Havemeyer Foundation
Monograph Series No. 1

Proceedings of the First Meeting of the

EUROPEAN EQUINE GAMETE GROUP (EEGG)

5th - 8th September 1999
Lopuszna, Poland

Editors: W. R. Allen and J. F. Wade

Havemeyer Foundation
Monograph Series No. 1

Proceedings of the First Meeting of the

EUROPEAN EQUINE GAMETE GROUP (EEGG)

5th - 8th September 1999
Lopuszna, Poland

Editors: W. R. Allen and J. F. Wade

Suites 3 & 4, 8 Kings Court, Willie Snaith Road, Newmarket, Suffolk CB8 7SG, UK

First published 2000

ISSN 1472-3158

Published by **R & W Publications (Newmarket) Limited**

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

CONTENTS

SESSION IV: OOCYTES - RECOVERY AND MATURATION

SESSION V: OOCYTES - FERTILISATION *IN VITRO*

SESSION VI: EMBRYO PRESERVATION

SESSION VII: COMMERCIAL DEVELOPMENT IN EQUINE EMBRYO TRANSFER

PREFACE

Dorothy Russell Havemeyer, the founder of the Dorothy Russell Havemeyer Foundation, Inc. was born in Rumson, New Jersey, on 24th July 1906. After graduating from Miss Porter's School, Farmington, Connecticut, she decided not to attend college. In 1958 she married Edward McConville. No children were born of the marriage. She continued to use her maiden name as well as her married name after her marriage. She died on 1st August 1985, survived by her husband.

For a number of years, Mrs McConville raced and bred horses in the States of New Jersey and Florida. She owned a farm in Spring Lake, New Jersey, until 1972 when she decided to sell it. During that season, she sold her horses at auction in Ocala, Florida. After those sales were completed, she never again owned horses and rarely attended the races.

In 1979 she founded The Dorothy Russell Havemeyer Foundation, Inc., an operating foundation. Its purpose is to conduct equine research and Mrs McConville was President until her death. During her lifetime the Foundation established its reputation for conducting 'cutting edge' scientific workshops. These were attended by a small number of participants who shared their knowledge, thoughts and ideas in an informal atmosphere. A list of the workshops conducted by the Foundation over the years follows this preface.

A generous bequest under Mrs McConville's Will has allowed the Foundation to expand its activities. It is at the forefront of the equine genome project, having conducted several international workshops on the subject. It also participates in the annual USDA International Plant and Animal Genome Conference. Other current projects include studies of equine neonatology, placentitis and behavioural problems. The results of its research programme have been published in a wide variety of journals, including Equine Veterinary Journal, Animal Genetics, Veterinary Immunology and Immunopathology and Veterinary Microbiology.

Mrs McConville was particularly proud of the barn dedicated to her at Cornell University, Ithaca, New York, known as The Dorothy Havemeyer McConville Barn. The Equine Genetics Center is situated at this barn which is the focus for the Foundation's research activities at Cornell. She felt an affinity for the College of Veterinary Medicine, Cornell University, because of care given to one of her horses as a patient at the Large Animal Hospital. Doug Antczak, VMD, PhD, Director of the James A. Baker Institute for Animal Health at Cornell, was the first Principal Investigator retained by the Foundation in 1981. Since then, Sue McDonnell, PhD (University of Pennsylvania, School of Veterinary Medicine, New Bolton Center, Pennsylvania), Mary Rose Paradis, DVM, MS (Tufts University, School of Veterinary Medicine, North Grafton, Massachusetts), W. R. (Twink) Allen (Thoroughbred Breeders' Association Equine Fertility Unit, Newmarket, England) and Michelle LeBlanc, DVM, PhD (University of Florida, VMTH Large Animal Clinic, Gainesville, Florida) have joined the roster of Principal Investigators under retainer to the Foundation.

Mrs McConville also felt strong ties to the University of Pennsylvania, School of Veterinary Medicine, New Bolton Center, which had also cared for one of her horses. In 1992 The Dorothy Russell Havemeyer Barn at New Bolton Center was named in her honour. The behaviour laboratory, where the Foundation's study on stallion behaviour is conducted, is based in the Havemeyer Barn.

The Foundation is justifiably proud of its research activities and its programme of workshops. This series of monographs has been launched to ensure that the information gained is disseminated as widely as possible.

