oviductal embryos, recovery of uterine embryos, and assessment of embryo development by ultrasound or production of a live foal.

The first foal was born from OT in the late 1980s (McKinnon *et al.* 1988); however, efficiency of the technique (one foal from 15 oocytes) was too low for effective use as a procedure to evaluate oocyte viability. However, in the mid 1990s, OT was used successfully to compare viability of oocytes from young and old mares. Carnevale and Ginther (1995) collected oocytes from the maturing follicles of young or old mares, cultured for completion of maturation and transferred into young inseminated recipients. In the study, 1–4 oocytes were transferred into the oviduct of each recipient, with the assumption that fertilisation rates would not be reduced when multiple oocytes were transferred into the recipient's oviduct. Pre-ovulatory follicles of recipients were aspirated to remove their own oocyte, and oocytes were transferred ipsilateral (n=5) or contralateral (n=8) to the side of aspiration. The study demonstrated a significant reduction in viability of oocytes collected from the follicles of old versus young mares with embryo development rates of 92%, 11/12 and 31%, 8/26, respectively. The authors also compared embryo

development rates after the exclusion of recipients, which were not pregnant with at least one embryonic vesicle, to minimise the effect of unsuitable recipients; however, groups were still significantly different (young vs. old, 11/12, 92% and 8/16, 50%). The study demonstrated that multiple oocytes could be transferred into a single recipient and that transfers could be done ipsilateral or contralateral to the side of the recipient's follicle. OT was used successfully to demonstrate that viability is reduced in oocytes from old mares.

For experimental transfers, recipients are usually cyclic; therefore, their own oocyte must be differentiated from transferred oocytes. In the previous study by Carnevale and Ginther (1995), the recipient's own oocyte was aspirated before transfer. However in a recent experiment (Carnevale *et al.* 2002), parentage testing was used to differentiate transferred versus ovulated oocytes, success of insemination before versus after transfer, and effect of media. Oocytes were collected from multiple donors, cultured in different media, and transferred into the oviduct contralateral to the recipient's own ovulation. Recipients were inseminated with semen from 2 different stallions, one prior to transfer and another after transfer, to determine if semen inseminated before or after transfer resulted in fertilisation of transferred oocytes. At 16 days after ovulation, embryonic vesicles were recovered and submitted for parentage verification. Embryo development rates were not different for transferred versus ovulated oocytes (32/44, 73% and 9/13, 69%), also suggesting that fertility was not reduced when multiple oocytes were fertilised or when fertilisation occurred in the oviduct contralateral to natural ovulation. Significant differences were determined for media. More oocytes were fertilised

by semen inseminated before versus after transfer (29/31, 94% versus 2/31, 6%). OT and parentage testing were combined to reduce recipient effects and minimise the number of recipients required for the project. Using these procedures, multiple factors were evaluated during the same experiment. Similar pregnancy rates for ovulated and transferred oocytes suggest that the procedures for OT are effective and do not result in reduced embryo development compared to normal ovulation.

When compared to oocytes collected from pre-ovulatory follicles, oocytes matured *in vitro* have more sources of variability for an individual oocyte. In one study (Scott *et al.* 2001), the developmental competence of oocytes matured *in vivo* or *in vitro* (collected from live mares or from slaughterhouse ovaries) or within the oviduct was compared. More ( $P < 0.05$ ) embryonic vesicles developed after the transfer of oocytes matured *in vivo* (9/11, 82%) than *in vitro* (live mares, 2/29, 7% and slaughterhouse ovaries, 4/40, 10%) or within the oviduct (0/27). OT was used successfully to demonstrate a significant reduction in developmental competency for oocytes matured *in vivo* versus *in vitro*. The transfer of immature oocytes into the oviduct did not result in pregnancies.

Hinrichs *et al.* (2002) transferred oocytes, matured in 2 media (maturation medium or follicular fluid), into the oviducts of inseminated recipients. Oocytes matured in one medium were transferred into one oviduct, and oocytes matured in another medium were transferred into the contralateral oviduct. Twelve or 25 oocytes were transferred per oviduct. The oviducts were recovered between 40 and 44 h after transfer and oocytes or embryos were recovered and evaluated. Of the transferred oocytes, 74% (87/118) were recovered; and 43 of 87 (49%) oocytes were not degenerate and appeared to be normally fertilised. Of the 87 recovered oocytes, 16 had cleaved to 2 or more cells (18%). The authors noted that the recovery rate was better when 12 versus 25

oocytes were transferred into the recipient's oviduct. Although fertilisation rates were high (76 to 77%) and not different between maturation groups, cleavage rates were significantly higher when oocytes were matured in maturation medium versus follicular fluid. The study demonstrated that *in vivo* fertilisation rates were high for oocytes matured *in vitro*. However, results suggested that some fertilised oocytes were delayed and may not continue to develop. Potentially, only a portion of the fertilised oocytes would develop to an embryonic vesicle that could be imaged by ultrasound.

OT has been used successfully to determine the viability of oocytes matured *in vivo* or *in vitro*. Recent results demonstrate that techniques for the transfer of oocytes are highly effective. The use of OT provided a method to assess early developmental competency after fertilisation of the oocyte while minimising the effects of *in vitro* culture.

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# SESSION 3:

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## **Ways to improve *in vitro* maturation**

**Chairman:**

***Katrin Hinrichs***



# IN VITRO MATURATION OF MAMMALIAN OOCYTES

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## INTRODUCTION

This paper will review the important features of oocyte maturation in large mammals with a particular emphasis on the bovine as it is actually the species where there is the most information.

Oocyte maturation is a natural process that occurs within the follicle in response to the surge of luteinising hormone (LH) and prior to ovulation. This phenomenon can be reproduced *in vitro* but it is not yet known if it mimics completely what happens *in vivo*. To better describe the changes that occur in the oocyte during culture, the maturation process should be divided in 3 aspects: the nuclear, the cytoplasmic and the molecular maturation.

## NUCLEAR MATURATION

The oocyte must resume meiosis to respond properly to fertilisation and this event is normally triggered by the LH surge *in vivo*. When immature bovine or most mammalian oocytes are released from their follicles and cultured in standard maturation media, they resume the first meiotic division without any special triggering agent (Edwards 1965).

Different hypotheses by which gonadotropins can stimulate meiotic resumption within the follicle have been proposed: 1) by decreasing the intrafollicular level of an oocyte maturation inhibitor (Downs and Eppig 1986); 2) by eliciting a maturation-inducing signal by the granulosa cells which over-rides the inhibitory milieu of the follicle (Eppig and Downs 1987); or 3) by blocking or disrupting the gap junctions between cumulus cells and the oocyte (Hyttel 1987). As proposed recently, somatic cells read the LH

signal and send a second messenger to the oocytes by 2 different mechanisms. LH can act either indirectly on the follicular wall inducing theca and/or granulosa cells to secrete a second messenger into the follicular fluid, or directly on the cumulus cells which communicate with the oocyte through gap junction (Mattioli and Barboni 2000). Cumulus cells react to the LH signal with a intracellular  $Ca^{2+}$  rise which diffuses through gap junctions into the oocyte (Mattioli *et al.* 1998; Mattioli and Barboni 2000). Soon after the LH peak, a transient rise of cAMP occurs within the oocyte. Therefore, cAMP can no longer be considered as a general inhibitor of maturation. Its regulatory role relies on the levels reached in cumulus cells and the oocyte.

The pre-ovulatory follicle also undergoes a series of changes during final maturation. The membrana granulosa cells cease to synthesise oestradiol, and the follicular wall luteinises shortly before ovulation, coinciding with a marked increase of progesterone production and extensive expansion of the cumulus (Dieleman and Blankenstein 1985). A major question is whether these maturational changes of the follicle are of any significance for the oocyte. The oocyte could also be largely on its own after receiving the signal to resume meiosis as mediated by the follicular cells directly or indirectly via the cumulus cells. Newly synthesised proteins are observed throughout maturation *in vivo* in oocytes incubated as cumulus-oocyte complexes (COCs) with major changes in protein patterns occurring around germinal vesicle breakdown (Kastrop *et al.* 1991a, 1992), and possibly, some peptides could originate from the cumulus cells. Also transcription in the cumulus cells might be needed for final maturation *in vivo*. During the first 2 h of *in vitro* maturation transcription in the COC is

necessary to drive resumption and completion of meiosis (Kastrop *et al.* 1991b). The immature oocyte itself at the germinal vesicle (GV) stage is capable of incorporating uridine suggesting synthesis of mRNA (Memili *et al.* 1998). During this culture period, oocytes undergo a series of cytoplasmic changes before the resumption of nuclear maturation leading to a variable competence of the resulting embryo (Moor *et al.* 1990).

## CYTOPLASMIC MATURATION

There is still a clear difference between ova obtained from *in vivo* maturation and oocytes matured in culture. Oocytes obtained by laparoscopy or surgery following *in vivo* maturation have shown a higher potential for development (Sirard and Lambert 1986; Bousquet *et al.* 1995). When comparing oocytes matured *in vivo* versus *in vitro*, no apparent differences are seen in the level of nuclear maturation, in the rates of fertilisation or cleavage, but rather in the developmental competence of the oocyte. These observations indicate that the cytoplasmic competence must be different between the *in vitro* and the *in vivo* matured oocyte.

During the period just before the LH surge and/or during the period between the LH surge and ovulation, the oocyte is thought to be able to respond to follicular factors that determine its subsequent developmental capacity. Furthermore, under the influence of yet unknown factors, the oocytes undergo a series of profound changes involving both the nucleus and cytoplasm during this time (Eppig 1996). These changes are essential for the formation of an egg having the capacity for fertilisation and development. However, COCs recovered from 2–6 mm follicles for *in vitro* maturation (IVM) lack this period of pre-ovulatory development, which is required for increasing the developmental competence of oocytes in comparison to oocytes matured *in vivo* (Hendriksen *et al.* 2000).

In the cyclic cow, final maturation of the ovulatory follicle is initiated by the pre-ovulatory LH surge. During the ensuing 24 h period, the oocyte undergoes progression of meiosis to metaphase II and several changes in cytoplasmic organisation, such as a continued development of the lipid store, reduction of the Golgi compartment, rearrangement of mitochondria and

alignment of the cortical granules along the oolemma (Kruip *et al.* 1983; Hyttel *et al.* 1986). The increased lipid compartment probably forms an essential energy pool for the oocyte to support development after fertilisation up to blastocyst stage (Betteridge and Fléchon 1988). Further, numerous ribosomes appear especially adjacent to the chromosomes (Kruip *et al.* 1983; Hyttel *et al.* 1986).

A number of ultrastructural and molecular changes occurring during oocyte development have been linked to developmental competence (Assey *et al.* 1994). Also, *in vitro* maturation has been associated with certain abnormalities in the oocyte (Hyttel *et al.* 1989). A previous study reported that bovine oocytes aspirated from dominant follicles before the LH surge display alterations in their nuclear and cytoplasmic morphology, which according to the author are a pre-requisite for the acquisition of full developmental competence. This would indicate that not only the final oocyte maturation (ie the processes occurring between LH surge and ovulation) is significant, but also the period preceding the LH surge may be important for the establishment of developmental competence. Differences have been reported between *in vivo* and *in vitro* matured oocytes which may explain the observed differences in developmental competence. Cumulus expansion is usually more extensive following maturation *in vivo* (Suzuki *et al.* 1996). In addition, there is a high degree of homogeneity amongst oocytes matured *in vivo* at the ultrastructural level; this contrasts with the ultrastructural heterogeneity exhibited by *in vitro* matured oocytes, even when a uniform population of the latter is selected period to *in vitro* maturation (De Loos *et al.* 1992).

In general, oocytes from 3–8 mm follicles from a heterogeneous population (De Loos *et al.* 1989) and show blastocyst formation rates *in vitro* which are about one-half of those of oocytes from ovulatory follicles (Hendriksen *et al.* 2000).

There are 2 possible explanations for the difference observed between the oocyte matured *in vivo* and *in vitro*: the culture conditions used or the intrinsic competence of the oocyte. It is certainly feasible to improve the culture conditions during maturation.

As shown by Eppig *et al.* (1994) the acquisition of oocyte developmental competence relies on nuclear and cytoplasmic maturation. These 2 events are more or less independently

programmed. The completion of meiotic maturation to metaphase II is observed about 95% of the time whereas the level of embryo production from *in vitro* matured oocytes is approximately 35%. Indeed, oocytes matured *in vitro* or *in vivo* have similar rates of nuclear maturation, fertilisation and cleavage; however, they clearly differ in their developmental potential (Sirard and Blondin 1996). While COCs for *in vitro* maturation (IVM) are usually obtained from follicles about 2–6 mm in size and about 4–10 days from any possible ovulation (Sirard *et al.* 1992), oocytes resuming meiosis *in vivo* originate from dominant follicles of about 15 mm in size (Pavlok *et al.* 1992). The IVM period lasts only 24 h, while the dominant follicles grow from 4 to 15 mm for approximately 5 days.

There is growing evidence that the follicle of origin is important in determining the quality of the oocyte. Follicles can be classified according to 2 primary criteria: follicle size, which determines its ability to respond to ovarian and systemic messages, and follicle health status evaluated in terms of a follicle's ability to ovulate a functional oocyte. The impact of these factors has been addressed using oocyte and embryo that have been cultured individually to establish a cause and effect relationship between follicular characteristics, oocyte morphology and the resulting developmental competence (Blondin and Sirard 1995). Also, it is clear that the size of the follicle is involved in oocyte and embryo competence (Fair *et al.* 1995).

It has been shown that intrinsic factors such as follicular diameter could influence the percentage of bovine embryos produced *in vitro* (Pavlok *et al.* 1992; Loneragan *et al.* 1994). Large follicles contain oocytes more capable of developing into blastocysts than smaller follicles. Another approach is to determine the effect of extrinsic factors, such as the time and temperature ovaries are stored before oocyte recovery on the rates of embryo production. Blondin and co-authors found that bovine oocytes derived from ovaries maintained at 35°C for 4 h yielded higher frequencies of blastocyst (Blondin *et al.* 1997a). Their data suggested that developmental competence was acquired shortly prior to *in vitro* maturation and depended on the handling conditions of postmortem ovaries. Our laboratory has also demonstrated that the 'coasting' period between hormonal stimulation and ovary collection (Blondin *et al.* 1997a) as well as the

time interval between ovary collection and oocyte aspiration (Blondin *et al.* 1997b) significantly improves the developmental potential of COC (Blondin *et al.* 2002).

The mammalian oocyte is surrounded by layers of cumulus cells, with the corona radiata being closest to the oocyte. Communication between the oocyte and the corona radiata is accomplished through cytoplasmic extensions across the zona pellucida, and also with the outer cumulus through gap junctions (De Loos *et al.* 1991; Allworth *et al.* 1993). Intercellular communication between the oocyte and surrounding follicle cells is of vital importance, first to keep the oocyte arrested at prophase I of meiosis and later to urge the oocyte to resume meiosis at the time of ovulation. Cumulus cells communicate with each other and with the oocyte by means of gap junctions between the cumulus cells and between the oocyte and cumulus cells (Furger *et al.* 1996). The intact state of the surrounding cumulus cells was demonstrated to be beneficial to the oocyte and subsequent embryo development (Blondin and Sirard 1995). Removal of the cumulus oophorus before IVM is detrimental for oocyte maturation in cattle (Chian and Niwa 1994). Therefore cumulus cells are considered to play an important role in oocyte maturation.

The beneficial effect of cumulus oophorus during IVM can be attributed to the formation of a favourable microenvironment (biochemical or metabolic) around the oocyte. Cumulus cells benefit bovine oocyte development during IVM either by secreting soluble factors, which induce developmental competence, or by removing inhibitory or toxic components from the maturation medium (Hashimoto *et al.* 1998). In addition, cumulus cells might have unknown promoting effects on subsequent oocyte development which may be attributable to intracellular changes such as pH or calcium ions (Mori *et al.* 2000). Another possible influence of cumulus cells during IVM of bovine oocytes might be that cumulus cells decrease the oxygen tension in the immediate vicinity of the oocyte as a result of the active metabolism of the cumulus cells (Ali *et al.* 2003).

## MOLECULAR MATURATION

One of the events involved in cytoplasmic maturation, is the storing of RNA in the cell

cytoplasm (Paynton and Bachvarova 1994). It is believed that the synthesis and storage of certain forms of mRNA and proteins during IVM and early embryonic development are necessary (Motlik and Fulka 1986; Thibault *et al.* 1987). These stored mRNA are so essential for the survival of the early bovine embryo, as the major activation of the zygotic genome is at the 8–16 cell stage, that they should be considered as a new chapter of competence, hence the molecular maturation. Thus, novel protein synthesis in the early bovine embryo relies mostly on pools of stored maternal RNA.

It is also important to evaluate external factors involved in the manipulation of oocytes before culture which can affect developmental competence. It has been shown that the differences between the *in vivo* and *in vitro* cultured oocytes were expressed 4–5 days post fertilisation. This long period supports the hypothesis that competent oocytes must store important factors either in the form of proteins or in the form of stable mRNA. When the oocytes are aspirated from the follicle, their ability to synthesise proteins is not affected, but they lose the capacity to make RNA in less than 2 h unless meiotic arrest is artificially maintained (Sirard and Coenen 1994). Since over the last 15 years, a wide variety of *in vitro* maturation conditions did not produce any significant improvements on the developmental rates of oocytes, it is logical to support the hypothesis that specific mRNA accumulation, rather than post translational change, is affected. This hypothesis suggests that competence is acquired before the beginning of nuclear condition, which starts a few hours after the aspiration from the follicle or the LH surge.

Recently, it has been shown in ruminants that the oocyte's capacity to become an embryo could be pre-programmed in the ovary. Indeed, when the right hormonal treatment is applied to a cow before follicular aspiration, it is possible to harvest immature oocytes that will almost all become embryos (Blondin *et al.* 2002). This hormonal treatment simply mimics the natural ovulatory process where FSH rises and then falls a few days before ovulation. The difference is that progesterone does not decrease to allow ovulation and the follicles start their progression towards atresia. But the right signals have been given to the oocyte and when removed from the compromised follicle, it can express its competence *in vitro*. This is a little different from the mouse where fully

grown oocytes have already acquired all the abilities to develop to embryos.

Although it is known that a given treatment in the animal induces competence in bovine oocytes, how do we have limited insight to the cause or the mechanism? Except for slight changes in the nucleolus morphology associated with the pre-maturation or oocyte capacitation event (Hyttel *et al.* 1997), there are no methods to distinguish between competent and incompetent oocytes other than IVF culture and embryo transfer. But the difference is present in the form of molecular memory. It is known that what happens during the last few hours prior to oocyte harvest will have a significant impact on further competence. This event must be memorised or stored in long life molecules. In most tissues, long term effects are mediated by gene expression, but the oocyte loses rapidly its transcriptional capacity upon maturation. The second most preferred mechanism to retain information is 'proteins' and possibly competent oocytes accumulate a number of specific and important factors for later use in embryogenesis (Rodman and Bachvarova 1976). But studies in vertebrate: *Xenopus* (McGrew *et al.* 1989), mouse (Huarte *et al.* 1992) or insects (*drosophila*) (Lieberfarb *et al.* 1996) indicate that stored mRNA might be the preferred form of information storage in oocytes. The problem with mRNA is related to its instability or half-life. In most somatic cells, the half-life of mRNA is from minutes to hours while in oocytes it can stay stable for days (Brower *et al.* 1981).

## CONCLUSIONS

A better understanding of the meiotic control mechanism and of the signalling from the follicle will be required to obtain improvement of developmental competence in most mammalian immature oocytes.

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# SESSION 4:

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## Evaluation of oocytes

**Chairman:**

***Sylvia Bedford***



# EFFECT OF DIFFERENT PRE-MATURATION TREATMENTS ON *IN VITRO* MATURATION OF EQUINE OOCYTES AND SUBSEQUENT COMPETENCE FOR NUCLEAR TRANSFER

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The low efficiency of the somatic cell nuclear transfer (NT) procedure requires that large numbers of meiotically competent equine oocytes must be available for maturation. *In vitro* maturation rates of equine oocytes are generally lower and more variable than those of other domestic species. In a recent study investigating oocyte meiotic competence as related to granulosa cell apoptosis, Dell'Aquila *et al.* (2003) reported that although follicle size was not significantly correlated to apoptosis, the proportion of oocytes maturing *in vitro* was significantly higher in oocytes issuing from apoptotic follicles than in those issuing from healthy follicles. Also, it was found that significantly more expanded than compact oocytes originated from apoptotic follicles. This supports previous studies that reported the compact oocytes having a lower meiotic competence (Alm and Hinrichs 1996; Hinrichs and Williams 1997) and slower maturation rate (Zhang *et al.* 1989; Hinrichs *et al.* 1993; Alm and Hinrichs 1996).

The first experiment was conducted to evaluate the nuclear status of equine oocytes matured *in vitro* using a sequential maturation medium. A second study was conducted to evaluate the effect of roscovitine pre-maturation in oocytes from

small equine ovarian follicles and to use these *in vitro* matured oocytes for equine NT procedures.

## EXPERIMENT I

Twenty-five cycling, mixed breed mature mares were selected for aspiration after  $\geq 28$  days of no stimulation during the 2002 breeding season. From a total of 81 oocytes recovered from 134 follicles (60% recovery, yielding a mean of 3.96 oocytes/mare), 71 were classified as good quality oocytes, allocated and grouped as shown in Table 1. Oocytes were exposed to pre-maturation medium (100% follicular fluid supplemented with 75  $\mu\text{M}$  roscovitine) for 0–24 h, followed by a 24–36 h exposure to maturation medium (TCM-199, 10% FBS, 5  $\mu\text{g/ml}$  of FSH, 10  $\mu\text{g/ml}$  of LH, 1  $\mu\text{g/ml}$  of  $\text{E}_2$ , 100 of  $\text{ng/ml}$  EGF and 100  $\text{ng/ml}$  of IGF 1). All oocytes were then stained with Hoechst 33342 and subjected to epifluorescent illumination to determine nuclear status (Table 2).