Gene Pranzo

President, Dorothy Russell Havemeyer Foundation

HAVEMEYER SCIENTIFIC WORKSHOPS

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

1995

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

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

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

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

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

1997

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

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

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

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

1998

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

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

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

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

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

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

1999

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

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

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

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

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

2000

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

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

EDITORS' FOREWORD

Many significant advances in the field of equine gamete research have been made by individual scientists, or groups of scientists, working within Europe. However, until now there has been no formal liaison between these individuals and groups which may have hampered progress and resulted in unnecessary duplication of effort.

This lack of communication was the motivation for forming the European Equine Gamete Group (EEGG) and it is to be hoped that, by facilitating a means of regular dialogue and active collaboration between all those working in Europe in this very specialised field, it will be possible to enhance research efforts considerably and expedite increases in understanding and knowledge of the subject.

It is particularly appropriate that the first meeting of EEGG has been held in the idyllic village of Lopuszna, in southern Poland, the ancestral home of Professor Marian Tischner. He, together with his old mentor the late Wladislaw Bielanski, were pioneers in the field of equine gamete technology and their classical studies in the 1960s and 1970s were a great source of encouragement for so many who are currently actively involved in the field.

The meeting was made possible by the generous financial support of the Havemeyer Foundation and sincere thanks are due to the Foundation Trustees, for their foresight in recognising the importance of the topic to the international horsebreeding industry. The Foundation has initiated a series of Monographs to publish information arising from its Workshop Meetings and we are very pleased that these proceedings represent the first in the series.

W. R. Allen
J. F. Wade

SESSION 1:

Stallion semen - research aspects

Chairman: W. R. Allen

INDUCTION OF THE ACROSOME REACTION BY PROGESTERONE BINDING TO A NON-GENOMIC RECEPTOR EXPOSED ON THE APICAL PLASMA MEMBRANE OF CAPACITATED SPERM CELLS

B. Colenbrander, R. Rathi, M. M. Bevers and B. M. Gadella*†

*Department of Equine Sciences, *Department of Farm Animal Health and †Department of Biochemistry and Cell Biology, Graduate School of Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 12, 3584 CL, Utrecht, The Netherlands*

The sperm acrosome reaction (AR) is a calcium dependent, exocytotic event required for fertilisation. The AR facilitates the penetration of the zona pellucida by spermatozoa and subsequent fusion of the sperm plasma membrane with the oocyte's oolemma. Ejaculated spermatozoa require a series of preparatory changes in order to undergo the AR. These physiological changes are collectively termed 'capacitation'. The capacitation process of stallion sperm involves complex changes in the composition and orientation of molecules at the surface of the sperm cell. These membrane changes relate to intrinsic membrane properties such as fluidity and permeability and are required for the induction of sperm hypermotility and increased affinity of sperm cells for the zona pellucida.

Capacitation can either occur *in vivo* during the passage of spermatozoa through the female genital tract, or *in vitro*, during incubation of washed spermatozoa under proper conditions. The AR of bovine, and *in vitro* capacitating human sperm suspensions is induced by adding follicular fluid from mature Graafian follicles.

In stallion sperm we observed similar changes induced by follicular fluid from mature follicles (fluid obtained by ovum pick up techniques). However, this potential was diminished when the follicular fluid was treated with charcoal in order to extract lipids and steroids from the fluid. The follicular fluid regained its full potential by re-addition of physiological concentrations of progesterone to the fluid. This fits well with previously published observations describing that addition of progesterone to an *in vitro* capacitation medium induced the AR in mouse and human sperm cells. Therefore, progesterone is thought to play a part in the induction of AR after capacitation although its receptor has not been identified or localised on the stallion sperm cell. Furthermore, the mechanism by which progesterone can induce the AR in sperm cells remains unknown. Normally steroid receptors are found in the nucleus and cytosol of somatic cells and steroid receptor binding will change gene expression patterns resulting in cellular responses. However, such a mechanism cannot be proposed for sperm cells because these cells lack an endoplasmic reticulum and ribosomes for protein synthesis. Also, the entire DNA in the nucleus is fully compacted to protamines so RNA synthesis and DNA duplification are completely blocked. In fact mRNA is absent from the cytosol. Although progesterone may diffuse through the plasma membrane and might bind to a cytosolic receptor involved in the induction of certain genes in somatic cells, it clearly will not have any effect on the gene expression of the sperm cell. Therefore, the progesterone has to elicit its AR inducing effect on capacitated sperm cells by other intracellular signalling pathways. Recently, non-genomic steroid receptors (including the progesterone receptor) have been characterised on the surface of diverse somatic cell types.