The pre-maturation of oocytes pooled from all follicle sizes in roscovitine with follicular fluid did not improve nuclear maturation rates obtained after permissive maturation. The highest maturation rate was obtained when oocyte maturation was conducted in maturation medium alone (Treatment

**TABLE 1: Sequential maturation of equine oocytes**

Treatment	No. oocytes	Time in pre-maturation medium (h)	Time in maturation medium (h)	Total time <i>in vitro</i> (h)
1	7	12–13	24	36–37
2	16	14–17	24	38–41
3	27	18–20	25	43–45
4	21	22–24	26	48–50
5	36	0	36	36

**TABLE 2: Nuclear status of *in vitro* matured equine oocytes**

Treatment	No. oocytes	GV (%)	GVBD (%)	MI (%)	MII (%)	Degenerate (%)
1	7	1 (14)	1 (14)	1 (14)	1 (14)	3 (43)
2	16	3 (19)	2 (12)	7 (44)	0 (0)	4 (25)
3	27	3 (11)	2 (7)	13 (48)	1 (4)	8 (30)
4	21	3 (11)	0 (0)	11 (52)	4 (19)	3 (14)
5	36	2 (5)	0 (0)	6 (17)	22 (61)	6 (17)

GV: Germinal vesicle; GVBD: Germinal vesicle breakdown.

**TABLE 3: Effect of oocyte source and roscovitine on maturation and nuclear transfer (NT)**

Treatment	No. oocytes	No. (%) mature	No. (%) enucleated	No. (%) reconstructed	No. (%) fused
Subordinate (IVM only)	24	12 (50)	9 (75)	7 (78)	5 (71) <sup>a</sup>
Small (IVM only)	100	66 (66)	30 (45)	22 (73)	12 (55) <sup>a</sup>
Small (Roscovitine + IVM)	93	53 (57)	30 (57)	20 (67)	0 <sup>b</sup>
Total	217	131 (60)	69 (53)	49 (71)	17 (35)

<sup>a,b</sup>Values in columns with different superscripts are different (Chi square,  $P < 0.05$ ).

5), indicating the incubation in follicular fluid with roscovitine for a prolonged period of time may have negatively affected not only cytoplasmic maturation but also the ability of the chromosomes to condense and resume the normal steps of nuclear maturation.

## EXPERIMENT II

Roscovitine has been shown to inhibit germinal vesicle breakdown without compromising further developmental competence in the cow (Mermillod *et al.* 2000). A second study was conducted to evaluate the effect of roscovitine pre-maturation in oocytes from small equine ovarian follicles and to use these *in vitro* matured oocytes for equine NT procedures. Twenty-nine mixed breed, cycling mares were aspirated for 6 consecutive times during the breeding season. Seven days prior to oocyte collection, follicle ablation was performed on all mares to initiate a new wave of follicles. Follicles were classified at the time of aspiration as pre-ovulatory (not used in this experiment), subordinate (>20 mm diameter) or small (<20 mm diameter).

A total of 217 oocytes were recovered from 346 follicles for an overall recovery rate of 63%. When classified according to follicular size, 71 subordinate follicles yielded 24 oocytes (34% recovery), whereas 193 oocytes were recovered from 275 small follicles resulting in a recovery

rate of 70%. Oocytes recovered from subordinate follicles were subjected to *in vitro* maturation (IVM) in TCM-199, 15% oestrous mare serum, 5 µg/ml of FSH, 10 µg/ml of LH and 1 µg/ml of E<sub>2</sub> for 36 h. Oocytes aspirated from small follicles were allocated randomly to IVM only or pre-treated for 48 h in roscovitine (75 µM) prepared in TCM-199 prior to IVM. Results are summarised in Table 3.

Significantly more couplets fused with ova from subordinate and small (IVM only) treatments compared with the small (roscovitine + IVM) treatment (71%, 55% and 0%, respectively). This could be because oocytes from the subordinate and small (IVM only) groups were held *in vitro* for 36 h whereas oocytes from the small (roscovitine + IVM) group were held *in vitro* for 84 h (48 h roscovitine pre-maturation plus 36 h IVM).

In this study, the pre-treatment of oocytes from small follicles in roscovitine did not have a significant effect on the proportion of oocytes reaching the metaphase II stage after maturation. This is in agreement with previous studies that have shown that, although roscovitine was effective in maintaining the oocytes in meiotic arrest, the nuclear maturation rates were not significantly affected by the pre-incubation treatment (Khatir *et al.* 1998; Mermillod *et al.* 2000; Ponderato *et al.* 2001; Franz *et al.* 2003). The beneficial effects of the permissive cytoplasmic maturation on oocyte maturation and embryo development still remain to be demonstrated.

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## GAMETE INTRAFALLOPIAN TRANSFER

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Advances in assisted-reproduction in the horse, such as embryo and oocyte transfer, have helped to obtain pregnancies from subfertile mares. Embryo transfer has been performed in valuable mares that cannot carry their pregnancies to term. Embryos are usually collected from donor mares between 6–8 days post ovulation and transferred into the uterus of synchronised recipients. Ovulation failure and unsuitable oviductal and/or uterine environments to support fertilisation and early embryonic development are common findings in mares that fail as embryo donors. These problems can be circumvented by performing oocyte transfer (OT). During OT, the oocyte is collected from a donor mare and transferred into the oviduct of an inseminated recipient. The success of OT depends on the quality of the oocyte, recipient and sperm. Best results with OT are obtained working with young donors and recipients, and using fresh semen from a fertile stallion inseminated into the uterus. In commercial OT programs, donor mares are usually old and have history of subfertility. Therefore, recipients must be inseminated with high quality semen from fertile stallions in order to maximise chances of pregnancy. However, in most cases, recipients are inseminated with cooled or frozen semen with variable fertility and sperm numbers, resulting in lower pregnancy rates compared to experimental results. Potentially, alternative assisted reproductive technologies could be used to improve the efficiency of oocyte transfer when semen of poor quality is available.

Minimal assisted-reproductive techniques are available to produce offspring from stallions with poor sperm quality or quantity. *In vitro* fertilisation (IVF) is not a repeatable procedure in the horse; and although fertilisation can be obtained *in vitro*

by intracytoplasmic sperm injection (ICSI), the procedure requires extensive expertise and equipment.

A potential assisted-reproductive technique to obtain pregnancies from subfertile stallions is gamete intrafallopian transfer (GIFT). GIFT involves the transfer of the oocyte and a low number of sperm into the oviduct of the recipient. During GIFT, sperm capacitation, as well as fertilisation and embryo development, occur within the recipient's reproductive tract. Sperm are deposited into the oviduct; therefore, sperm bypass the uterus and the utero-tubal junction, and selection of a morphologically normal sperm population by the female reproductive tract does not occur. Separation of sperm from seminal plasma, debris and contamination, and selection of a population of motile sperm from the ejaculate can be obtained using different methods *in vitro*. Percoll has been used to separate semen in most sperm preparations for GIFT in mares. Because a low number of sperm ( $2$  to  $5 \times 10^5$  motile sperm) is used and because sperm are deposited near the site of fertilisation, GIFT could potentially be used to produce offspring from subfertile stallions, frozen semen, or sperm that have been sex-selected.

GIFT has been performed in the horse using fresh, cooled and frozen semen. Initial attempts to perform GIFT in the horse used fresh semen from fertile stallions and resulted in success rates ranging from 27–82% (Carnevale *et al.* 2000; Coutinho da Silva *et al.* 2002). In these experiments, raw semen was centrifuged through Percoll, resuspended and transferred into the oviduct with oocytes. These results demonstrated that high fertilisation rates could be achieved by depositing low numbers of fresh sperm and oocytes into the oviduct of mares. However, in a

commercial setting, potentially cooled and frozen semen would be more frequently available; and therefore, use of cooled and frozen semen for GIFT was investigated in subsequent experiments.

Experiment I compared embryo development rates obtained after using fresh or frozen semen for GIFT versus OT using fresh semen. Semen used for GIFT was either frozen in Lactose-EDTA prior to the experiment or collected approximately 1 h before transfer. Fresh and frozen-thawed semen were centrifuged through Percoll, washed and resuspended in Emcare holding solution (ICP, Auckland, New Zealand). Within less than 5 min of transfer,  $2 \times 10^5$  motile sperm (5  $\mu$ l) were added to holding medium containing the oocytes. For intra-uterine inseminations, fresh semen ( $1 \times 10^9$  progressively motile sperm) was diluted with E-Z Mixin 'CST' (Animal Reproduction Systems, California, USA) and inseminated at approximately 12 h before and 2 h after OT.

In Experiment II, embryo development rates after GIFT or OT using cooled semen were compared. After semen collection,  $1 \times 10^9$  progressively motile sperm were diluted with E-Z Mixin 'CST' and stored for approximately 24 h at 5°C. Approximately 1 h before GIFT, cooled semen was processed using one of 3 methods. For the first method, semen was warmed in a water bath at 25°C for 10 min and no centrifugation was performed. For the second method, warmed semen was centrifuged through Percoll and the sperm pellet was resuspended in holding medium. In the third method, cooled semen was initially centrifuged and the pellet was resuspended in holding medium. An aliquot of the sperm suspension was then centrifuged through Percoll as described above. After preparation of sperm by each of the 3 methods,  $2 \times 10^5$  motile sperm were added to holding medium containing the oocytes. For intra-uterine inseminations, recipients were inseminated approximately 15 h before and 2 h after transfer with  $1 \times 10^9$  cooled-stored sperm.

In Experiments I and II, oocytes were collected from the pre-ovulatory follicle of donor mares at approximately 22 h after hCG administration. Oocytes were cultured in TCM-199 supplemented with 10% FCS, 0.2 mM pyruvate, and 25  $\mu$ g/ml gentamicin sulphate for approximately 17 h in an atmosphere of 6% CO<sub>2</sub> in air at 38.5°C. After culture, oocytes were placed in a droplet of holding medium, loaded in a fire-

polished glass pipette with (GIFT) or without (OT) sperm and transferred into the oviduct of recipients.

In Experiment I, embryo development rates were similar ( $P > 0.05$ ) when fresh sperm were used for GIFT (9/11, 82%) or OT (8/14, 57%). However, embryo development rates were lower ( $P < 0.05$ ) after GIFT using frozen sperm (1/12, 8%).

In Experiment II, the first, second and third methods of sperm preparation for GIFT resulted in similar ( $P > 0.05$ ) embryo development rates (1/6, 17%; 1/5, 20%, and 2/5, 40%, respectively). Because no differences were detected, results were combined and compared to OT using cooled semen. Embryo development rates were higher ( $P < 0.05$ ) when cooled semen was used for OT (19/23, 83%) compared to GIFT (4/16, 25%).

High embryo development rates were obtained using fresh sperm for GIFT. In Experiment I, lower embryo development rates were obtained using frozen semen for GIFT. Fertility of cryopreserved sperm is usually lower than fresh sperm (Samper *et al.* 1991). Procedures used to cryopreserve semen (dilution, cooling, freezing, and thawing) damaged the plasma membrane of sperm (de Leeuw *et al.* 1991; Parks and Graham 1992), causing changes in membrane fluidity and loss of membrane-selective permeability. These changes resulted in a reduced ability of frozen-thawed sperm to bind to oviductal epithelial cells and zona pellucida (Dobrinski *et al.* 1995). Changes caused by cryopreservation could result in a sperm population with a reduced ability to form the sperm reservoir in the oviduct and, consequently, reduced lifespan and ability to fertilise the oocyte. In addition, sperm were deposited into the oviduct and the effects of site of sperm deposition on the interaction between sperm, oviduct and oocyte are not known. Further research is required to determine differences in fresh versus cryopreserved sperm when placed within the oviduct.

In Experiment II, cooled semen used for GIFT was processed by 3 different methods. Embryo development rates were similar among methods of sperm preparation; therefore, centrifugation of sperm and the use of Percoll did not appear to affect sperm function, although experimental numbers were low. Embryo development rates were lower when cooled sperm were used for GIFT compared to OT. Fertility of

cooled semen was high when sperm were deposited into the uterus. However, even when sperm were not centrifuged (first method) and placed within the oviduct, embryo development rates were lower. Therefore, results suggested that sperm interactions with oviduct and oocyte might be different when sperm are deposited in the uterus or oviduct. Components of cooling and freezing extenders could have interfered with sperm binding to oviductal epithelium or zona pellucida. Studies are in progress to determine the effects of site of sperm deposition and extender on the interactions between sperm, oviduct and oocyte.

GIFT has the potential to be a successful and practical assisted-reproductive technique for subfertile stallions with low sperm numbers. However, GIFT has not yet been performed with sperm from subfertile stallions and, to date, only fresh semen could be used for optimal success rates. Further research is necessary to investigate the use of GIFT for subfertile stallions and to improve the success of GIFT using cooled and frozen semen.

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# BLASTOCYST DEVELOPMENT OF *IN VITRO* AND *IN VIVO* MATURED EQUINE OOCYTES FERTILISED BY INTRACYTOPLASMIC SPERM INJECTION (ICSI)

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While meiotic competence of equine oocytes has been fairly well studied, little information is available on factors affecting their developmental competence, which is evaluated by assessing the ability of the oocyte to develop to the blastocyst stage and beyond after fertilisation. Due to the poor results with *in vitro* fertilisation in the horse, there has historically been no efficient way to fertilise oocytes to evaluate development. Introduction of intracytoplasmic sperm injection (ICSI), which was improved further by use of the piezo drill, resulted in a method that can repeatedly give high fertilisation and cleavage rates (69–89%; Choi *et al.* 2002; Galli *et al.* 2002). However, the *in vitro* blastocyst development rate after piezo-driven ICSI, as after standard ICSI, remains low. Little is known about culture methods for equine embryos. It is therefore difficult to determine whether the observed low blastocyst development is due to problems with oocyte developmental competence, fertilisation techniques, or embryo culture systems. Rates of blastocyst development in *in vitro* equine embryo culture systems have been low both when embryos are cultured in semi-defined medium such as G1.2/2.3 or synthetic oviductal fluid (9–14% (combination of morula and blastocyst); Galli *et al.* 2002; Choi *et al.* 2003a) or when they are cultured in co-culture systems (2–3%; Dell'Aquila *et al.* 1997; Guignot *et al.* 1998). Li *et al.* (2001) reported that co-culture with a cumulus cell monolayer improved development of equine ICSI embryos, but only of embryos resulting from oocytes that had been matured in co-culture (14% blastocysts). A major factor in success of *in vitro* culture systems in other species has been manipulation of glucose concentrations during the different stages of embryo development. High concentrations of glucose have been shown to be detrimental to early embryo development, whereas

later development (after Day 4) requires glucose. Choi *et al.* (2003b) previously established the ability of a modified Chatot, Ziomek, Bavister (CZB) medium (CZB-C) to support equine embryo development *in vitro* for 4 days. The authors evaluated the effect of glucose concentration on equine embryos by culturing ICSI zygotes in CZB-C with either low (0.55 mM) or high (5.5 mM) glucose. Both glucose concentrations resulted in high cleavage rates (75–82%) and similar embryo development at 4 days (Choi *et al.* 2004).

This study investigated the intrinsic developmental competence of *in vitro* matured oocytes by evaluating blastocyst development *in vivo*, after transfer to the oviduct. Subsequently, we evaluated *in vitro* culture of ICSI zygotes, derived from the same source, in two culture systems: 1) modified CZB-C + BSA with 0.55 or 5.5 mM glucose for 72 h, followed by increasing or static glucose concentrations, respectively; and 2) culture in DMEM/F-12 + 10% FBS with or without equine oviductal epithelial explants. *In vitro* development of zygotes derived from ICSI of *in vivo* matured oocytes was also examined, by culture in DMEM/F-12 + 10% FBS for 7.5 days.

Oocytes were collected from slaughterhouse-derived ovaries, matured for 24 h, and oocytes with a polar body were fertilised by injection with frozen-thawed sperm using a piezo drill as described previously (Choi *et al.* 2002). Transfer of presumptive zygotes to the oviduct was performed via standing flank laparotomy (Hinrichs *et al.* 1998). At 7.5 to 8.5 days after transfer, the uteri were flushed for embryo recovery in the standing mare. The mares were then euthanised and the uterus, ovaries and oviducts were removed. The oviducts were flushed using 50 ml of Dulbecco's PBS, and the embryos recovered were evaluated for stage of development. Embryo development

*in vitro* was assessed at 7.5 days. To compare development by oocyte source, *in vivo* matured oocytes were recovered from the pre-ovulatory follicles of mares after gonadotropin stimulation and were subjected to ICSI, then assessed after 7.5 days embryo culture *in vitro*.

Of 132 zygotes transferred to the oviducts of 3 recipient mares, 69 (52%) were recovered; 48 from the uterus and 21 from the oviducts. Of these, 25 (36%) were expanded blastocysts with a blastocele and capsule. All blastocysts were recovered from the uterus. In the *in vitro* culture experiments, none of the zygotes cultured in CZB-C + BSA developed to the blastocyst stage, regardless of glucose concentration. The rate of blastocyst formation in DMEM/F-12/FBS with oviductal explants was 16% and in DMEM/F-12/FBS alone was 15%. When *in vivo* matured oocytes were fertilised and cultured in DMEM/F-12/FBS, 6 out of 8 (75%) cleaved and one (13%) developed to the blastocyst stage.

These results indicate that *in vitro* matured equine oocytes are competent to form around 35% blastocysts under optimal culture conditions, ie *in vivo*. Culture in modified CZB did not support development to the blastocyst stage. The rate of development *in vitro* in DMEM/F-12 is higher than has been previously reported without co-culture, but is only half of that obtained after oviductal transfer. Transfer of ICSI zygotes to the oviduct provides components that support higher blastocyst development than does *in vitro* culture. This result is consistent with the previous report that average nucleus number of ICSI embryos transferred to the oviducts was twice that of *in vitro* cultured embryos at 96 h (Choi *et al.* 2002). However, Scott *et al.* (2001) reported significantly lower pregnancy rates at Day 15 after oviductal transfer of *in vitro* matured oocytes than after transfer of *ex-vivo* collected pre-ovulatory oocytes (10 vs. 82% respectively). This difference in results for *in vitro* matured oocytes might be related to the selection of oocytes before and after maturation, and methods of *in vitro* maturation between laboratories.

In the present study, only one embryo out of 8 *in vivo* matured ICSI oocytes developed to the blastocyst stage, which is equivalent to the development obtained from *in vitro* matured oocytes. This indicates that the major cause of poor blastocyst development is the ICSI technique or *in vitro* embryo culture, rather than defects of developmental competence in *in vitro* matured oocytes.

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# SESSION 5:

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## Cooling and freezing

**Chairman:**

***Jason Bruemmer***



# CRYOPRESERVATION OF EQUINE OOCYTES

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## INTRODUCTION

Unlike embryo freezing cryopreservation of oocytes is not considered an established technology. However there are many good reasons for wanting to preserve oocytes. Cryopreservation of oocytes from live mares or mares that have died will allow storage of valuable genetic material. This would eliminate the need to fertilise those oocytes immediately or discard unused oocytes. Cryopreservation of oocytes is particularly important for endangered species and for women who want to harvest genetic material prior to treatment for diseases, such as cancer. Also, there are ethical and legal reasons for wanting to freeze human oocytes instead of embryos.

There are 2 types of freezing procedures, slow rate cooling and vitrification. Slow rate cooling works better for oocytes that are not sensitive to chilling injury, in species such as the mouse, cat, rabbit and human, but not for the pig, cow and more than likely horses (Ledda *et al.* 2001). Advantages of slow rate cooling are that a low concentration of cryoprotectants is generally used and that the oocytes are slowly dehydrated. The disadvantage is the possibility of chilling injury due to ice crystal formation (Vajta 2000). An alternative is vitrification, in which rapid freezing results in solidification of a solution at a low temperature without ice crystal formation. The advantage of vitrification is the elimination of ice crystal formation, however all other types of injuries are increased by vitrification. The oocytes are exposed to very high concentrations of cryoprotectants.

Studies on cryopreservation of equine oocytes have been extremely limited. Hochi *et al.* (1994, 1996) reported on cryopreservation of equine

oocytes using ethylene glycol. Their study evaluated the survival rates of *in vitro* matured equine oocytes using either the slow cool protocol or vitrification methods. The survival rates were extremely low for both methods, 16 and 17%, respectively. Vitrification of mouse, cattle and human oocytes has resulted in production of live offspring (Schellander *et al.* 1994; Men *et al.* 1997). Some of the advances with oocytes from other species include the use of combination of cryoprotectants, stepwise addition and removal of cryoprotectants, and the addition of macromolecules to the cryoprotectant solution. The use of open pooled straws, (Vajta *et al.* 1997) or electron microscope grids (Arav and Zeron 1997) has allowed for the ultra rapid freezing of oocytes, which appeared to improve the survival.

This report reviews the results of studies on the cryopreservation of equine oocytes. Some of the factors that affect the viability of oocytes after freezing and thawing in both the equine and other species will also be discussed.

## VITRIFICATION OF IMMATURE AND MATURE EQUINE AND BOVINE OOCYTES

The objective of this study was to compare viability of immature and mature equine and bovine oocytes vitrified in 5 M ethylene glycol + 18% ficoll + 0.5% sucrose using open-pulled straws (Hurt *et al.* 2000). Equine and bovine oocytes were collected from slaughterhouses. Oocytes were assigned to one of 3 treatment groups/species using 4 batches of ovaries. Equine and bovine oocytes were vitrified immediately after collection (immature group), were vitrified after culture (mature group) or were not vitrified and matured with the same protocol (controls).

Equine oocytes were cultured for 36–40 h and bovine oocytes were cultured for 24 h. The vitrification solution was 2.5 M ethylene glycol + 18% ficoll + 0.5 M sucrose for 30 s at 39°C. The oocytes were then transferred to 5 M ethylene glycol and immediately pulled into the open end of open-pulled straw, which was then submerged in liquid nitrogen. Straws were not sealed. For the first 2 batches of oocytes only nuclear maturation was determined, whereas for the last 2 batches oocytes both nuclear and cytoplasmic maturation were determined. Live/dead determination was made using Hechst 33342 staining and chromatin configuration was evaluated in the oocytes stained with 1% orcein and 45% acetic acid. For cytoplasmic maturation cortical granule distribution was analysed by using confocal microscopy.

After vitrification, equine oocytes had maturation rates to 30, 40 and 46% for the immature, mature and non-vitrified control groups, respectively. Bovine oocytes had maturation rates to MII after vitrification of 60, 70 and 77% for the same 3 groups respectively. Live/dead rates for the 3 groups of equine oocytes were similar but the rates were lower than those for bovine oocytes. Only 25, 30 and 45% of the equine oocytes in the immature, mature, and control groups displayed mature cortical granule distribution. Based on the results of this study, both immature and mature equine and bovine oocytes survived cryopreservation using vitrification and open-pulled straws. However, survival rates were lower for equine than for bovine oocytes.

#### **EFFECT OF SUCROSE OR TREHALOSE ON VITRIFICATION OF EQUINE OOCYTES 12 OR 24 H AFTER THE ONSET OF MATURATION**

The objectives of this study were to determine if the addition of sucrose or trehalose would minimise the toxicity effect of cryoprotectants on equine oocytes and to determine the effect of stage of maturation on survival of equine oocytes after cryopreservation (MacLellan *et al.* 2001). Oocytes were matured in maturation medium EMM1 for 12 or 24 h. Cryoprotectants were loaded in 3 steps, 5% DMSO and 5% ethylene glycol for 30 s, 10% DMSO and 10% ethylene glycol for 30 s, and 20% DMSO and 20% ethylene glycol with either

0.65M sucrose or 0.065M trehalose for 20 s before being loaded on to a nylon cryoloop. Oocytes were thawed using 3-step dilutions with 0.25M, 0.188M, 0.125M sucrose or trehalose. Oocytes in the 12 and 24 h groups were cultured in EMM1 for 14 or 2 h post thaw, respectively, before undergoing intracytoplasmic sperm injection (ICSI). Non-vitrified oocytes matured for 26 h were used as controls. All oocytes were activated with 10 µM ionomycin for 5 min and then cultured for 20 h before being fixed. Oocytes vitrified in trehalose at 12 h had lower rates of polar body formation than control groups. Fertilisation rates were similar for both control and vitrified oocytes. Equine oocytes vitrified in either sucrose or trehalose at 12 or 24 h evaluated by ICSI appeared viable.

#### **PREGNANCIES FROM VITRIFIED EQUINE OOCYTES COLLECTED FROM SUPER-STIMULATED AND NON-STIMULATED MARES**

This experiment compared embryo development rates after transfer of vitrified and non-vitrified oocytes into inseminated recipient mares (MacLellan *et al.* 2002). Oocytes were collected by transvaginal ultrasound guided follicular aspiration 24 h after administration of hCG. Oocytes for control transfer were cultured 14–16 h in EMM1 before transfer, whereas, oocytes for cryopreservation were placed into culture media for approximately 2–4 h before vitrification. The recipient mares were inseminated with 2 billion progressively motile sperm 15–18 h before transfer of oocytes. The ovaries and oviducts of recipients were exposed by standing flank laparotomy and the oocytes transferred into the oviduct. For vitrification the oocytes were placed in the cryoprotectant solution in 3 steps as described by Hurtt *et al.* (2000). Oocytes were stored up to 2 months before transfer. They were then thawed and diluted in 3 steps and cultured for 10–12 h in EMM1 before transfer into inseminated recipients.

Of the 26 oocytes vitrified, 19 were considered morphologically normal after thawing and culture. Of the 20 oocytes collected from non-stimulated mares, 12 were vitrified and 8 were transferred as controls. After thawing, 10 of the 12 were morphologically intact and transferred in recipients, resulting in one embryonic vesicle on

Day 16 (1 of 12, 8%). Fourteen oocytes from super-stimulated mares were vitrified and 4 were transferred as controls. After thawing, 9 of the 14 oocytes were morphologically intact and transferred into recipients resulting in 2 embryonic vesicles on Day 16 (2 of 14, 14%). In control transfers, 7 or 8 oocytes from non-stimulated mares and 3 or 4 oocytes from super-stimulated mares resulted in embryonic vesicles on day 16. The 2 pregnancies from vitrified oocytes resulted in healthy foals. Apparently this is the first report of foals being born from vitrified equine oocytes.

## DISCUSSION

In these studies it appeared that both the immature and mature equine oocytes could be vitrified, thawed and survive. However, there seems to be a trend towards better survival for vitrification of mature oocytes. This would agree with studies in other species, in which mature oocytes were more resistant to cryopreservation. *In vitro* matured cat oocytes froze better than immature oocytes (Luvoni and Pellizzari 2000). Shaw *et al.* (2000), reported that chilling injury affects microtubules, cytoskeletal organisation, zona pellucida and the meiotic spindle. Several approaches have been used to minimise the chilling injury in cryopreservation of oocytes, these include the selection of the permeable cryoprotectants. For oocytes that are frozen with a slow cool rate protocol, propylene glycol and DMSO are the most commonly used. However, for vitrification generally, a combination of cryoprotectants are used. Hochi *et al.* (1994), demonstrated that ethylene glycol was the best cryoprotectant for freezing equine oocytes. Several studies have demonstrated the advantage of replacing some of the sodium in the media with choline. This seems to be particularly advantageous for cryopreservation of mouse oocytes (Stachecki and Willadsen 2002) and human oocytes (Quintano *et al.* 2002). Another approach to decrease the toxicity of cryoprotectants is a step-wise addition and removal of the cryoprotectant. Generally the cryoprotectant solution is added in 3 steps and removed in 3 steps. This minimises the time that the oocyte is in contact with high concentrations of the cryoprotectants. One factor that has made vitrification of oocytes so successful is the use of containers such as open-pulled straws or cryoloops. These allow for extremely rapid

cooling rates. In the bovine, Maverides and Morroll (2002), reported a 54% survival of bovine oocytes after slow cooling versus a 90% survival of bovine oocytes loaded on cryoloops and vitrified. Lane and Gardner (2001) also reported higher survival rates for mouse oocytes frozen on cryoloops and vitrified versus those that were slow cooled. The use of open-pulled straws and cryoloops in studies conducted in our laboratory also allowed for a relatively high survival of oocytes that were vitrified and thawed. Blastocyst rates after fertilisation of cryopreserved bovine oocytes using open-pulled straws and vitrification nearly approached the value of unfrozen controls (Vajta 2000).

The criteria chosen for evaluation of oocytes after freezing and thawing are extremely important. In the studies conducted in this laboratory 3 different approaches were used, staining for nuclear maturation, staining for cytoplasmic maturation, based on cortical granule migration and transfer into recipient's oviduct. Establishment of pregnancy in a recipient is a more rigorous criterion than staining techniques. The frozen thawed equine oocyte must have both nuclear and cytoplasmic maturation. Although initial studies indicated that nuclear maturation was reasonable, relatively poor cortical granule migration was found in the current study. This may have been caused by premature release of cortical granules as the result of the cryoprotectants or the fact that these oocytes were not cytoplasmically mature. Another technique that is extremely useful for evaluation of the viability of oocytes is the use of ICSI. This technique has been used in several species including the horse as a means of assessing viability of the oocyte. Since *in vitro* fertilisation in the horse is not a reliable technique, ICSI has become the best *in vitro* technique for evaluating the oocyte. It also has the advantage of inducing fertilisation in oocytes that may not be capable of fertilisation because of zonal hardening. One of the suggested problems with frozen/thawed oocytes is the change in the permeability in the zona pellucida.

More studies need to be conducted on cryopreservation of equine oocytes. Procedures that have been used in other species should be applied to freezing of equine oocytes. The low pregnancy rate obtained after transfer of frozen/thawed equine oocytes into the oviduct of inseminated recipients would indicate that the procedures for freezing equine oocytes have not been optimised.

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# EFFECT OF CRYOPRESERVATION ON THE CYTOSKELETAL INTEGRITY OF EQUINE EMBRYOS

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## INTRODUCTION

Reasonable pregnancy rates can be achieved after transfer of frozen-thawed equine embryos (55–60%: Lascombes and Pashen 2000), if embryos are small when frozen (<250  $\mu\text{m}$ : Slade *et al.* 1985). Expanded blastocysts tolerate cryopreservation poorly. Legrand *et al.* (2000) proposed that the acellular capsule formed around the embryo during blastulation reduces cryopreservability by impeding cryoprotectant penetration, a theory supported by high pregnancy rates (6/8) obtained after partially digesting the capsule around expanded blastocysts with 0.2% trypsin prior to freezing. Subsequent studies have yielded lower pregnancy rates (3/11, Legrand *et al.* 2002; 0/14, Maclellan *et al.* 2002).

Another potential problem during freezing is the formation of ice crystals that disrupt the cell membrane or cytoskeleton (Dobrinsky 1996), where the latter is the network of actin (microfilament), tubulin (microtubules) and intermediate filaments that organises and supports a variety of cellular events. Stabilising the actin cytoskeleton with the reversible actin-polymerisation inhibitor, cytochalasin B (cyto-B), has been reported to reduce cytoskeletal damage and to improve the viability of frozen pig embryos (Dobrinsky 2000).

The aim of the current experiment was to determine whether either partial capsule digestion with trypsin, or actin filament depolymerisation with cyto-B really protect against cell and cytoskeletal damage during the cryopreservation of horse embryos.

## MATERIALS AND METHODS

### *Embryo collection*

On Day 7 after ovulation, embryos were collected from inseminated Dutch Warmblood mares by

non-surgical uterine lavage with Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5% (v/v) fetal bovine serum (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands). Embryos were assessed for quality and their diameter was recorded before transportation to the laboratory in ovum culture medium (OCM; ICN Biomedicals, Zoetermeer, The Netherlands). Only embryos classed as Grade 1 or 2 (excellent or good) were used in further experiments.

### *Experimental design*

After washing in PBS, embryos were stained with the membrane impermeable DNA stain, 4,6-diamino-2-phenyl-indole (DAPI), to allow counting of dead cells by immunofluorescence microscopy. Embryos were assigned randomly to one of 3 freezing treatments: 1) a conventional 4-step, 10% glycerol protocol ( $n = 14$ ); 2) 15 min immersion in 0.2% w/v trypsin in PBS prior to cryoprotectant exposure ( $n = 11$ ); and 3) 7.5  $\mu\text{g}/\text{ml}$  cyto-B added to the glycerol solutions ( $n = 11$ ). A further 10 non-frozen control embryos were fixed in 4% paraformaldehyde immediately after examination for dead cells.

### *Cryopreservation*

Embryos were exposed for 10 min to each of 4 concentrations of glycerol in PBS (2.5, 5, 7.5 and 10%) at room temperature (27°C). Thereafter, they were loaded into 0.25 ml straws and frozen using a programmable machine (Planer Kryo 10 Series II, Sunbury-on-Thames, England). Initially, embryos were cooled at 1°C/min down to -6°C, where they were held for 10 min and seeded. Subsequent cooling was at 0.3°C/min down to

-33°C, after which the straws were plunged into liquid nitrogen.

**Assessing viability and actin cytoskeleton integrity**

After thawing, the embryos were stained with DAPI, and the dead cells were counted using an immunofluorescence microscope. The embryos were then fixed overnight in 4% paraformaldehyde and stored at 4°C in PBS. Prior to staining, embryos were permeabilised using 0.1% Triton X-100. Next, they were labelled with Alexafluor 488 Phalloidin (Molecular Probes Europe BV, Leiden, The Netherlands), to visualise the actin cytoskeleton, and relabelled with DAPI to allow counting of total cell numbers. Finally, stained embryos were mounted on glass slides with an antifade medium (Vectashield, Vector Lab., California, USA) and sealed under the coverslip using nail polish. Examination of cytoskeletal architecture was performed using a Multiphoton Excitation Microscopy system (Bio-Rad Radiance 2100MP) mounted on a Nikon TE300-inverted microscope.

**Statistical analysis**

Differences in embryo diameter and percentage of dead cells were compared between groups using one-way ANOVA, after Bonferroni correction. Actin cytoskeleton quality was scored subjectively on a scale of I to III, where Grade I was typified by sharp staining of cell borders. Grade II staining was characterised by gross maintenance of cell outlines, but patchy staining with small clumps also visible in the cytoplasm. A Grade III cytoskeleton had large areas lacking staining with much of the visible actin agglomerated in clumps. Between groups differences in actin cytoskeleton quality were compared using non-parametric (Kruskal-Wallis and Mann-Whitney U) tests. In all cases, differences were considered statistically significant when  $P < 0.05$ .

**RESULTS**

Mean ( $\pm$  sem) embryo diameter before freezing did not differ between the experimental groups ( $391 \pm 28 \mu\text{m}$ ; range 160–1,000 mm;  $P = 0.63$ ). Two of 14 (14.3%) conventionally-frozen and 3 of 11 (27.3%) trypsin-treated embryos fractured during thawing, and only their actin cytoskeleton quality could subsequently be analysed. The mean percentage of

dead cells after thawing did not differ between the conventional, trypsin and cyto-B treated groups ( $14.6 \pm 2.4$ ,  $9.6 \pm 2.8$  and  $9.7 \pm 2.4$  respectively;  $P = 0.27$ ), but was significantly higher than in non-frozen control embryos ( $0.15 \pm 0.6\%$ ;  $P < 0.001$ ). Embryos  $>300 \mu\text{m}$  in diameter had a significantly higher percentage of dead cells post thaw than those  $<300 \mu\text{m}$ , if they were frozen without additional treatment ( $19.0 \pm 2.9$  vs  $8.4 \pm 2.1\%$ ;  $P = 0.023$ ; Fig 1). The effect of blastocyst size was less marked if embryos were treated with either trypsin ( $13.8 \pm 4.9\%$  vs  $5.4 \pm 0.7\%$ ;  $P = 0.141$ ) or cyto-B ( $12.4 \pm 5.5$  vs  $8.1 \pm 2.3\%$ ;  $P = 0.420$ ) before freezing.

Eight of 10 freshly fixed embryos had a Grade I cytoskeleton. Freezing and thawing adversely affected cytoskeleton quality such that only 4 of 11 (36.4%) trypsin-treated and no conventionally frozen or cyto-B treated embryos had a Grade I cytoskeleton (Fig 2). All frozen-thawed cyto-B treated embryos had a mottled, Grade II cytoskeleton.

**DISCUSSION**

Compared to fresh embryos, frozen-thawed embryos had high proportions of dead cells. The percentage of dead cells did not differ between freezing protocols, but smaller embryos ( $\leq 300 \mu\text{m}$ ) tended to suffer less damage than larger embryos. While conventionally frozen large embryos had significantly more dead cells than their smaller counterparts, the difference was not significant if embryos were pre-treated with either trypsin or cyto-B. This suggests that both trypsin and cyto-B treatment protected primarily the larger embryos. Freezing and thawing clearly

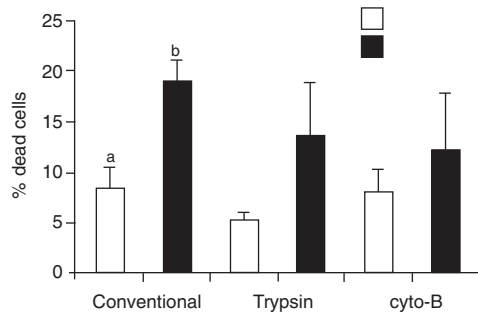


Fig 1: The percentage of dead cells after freezing and thawing of small ( $<300 \mu\text{m}$ ) or large ( $>300 \mu\text{m}$ ) horse embryos with or without pre-treatment with trypsin or cytochalasin B. <sup>a,b</sup>Labels denote values that differ significantly.

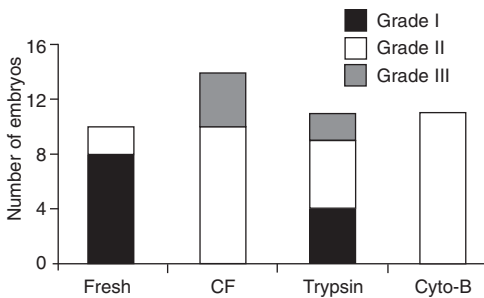


Fig 2: Actin cytoskeleton quality of horse embryos soon after collection (fresh) or following freezing and thawing conventionally (CF) or after trypsin or cyto-B pre-treatment.

tended to disrupt the actin cytoskeleton of horse embryos. However, while there was no significant effect of treatment on post thaw mean cytoskeleton quality, Grade I cytoskeletons were only seen in embryos pre-treated with trypsin (36%). This suggests that trypsin treatment protected against actin filament disruption and it is possible that thinning the capsule really does enhance cryoprotectant entry and reduce the risk of intracellular ice crystal formation.

While the ability of cyto-B to protect against cytoskeleton damage was superficially disappointing, examination of fresh cyto-B treated embryos demonstrated that the actin pattern observed was a function of cyto-B treatment *per se* rather than an effect of cryopreservation. Incubating these embryos for 4 but not 2 h without cyto-B was sufficient to allow recovery of a normal actin filament pattern. Thus, to properly assess the end effect of cyto-B treatment on the cytoskeleton, embryos should be incubated for around 4 h after thawing. It therefore remains to be determined if cyto-B treated horse embryos are able to repolymerise their actin filaments after cryopreservation, although the fact that pregnancies have resulted from cyto-B treated frozen-thawed embryos (Maclellan *et al.* 2002) indicates that recovery is possible. With regard to the efficacy of cyto-B treatment, the mottled actin pattern observed was strikingly different to the complete dispersal of the cytoskeleton seen in pig embryos treated identically (Dobrinsky *et al.* 2000). This suggests that the cyto-B treatment used may be insufficient for complete actin filament depolymerisation in horse blastocysts.

While trypsin pre-treatment appeared to improve the cytoarchitecture of frozen

blastocysts, it also resulted in a stickier, difficult-to-handle capsule. Given that the capsule appears to be essential to conceptus survival *in vivo* (Stout *et al.* 1997), it is speculated that the poor pregnancy rates reported recently for trypsin-treated frozen embryos (Legrand *et al.* 2002; Maclellan *et al.* 2002) may have resulted from loss of the altered capsule during transfer.

In conclusion, freezing/thawing disrupts the cytoarchitecture of equine embryos, particularly those >300 µm in diameter. Pre-treating larger blastocysts with trypsin or cyto-B appears to reduce the rate of cell death and, in the case of at least trypsin, reduce actin cytoskeleton disruption.

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# SESSION 6:

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## Capacitation of sperm from various species

***Chairman:***

***Mats Troedsson***



# BICARBONATE DEPENDENT CAPACITATION OF MAMMALIAN SPERM CELLS: A COMPARATIVE OVERVIEW

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## INTRODUCTION

This paper deals with the effects of bicarbonate induced signalling pathways on the plasma membrane lipid organisation, downstream protein signalling, and their role in sperm-egg interactions. It also provides an overview of results indicating that the bicarbonate responses are not related with cell death or apoptosis. Data will be presented showing that only the sperm cells that functionally completed maturation in the epididymis are sensitive to bicarbonate, whereas, immature sperm fail to respond to this physiological challenge. Therefore, it is important to analyse selectively the responsive sperm subpopulations when studying sperm capacitation. Finally, data will be presented indicating that bicarbonate induced signalling responses differ within the diverse sperm structures (eg the tail versus the head). Dissecting sperm structures and signalling areas from each other deserves more attention in sperm capacitation research. The presented data are obtained from a variety of animal species and only in part from stallion sperm. However, despite some species to species variations (most notably in kinetics rather than sequence of events), the basic bicarbonate mediated sperm responses are similar in most Eutherian species including the equines. The authors' aim is to provide a comparative experimental overview in bicarbonate mediated sperm capacitation. It is hoped that the provided information will enable better understanding of the complex biochemical nature of the process of bicarbonate involvement in mammalian sperm capacitation.

## BICARBONATE AND PROTEIN KINASE A ACTIVATION

During the fertilisation process, 2 fusion events must take place in the sperm cell: exocytosis of the

secretory granule known as the acrosome, which overlies the anterior portion of the condensed nucleus (head), and fusion of the sperm cell with the oocyte leading to fertilisation. Such fusion events imply that at the time of fertilisation, the sperm plasma membrane must be in a metastable fusible state. During spermiogenesis, sperm maturation, and storage, however, the spermatozoon must remain stable. Thus a preparatory process of membrane destabilisation must take place following the sperm's emergence from the female genital tract and prior to its encountering the egg. This process is known in mammals as capacitation (for review see Flesch and Gadella 2000). *In vivo*, capacitation takes place in the female reproductive tract, but it can be induced *in vitro* by incubation of the spermatozoa in specially developed media. The component that appears particularly responsible for inducing capacitation in these media is bicarbonate, which acts by stimulating a special form of a soluble adenylyl cyclase (sAC) abundant in sperm (Litvin *et al.* 2003). The resultant increased levels of cAMP activate a protein kinase A (PKA)-dependent protein phosphorylation cascade that is operative extremely rapidly after initiation of the bicarbonate challenge (within minutes, Harrison and Miller 2000). It is most likely that the bicarbonate sensing sAC form is also involved in sperm capacitation *in vivo*, in the oviduct, with relatively high bicarbonate levels (approximately 20 mM) compared to the relatively low levels of bicarbonate in sperm containing fluids from the epididymis or in the ejaculate (<1 mM).

## BICARBONATE AND MEMBRANE FLUIDITY

Bicarbonate induced a rapid increase in merocyanin fluorescence indicating that the phospholipid packing of the sperm plasma

membrane became disordered by this treatment. This disordering causes higher membrane fluidity and enables intercalation of merocyanin into the hydrophobic core of the sperm plasma membrane. The bicarbonate response is relatively fast (<10 min) and involves the sAC/cAMP/PKA signalling pathway (Harrison and Miller 2000). Only a subpopulation of live sperm cells showed the merocyanin response whereas 40–80% of the intact cells showed no response. Interestingly, immature sperm cells (containing cytoplasmic droplets) did not show the bicarbonate induced merocyanin response, indicating that high merocyanin staining is dependent on complete epididymal maturation (Flesch *et al.* 2001a). Similar results were found for bicarbonate challenged stallion sperm although kinetics were considerably slower (Rathi *et al.* 2001). In equine and human sperm the proportion of cells with the merocyanin 540 response correlated very well with the proportion of cells with chlorotetra cyclin changes that were characteristic for sperm capacitation (Rathi *et al.* 2001; de Vries *et al.* 2003). Again merocyanin responses as well as capacitation-specific changes in chlortetracyclin staining only took place on mature sperm cells and were absent in cytoplasmic droplet bearing sperm cells.