The localisation of the progesterone receptor, as well as the cellular responses to progesterone (progesterone induced AR in capacitated sperm cells), was studied using stallion semen as a model. Progesterone conjugated to a fluorescein-albumin complex (P-BSA-FITC) in combination

with a vital stain ethidium homodimer was applied to visualise the presence of the progesterone receptor on living spermatozoa.

Alternatively, indirect immunofluorescence localisation of a monoclonal antibody (C-262) for the intracellular progesterone receptor was studied. Immunogold labelling enabled ultralocalisation of P-BSA-FITC or C-262 using electron microscopy. The dynamic changes in labelling patterns on sperm cells were monitored by fluorescence microscopy and flow cytometry during a 5 h capacitation period. An increasing number of viable cells showed affinity for P-BSA-FITC at the acrosomal plasma membrane region of the sperm head, while a decreasing number of viable cells was not labelled. This was due to a capacitation dependent release of a post epididymal coating factor of the progesterone receptor. The P-BSA-FITC binding could be blocked by pre-incubation with unconjugated progesterone in a dose-dependent manner showing competition for the same binding site.

Furthermore, BSA-FITC (ie without conjugated progesterone) did not have any affinity for stallion sperm cells. These observations made us confident that P-BSA-FITC binds to a progesterone receptor on the plasma membrane of sperm cells (the BSA-FITC moiety of P-BSA-FITC is membrane insoluble) and only via the progesterone moiety of the P-BSA-FITC reporter probe. Incubation of capacitated sperm suspensions with 3.2 µM progesterone did not induce sperm hypermotility but resulted in increased affinity for the zona pellucida and induced the AR (a similar effect was elicited by P-BSA-FITC). Therefore, coupling of progesterone to its receptor on the sperm plasma membrane seems to be an important step during sperm capacitation.

From studies on human and mouse sperm cells it has been established that progesterone induces the entry of calcium from the extracellular environment into the sperm cytosol by opening voltage dependent calcium channels (probably the T-type). This influx of calcium is an absolute requisite for the initiation of membrane fusions involved in the AR. Most likely the T type voltage dependent calcium channels are opened after a depolarisation of the sperm plasma membrane. Currently, it is believed that progesterone after coupling to its receptor induces this depolarisation because the receptor communicates or forms a complex with a GABAa receptor-like Cl- channel. It should be mentioned that the sperm cell will physiologically encounter an environment with very high progesterone concentrations at the site of fertilisation.

During ovulation the oocyte is released into the oviduct from a follicle filled with a fluid that contains very high levels of progesterone. The zona pellucida is, therefore, impregnated with progesterone. Probably, progesterone has a synergistic effect on the AR induction by zona proteins. In order to get more fundamental insights into how progesterone induces AR, we would like to isolate and characterise the molecular structure of its receptor in the plasma membrane.

Conclusively, an increasing amount of stallion spermatozoa exposes a plasma membrane progesterone receptor during *in vitro* capacitation treatment. The coupling of progesterone to its receptor is an important step toward the AR induction. The exposure of the progesterone receptor and the subsequent coupling of progesterone are probably involved in sperm binding to the zona pellucida as well as zona-induced AR.

ACKNOWLEDGEMENTS

Dr B.M. Gadella is supported by the Royal Dutch Academy of Sciences and Arts (KNAW).

THE EFFECT OF SEMINAL PLASMA ON THE MOTILITY AND CRYOPRESERVABILITY OF HORSE EPIDIDYMAL SPERM

T. A. E. Stout*, L. H. A. Morris, X. Li and W. R. Allen

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

INTRODUCTION

The epididymes of the stallion contain large numbers of morphologically normal and viable spermatozoa and they therefore represent an available source of germ plasm in the event of death or castration. Unfortunately, these sperm exhibit very poor motility which makes them unsuitable for conventional intrauterine insemination. This probably results either from their lack of exposure to specific 'activating factors' present in seminal plasma or a failure to dilute or remove 'quiesence-inducing factors' present in epididymal secretions. Not surprisingly, Braun *et al*. (1994) found that exposure of stallion epididymal spermatozoa to seminal plasma markedly improved their motility. However, the effect of seminal plasma on the cryopreservability of stallion epididymal spermatozoa has not been investigated and is much more difficult to predict. It is generally believed that seminal plasma is detrimental to sperm survival after freezing and thawing and hence, prior to conventional cryopreservation of stallion semen, the bulk of the seminal plasma is removed by centrifugation. However, Aurich *et al.* (1996) found that treating the semen of 'poor-freezing' stallions with the seminal plasma of 'good-freezing' stallions markedly improved post thaw sperm motility. The aim of the present experiment was to determine if seminal plasma recovered from stallions exhibiting good sperm motility and post freeze fertility, would improve the motility and freezeability of epididymal spermatozoa.