### **BICARBONATE AND PHOSPHOLIPID ASYMMETRY**

The increased membrane fluidity (detected as increase in merocyanin staining) may be linked to an altered bilayer distribution of phospholipids. To test this possibility sperm cells were loaded with fluorescent phospholipid analogues and their bilayer distribution was measured (Gadella and Harrison 2000). In bicarbonate depleted cells phosphatidylethanolamine (PE) and phosphatidylserine (PS) analogues were very rapidly sequestered into the inner leaflet of the plasma membrane whereas sphingomyelin (SM) and phosphatidylcholine (PC) analogues remained in the outer lipid leaflet of the sperm plasma membrane (Gadella *et al.* 1999). Bicarbonate induced a dramatic change in transbilayer movements of all of these phospholipid analogues indicating phospholipid scrambling (Gadella and Harrison 2000). In mature sperm cells PS and PE were exclusively exposed in the apical surface area of the head in response to the bicarbonate challenge (Gadella and Harrison 2002). Immature sperm cells

did not show this bicarbonate response but in some cases showed PE or PS staining at cytoplasmic droplets (which was bicarbonate-independent). Bicarbonate induced PE exposure correlated exactly with the merocyanin response (similar subpopulation of cells similar kinetics), and was dependent on the sAC/cAMP/PKA signalling pathway. The exposure of PE and PS was not related to apoptosis as: i) Broad-spectrum caspase inhibitors failed to inhibit the bicarbonate effect; ii) Signs of mitochondrial or DNA damage (typical for cell apoptosis) were not detected after bicarbonate stimulation of sperm (Gadella and Harrison 2002); iii) Effector caspases were detected in human ejaculates but only appeared on cytoplasmic droplets of immature sperm cells (explaining the bicarbonate independent exposure of PE and PS on these structures) or on contaminating somatic cells. Active and inactive caspase forms were absent from functional matured sperm cells (de Vries *et al.* 2003). Recent data also indicated that human sperm cells do contain a testis-specific 25 kDa isoform of phospholipid scramblase. Current research focusses on the regulation of the activity of this enzyme that is thought to be responsible for the collapse of lipid asymmetry. Interestingly, scramblase appears to be located over the entire sperm surface while PE and PS exposure is limited to the apical sperm area (de Vries *et al.* 2003). This may indicate that bicarbonate elicits signalling responses that differ between the diverse structures: in the apical side of the sperm head it leads to phospholipid scrambling, in the tail it leads to tyrosine phosphorylation and thus induces hypermotility (see section on bicarbonate and tyrosine phosphorylation).

### **BICARBONATE AND CHOLESTEROL**

The lateral distribution of cholesterol can be monitored by labelling sperm cells with filipin, a fluorescent antibiotic that complexes with free sterols. In the absence of bicarbonate the entire sperm surface of the sperm head showed filipin labelling. However, bicarbonate (without albumin) induced a shift of these complexes to the apical membrane area and a clearance at the equatorial and post acrosomal head area (Flesch *et al.* 2001a) that again depended on sAC/cAMP/PKA signal transduction pathway. Moreover, the lateral redistribution of cholesterol was only found in merocyanin positive cells. In porcine sperm cholesterol redistribution occurred at much slower rates (>90 min) when compared to the membrane



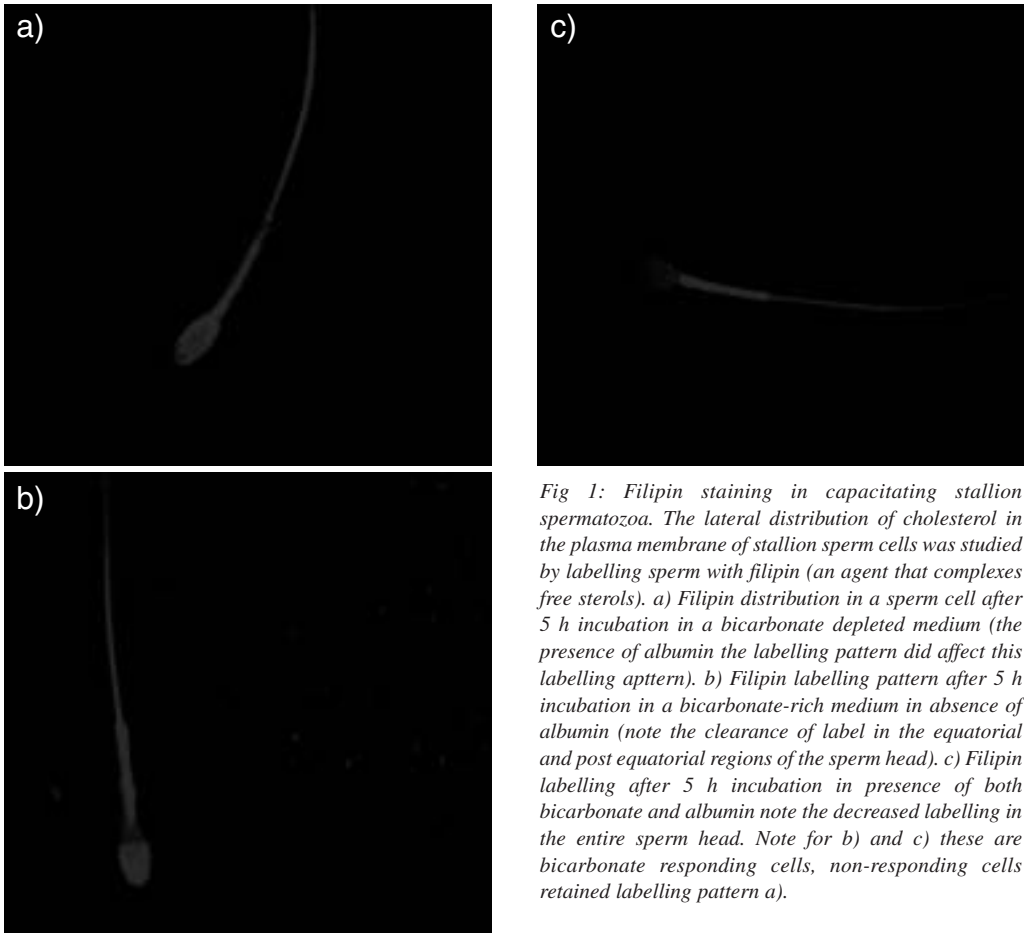


Fig 1: Filipin staining in capacitating stallion spermatozoa. The lateral distribution of cholesterol in the plasma membrane of stallion sperm cells was studied by labelling sperm with filipin (an agent that complexes free sterols). a) Filipin distribution in a sperm cell after 5 h incubation in a bicarbonate depleted medium (the presence of albumin the labelling pattern did affect this labelling apttern). b) Filipin labelling pattern after 5 h incubation in a bicarbonate-rich medium in absence of albumin (note the clearance of label in the equatorial and post equatorial regions of the sperm head). c) Filipin labelling after 5 h incubation in presence of both bicarbonate and albumin note the decreased labelling in the entire sperm head. Note for b) and c) these are bicarbonate responding cells, non-responding cells retained labelling pattern a).

fluidity changes (<10 min). In the presence of albumin, cholesterol can be extracted from sperm cells. Filipin labelling and lipid composition analyses of bicarbonate treated sperm cells showed that the cells with the merocyanin response actually were depleted from cholesterol in contrast to non-responding cells that showed overall filipin staining and unaltered cholesterol levels. Similar filipin labelling changes were found on mature equine sperm after a 5 h bicarbonate challenge, and again equine sperm responds with slower kinetics to bicarbonate when compared to porcine sperm; (for labelling see Fig 1). Albumin did not extract other lipids from sperm cells (Flesch *et al.* 2001a). Since cholesterol is highly concentrated in membrane rafts (for review see Zajcowski and Robbins 2002), the effect of bicarbonate on cholesterol redistribution and depletion may well be linked to the induced clustering membrane microdomains into a raft (model proposed by

Flesch *et al.* 2001a) or alternatively induce disruption of membrane rafts in the apical head area (model proposed by Visconti *et al.* 2002). At any rate the altered raft organisation may be the driving force for cholesterol redistribution and depletion. Indeed recent data from our laboratory indicate that media containing bicarbonate and albumin induce a redistribution of the membrane raft marker proteins flotillin and caveolin within the sperm head plasma membrane: an overall punctuated distribution in the anterior acrosomal region shifts (unprimed cells) to a more diffuse but tight apical distribution after induction of capacitation *in vitro* (van Gestel *et al.* 2004).

#### BICARBONATE AND LATERAL POLARITY OF GLYCOLIPIDS

In the mid 90s it was noted that the sperm-specific glycolipid (seminalipid) was actually only present in

the outer lipid leaflet of the sperm plasma membrane in a number of mammalian species including equine sperm (Vos *et al.* 1994). Moreover, this lipid was located in the apical membrane area in freshly ejaculated sperm, and bicarbonate induced its lateral migration to the equatorial surface of the sperm head (Gadella *et al.* 1994, 1995). In these days almost nothing was known about lipid-enriched microdomains in biomembranes. However, with the current understanding of membrane rafts, the authors' would like to bring these old data in a new perspective.

Glycolipids are marker lipids of membrane rafts and probes that bind to the ganglioside GM1 and are routinely used to monitor membrane rafts in somatic cells. Sperm cells are devoid of GM1 but contain high levels of seminolipid. Recent data from our laboratory show seminolipid as a glyco glycerolipid, in contrast to the glycosphingolipids (such as GM1) because seminolipid does not prefer membrane rafts. Seminolipid is virtually absent from the detergent resistant membrane fraction (van Gestel *et al.* 2004). Bicarbonate driven escape of seminolipid from the apical membrane area therefore forms additional evidence for the idea that bicarbonate induces alterations in the organisation of sperm membrane rafts. Seminolipid is believed to stabilise the lipid bilayer of the sperm plasma membrane and may indeed in unprimed cells at the apical head area prevent the acrosome reaction under low bicarbonate levels. After migration to the equatorial segment seminolipid's protective role in the apical head area is diminished and the local membrane destabilisation will render this area fusogenic for the acrosome reaction when triggered by the zona pellucida. In contrast the equatorial segment needs to remain stable for the later fertilisation fusion and indeed this is exactly the surface area where seminolipid is concentrating after capacitation (before the acrosome reaction, Gadella *et al.* 1995). Note that this is also an area where phospholipid scrambling is not induced by bicarbonate.

### **BICARBONATE AND TYROSINE PHOSPHORYLATION**

Bicarbonate induces an increase in tyrosine phosphorylation. In contrast to the lipid rearrangements most pronounced tyrosine

phosphorylation appeared in the sperm tail. New data obtained in bicarbonate stimulated human sperm cells indicated that >98% of the PE exposing cells showed very clear tyrosine phosphorylation on the sperm tail (de Vries *et al.* 2003). Most likely fibrous sheath proteins are phosphorylated and this modification is known to induce hypermotility (Turner *et al.* 1999). Only less than 2% of the non-exposing cells % showed this response. In fact in non-sorted cells in the absence of bicarbonate 12% showed tyrosine phosphorylation and this proportion increased up to approx 43% after a 4 h bicarbonate stimulation period (in both cases similar proportions of cells showed PE exposure). The tyrosine phosphorylation response emerged at a relatively slow rate (> 30 min). Tyrosine phosphorylation can be induced much faster and in absence of bicarbonate by adding pharmaceutical drugs like cAMP analogues, phosphodiesterase inhibitors and cholesterol depletors (like cycloextrin). Despite some other interpretations in the literature (for instance by Visconti *et al.* 1999) these pharmaceutical drugs all act downstream of bicarbonate's principle and initiatory capacitation action: the physiological activation of sAC.

Bicarbonate induced tyrosine phosphorylation is relatively slow in the natural situation as it depends on membrane changes and removal of cholesterol (these changes are indirectly guided by bicarbonate as mentioned in the sections above). Bicarbonate dependent tyrosine phosphorylation was also observed in proteins of the apical plasma membrane (Flesch *et al.* 1999). Interestingly, 2 of these proteins showed affinity for the zona pellucida but only after tyrosine phosphorylation (Flesch *et al.* 2001b). Currently, using proteomic strategies we are identifying these primary zona-binding proteins. Interestingly, zona binding proteins identified so far appear to recover from apical membrane preparations and are also found in purified membrane raft fractions (Flesch *et al.* 1998; van Gestel *et al.* 2004). Therefore, the above described bicarbonate-driven raft dynamics may well relate to induced tyrosine phosphorylation and 'capacitate' the sperm zona binding.

### **BICARBONATE AND THE ACROSOME REACTION**

Bicarbonate signalling is preceding and coupled with progesterone signalling during stallion sperm

capacitation (but also for other mammalian species studied): Progesterone only elicited responses to the bicarbonate responsive sperm cells (by inducing tyrosine kinase and protein kinase C, Rath *et al.* 2003). We believe that progesterone and the sperm's interaction to the zona pellucida are events that are not part of capacitation but the result of this process. Instead of this, progesterone and the zona pellucida are involved in the induction of the acrosome reaction and only succeed in doing this to full bicarbonate-capacitated cells. The zona pellucida of a just ovulated oocyte is probably impregnated with progesterone (present in high concentration in the follicular fluid of the Graafian follicles). Therefore, the effects of progesterone on bicarbonate primed sperm cells may well be relevant for the zona induced acrosome reaction. We may note that under correct *in vitro* capacitation conditions (see the practical consideration section) bicarbonate does not itself induce the acrosome reaction (Gadella and Harrison 2002). Progesterone, the zona pellucida or artificial inducers like calcium ionophore, lysophosphatidylcholine can be used to evoke the transient rise in intracellular  $Ca^{2+}$  in bicarbonate responsive cells in order to induce the acrosomal fusions (probably the lipid changes in responsive cells makes them more vulnerable for these components; Flesch and Gadella 2000). Bicarbonate is simply needed to get the sperm cell to bind to the zona pellucida and to render the apical head plasma membrane fusogenic. Interestingly, sperm cells contain all SNARE members for 20S fusion protein-complex formation and it was shown recently that bicarbonate induces relocation of these members exactly to the area where the acrosome fusions will be initiated (de Vries *et al.* 2004). In fact SNARE redistribution seems to match redistribution of raft marker proteins.

### SOME PRACTICAL CONSIDERATIONS FOR THE SPERMATOLOGIST STUDYING CAPCITATION

i) Sperm capacitation is a lengthy process in which early changes take place as rapidly as 1 min whereas full capacitation is accomplished within hours (approximately 5 h in equines). Therefore, one cannot design a single capacitation assay, and the existing assays discriminate non-responding

from responding cells at different time intervals of capacitation (see Flesch *et al.* 2001a).

ii) Chlortetracyclin is the classical stain used to detect sperm acrosome reaction as well as sperm capacitation. This probe binds in a calcium dependent manner to the surface of sperm cells and this intrinsic molecular property makes chlortetracycline useless for discriminating the calcium dependent and independent pathways leading to capacitation (see Rath *et al.* 2001)

iii) Capacitation *in vitro* is performed at physiological temperature (38°C) in a tube that is placed under a 5%  $CO_2$  humidified atmosphere. It is considered generally that capacitation destabilises sperm cells and indeed cooling bicarbonate-capacitated sperm cells only slightly to 30°C will already result in cell deterioration and false acrosome reactions (see Gadella and Harrison 2000). However, a careful researcher who maintains sperm cells at the physiological incubation conditions can keep the cells alive for hours without notable cell deterioration or acrosome reaction. The major reason why some researchers score acrosome reactions (or disruption) as a result of capacitation is due to manipulation of sperm post capacitation.

iv) In line with this, non-physiological 'inducers of capacitation and acrosome reaction' such as  $Ca^{2+}$ -ionophore, cyclodextrin and lysophosphatidylcholine do not induce capacitation but selectively disrupt membranes in the bicarbonate responsive cells (by virtue of the lipid modifications described). This again results in detection of a false acrosome reaction and will not shed new light on the mechanisms involved in the physiologically induced acrosome reaction.

### CONCLUSION

Capacitation has long been known to involve major modifications of the sperm's plasma membrane, and bicarbonate has been shown to be a key capacitating agent. Our studies have shown that bicarbonate has a primary early role in bringing about the important changes in membrane lipid architecture, and we have identified the initial steps of signalling involved. Although kinetics in the sequence of capacitation responses differs from species to species it appears

that early responses to bicarbonate involve PKA activation, hypermotility, membrane fluidity, and membrane lipid scrambling. On the longer term PKA mediated effects will cause lateral redistribution of lipids and membrane proteins involving dynamic reorganisation of raft microdomains leading to cholesterol reorganisation and depletion. Additional sperm signalling pathways will be activated ultimately leading to the full capacitation of sperm. It is also clear that due to all these membrane modifications the fully capacitated sperm cell obtains enhanced affinity for the zona pellucida and is primed to undergo the agonist induced acrosome reaction: lipid alterations and the concentration of primary zona binding proteins as well SNARE proteins in the apical head membrane are probably responsible for these alterations. It is hoped that this paper will help in obtaining solid experimental data, and that this will serve the research community further in understanding the relation of bicarbonate mediated sperm capacitation and equine fertilisation.

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## MEDIA REQUIREMENTS FOR STALLION SPERM CAPACITATION

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### INTRODUCTION

Capacitation has been defined as the series of molecular changes that ejaculated sperm must undergo to become capable of fertilisation; it has long been known that this process naturally occurs within the reproductive tract of the female (Chang 1951). Recent research has advanced our knowledge in the molecular pathways linked with capacitation in laboratory and domestic species other than the horse (reviewed by Visconti *et al.* 2002). Although these pathways are still the subject of intense study, it is well characterised that incubation of mouse (Visconti *et al.* 1995b), bovine (Galantino-Homer *et al.* 1997), human (Leclerc *et al.* 1996; Osheroff *et al.* 1999), hamster (Devi *et al.* 1999; Visconti *et al.* 1999) and porcine (Tardif *et al.* 2001) sperm in conditions conducive to capacitation results in an increase in cAMP dependent protein tyrosine phosphorylation. Upstream from these events is a release of cholesterol from the sperm membrane that changes its permeability to  $\text{Ca}^{2+}$  and bicarbonate ( $\text{HCO}_3^-$ ), as well as to anions/cations that confer changes in sperm membrane potential (reviewed by Visconti *et al.* 2002).

Since capacitation occurs *in vivo* within the female's oviduct around the time of ovulation, media used for *in vitro* sperm capacitation typically mimic the composition of follicular fluid. Thus it is clearly established that  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and bovine serum albumin (BSA) are required to optimise capacitation conditions in

sperm from laboratory species (Visconti *et al.* 1995a). Bovine serum albumin appears to function as a membrane cholesterol acceptor, since its function may be substituted by chemicals known to induce membrane cholesterol efflux (ie cyclodextrins; Visconti *et al.* 1999). This results in a change in membrane fluidity that may activate channels responsible for  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  influx, both of which may initiate the cAMP-mediated signalling cascade, ultimately leading to an increase in tyrosine residue phosphorylation events (reviewed by Visconti *et al.* 2002).

Incubation conditions conducive to stallion sperm capacitation have not been evaluated critically. Furthermore, little is known about the molecular pathways linked to this process in stallion sperm. One of the problems with research in stallion sperm capacitation is the inconsistency in media, experimental conditions and endpoint evaluations so far reported (Meyers *et al.* 1996; Pommer *et al.* 2002; Rathi *et al.* 2003). Only two reports have addressed the molecular pathways linked to stallion sperm capacitation. Pommer *et al.* (2002) reported that stallion sperm incubated with reagents known to stimulate cAMP dependent pathways underwent significant levels of protein tyrosine phosphorylation. Conversely, a more recent report shows that progesterone-induced acrosome reaction, but not capacitation, is protein tyrosine kinase dependent in stallion sperm (Rathi *et al.* 2003). The slight discrepancy between these 2 reports is probably due to different endpoint evaluation, although this requires further investigation. Therefore, our long-term objectives are: 1) To evaluate media composition and incubation conditions promoting stallion sperm capacitation; and 2) to investigate molecular pathways leading to sperm capacitation in the horse.

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## MATERIALS AND METHODS

### Capacitating media

Two different media are being evaluated in the authors' laboratory: 1) Sperm TALP (sTALP; 100 mM NaCl, 3.1 mM KCl, 0.4 mM MgCl<sub>2</sub>, 10 mM Hepes, 21.6 mM Sodium lactate, 25 mM NaHCO<sub>3</sub>, 1 mM sodium pyruvate, 0.3 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>); and 2) Biggers, Whitten and Whittingham (BWW; 103.85 mM NaCl, 4.78 mM KCl, 2.44 mM MgSO<sub>4</sub>, 1.17mM KH<sub>2</sub>PO<sub>4</sub>, 5.55 mM glucose, 0.25 mM sodium pyruvate, 1.17 mM calcium lactate, 21.55 mM sodium lactate). Media were used with and without supplementation of 7 mg/ml BSA and/or 1 mM dbcAMP + 100 μM IBMX.

### Sperm processing and incubation

Semen was collected from one stallion of proven fertility. After collection, semen was diluted 1:1 with the corresponding medium containing 20 mM Hepes without NaHCO<sub>3</sub>. Extended semen was centrifuged at 100 g for 5 min to remove debris and dead sperm; then, the supernatant containing mostly motile sperm was centrifuged at 300 g for 12 min. Pellets were resuspended with the corresponding capacitating media at 25 × 10<sup>6</sup> sperm/ml and incubated in Eppendorf tubes at 38°C in a 5% CO<sub>2</sub> atmosphere.

### Tyrosine phosphorylation assays

These were performed as described in Visconti *et al.* (1995a). Briefly, incubated sperm were washed in PBS, resuspended in sample buffer without mercaptoethanol and boiled for 5 min. After centrifugation at 5,000 g for 3 min, the supernatant was collected, boiled in the presence of 5% 2-mercaptoethanol for 5 min, and then subjected to SDS-PAGE in 12% gels. After electrophoretic transfer of proteins to Immobilon P (30V, 1 h, 4°C), immunodetection of proteins was performed using a monoclonal antibody against phosphotyrosine residues (clone 4G10; UBI) and enhanced chemiluminescence detection using an ECL kit (Amersham Corp).

### Experiments

In Experiment 1, tyrosine phosphorylation assays were compared for sperm incubated in spTALP or

BWW with and without BSA. Sperm were incubated with BSA for 0, 2 and 4 h and without BSA for 2 and 4 h.

In Experiment 2, sperm motility and tyrosine phosphorylation assays were compared for sperm incubated in BWW with and without BSA and/or dbcAMP+IBMX.

## PRELIMINARY RESULTS AND CONCLUSIONS

Two replicates for each experiment have been completed. In Experiment 1, incubation of sperm in media without BSA resulted in decreased levels of protein phosphorylation. Bands of tyrosine phosphorylated proteins were more evident in samples incubated in BWW+BSA than sperm in TALP+BSA. Tyrosine phosphorylation was especially evidenced in a ≈65 KD protein, increasing from 2 to 4 h; this was not seen for non-incubated sperm or sperm incubated without BSA. In Experiment 2, sperm incubated in BWW with and without cAMP, also underwent a time-dependent increase in tyrosine phosphorylation, peaking between 2 and 4 h. Furthermore, cAMP compensated for BSA absence.

These findings suggest that a cAMP dependent pathway is involved in changes leading to stallion sperm capacitation, and that BSA and probably NaHCO<sub>3</sub> in the medium are required to achieve phosphorylation in tyrosine residues. Further media requirements and narrowing of conducive molecular pathways require additional investigation.

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# SESSION 7:

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## Transported testicles and freezing epididymal sperm

***Chairman:***

***Marco Alvarenga***



# THE POTENTIAL USE OF EPIDIDYMAL SPERM FROM BULLS AND STALLIONS FOR ASSISTED FERTILISATION

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## INTRODUCTION

Interest in using epididymal sperm from domestic and non-domestic species is increasing because of the inherent value of many individuals who die before making a contribution to the genetics of a their own animal population. Utilisation of epididymal sperm is also important for endangered species to insure preservation of genetic variability within closed captive populations. The ability to use epididymal sperm from males culled in zoos, parks, or in the wild, could preserve their 'wild' DNA for introduction into captive populations.

Research is now underway to determine the feasibility of collecting viable epididymal sperm from various domestic and non-domestic species. Studies have been conducted in this laboratory in an attempt to determine the time constraints for collecting viable epididymal sperm from domestic (rat, cat, dog, bull, buck and stallion) as well as exotic species (Cape buffalo, zebra and various antelope species). Epididymal sperm has also been collected in these studies at 0, 1, 2 and 3 days post mortem and remained relatively motile.

Livestock producers with economically valuable breeding males have frequently asked for assistance from professionals when their animals can no longer mate naturally or die before completing their own breeding schedule. Unfortunately, offspring from artificial insemination (AI) and *in vitro* fertilisation (IVF) using sperm collected from the epididymis has only been reported in several farm animals, including the goat (IVF) and the horse (AI). Cattle offspring born from AI with epididymal sperm have not been verified in scientific reports. To date, the primary limiting factors to this approach include: the total volume of sperm harvested from epididymides

from a single male is often low for in-field AI and methods need to be developed to prepare harvested sperm for success in AI, IVF or intracytoplasmic sperm injection (ICSI). Offspring from ICSI have been reported in cattle (Goto *et al.* 1990; Hamano *et al.* 1999) and horses (Cochran *et al.* 1998; McKinnon *et al.* 1998). Currently, successful IVF and/or ICSI procedures are not available to the livestock producers for their farm animals. The objective of this paper is to summarise some of the recent studies conducted at this laboratory on bull and stallion epididymal sperm in an effort to reduce the limitations of this assisted reproductive technology.

## STORING BULL AND STALLION EPIDIDYMAL SPERM IN THE TESTIS AT 4°C

### *Bovine epididymal sperm stored in the testis at 4°C for 24, 48, 72 and 96 h*

The objective of the first study was to determine the 'window of opportunity' for collection of bovine epididymal sperm from testes stored under refrigeration over different periods of time. Bovine testicles were collected as pairs (n=30) from mature beef bulls at an abattoir and transported overnight to the laboratory at 4°C. Upon arrival, one of the testicles of a pair was stored at 4°C for 48, 72 or 96 h, while the other testicle (control) was immediately processed (24 h time point). Percent progressively motile (PPM) and percent live (PL) sperm, evaluated by Eosin-B/Fast Green stain, were analysed by the same technician for each sample at each time period (24, 48, 72 and 48 h).

Results from this study indicate that PPM for epididymal sperm had a significant decline from

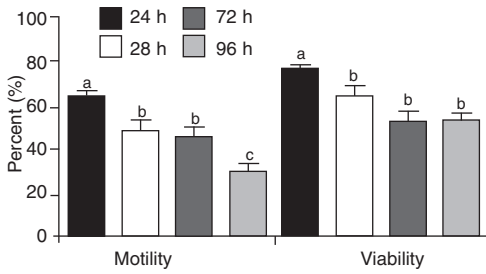


Fig 1: Mean percent ( $\pm$ SE) progressively motile and viable bovine epididymal sperm after storage in the testicle at 4°C for 24, 48, 72 and 96 h post mortem. <sup>a,b,c</sup>Columns with different superscripts are significantly different ( $P < 0.05$ ).

24 to 48 h and a significant decline from 72 to 96 h (65, 49, 46 and 30% mean PPM, respectively) (Fig 1). Live sperm were obtained from samples stored throughout the experiment as indicated by mean PL for 24, 48, 72 and 96 h of 77, 65, 53 and 54%, respectively, with a significant decline from 24 to 48 h. Epididymal sperm collected from testes stored at 4°C retained acceptable motility as late as 72 h and gave all indications of maintaining membrane integrity up to 96 h post mortem.

**Equine epididymal sperm stored in the testis at 4°C for 24, 48, 72 and 96 h**

A series of studies are now underway in this laboratory to evaluate if cooled epididymal sperm from stallions can be harvested post castration and used to produce a live foal. The objective of this study was again to identify a ‘window of opportunity’, as with that for bulls in the first study, for collection of epididymal sperm that could be used for subsequent *in vitro* production of embryos. Equine testicles were collected as pairs (n=17) from mature stallions at an abattoir and transported overnight to the laboratory at 4°C. Upon arrival, one of the testicles of a pair was stored at 4°C for 48, 72 or 96 h while the other testicle (control) was immediately processed and the epididymal sperm used as the 24 h treatment group (24 h time point).

Results indicate that PPM for epididymal sperm had a significant decline from both 24 and 48 to 72 through 96 h (57, 42, 34 and 32% mean PPM, respectively; Fig 2). Potentially viable sperm were obtained from all samples stored throughout the experiment, as indicated by mean PL for 24, 48, 72 and 96 h of 75, 71, 68 and 65%,

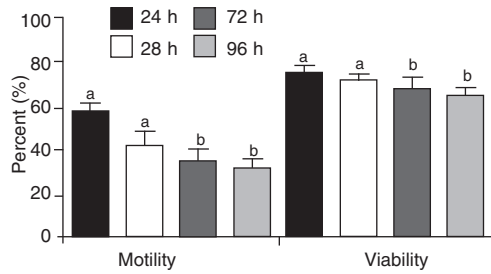


Fig 2: Mean percent ( $\pm$ SEM) progressively motile and viable equine epididymal sperm after storage in the testicle at 4°C for 24, 48, 72 and 96 h post mortem. <sup>a,b</sup>Columns with different superscripts are significantly different ( $P < 0.05$ ; from James et al. 2002).

respectively. There was a significant decline in viable sperm from the 48 h to the 72 h time points. Epididymal sperm collected from the testis stored at 4°C, as with bull, appear to retain adequate motility and maintained membrane integrity up to 96 h post mortem.

It is interesting to note that the percent progressive motility pattern for epididymal sperm after storage in the testicle at 4°C for 24, 48, 72 and 96 h of bulls was similar to the pattern noted for stallions. Correspondingly, when membrane integrity was evaluated on epididymal sperm after storage in the testicle at 4°C for 24, 48, 72 and 96 h for bulls and stallions, the pattern was less similar for sperm viability over time points. The percent of stallion epididymal sperm with intact plasma membranes was greater over a longer period of time when the sperm remained in the epididymides of the testes of stallions compared with sperm remaining in the testes of bulls.

**IN VITRO FERTILISATION WITH EPIDIDYMAL SPERM**

Over the years, successful procedures have been developed for *in vitro* maturation (IVM) and IVF of oocytes from cattle, sheep, goats and swine. This technology has allowed the production of a large number of embryos in the laboratory from frozen-thawed sperm and oocytes harvested from cattle ovaries obtained from an abattoir.

For over 2 decades researchers have been fine-tuning IVF procedures for cattle. The IVF technology has only been commercially available at embryo transplant stations for dairy and beef cattle producers since the early 1990s. From good

quality oocytes harvested from bovine ovaries, one would expect a 90% IVM rate and >85% fertilisation and cleavage rates. It is expected that 35–50% of the resulting IVF-derived embryos reach the morula and blastocyst stage of development for transfer to recipient females on Day 6, 7 or 8 of the oestrous cycle. The pregnancy success rate for good quality IVF-derived embryos is expected to range from 50–65%. Although viable healthy calves have been successfully produced from frozen-thawed IVF-derived embryos, the success rate is generally lower if IVF-derived frozen-thawed embryos are transferred to recipient females. To the authors' knowledge, IVF-derived offspring resulting from either fresh or frozen-thawed bovine epididymal sperm has not been verified in the scientific literature to date.

Several IVF-derived foals have been reported in France over a decade ago. During the last decade various research groups have been working to further develop and refine this *in vitro* procedure. More recently good success has been reported on *in vitro* maturation of abattoir-derived equine oocytes, which may offer new hope for developing an effective IVF procedure for the horse in the future.

#### ***IVF with cryopreserved bovine epididymal sperm stored in the testis at 4°C for 24 and 48 h***

The objective of this study was to evaluate if cryopreserved bovine epididymal sperm could be used for the production of IVF-derived cattle embryos. Cryopreserved samples of bovine epididymal sperm were collected during the first experiment. Samples from 3 beef bulls with a pre-freeze PPM of >50% from testes stored at 4°C for either 24 h or 48 h prior to freezing were used to determine feasibility of using frozen-thawed epididymal sperm for IVF. Bovine oocytes and sperm from these 3 post mortem bulls and frozen-thawed ejaculated sperm from one control bull of known IVF fertility were subjected to a standard bovine laboratory IVF protocol.

Bovine oocytes were collected from the abattoir and shipped overnight at 39°C in maturation medium. After 22 h of IVM, the oocytes (n=615) were prepared for a standard bovine IVF procedure. Sperm samples were thawed for 1 min in a water bath and placed in 3 ml of Sperm TL medium. Sperm were washed and centrifuged to remove cryoprotective agents. After

centrifugation, each sperm pellet was re-suspended and 50 µl of the sperm mixture was placed in 50 µl microdroplet of fertilisation medium containing 15 to 20 ova each. *In vitro* embryo development was then assessed at 24 h intervals.

In summary, the ejaculated sperm used from the bull of known IVF fertility had a cleavage rate of 83% and blastocyst development rate of 35%, both within the normal range for bovine IVF laboratories. In contrast, epididymal sperm from testes stored for 24 h at 4°C prior to freezing had a cleavage of 26% and a blastocyst development rate of only 6%, both considerably lower ( $P<0.05$ ) than that of the control ejaculated sperm used for IVF. In addition, epididymal sperm from testes stored for 48 h at 4°C prior to freezing had a cleavage rate of 23% and a blastocyst development rate that was even lower for epididymal sperm stored in the testis for 24 h at <2%.

These results indicate that frozen-thawed epididymal sperm from testes stored for 24 h at 4°C prior to freezing can produce IVF-derived blastocysts for embryo transfer. Although there were a few blastocysts that developed from epididymal sperm from testes stored for 48 h at 4°C prior to freezing, the rate was so low (<2%) in this study, this protocol would have poor odds of producing an IVF offspring.

#### ***IVF-derived embryos using cryopreserved ejaculated and epididymal bovine sperm***

In the fourth is this series of studies, cryopreserved bovine epididymal sperm were used to determine if blastocysts could be produced using standard IVF procedures. Sperm were aspirated manually from the cauda epididymidis of each testis with a needle and 10 ml plastic syringe from a sedated, mature, fertile dairy bull. Semen was then extended and cryopreserved in Brackett-Oliphant medium-based egg yolk buffer. Bovine oocytes were obtained from a commercial source and allowed to mature for 23 h *in vitro* before insemination using a standard bovine heparin-based IVF protocol. All oocytes (n=701) were inseminated on the same day with either frozen-thawed ejaculated sperm previously frozen from the same dairy bull or with his frozen-thawed aspirated epididymal sperm.

Using a 2 × 2 factorial arrangement, the ejaculated sperm and the epididymal sperm were treated with either heparin or not treated with heparin prior to IVF. After a 12 h insemination

**TABLE 1: Using frozen-thawed epididymal or ejaculated sperm from the same bull to produce IVF-derived bovine embryos *in vitro***

Sperm origin + Sperm treatment	Oocytes (n)	Cleavage no. (%)	8-cell no. (%)	Blastocysts no. (%)
Ejaculated + Heparin	181	113 (62%)	56 (32%)	39 (21%)
Ejaculated + No Heparin	158	67 (42%)	35 (22%)	25 (16%)
Epididymal + Heparin	202	45 (22%)	18 (9%)	3 (1%)
Epididymal + No Heparin	160	29 (18%)	7 (4%)	2 (1%)

**TABLE 2: IVF-derived bovine embryos from cooled epididymal and ejaculated bull sperm**

Treatment group	No. of oocytes	No. cleaved (%)	Blastocysts (%)	HBLST* (%)
Epididymal sperm + IVF	307	277 (90)	113 (37)	70 (23) <sup>1</sup>
Ejaculated sperm + IVF	288	259 (90)	92 (32)	77 (27)

\*HBLST = hatched blastocyst.

<sup>1</sup>The number and percent does not included the embryos selected for transfer (n=28).

interval, embryos were cultured in CR1aa medium in a 5% CO<sub>2</sub> in humidified air at 39°C for 7 or 8 days.

The results from this study indicate that cryopreserved bovine epididymal sperm can produce IVF-derived blastocysts but at much lower rates than that of cryopreserved ejaculated sperm (Table 1). The cleavage and blastocyst development rates from frozen-thawed epididymal sperm in this study had a similar *in vitro* embryo production pattern to those from frozen-thawed epididymal sperm from bovine testes stored for both 24 h and 48 h at 4°C prior to freezing and then used for IVF in Experiment III. These results also suggest that cryopreserved ejaculated sperm not treated with heparin prior to IVF can successfully produce IVF-derived bovine embryos.

#### ***Pregnancies from IVF with epididymal sperm stored in the testis at 4°C for 24 h***

The objective of the most recent study was to determine if pregnancies could be established using cooled bovine epididymal sperm for IVF. Pairs of testicles from beef bulls were obtained from a commercial source (4°C) and transported overnight to the laboratory. After arriving at this station, sperm were harvested from the cooled testes (n=6) at 24 h post mortem. The cauda

epididymides were minced in Brackett-Oliphant medium and allowed to incubate at 39°C for 20 min in a 5% CO<sub>2</sub> in humidified air.

Oocytes obtained from a commercial source were also shipped to the laboratory via overnight express service. After arrival, oocytes (n=595) were randomly allotted to 2 treatment groups as follows: Treatment A, cooled epididymal sperm was used in a standard laboratory IVF procedure and in Treatment B, frozen-thawed ejaculated sperm from a mature bull of known IVF fertility (control) was used in a standard IVF procedure. A 50 µl dose of the sperm mixture at a concentration of 1 × 10<sup>6</sup> sperm/ml was placed into 50 µl insemination microdroplets containing oocytes for 18 h of incubation. The developing embryos were cultured in CR1aa medium in a 5% CO<sub>2</sub> in humidified air at 39°C for 7 days.

In summary, no significant difference was detected in the number of Day 7 blastocysts produced from IVF between the epididymal and the ejaculated sperm treatment groups (Table 2). Blastocysts were non-surgically transferred to synchronized beef cows on Days 6 to 8 post oestrus. Five singleton pregnancies were detected in 11 recipient females (45%) and verified by heartbeats using ultrasonography at 30, 60 and 90 days of pregnancy. All 5 of the recipient females remain pregnant at this time (>260 days).

The results from this study indicate that oocytes inseminated *in vitro* with bovine epididymal sperm stored in testes at 4°C for 24 h can produce viable pregnancies. With refinement of the sperm preparation protocol, it now appears that epididymal sperm stored in bovine testes for 24 h at 4°C can be considered when attempting to propagate germplasm from post mortem bulls.

## SUMMARY

It has been proposed that the ability to collect and cool or cryopreserve sperm from the epididymis and use it successfully for AI and IVF may prove to be a useful technique for preserving genes of valuable males. There has been success with this approach although primarily in laboratory animals, several farm animals (Barker and Gandier 1957; Blash *et al.* 2000) and humans. After conducting a series of studies on evaluating epididymal sperm from bulls and stallions in this laboratory, it is clear that many of the limitations relating to this approach to assisted reproductive technology will be a challenge in the years to come.

In first 2 experiments, it was noted that both bovine and equine epididymal sperm collected from testes stored for 24, 48, 72 or 96 h at 4°C remain relatively motile as late as 72 h and membrane integrity was maintained up to 96 h post mortem, especially with stallion sperm. However, when the same experimental approach was used in a third experiment and the epididymal sperm from bulls was subjected to standard IVF procedure, the frozen-thawed ejaculated sperm used from the bull had a cleavage rate of 83% and blastocyst development rate of 35% (both within the normal range for IVF laboratories) and compared with epididymal sperm from testes stored for 24 h at 4°C had a cleavage of 26% and a blastocyst development rate of 6%, both considerably lower than that of the control ejaculated sperm used for IVF. Also, epididymal sperm from testes stored for 48 h at 4°C had a cleavage rate of only 23% and blastocyst development rate that was even lower for epididymal sperm stored in the testis for 24 h at less than 2%.

When epididymal sperm aspirated from the testes of a fertile live bull was frozen and later thawed and used with a standard IVF procedure in a fourth experiment, the cleavage and blastocyst

development rates from frozen-thawed epididymal sperm was markedly reduced from frozen-thawed ejaculated sperm from the same bull. The frozen-thawed epididymal sperm aspirated from the bull had a similar pattern to those from epididymal sperm from testes stored for both 24 and 48 h at 4°C prior to freezing followed IVF.

These results indicate that bovine epididymal sperm from testes stored for 24 h at 4°C can produce IVF-derived blastocysts for transfer and, therefore, should not be totally overlooked as offering hope in the effort to produce offspring from genetically valuable bulls. Although there were a few blastocysts that developed from epididymal sperm from testes stored for 48 h at 4°C, the rates was so low in this study, this protocol would have poor odds of producing an IVF offspring.

The results from the most recent study with epididymal sperm collected from testis stored at for 24 h at 4°C prior to IVF indicate that oocytes inseminated *in vitro* with bovine epididymal sperm can produce viable pregnancies. Five of 11 recipient cows (45%) are now carrying viable IVF-derived pregnancies at >260 days of gestation. With refinement of the sperm preparation protocol, it now appears that cooled epididymal sperm stored in bovine testes for 24 h at 4°C can be considered when attempting to propagate germplasm from post mortem bulls.

Although epididymal sperm collected from bovine testes stored at for 24 or 48 h at 4°C prior to freezing produced lower IVF-derived embryo development rates compared with cryopreserved ejaculated sperm, it should be noted that fresh, cooled and cryopreserved epididymal sperm has been used successfully in several species for embryo production. Thus, the collection and storage of epididymal sperm should not be overlooked for use in ICSI when attempting to salvage germplasm from genetically valuable bulls.

Although several IVF-derived foals have been reported in France over a decade ago (Palmer *et al.* 1991) this *in vitro* reproductive technology is presently not available for use by horse producers. In recent years, good success has been reported using ejaculated sperm and ICSI to produce good quality blastocysts and live offspring from *in vitro* and *in vivo* collected equine oocytes (Squires *et al.* 1996; Cochran *et al.* 1998; McKinnon *et al.* 1998). Results from our study show that equine epididymal sperm collected from the testis stored at 4°C appears to

retain adequate motility and maintains membrane integrity up to 96 h post mortem. Efforts are now underway to use epididymal sperm collected from testes stored for 24 h at 4°C and use ICSI in an effort to produce live foals.

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# FREEZING EPIDIDYMAL SPERM FROM TRANSPORTED TESTICLES

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## INTRODUCTION

The stallion's cauda epididymides should contain sperm capable of fertilisation, thus providing a significant source of salvageable genetic material in the case of elected castration or death. Successful cryopreservation of epididymal sperm is well documented (Barker and Grandier 1957; Morris *et al.* 2002). Unfortunately, the timing and often location of the castration or post mortem tissue harvest does not lend itself well to sperm collection and preservation. For these reasons, researchers have been developing methods to: a) successfully preserve and transport the testicular and epididymal tissue; b) collect and cryopreserve the sperm cells; and c) effectively inseminate mares with the cryopreserved epididymal sperm. These methods are not independent as each is an integral part of the other. Although storage and transport techniques have not yet been maximised, most research demonstrates that transport at 4–5°C for 24 h appears to result in sperm motility similar to that of cells processed immediately both neat and following conventional cryopreservation (Bruemmer *et al.* 2002; Janes *et al.* 2002). The factors that influence survival of frozen epididymal sperm also appear to be similar to those of ejaculated sperm, with one potential exception. Currently, reports indicate that stallion differences and initial sperm quality are the major factors effecting survival. The potential difference between epididymal and ejaculated sperm may be in the presence and quantity of seminal plasma or more specifically seminal plasma proteins.

## MATERIALS AND METHODS

### *Sperm treatment*

Semen from 9 light horse breed stallions ranging in age from 3–17 years of age was used for this

experiment. Ejaculated semen from each stallion was used as its own control. Semen from stallions was collected at least twice within 72 h of the control collection. Ejaculated semen was divided into 2 aliquots and frozen in one of 2 commercially available extenders MFR5 or L-EDTA (Animal Reproduction Systems, California, USA) and frozen according to standard protocol. Each stallion was then castrated. Semen from one testicle was used as the immediate control while the opposite testicle was cooled and stored for 24 h prior to semen harvest and freezing. Sperm from the immediate (0 h) and cooled (24 h) epididymal collections were divided into 4 aliquots and frozen in either extender and with or without seminal plasma (SP; 0.5 ml/1.6 × 10<sup>9</sup> cells) resulting in 8 treatments. As follows: 1) 0 h L-EDTA –SP; 2) 0 h FR5 –SP; 3) 0 h L-EDTA +SP; 4) 0 h FR5 +SP; 5) 24 h L-EDTA – SP; 6) 24 h FR5 –SP; 7) 24 h L-EDTA +SP; and 8) 24 h FR5 +SP.

Sperm cells were collected and frozen following dissection and retrograde flushing of the cauda epididymis as previously described (Bruemmer *et al.* 2002). Cells were packaged at 400 × 10<sup>6</sup>/ml in 0.5 ml PVC straws and stored in liquid nitrogen until analysis.

### *Post thaw analysis*

Motility parameters were determined via computer assisted sperm analysis at the time of collection, immediately post thaw and 30 min post thaw. Additionally both viability and acrosome integrity were assessed by way of flow cytometry.

## RESULTS

Motility parameters of frozen-thawed sperm (both ejaculated and epididymal) were consistently lower than those measured in their fresh ejaculated controls. These post thaw parameters

were not different from each other, however. Both percent of total and progressively motile cells remained constant regardless of time of collection, extender used or the presence or absence of seminal plasma. Likewise, sperm membrane integrity and acrosomal integrity were similar across treatments.

## CONCLUSION

Sperm cells can be harvested from cauda epididymides after a 24 h cooling a storage period. This procedure should allow for preservation of certain genetics of particularly valuable animals in situations of catastrophic injury or death.

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# SESSION 8:

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## Semen freezing

**Chairman:**

***Barry Ball***



# FUNDAMENTAL CRYOBIOLOGY

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## INTRODUCTION

Understanding the biophysics of low temperature effects on cellular systems and utilising this knowledge to develop improved cryopreservation protocols are the principal aims of fundamental cryobiology. The 3 parameters available for control: extracellular solute concentration, its constituents of permeating and non-permeating solutes, and the rate of temperature change (both cooling and warming), give cryobiologists powerful tools to regulate the physical/chemical conditions inside and outside of the cell, and allow an adequately prepared investigator to develop optimised protocols to cryopreserve specific cell types. Many investigators, however, avoid the sometimes tedious process of determining cellular permeabilities at different temperatures, and instead try to empirically obtain successful freezing protocols. This methodology has proven

successful for several cell types but, often, severe limitations are quickly encountered. For example, an empirically derived methodology for the cryopreservation of mouse sperm was developed by Tada *et al.* (1990) which seems effective for certain strains but, for more sensitive inbred strains, no reasonably efficient protocol has been developed. This highlights an important complication of cryobiology: the variability in successful freezing protocols lies not only in different cell types (Fig 1), but in the same cell types from different species. Thus a thorough understanding of the fundamental cryobiological ramifications of cooling a cell below physiological temperatures is necessary. Overcoming the obstacles to achieve this, however, is often quite difficult.

There are 2 basic sources of cell damage (intracellular ice formation and solute damage) that must be avoided when freezing cells and 2

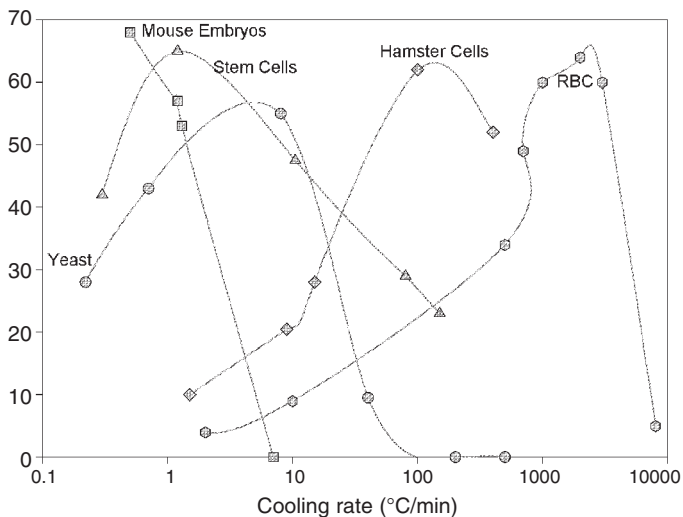


Fig 1: Freezing survival of different cell types at various cooling rates (redrawn from Mazur 1977).

methodological tools with which to avoid them (Mazur *et al.* 1972). Of the 2 sources, intracellular ice formation is the best understood and therefore the one for which we have the best tools to prevent. The second is less well understood and more subtle but, in principle, just as problematic. For example, during freezing the osmotic stresses placed on a cell and its membrane extend well beyond normal physiological conditions, exposing a cell to media either greatly hypertonic or greatly hypotonic, sometimes forcing the cell to shrink or swell far beyond its limits (osmotic tolerance limits). Also, the effect of specific chemical toxicity of high concentrations of cryoprotective agents such as glycerol or DMSO or extracellular electrolytes must be addressed.

The damage caused by intracellular ice and solute effects can be alleviated by: 1) controlling the initial composition of the freezing media; 2) controlling the rates of cooling and warming, which can vary from less than 0.5°C/min to over 10,000°C/min; and 3) controlling the temperature at which the cells are plunged into liquid nitrogen. The challenge lies both in the differences in cryobiological characteristics among cell types and the interactions among the various steps in the cryopreservation process (eg CPA type and concentration, cooling and warming rates). For example, when cryopreserving cells, the choice of CPA and CPA concentration affect the rate at which cells can be frozen successfully. This determines the temperature at which the cells should be plunged into liquid nitrogen, which, in turn, determines the optimal warming rate.

It is likely that, left to purely empirical study, little progress in this field would have been made since the early 1950s. However, following the Mazur (1966) report on the use of quantitative approaches to characterise cell responses to low temperatures, cryobiology has taken a more fundamental and theoretical approach, enabling accurate predictions of optimal cooling rates, media contents and concentrations. Mathematical models of the osmotic behaviour of cells have allowed the prediction of cellular water content and ensuing intracellular concentration at low temperatures. Further models have been developed to predict the probability that intracellular ice will form given specific intracellular and extracellular conditions. These models provide a reasonably complete and accurate picture of intracellular water content and provide predictive models which can be tested experimentally. Left with only

the less theoretically understood solution effects, several significant observations can be made which further increase cryopreservation success. First, osmotic tolerance limits of a cell, the extents to which a cell can shrink or swell and still remain viable, can be derived and used to develop optimal methods for addition and removal of CPAs (Critser *et al.* 2002). Both theoretical predictions and empirical results suggest that for both freezing rates and solute concentration there exists an inverted 'U' which can be optimised (see Fig 1). Thus by combining the optimal curves for freezing rates and solute concentration researchers can theoretically optimise protocols and minimise empirical trial and error approaches.

## THEORY

### *Osmotic tolerance*

Volume excursions upon addition and removal of CPAs can be lethal, especially during non-equilibrium cooling which often requires higher concentrations to protect from the intracellular ice and extracellular electrolyte concentration. These concentrations cause a cell to shrink or swell during addition or removal of CPAs, sometimes beyond its physical limits. The knowledge of these limits, in conjunction with other biophysical parameters, can be used to devise a safe protocol for adding and removing CPAs. For example, if a cell has a low permeability to CPAs, the initial volume excursion when the cell is placed in solution containing these CPAs will be high as the CPA will slowly cross the membrane and equilibrate the cell. Often these higher volume excursions are ameliorated by multi-step additions or dilutions, in which the cells are gradually exposed to higher and higher (or lower and lower) concentrations until a final concentration is achieved. Figure 3 is a theoretical example of volume excursions versus intracellular concentration over a 3-step addition. These limits are species and cell specific. For example, boar spermatozoa can swell to within only 1.02 times and shrink to within only 0.97 times their isotonic volume and maintain >70% motility (Gilmore *et al.* 1998). Canine pancreatic islets, on the other hand, have a broader tolerance range of 1.53 to 0.6 times their isotonic size (Zieger *et al.* 1999).

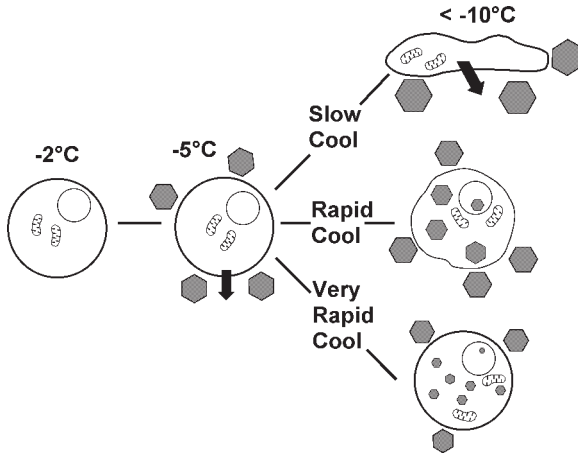


Fig 2: Cellular biophysical behavior at various cooling rates. Note the different sized hexagonal ice crystals for different cooling rates. Slow cooling allows cells to dehydrate, and only extracellular ice forms. Rapid cooling allows little time for dehydration and exposes cells to large, damaging intracellular ice crystals. Very rapid cooling allows almost no dehydration and the intracellular ice crystals, if formed, are small and relatively innocuous (redrawn from Mazur 1977).

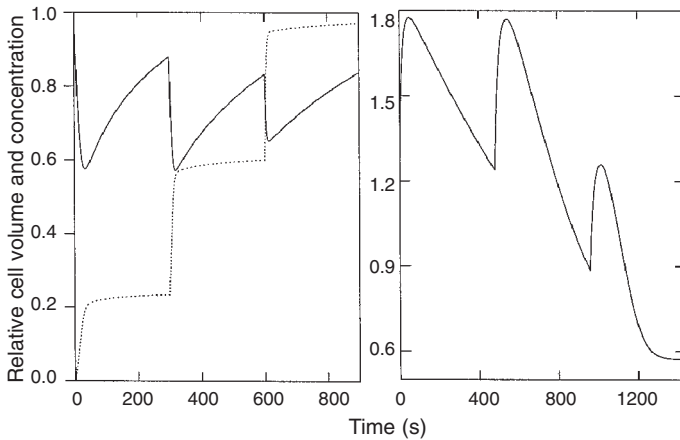


Fig 3: Calculated volume (solid line) and CPA concentration (dotted line) for a 3 step addition (left panel) and dilution (right panel) (redrawn from Agca et al. 2000).

### Intracellular ice

Intracellularly, the cytoplasm can supercool several degrees. In other words, the water in the cell can be lowered to less than  $0^{\circ}\text{C}$  and remain unfrozen. This supercooling allows cells such as embryos to be stored unfrozen at temperatures below freezing for an extended period and be revived with much success (Mazur 1990). Unfortunately, this low temperature is not enough to prevent cellular degradation over a few days and thus is not a viable alternative to deep-freeze cryopreservation in liquid nitrogen (Mazur 1966). The degree of this supercooling depends on several factors, including the presence of ice nucleators, molecules which mimic the molecular structure of ice, expediting the 'building' of an ice lattice, and the best nucleator of ice is ice itself

(Mazur 1966). To facilitate extracellular freezing before supercooling, ice is initiated by 'seeding' the extracellular solution with a cold needle, tweezers or other implement. Intracellularly, however, there is an absence of these ice nucleators (Mazur 1966). Thus, the cytoplasm will not usually freeze unless ice penetrates the membrane. Surprisingly, the cell membrane acts as a fairly sufficient barrier to extracellular ice well below  $0^{\circ}\text{C}$ .

There are 3 fates which can befall the intracellular water (Fig 2). Embryos cooled at higher cooling rates (above  $10^{\circ}\text{C}/\text{min}$ ) become supercooled, and below their nucleation temperature large ice crystals form (for embryos this is around  $-33^{\circ}\text{C}$ ). These are damaging ice crystals, associated with essentially no survival (Polge 1977). Embryos cooled at moderate

cooling rates (2–10°C/min) often supercool above their nucleation temperature, but are not supercooled below their nucleation temperature. This cooling rate tends to form small, less damaging, ice crystals which are not lethal, but upon warming may reform (re-crystallise) into larger, more damaging crystals. Finally, at equilibrium cooling rates (the focus of this chapter), if the cooling rate is allowed to be relatively slow (usually less than 0.5°C/min is sufficient), the amount of supercooling is small because the cell will have time to equilibrate internal and external chemical potentials and, instead of supercooling or freezing, water will leave the cell. This is driven by the osmotic pressure which is induced by pure water freezing (precipitating) in the extracellular solution, increasing the solution tonicity and decreasing the chemical potential of the extracellular solution. Thus, in order to maintain equilibrium, water flows out via exosmosis, causing the cell to shrink and reducing the intracellular chemical potential. This has several consequences. It increases the intracellular solute concentration and decreases the amount of freezable water inside the cell, depressing the freezing temperature further and easing the potential for damage due to ice formation.

Embryos frozen at near equilibrium cooling rates are dehydrated significantly, often to a small fraction of their osmotically active initial cell water volume, and successful freezing at these rates based on membrane integrity and initial cell division is quite high (Mazur 1990). At these cooling rates and solute concentrations, the remaining intracellular solution (cytoplasm plus CPA) forms an amorphous glass-like solid undergoing vitrification. Lacking the ice crystals which damage cells, this type of freezing occurs when water viscosity is very high and, under stringent thawing conditions, can avoid devitrification (ie avoid ice crystal formation) upon warming. However, as extended exposure to high solute concentrations (eg electrolytes) at equilibrium cooling rates can potentially damage on cell survival rates, usually there is a superior cooling rate which minimises exposure time to solutes, but avoids large intracellular ice crystal formation.

### *Solute effects*

The primary advantage of equilibrium freezing is that the probability of intracellular ice formation is

significantly reduced. The limitation is that many cells are sensitive to the high concentrations of solutes occurring at relatively slow cooling rates which may cause cell death. For example, at sub-zero temperatures during slow freezing, solution osmolality increases linearly with decreasing temperature when pure water precipitates out of the extracellular solution as ice. This increase in osmolality causes the cells to shrink dramatically. As the cells undergo this dehydration, increasing solution osmolality may cause cellular disruption, indicated by leakage of ions or other typically non-permeating solutes (Meryman 1968).

Another source of potential cell damage is the chemical toxicity of high concentrations of electrolytes, proposed by Lovelock (1953). Mazur (1977) refined Lovelock's argument by asserting that the damage from electrolytes does not come from intracellular but from extracellular electrolyte concentration. This dangerous solute concentration, however, may be counteracted by the addition of permeating CPAs such as glycerol or ethylene glycol.

The benefits of these compounds lie in the additive quality of osmolality, which allows them to reduce the relative concentration of electrolyte in the solution through their colligative action. For example, the concentration of initially isosmotic NaCl solution at -10°C is about 2.8 molal, whereas the concentration of the same solution with 1 M-glycerol is 0.66 molal (Mazur 1977). This reduction of electrolyte concentration contributes to cell survival, has the additional benefit of initially dehydrating the cell, but adds to the complications due to the osmotic challenges that the cell faces before and after freezing during addition and subsequent removal of CPAs.

## CONCLUSIONS

Slow, equilibrium freezing of embryos is vital to avoid dangerous intracellular ice formation, but also contributes to higher, potentially hazardous solute concentrations in the extracellular solution. The dangers from these concentrations, however, are predictable and avoidable with a rigorous theoretical and methodological cryobiological approach. With the knowledge of several basic permeability parameters, a fairly successful freezing protocol, including media contents and cooling rates, can be deduced.



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# DOES CELLULAR INJURY RESULTING FROM CRYOPRESERVATION SHARE TRAITS WITH SPERM CAPACITATION?

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## INTRODUCTION

Conventional cryopreservation provides numerous routes for reversible and irreversible cellular damage to sperm cells. Cryopreservation requires the exposure of spermatozoa to extreme variations in temperature and osmolality. When a solution is cooled below the freezing point, pure water crystallises out as ice, so that the solutes dissolved in the remaining liquid water fraction increase the osmotic strength of the extracellular space. This creates osmotic pressure changes in the unfrozen fraction that affects the cells. It is generally recognised that the duration of exposure to such events should be minimised for optimal cell survival, implying that the cooling rate should be rapid. However, the cooling rate must be slow enough to allow water to leave the cells by osmosis, preventing lethal intracellular ice formation. In addition, the cryopreservation process induces cellular changes that result in membrane lipid peroxidation that has been shown to be both beneficial and injurious to spermatozoa.

In somatic cells, several mechanisms of cellular injury have been proposed which lead to necrosis and apoptosis (Cotran *et al.* 1999). Necrosis has been defined as a pathologic response to cellular injury from the extracellular environment that could include thermogenic stress, chemical agents, infectious agents, immunologic reactions, nutritional imbalances etc. Apoptosis, in contrast, is a pre-programmed type of cell death and is associated with elimination of unwanted host cells through an internally programmed series of events affected by a defined set of gene products. It is characterised by condensation of cell cytoplasm, membrane blebbing and a specific nuclear change resulting in

the formation of apoptotic bodies. Features of apoptosis have been described during various phases of spermatogenesis but it has not been determined whether the terminally differentiated sperm cell also undergoes apoptotic change.

The physiological process of capacitation, a complex of spermatozoal events that are prerequisite for successful fertilisation, has several features in common with cell necrosis and apoptosis and is dependent on cellular integrity for completion of this process. Studies in the authors' laboratory have focused on the phenomenon known as 'cryocapacitation' by which capacitation-like changes have been associated with cryoinjury. Recent research from this laboratory, suggests that equine sperm undergoing low-temperature storage experience characteristics of premature capacitation (Bedford *et al.* 2000, Pommer *et al.* 2002), membrane peroxidation (Baumber *et al.* 2003), osmotic damage (Linfor *et al.* 2002), and DNA damage (Linfor and Meyers 2002).

## DNA FRAGMENTATION

The nature of sperm DNA damage has not been clearly determined. Two theories have been advanced to explain the appearance of DNA anomalies in ejaculated and frozen sperm. The first theory suggests that endogenous nicks in DNA occur during specific stages of spermiogenesis and an alternative theory has been proposed in which the presence of endogenous DNA nicks is characteristic of programmed cell death, or apoptosis, followed by elimination of defective germ cells. The single cell gel electrophoresis method (SCGE, or Comet, assay) is one of the most sensitive methods of detecting

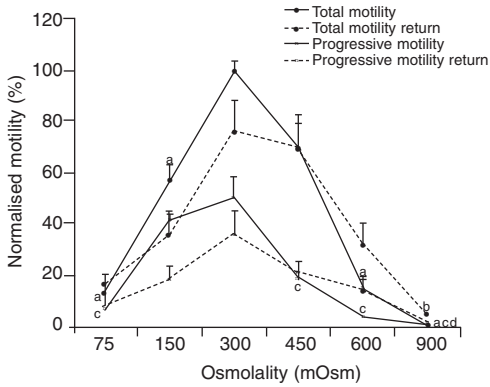


Fig 1: Sperm samples were incubated for 10 min isosmolar or anisosmolar TALP and motility was analysed using a CASA system. Samples were then diluted with isosmolar TALP, incubated 5 min, and motility was analysed again. Letters (a–d) denote significant difference ( $P < 0.05$ ) from control (300 mOsm/kg) within each treatment group, respectively;  $n = 5$  experiments, 3 stallions.

DNA fragmentation/strand breaks and has been used to detect DNA damage in individual sperm cells (the SCSA cannot be used to evaluate individual cell DNA damage). In addition to factors that affect sperm survival, it is increasingly evident that ‘sub-lethal’ damage to sperm is an important limitation to successful cryopreservation in the horse. Data from this laboratory suggest that the initial cooling of equine semen to 4°C that occurs prior to cryopreservation, as well as during cooling of transported semen, is capable of inducing significant fragmentation of sperm DNA. Furthermore, recent studies have extended the occurrence of DNA damage to that of cryopreservation suggesting that conventional cryopreservation is capable of inducing significant DNA fragmentation to stallion sperm (Baumber *et al.* 2003). The observed degree of sperm DNA damage could influence the fertility of certain stallions, although sperm populations are significantly heterogeneous with regard to sperm morphology and biochemical function. DNA fragmentation has been demonstrated to initiate apoptosis in certain somatic cell types (Cotran *et al.* 1999) and could be a signal in sperm.

### OSMOTIC STRESS

The rate at which osmotic volume regulation, and hence, cooling, warming, or response to stressors,

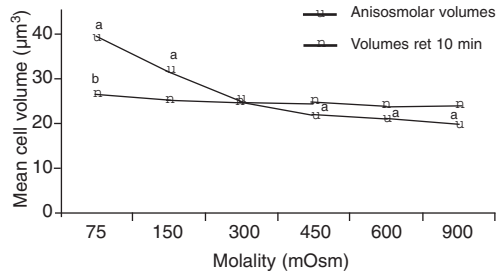


Fig 2: Sperm samples were incubated for 10 min at room temperature in isosmolar or anisosmolar buffer. MCV was then determined using a Beckman Coulter Counter (Z2). In separate tubes, after the initial 10 min incubation, samples were diluted with isosmolar buffer and incubated for 10 min before volume was determined. *a* denotes significance from control (300 mOsm) volume. *b* denotes significance from control (300 mOsm) for the returning values of volume.  $n = 3$ , 3 stallions.

may take place is highly dependent upon the cell’s hydraulic conductivity (water permeability),  $L_p$ . Water permeability has been shown to be dependent on species, temperature, cryopreservative, and ice crystal formation and there is evidence that plasma membrane permeability is regionally variable in sperm. It has been suggested that lipid packing structure is altered by intramembranous cryoprotectants and may be a mechanism whereby  $L_p$  and signal transduction processing could be altered during cooling. Studies in the authors’ laboratory have suggested that exposure of sperm to variations in temperature and medium resulted in differences in the ability of stallion sperm to volume-regulate (Pommer *et al.* 2002; Fig 1). This suggests that sperm survival in the female genital tract as well as following cryopreservation is dependent on the quality of the fluid extracellular environment and may be ultimately stallion-dependent. Using the Coulter Counter method, sperm cell volume can be measured accurately in response to hyposmotic (HOS) conditions, as in the HOS test, but precise measurements can be taken for target cell populations. Studies in the authors’ laboratory have shown that the mean cell volume, or MCV, of isotonic equine sperm was 24.4  $\mu\text{m}^3$ . Equine sperm cell volume had not been previously reported; therefore results could not be compared to other studies (Fig 2). Furthermore, equine spermatozoa behaved as linear osmometers in the range of 150–900 mOsmol/kg ( $r^2 = 0.999$ ) with 70.7% of the total cell volume ( $V_b$ ) (both solids and water) being osmotically inactive. When a cell

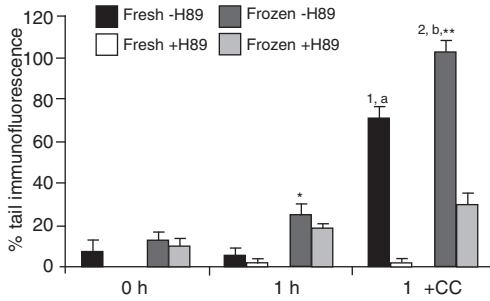


Fig 3: Percentage sperm tail immunofluorescence of phosphorylated tyrosine residues after a 1 h incubation of activated (1.2 mM db cAMP, 1 mM caffeine; CC) and non-activated sperm versus control (0 h). Fresh and frozen-thawed sperm were incubated in BWW medium at 37°C in humidified 95% air and 5% CO<sub>2</sub> atmosphere. Treatment included incubation with 60 μm H-89 versus control (no H-89). Values are expressed as mean percentage ±sem, n=3. Numbers (1,2) denote a significance over time, letters (a,b) denote significance between treatments and asterisks (\*,\*\*) denote significance between fresh and frozen-thawed samples. Values were considered significant when P<0.05.

behaves as an ideal osmometer, the volume of osmotically available water contained in the cell, in our case almost 30% of total sperm volume in isosmotic conditions, will be inversely related to the osmolality of non-permeable solutes in the external medium. In addition, the observed linear osmotic behavior included hypo- and hyperosmolar conditions (150–900 mOsmol/kg), suggesting that the values for exosmotic and endosmotic hydraulic flows were similar in the tested osmolality range.

It was also observed that MCV returned to control levels in light of the failure of sperm motility to return to control levels. It is likely that the 10 min anisomotic stress induced sub-lethal damage to equine sperm. Although the appearance of the cells was normal using light microscopy, and the cell volume returned to normal, there were significant detrimental changes in sperm motility. In ongoing experiments, the authors have observed that variations in anisomolality result in changes in intracellular signalling (tyrosine phosphorylation), viability, and motility.

### CELL SIGNALLING (TYROSINE PHOSPHORYLATION)

Initial events in sperm capacitation are increases in intracellular calcium, bicarbonate, and hydrogen

peroxide, which collectively activate adenylyl cyclase to produce cyclic AMP (cAMP). The stimulation of cAMP then activates protein kinase A (PKA) that, in turn, phosphorylates a number of proteins. It has been suggested that protein tyrosine phosphorylation mediates a variety of cellular functions such as growth regulation, cell cycle control, cytoskeleton assembly, ionic current regulation, and receptor regulation and is an essential downstream component of capacitation (Visconti *et al.* 1998). Recent studies suggest that reactive oxygen species (ROS) and osmotic stress may contribute to membrane leakiness and subsequent increases in intracellular calcium concentration and downstream phosphorylation cascades (Baumber *et al.* 2002; Pommer *et al.* 2003). We, and others, have also demonstrated that one consequence of cryoinjury to sperm is that of excessive levels of protein tyrosine phosphorylation within the sperm tail region (Cormier *et al.* 1997; Bailey *et al.* 2000; Linfor *et al.* 2002; Pommer *et al.* 2003).

### THERMOTROPIC LIPID PHASE TRANSITIONS

Plasma membranes respond to temperature changes through lipid phase transitions. Evidence that cold shock damage to mammalian and invertebrate sperm cells is mediated by lipid phase transitions has been reported (Drobnis *et al.* 1993). Sperm from most species are susceptible to varying degrees of cold shock in which rapid cooling above 0°C confers major structural and functional damage to sperm. In addition to physical membrane disruption, cooling sperm below the lipid phase transition temperature may disrupt membrane enzyme systems including ATPases. Several studies have demonstrated that thawed sperm display increased intracellular calcium concentration and this is indicative of poor control of calcium regulation. Lipid membrane phase transitions of equine and non-human primate sperm have been studied in this laboratory using Fourier Transform Infrared Spectroscopy (FTIR). This method provides a sensitive, non-invasive probe of lipid packing within the cell membrane and is detected by melting trends of membrane lipids. It is known that when cells lose membrane cholesterol, the membrane becomes increasingly fluid and this has been shown experimentally using methyl β cyclodextrin to extract membrane cholesterol. We

have similarly shown that equine sperm display similar fluidity increases under these conditions. Our preliminary data suggest that capacitating sperm also show a similar tendency for increased fluidity and leakiness. Stallion sperm demonstrate distinct suprazero phase transitions of membrane lipids (at which the cell membrane transitions from primarily gel phase to liquid crystalline) at approximately 10°C and 30°C. These temperatures indicate conditions at which sperm are unstable in aqueous solutions and could be particularly susceptible to hyperosmotic damage by becoming leaky. In addition, exposure of sperm to low temperatures reveals membrane lipid remodelling in that membrane raft domains (domains enriched in cholesterol and sphingomyelin) have displayed reversible aggregation. Aggregation of membrane rafts has been linked to alterations in intracellular signalling in somatic cell types and is likely to be involved in sperm capacitation and response to cellular stress.

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# THE USE OF ALTERNATIVE CRYOPROTECTORS FOR FREEZING STALLION SEMEN

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## INTRODUCTION

Recent acceptance of the use of frozen equine semen by important horse breed associations such as Arabian, Quarter Horse and American Paint Horse has renewed and stimulated the interest in this technique. However, it has been estimated that only 30–40% of stallions produced semen suitable for cryopreservation, and a consistent between breeds variation in sperm freezeability is also observed (Alvarenga *et al.* 1996).

Glycerol, the most common cryoprotector used to freeze stallion semen, has a detrimental effect on the fertility of fresh cooled equine semen (Pace and Sullivan 1975). The toxic effect of glycerol on sperm has been reported by many authors, and reviewed by Fahy *et al.* (1990). The presence of glycerol can be an important factor associated with the variations in freezeability and fertility of frozen stallion semen.

The sensitivity of sperm to the detrimental effects of glycerol is species dependent. For some animals, such as rabbits, chickens and fish, glycerol has a contraceptive effect and amides have been shown to be a good alternative cryoprotector for freezing semen from these species.

Keith (1998), when comparing different amides as cryoprotectants for equine semen, observed that between the amides methylformamide and dimethylformamide resulted in highest percentage of motility, however the sperm preservation was similar to glycerol. Alvarenga *et al.* (2000) compared glycerol, DMSO, ethyleneglycol and dimethylformamide, showing that for most stallions dimethylformamide was superior for preserving semen motility after thawing, especially for stallions with poor sperm motility after freeze-

thaw with glycerol ('bad freezers'). Medeiros *et al.* (2002) compared several amides when freezing stallion semen and observed that all amides were superior to glycerol in their ability to preserve sperm cells from cryodamage.

Based on this, several experiments were conducted in this laboratory that aimed to study the use of different kinds of amides in freezing stallion semen, with special attention to stallions with poor sperm freezeability when sperm were frozen in glycerol ('bad freezer' stallions).

## EXPERIMENTAL DESIGN

In the first experiment one ejaculate from 55 stallions was used to verify variations between Stallions and breeds (Quarter Horse [QH], Warmblood, Mangalarga, Arabian, Campolina and Lusitano), using DF (5%) or GLY (5%) as cryoprotectors.

In the second experiment 3 kinds of amides were compared (dimethylformamide at 5%, dimethylacetamide at 3% and methyl formamide at 5%) for the cryopreservation of semen of 18 stallions from the Mangalarga breed.

A fertility trial with frozen semen of a glycerol 'bad freezer' stallion was conducted. The semen from this stallion was frozen in INRA 82 extender with DF or GLY. The total motility after thaw for the batches used to inseminate the mares was, on average, 18% and 48 % for GLY and DF, respectively. Fifteen mares were inseminated in each group with 800 million sperm.

## GENERAL PROCEDURES

All the ejaculates with initial motility greater than 60% were diluted 1:1 with milk base extender,

split and centrifuged at 600 g for 10 min. After the centrifugation, the supernatant was removed, the remaining pellets resuspended and adjusted for 100 million sperm/ml with milk-egg yolk extender (INRA 82) containing the different cryoprotectors: I) 5% glycerol (GL); II) 5% dimethyl-formamide (DF); III) 5% methyl-formamide (MF); IV) 3% dimethyl-acetamide (DA). The straws were cooled at 5°C for 2 h and frozen in liquid nitrogen vapour for 15 min before being plunged into liquid nitrogen. Straws were thawed for examination by immersion in a 46°C water bath for 20 s followed by immersion at 37°C water bath for 60 s.

The total post thaw percentage of sperm motility was evaluated using CASA (Hamilton Thorn motility analyser-IVOS 10), with a minimum of 5 fields and 500 cells being evaluated.

To evaluate cell viability fluorescent probes were used. Stains used were carboxyfluorescein and propidium iodide (PI).

Differences in motion parameters between cryoprotectant treatments were determined by analysis of variance using SAS system.

## RESULTS

In Experiment 1, the comparison between GLY and DF showed a higher total motility using DF. The progressive motility was also higher in the DF Group. DF was superior to GLY in 40 out of 55 stallions studied. The percentage of stallions with good post thaw motility (>40%) was 38% (21/55) for the GLY group and 80% (44/55) for the DF group.

The comparison of data from all stallions showed that DF was superior ( $P < 0.05$ ) for post thaw total motility (50% × 33%) and progressive motility (19% × 15%).

The differences favourable to DF were more evident when only data from 'bad freezer' stallions were compared. Stallions were classified as 'bad freezers' based on total motility (<20%) after sperm were frozen-thawed with glycerol. For 'bad freezer' stallions (20/55) the greatest differences ( $P < 0.05$ ) were observed in total motility (38% × 11%), progressive motility (12% × 3%) and percentage of rapid cells. The other motility parameters evaluated (VAP, VCL, VSL) were also higher when DF was used ( $P < 0.05$ ). For

14 stallions classified as 'regular freezers' (20–40% of post thaw motility) the differences were also favourable to DF for the parameters total motility (51% × 31%) and progressive motility (18% × 11%).

The comparison between breeds showed that most stallions from the Mangalarga Marchador Breed had poor post thaw motility when GLY was used. With the use of DF a smaller difference in freezeability between stallions was observed, and a higher number of stallions were classified as 'good freezers' in all breeds studied. The percentage of stallions with more than 40% of motile sperm cells after thaw with the use of DF and GLY was respectively, 64% and 11% for Mangalarga; 75% and 50% for QH and 84% and 53% for Warmblood stallions.

In Experiment 2 where only Mangalarga stallions were used, the use of dimethylformamide and methylformamide significantly improved most of parameters evaluated when compared with glycerol and dimethyl-acetamide. After thawing the percentage (%) of total and progressive motile sperm analysed using CASA were 42,75 and 13,50; 37,62 and 14,87; 30,25 and 12,12; 15,56 and 5,62 for DF, MF, DA, GLY, respectively.

A significant improvement in fertility was observed in a fertility trial comparing DF and GLY, with frozen semen from one Mangalarga stallion. From 15 mares inseminated with semen frozen with DF 6 (40%) were diagnosed as being pregnant, however in the GLY group no pregnancies were observed.

## CONCLUSIONS

The results of these experiments showed that the amides protected stallion sperm from cryo-damage better than glycerol with a better improvement in post thaw semen quality in stallions classified as 'bad freezers'. The preliminary fertility trial indicated that the amides can be less contraceptive than glycerol.

Further studies are necessary to explain why amides protect stallion sperm.

## ACKNOWLEDGEMENTS

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# CLINICAL FERTILITY DATA FOR MARES INSEMINATED WITH FROZEN SEMEN: EFFECTS OF TIMING AND FREQUENCY OF INSEMINATION

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A major limiting factor to the widespread application of frozen semen is the cost associated with the intense management of mares being inseminated. It is generally recommended that frozen stallion semen be inseminated within 12 h prior to or within 8 h after ovulation. The presumed shortened life-span for frozen-thawed stallion spermatozoa in the mare reproductive tract combined with the 'by the dose, no guarantee' system of marketing semen has led to the practice of 3 to 4 times per day examinations of mares inseminated with frozen semen. Currently, Select Breeders Service recommends use of a timed insemination protocol for cost-effective management of mares bred with frozen semen. This protocol involves daily ultrasonographic examinations during oestrus, induction of ovulation using hCG or deslorelin following detection of a >35 mm follicle and insemination at 24 and 40 h post injection. Using this insemination schedule, mares that ovulate 18 to 52 h after administration of the ovulatory agent will have had sperm deposited in the reproductive tract within 12 h prior to ovulation or within 6 h after ovulation or both. In a clinical trial in Italy (Reger *et al.* 2003) 26 of 34 mares conceived (76%) after 2 timed inseminations vs. 15 of 21 (71%) conceiving following a single insemination within 6 h post ovulation. In a controlled study in Colorado, (Reger *et al.* 2003) also reported no difference in embryo recovery rates for mares inseminated once within 6 h post ovulation with  $800 \times 10^6$  total frozen thawed sperm (60%) vs. mares inseminated twice at 24 and 40 post deslorelin with  $400 \times 10^6$  total sperm per insemination (55%). Matthews (unpublished) reported a per-cycle pregnancy rate of 46% (157 cycles) for mares inseminated twice with  $200 \times 10^6$  total frozen-thawed sperm using a video-endoscope and timed inseminations.

## 2002

Data was collected from Select Breeders Service affiliated laboratories for 167 mares inseminated with frozen semen during the 2002 breeding season. These mares were inseminated with semen from numerous different stallions whose semen was frozen by various laboratories in the US and Europe. The overall first cycle and seasonal pregnancy rates were 46% and 75% respectively. No difference was observed in first cycle pregnancy rates between mares inseminated once (50%) or multiple times (47%) per cycle.

## 2003

A retrospective study was conducted at the conclusion of the 2003 breeding season to examine pregnancy rates and the effects of insemination strategies for frozen semen distributed commercially throughout the U.S. from the author's Maryland laboratory. Surveys were included along with each of the 535 shipments of frozen semen sent in 2003. Information was requested on each of the mares as well as details concerning insemination dates and times, use of ovulating drugs, time of ovulation and fertility results. Results for 217 mares and 332 cycles were reported. These mares were inseminated with semen of varying quality from 54 different stallions of numerous breeds. Generally, insemination doses contained a minimum of 800 million total sperm and exhibited a minimum post thaw progressive motility of 30%. The number of progressively motile sperm per insemination dose ranged from approximately 240 to 600 million. Complete survey data for all 332 cycles was not reported. Therefore, we have

**TABLE 1: Fertility of frozen semen**

Cycle	No. cycles	No. conceptions	Conception rate (%)
1st	217	126	58.1
2nd	83	34	41.0
3rd	24	11	45.8
4th	6	3	50
5th	2	1	50
Total	332	175	52.7

**TABLE 2: Effects of single vs. multiple AI on conception rates for frozen semen**

	Cycles	Conceptions	Conception rate (%)
One AI	132	68	51.5
Multiple AI <sup>1</sup>	151	78	51.7

<sup>1</sup>Of the 151 multiple AI cycles, 128 were inseminated twice.

**TABLE 3: Effect of age on conception rates for frozen semen**

	Age				
	3-6	7-11	12-16	>16	Unknown
Cycles	57	77	67	31	100
Conceptions	33	42	36	15	49
Concentration rate (%)	57.9	54.5	53.7	48.4	49.0

**TABLE 4: Effect of reproductive status on conception rates for frozen semen**

	Status					
	Maiden	Foaling	Barren	Not bred <sup>1</sup>	Donor <sup>2</sup>	Unknown
Cycles	48	56	65	41	33	89
Conceptions	28	33	33	18	14	49
Concentration rate (%)	58.3	58.9	50.8	43.9	42.4	55.1

<sup>1</sup>Mares not bred in the previous season but had been pregnant in the last season they were bred

<sup>2</sup>Mares used successfully as embryo donors in the previous season

evaluated the various factors affecting conception rates based only on the cycles for which all pertinent data was available. Conception rates are reported rather than pregnancy rates as many of the mares were embryo donors or pregnancy data beyond 14 or 35 days was not provided. For this study a positive result was recorded if an embryo was recovered or an embryonic vesicle was observed using ultrasonography >13 days post ovulation.

## RESULTS

Of the 217 mares bred, 126 (58.1%) conceived on the first cycle of breeding. Overall the conception rate per cycle was 52.7% (Table 1). There was no effect of the number of inseminations on the fertility of frozen semen from 283 cycles for which data were available (Table 2). The effects of age and reproductive status on conception rates are listed in Tables 3 and 4. As reported by others

(Barbacini *et al.* 1999) old mares (>16 years) tended to have a lower conception rate than younger mares (< 16 years) and maiden and foaling mares tended to have higher conception rates than mares of other reproductive status. Table 5 shows the effect of single vs. multiple inseminations on young (3-11years) and old (12-20 years) mares. Interestingly, conception rates tended to be slightly higher in the younger mares when multiple inseminations were used but for older mares, rates were higher with single inseminations.

The conception rates for 7 different insemination strategies are given in Table 6. Three of the strategies used a single insemination and 4 used multiple inseminations. The vast majority of mares were inseminated either with a single post ovulation insemination or with 2 inseminations, one pre- and one post ovulation. There was no difference in the conception rates for mares inseminated with these 2 techniques.

**TABLE 5: Effect of mare age and number of inseminations on conception rates for frozen semen**

	Age (years)			
	3–11		12–20	
	Single AI	Multiple AI	Single AI	Multiple AI
Cycles	40	63	45	41
Conceptions	20	38	26	17
Concentration rate (%)	50.0	60.3	57.8	41.5

**TABLE 6: Effect of insemination strategy on conception rates for frozen semen**

	AI strategy <sup>1</sup>						
	One AI			Multiple AI			
	A	F	G	B	C	D	E
Cycles	93	11	12	104	9	6	10
Conceptions	44	8	7	50	5	1	8
Concentration rate (%)	47.3	72.7	58.3	48.1	55.6	16.7	80.0

<sup>1</sup>A: One AI, post ovulation

F: One AI, pre-ovulation within 12 h of ovulation

G: One AI, pre-ovulation within 24 h of ovulation

B: Two AI's, one pre- and one post ovulation

C: Multiple AI's pre-ovulation, last within 12 h of ovulation

D: Multiple AI's pre-ovulation, last within 24 h of ovulation

E: Multiple AI's pre-ovulation and one AI post ovulation

## DISCUSSION

Conception rates for 2003 are higher than those reported previously by this laboratory (Loomis 2001) for commercial frozen semen distributed domestically during the 1999 and 2000 breeding seasons (49.4% first cycle conception rate for 340 mares) but comparable to the results for exported semen during the same time period (53.5% first cycle conception rate for 536 mares bred). Pregnancy rates of greater than 50% per cycle have been reported by others. Barbacini *et al.* (2000) reported similar per cycle pregnancy rates (53.6% and 52.3%) for mares bred with frozen semen in Italy during the 1999 and 2000 seasons respectively. Vidament *et al.* (2000) reported a per cycle pregnancy rate of 54% for 446 mares inseminated with frozen semen in France. Samper (2001) reported a first cycle pregnancy rate of 56.7% for 578 mares inseminated with frozen semen between 1991 and 2000.

The purpose of recommending a timed insemination protocol employing 2 inseminations per cycle is to provide a simple and effective way to manage mares being bred with frozen semen. It is a commonly held belief that mares bred with frozen semen need to be examined 3–4 times a day during the periovulatory period so that a single

dose of frozen semen can be inseminated within 6–8 h after ovulation. This usually requires that mares are boarded at a clinic or that late night farm calls must be made by the practitioner to insure that the post ovulation insemination is performed within the critical 6–8 h window after ovulation. The cost in veterinary care to the mare owner is substantial and often discourages them from utilising frozen semen. Stallion owners who sell semen by the dose for hundreds or even thousands of dollars are forcing the mare owners to utilise this type of protocol because the cost of the veterinary care is less than the cost of the additional semen required for a 2-dose timed protocol. However, many stallion owners provide multiple doses per cycle and are paid per pregnancy, in this case using frozen semen as just another mechanism to achieve a pregnancy. Even in this situation many practitioners believe that they can only achieve acceptable pregnancy rates with frozen semen if the mares are managed with multiple daily examinations around the time of anticipated ovulation. These data support the theory that 2 inseminations timed to occur both before and after ovulation yield comparable conception rates to a single post ovulation insemination. This is in agreement with data recently published by Sieme *et al.* (2003). In this study, mares inseminated twice

per cycle at 24 h intervals had a 50% per cycle pregnancy rate and mares inseminated once averaged 42% per cycle pregnancy rate. Pregnancy rates for mares inseminated once within 12 h prior to ovulation or once within 12 h post ovulation were 41% and 50%, respectively. Samper (2001) and Vidament (1997) also reported that pregnancy rates with frozen semen were higher when mares were inseminated more than once per cycle.

A 2-dose timed insemination protocol allows a practitioner to examine mares once daily during normal hours without compromising fertility. However, use of this protocol may not be appropriate for all breeding situations. For example, mares that are susceptible to post breeding endometritis such as older or barren mares may require a more intense management scheme in order to minimise invasion of the susceptible uterus. Older mares may also be less responsive to ovulatory agents such as hCG (Barbacini *et al.* 2000). Although not examined directly in this study, the lower conception rate for older mares bred with multiple vs. single inseminations may be due to these factors.

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# THE USE OF HYSTEROSCOPY FOR THE APPLICATION OF STALLION FROZEN SEMEN IN COMMERCIAL PROGRAMMES

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## INTRODUCTION

Recent acceptance of the use of frozen semen by important horse breed associations has stimulated interest in its application by the horse industry. However, the fertility of frozen semen is still low - on average 2 or 3 cycles and a large number of doses are needed to result in pregnancy. The number of doses produced by a stallion ejaculate is very low (4–8) so only a limited number of doses can be stored.

A low dose artificial insemination (AI) technique using endoscopy has been shown to be effective in horses (Morris *et al.* 2000) and pigs (Martinez *et al.* 2000). Good fertility rates (60%) were achieved by researchers from England with the deposition, by endoscopic AI, of only 10 million sperm on the mare's utero-tubal papilla.

This study examined the potential use of low-dose hysteroscopic insemination for the application of frozen semen in 2 commercial artificial inseminations centres.

## PRELIMINARY EXPERIMENTS

Two preliminary experiments with negative (low dose AI uterine body) and positive control (conventional dose AI uterine body) groups using low dose AI for fresh and frozen stallion semen were designed. These experiments were based on the fact that in the most of experiments on hysteroscopic AI no control groups (low dose and normal dose in uterine body) were included.

In the first experiment fresh semen from one fertile stallion was used. Twelve mares aged between 5–12 years. were used and the same mares were used in all 3 experimental groups: Group 1, GF1 (n=12); hysteroscopic insemination using 10 × 10 million motile spermatozoa onto the UTJ, in

Group 2, GF2 (n=12); conventional insemination depositing 10 million motile spermatozoa in the uterine body and Group 3, GF3 (n=12); conventional insemination (uterine body) with 400 million motile spermatozoa. Ovarian follicular activity was monitored twice weekly by transrectal ultrasonography, until detection of oestrus and then daily thereafter. Mares were inseminated every 48 h after detection of a 35 mm follicle until ovulation. Semen was diluted with Kenney extender to adjust the final concentration. The semen used had 65–70% total motility and 30–35% of progressive motility. The pregnancy rates of GF1, GF2 and GF3 were 41.67%, 16.67% and 50% respectively and there was no difference between the groups (P>0.05). The pregnancy rate of GF1 was similar to GF3 using only 2.5% of the conventional dose.

In the second experiment frozen thawed semen from one fertile stallion was used. Twenty-four mixed breed mares aged between 5–12 years were assigned to one of 3 experimental groups: Group 1, G1 (n=12), hysteroscopic insemination onto the UTJ using 10 million motile spermatozoa in Group 2; G2 (n=12); conventional insemination (uterine body) with 400 million motile spermatozoa. The semen used had on average 40% total and 20% of progressive post thaw motility. Conception rates of 33.3% (4/12) and 0% (0/12) were achieved for G1 and G2 respectively, however no differences were observed between the groups (P>0.05).

Based on these results, the authors decided to use endoscopic AI for the application of frozen semen in 2 commercial equine reproduction centres.

## MATERIALS AND METHODS

Imported frozen semen (Netherlands, France and Belgium) from 15 different Warmblood stallions

was used in 2 AI centres located in Sao Paulo State-Brazil.

Mares were inseminated close to ovulation (6 h pre- or post ovulation) with 2 0.5 straws, each straw contained between 50–75 million total sperm cells, with at least 30% of motile cells.

Mares were inseminated through hysteroscopy as described by Morris *et al.* (2000). After thawing, the insemination dose was aspirated into a polypropylene tube, connected to a disposable syringe that was previously inserted into the working channel from the flexible endoscope.

The operator's hand inserted the tip of the endoscope through the vagina and cervix into the uterus. In Centre A the endoscope was guided to the tip of the uterine horn ipsilateral to the ovulation by rectal palpation and the procedure was monitored with a video to visualise of the utero-tubal papilla. In Centre B, the procedure was monitored without video, air was used to dilate the lumen to enable visualisation of the bifurcation, then the endoscope was passed along the uterine horn ipsilateral to ovulation to the tip of the horn where the papilla is located. After the visualisation of the papilla the semen was gently deposited. The air was aspirated during the removal of the endoscope. Mares were treated with oxytocin (20 UI) 6 and 12 h after the procedure.

Embryo flushes were performed 8 days after ovulation and non-surgically transferred to synchronised recipients. Pregnancy was determined on Day 15 post ovulation by ultrasonography per rectum. Differences between percentages were tested with chi-square analysis.

## RESULTS

A total of 61 (58.6%) embryos were collected from 104 ovulations. No differences ( $p>0.05$ ) were observed in embryo recovery rates between the 2 centres. The embryo recovery rate was 62.8% (32/48) and 51.7% (29/56) for Centre A and B, respectively.

The pregnancy rates at 15 days after ovulation for mares inseminated by endoscopy and kept pregnant was 54.5% (12/22) and 52.5% (22/40) for Centre A and B, respectively ( $P>0.05$ ).

The procedure was well tolerated by all mares and no uterine problems were observed. Fertility rates among stallions ranged from 0 to 70% (Table 1).

**TABLE 1: First cycle fertility rates after endoscopic insemination with frozen semen from individual stallions**

Stallion	Ovulations	Embryos or pregnancies	Fertility rates
A	14	10	71.4%
B	19	10	52.6%
C	35	19	54.2%
D	47	30	63.8%
E	10	04	40.0%
F	09	05	55.5%
G	05	02	40.0%
H	06	04	66.6%
I	04	03	75.0%
J	05	02	40.0%
L	03	03	100%
M	01	01	100%
N	01	00	0.0%
O	03	00	0.0%
P	04	02	50.0%
TOTAL	166	95	57.22%

## DISCUSSION

The overall conception rate of (57.2%) achieved using 2 straws (100–150 million total sperm) was satisfactory when compared with the pregnancy rates per cycle (20–50%) reported for a high percentage of stallions, using 8 straws (400 million sperm) in french AI programmes, using a large number of mares (Vidament *et al.* 1997, 2000). There was a large variability in fertility rates between stallions (0–70%) similar to results from others (Vidament *et al.* 2000).

It is concluded that hysteroscopic insemination provided satisfactory fertility rates and is a viable alternative for commercial application of horse frozen semen.

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# CRYOPRESERVATION OF EQUINE SPERMATOZOA ALTERS THE EXPRESSION OF SPERM PORTEIN 22 KDA (SP22)

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## INTRODUCTION

There is evidence that sperm surface proteins are involved in fertilisation and that they may serve as fertility markers (Samper *et al.* 1995; Meyers *et al.* 2000). Several sperm proteins have been suggested to be involved in fertilisation based on sperm-oocyte binding *in vitro*, but SP22 (sperm protein 22 kDa) is the only membrane bound sperm protein that has been associated with fertility in both *in vitro* and *in vivo* studies (Kleinfelter 1997, 2002). SP22 is a member of a highly conserved gene family, and the expression of this protein on spermatozoa has been found in many species including rats, bulls, and human (Welch *et al.* 1998). The expression of SP22 is correlated with fertility in rats, but has not previously been investigated in stallions. The objective of this report was to investigate if SP22 is expressed on equine spermatozoa, and to determine its localisation in fresh and cryopreserved semen.

## MATERIALS AND METHODS

Eighteen semen samples from 3 fertile stallions were collected and divided into 2 treatment groups: a) fresh semen; and b) cryopreserved semen. Semen undergoing cryopreservation was subjected to a standard cryopreservation and thawing protocol. Spermatozoa were immunocytochemically stained using a primary SP22-antibody, visualised by an anti-sheep FITC secondary antibody. A sample without a primary antibody served as control. Localisation patterns of SP22 on spermatozoa were analysed using fluorescent microscopy. The percentages of

spermatozoa with a specific staining pattern were determined by counting 100 sperm in each sample. Localisation patterns of SP22 within each treatment group were analysed using Kruskal-Wallis one-way ANOVA, and different patterns between the treatments were analysed using a Wilcoxon signed rank test.

## RESULTS

SP22 was detected on spermatozoa in all test samples but not control samples. Four different ( $P < 0.01$ ) localisation patterns of SP22 were observed in fresh semen: (1) overlying the acrosome and equatorial region ( $69\% \pm 3$ ), (2) overlying the acrosome ( $13\% \pm 2$ ), (3) equatorial region ( $13\% \pm 3$ ), and (4) neck ( $5\% \pm 1$ ). There was a significant difference in the localisation pattern of SP22 between fresh and frozen/thawed semen ( $P < 0.01$ ). The most common localisation pattern in frozen/thawed samples was (1) equatorial region ( $47\% \pm 3$ ), followed by a pattern not found in fresh semen (2) scattered over the head;  $18\% \pm 4$ , (3) overlying the acrosome ( $16\% \pm 6$ ), (4) overlying the acrosome and equatorial region ( $15\% \pm 3$ ), and the least common pattern was (5) neck ( $5\% \pm 1$ ;  $P < 0.05$ ).

## CONCLUSION

The altered expression and scattering of SP22 over the sperm head in frozen/thawed samples suggests that SP22 may detach from spermatozoa during the freeze/thaw process. The biological importance of SP22 relocation on equine spermatozoa following cryopreservation was not determined.

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# SESSION 9:

## Oviductal interaction

**Chairman:**

***Juan Samper***



# EFFECTS OF OXIDATIVE STRESS ON EQUINE SPERMATOZOA DURING CRYOPRESERVATION

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## INTRODUCTION

Most protocols currently in use for cryopreservation of equine semen result in the potential exposure of sperm to damage attributed to reactive oxygen species (ROS). Removal of seminal plasma during semen processing removes much of the antioxidant capacity present in semen and may increase the susceptibility of sperm to oxidative damage. Freeze-thaw damage to equine sperm increases ROS generation primarily as the superoxide anion via a calcium-dependent, NADPH oxidase (Ball *et al.* 2001). Although superoxide is the primary ROS generated, it appears that many of the effects related to oxidative stress are mediated by hydrogen peroxide (Baumber *et al.* 2000), which forms rapidly by dismutation of the superoxide anion.

## SPERM CAPACITATION

Low-level generation of ROS induce capacitation of equine sperm that is characterised by an increase in tyrosine phosphorylation as well as an increased acrosomal exocytosis in response to a progesterone agonist (Baumber *et al.* 2003b). When equine sperm were incubated with an ROS generating system (xanthine and xanthine oxidase [X-XO]) there was an increase in capacitation. Likewise, exposure to NADPH increased sperm capacitation as determined by response to a progesterone agonist. The addition of catalase or superoxide dismutase reduced the response indicating that both hydrogen peroxide and superoxide were involved in sperm capacitation induced by low-level ROS generation.

More recently, we have investigated the generation of ROS by sperm during capacitation *in*

*vitro*. In this experiment, equine spermatozoa were diluted 1:1 in BWW and separated from seminal plasma by centrifugation across a Percoll density gradient (80:40%). Sperm were resuspended in BWW or BWW with dibutyl cAMP (1.2 mM) and caffeine (1.0 mM) and incubated at 38°C for 200 min (Pommer *et al.* 2003). At the initiation of incubation (T0) and after the end of incubation (T200) sperm were labelled with dihydroethidium (DHE - 2.0 mM) and calcein AM (7.8 nM) for determination of superoxide anion production (Benov *et al.* 1998) and viability (Braut-Boucher *et al.* 1995), respectively. The application of these

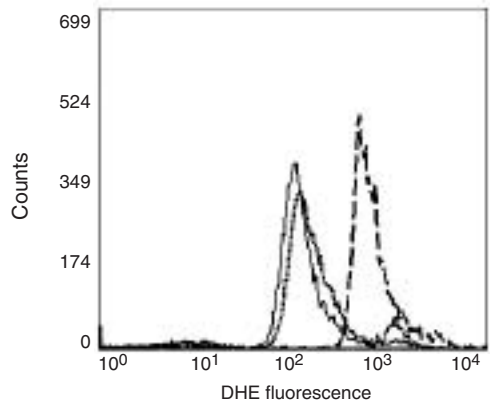


Fig 1: Single parameter histogram showing the effect of xanthine-xanthine oxidase (X-XO) on fluorescence of dihydroethidium (DHE) in live equine sperm. Sperm were loaded with DHE and Calcein AM and treated with X-XO (-----), X-XO and superoxide dismutase (.....) or untreated (\_\_\_\_). After 15 min incubation, sperm were analysed by two-parameter flow cytometry, and live (calcein positive) sperm were characterised for DHE fluorescence. The addition of X-XO increased DHE fluorescence compared to control while the addition of X-XO and SOD returned DHE fluorescence to control values.

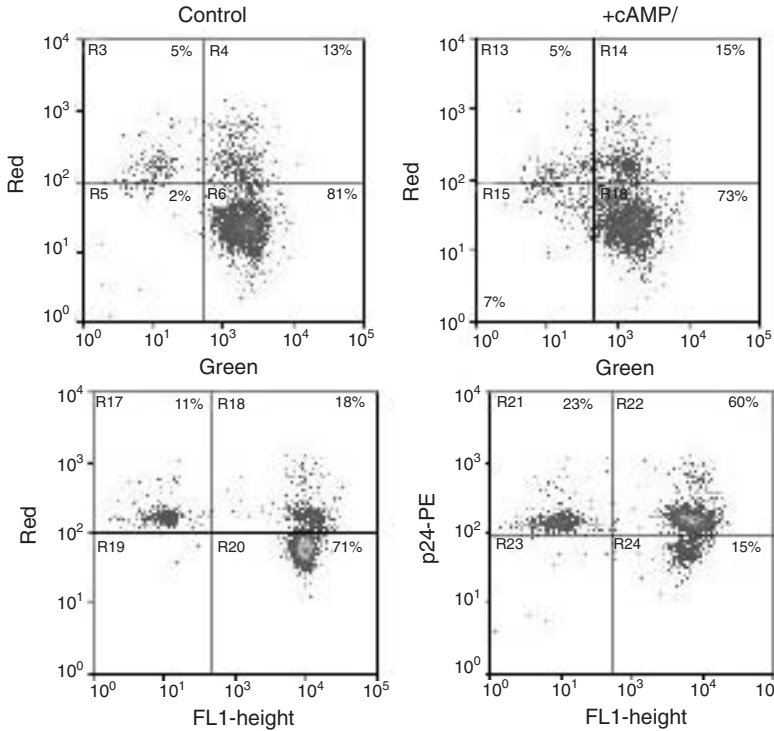


Fig 2: Flow cytometric analyses of equine sperm during capacitation *in vitro*. Equine sperm were incubated in BWB with 1.2 mM db-cAMP and 1.0 mM caffeine or without (control) activators for 200 min. At Time 0 (top panels) and Time 200 (bottom panels), sperm were labelled with dihydroethidium (red) for detection of superoxide anion and calcein AM (green) for assessment of viability. In the presence of cAMP and caffeine, there was an increase in superoxide generation during capacitation as represented as the proportion of live sperm with increased DHE fluorescence (upper right quadrant).

2 probes and flow cytometric analysis allows determination of superoxide generation within live, equine sperm (Fig 1). Evaluation of samples by 2-parameter flow cytometry revealed 2 subpopulations of viable (CAM +) sperm with an increase in the proportion of viable sperm with elevated superoxide generation during capacitation *in vitro* (Fig 2). These data provide further evidence that ROS are important in signalling events during capacitation of equine spermatozoa.

Equine sperm more readily undergo capacitation after cryopreservation as measured by tyrosine phosphorylation compared to sperm prior to cryopreservation (Pommer *et al.* 2003). Although the mechanism of this 'cryocapacitation' has not been clearly defined, it is speculated that generation of ROS by equine sperm after cryopreservation may play a role in this process based upon previous observations that ROS enhance sperm capacitation and that freeze-thaw damage to equine sperm increases ROS generation.

## EFFECT OF ROS AND CRYOPRESERVATION ON SPERM DNA

Beyond effects related to sperm capacitation, generation of ROS also results in an increase in DNA fragmentation in equine sperm (Baumber *et al.* 2003a). Generation of ROS via the X-XO system resulted in a dose-dependent increase in DNA fragmentation which was reduced by the addition of catalase or glutathione peroxidase, but not by superoxide dismutase (SOD; Baumber *et al.* 2003a). These findings suggest that hydrogen peroxide rather than the superoxide anion is responsible for DNA fragmentation induced by ROS.

DNA fragmentation also increased after cryopreservation of equine sperm (Baumber *et al.* 2003a), and it appears likely that ROS may contribute to these changes during semen freezing. However, definitive evidence for the role of ROS in the 'cryocapacitation' and DNA damage observed in equine sperm during freezing and thawing remains to be established.

## EFFECT OF ANTIOXIDANTS AND ENZYME SCAVENGERS ON CRYOPRESERVED EQUINE SPERM

Attempts to reduce damage to equine sperm during freezing and thawing by the addition of enzyme scavengers, lipid- or water-soluble antioxidants have met with limited success. Equine sperm were frozen with the addition of catalase (200 U/ml), superoxide dismutase (200 U/ml), glutathione (10 mM), ascorbic acid (10 mM),  $\alpha$ -tocopherol (1 mM) or vehicle control for  $\alpha$ -tocopherol (0.5% ethanol). Enzyme scavengers and antioxidants did not improve post thaw sperm motility, DNA fragmentation, acrosomal integrity, viability or mitochondrial membrane potential. Interestingly, superoxide dismutase actually resulted in a significant ( $P < 0.05$ ) increase in DNA fragmentation, attributable to generation of hydrogen peroxide by this enzyme. The effect of SOD on DNA fragmentation was probably due to an increased generation of hydrogen peroxide from superoxide as demonstrated after the addition of X-XO.

In summary, cryopreservation of equine sperm is associated with capacitation-like changes as well as DNA fragmentation that may be associated with a reduction in the longevity and fertility of frozen-thawed equine sperm. Similar changes can be induced in equine sperm by the low-level generation of ROS, and it can be inferred that ROS contribute to oxidative damage to equine sperm during cryopreservation. However, studies to date have not demonstrated a positive effect of addition of enzyme scavengers, lipid- or water-soluble antioxidants on these parameters after cryopreservation of equine sperm.

## ACKNOWLEDGEMENTS

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## ROLE OF SEMINAL PLASMA IN FREEZING EQUINE SPERMATOZOA

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### INTRODUCTION

Seminal plasma consists of fluid produced in the rete testis, epididymis, and the accessory sex glands. The chemical contribution to seminal plasma from these locations varies, but is important to sperm development and maturation in the male reproductive tract. However, seminal plasma has been shown to be detrimental to sperm motility and viability during long-term storage, and is therefore routinely removed when semen is processed for cryopreservation. There is evidence that seminal plasma may play a role in the female tract. In addition to being a vehicle for spermatozoa into the female reproductive tract, seminal plasma has been shown to have a positive influence on fertility. Enhanced sperm transport, hastening of ovulation, and increased blood flow to the uterus and oviducts have been associated with the presence of seminal plasma in the female tract (Clause 1989; Weitze *et al.* 1990; Willmen *et al.* 1991; Bollwein *et al.* 2001). Seminal plasma has also been shown to regulate various function of the immune system, and it appears to be an important factor in the regulation of breeding-induced endometritis (Troedsson *et al.* 1995).

### BREEDING-INDUCED ENDOMETRITIS

*In vitro* and *in vivo* studies suggest that equine spermatozoa activate complement in uterine secretion, which results in an influx of polymorphonuclear neutrophils (PMNs) into the uterine lumen (Troedsson *et al.* 1995). Activated PMNs bind to spermatozoa in the presence of complement factor C3b, and a recently described complement independent mechanism (Dahms and Troedsson 2002). Following binding, the

spermatozoa are phagocytosed by the PMNs. During the activation of PMNs, prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) is released from the cell membrane, resulting in myometrial contraction (Troedsson *et al.* 2001). Uterine contractions are believed to physically remove intrauterine fluid and harmful products that have been released during inflammation. While breeding-induced endometritis is a physiological reaction to semen, it needs to be transient to prevent interference with pregnancy. A function of seminal plasma may be to act as an inflammatory modulator in the uterus, which may be of importance for the transient nature of breeding-induced endometritis. This could potentially become a problem when frozen/thawed semen is used, since most of the seminal plasma is removed when processing semen for cryopreservation.

### ROLE OF SEMINAL PLASMA

Seminal plasma has a suppressive effect on complement activation, PMN-chemotaxis and phagocytosis (Troedsson *et al.* 2000). As a consequence of this suppression, the duration of breeding-induced uterine inflammation was found to be shorter when seminal plasma was included in an insemination dose, compared to when all seminal plasma is removed and replaced by a commercial semen extender (Troedsson *et al.* 2001). Clinical observations suggest that a marked and prolonged breeding-induced endometritis often follows insemination with frozen/thawed semen. It is reasonable to believe that removal of seminal plasma before freezing of the semen, may contribute to the increased duration of the induced endometritis.

Another function of seminal plasma in breeding-induced endometritis may be to protect

spermatozoa from being phagocytosed and destroyed when introduced to an inflammatory environment. PMNs interfere with sperm motion characteristics, and motile sperm cells appear to bind to PMNs forming large clusters of PMN/spermatozoa (Alghamdi *et al.* 2001). Addition of seminal plasma reduces the binding between spermatozoa and inflammatory cells *in vitro* (Troedsson *et al.* 2002). The clinical importance of this finding is supported by an *in vivo* study, in which removal of all seminal plasma in the presence of an active breeding-induced endometritis was shown to be detrimental to fertility (Troedsson *et al.* 2002). Interestingly, the fertility was restored to normal levels if seminal plasma was added to the insemination dose. The protective role of seminal plasma in PMN-phagocytosis of spermatozoa is confined to a heat labile component of seminal plasma that could not be removed by charcoal treatment (Dahms and Troedsson 2002). Using ammonium sulphate to precipitate proteins from other components of seminal plasma, inhibition of binding between PMNs and spermatozoa could only be found in the proteinaceous fraction (Alghamdi *et al.* 2003). It would be important to know how much seminal plasma is needed for an insemination dose to maintain its immuno-modulatory effect and its protective function on spermatozoa in the uterus.

## CONCLUSION

Fluid from rete testis and different sections of the epididymis is important for normal development and maturation of spermatozoa in the testes and epididymis, but once ejaculated, seminal plasma is detrimental to the viability of spermatozoa during long term storage. In addition, seminal plasma has an important role in the modulation of breeding-induced inflammation and sperm elimination from the female reproductive tract. It appears to be important to maintain a minimum volume of seminal plasma that effectively modulates inflammation and protects spermatozoa in the uterus, without being detrimental to the viability

of spermatozoa when semen is processed for cryopreservation.

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