MATERIALS AND METHODS

Immediately after elective castration, the testes of 8 2-year-old, one 3-year-old and one 6-year-old stallions were recovered and the cauda epididymes dissected free. Spermatozoa were flushed from each cauda using 8 ml of either a skim-milk extender or a 1:1 mixture of extender and seminal plasma that had been prepared by centrifuging the gel-free ejaculates of 3 fertile stallions at 4,000 *g* for 15 min. The motility of both the raw epididymal spermatozoa and those flushed out of the epididymis with the extender was assessed and smears were stained with nigrosin-eosin and propidium iodide for the assessment of, respectively, morphology and viability. The extended semen was centrifuged at 800 *g* for 10 min and the sperm pellet resuspended in a diluent containing 20% v:v egg-yolk and 5% v:v glycerol. Sperm motility was re-assessed before the extended semen was loaded into 0.5 ml straws which were frozen in liquid nitrogen vapour and then plunged into liquid nitrogen. Subsequently, one straw from each treatment for each stallion was thawed and the motility, morphology and viability of the sperm re-assessed.

RESULTS

Seminal plasma had a dramatic effect on sperm motility and, overall, it increased the mean total progressive motility (TPM) estimate of epididymal spermatozoa from 12 to 48%. Although sperm motility parameters were only measured subjectively, the most obvious components of the improved TPM were marked increases in both the velocity and progessiveness of sperm movement. This enhancement of motility conferred by seminal plasma was still evident after centrifugation and freeze-thawing although the effect was less pronounced (18% versus 10%). By contrast, exposure to seminal plasma did not affect either the morphology or the viability of epididymal spermatozoa, before or after freeze-

thawing. Marked differences between both pre- and post thaw motility of epididymal spermatozoa were evident between the stallions from which the testes originated.

Conclusions

In summary, seminal plasma improved the motility of epididymal spermatozoa dramatically and this effect was maintained partially after cryopreservation. Because sperm viability was not altered, it is likely that seminal plasma induced motility in live, but quiescent, sperm. It may, therefore, be useful to pre-treat epididymal sperm destined for cryopreservation with seminal plasma although, as the motility enhancing effects of human seminal plasma seem to be lost after freezing and thawing (Check *et al*. 1991) it would be interesting to examine the effects of treating epididymal sperm with fresh seminal plasma after, rather than before, freezing and thawing. The marked inter-stallion difference in epididymal sperm motility and freezeability was interesting and it suggests that some between-stallion differences in semen quality have their origin prior to ejaculation.

References

Aurich, J.E., Kuhne, A., Hoppe, H. and Aurich, C. (1996) Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. *Theriogenol.* **46**, 791-797.

Braun, J., Torres-Boggino, F., Hochi, S. and Oguri, N. (1994) Effect of seminal plasma on motion characteristics of epididymal and ejaculated stallion spermatozoa during storage at 5°C. *DTW Dtsch Tierarztl Wochensch*r. **101**, 319-22.

Check, D.J., Check, J.H. and Bollendorf, A. (1991) Fresh versus frozen seminal plasma for enhancing sperm motility in asthenozoospermic males. *Arch Androl* **26**, 79-81.

PRE-OVULATORY STORAGE OF STALLION SPERMATOZOA IN THE FALLOPIAN TUBE ISTHMUS

R. H. F Hunter

Faculty of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

The principal focus of this paper is the site of storage of a fertilising spermatozoon during the protracted pre-ovulatory interval in the mare. In particular, it questions the extent to which sperm transport strategies in equids parallel those in other mammalian species with intra-uterine accumulation of the ejaculate during a prolonged period of coitus. The paper also considers whether the mare's Fallopian tube acts rather precisely to regulate ad-ovarian sperm passage before ovulation, as is now known to be the case in laboratory rodents, ruminants such as sheep and cattle, and in pigs. A related aspect concerns ovarian control mechanisms that influence peri-ovulatory sperm activation and release from storage sites, and the question of whether these mechanisms function predominantly in a unilateral (ipsilateral) manner. This is not to deny that systemic endocrine influences are also at play, but *local* vascular and lymphatic pathways would offer a more sensitive and incisive means of controlling events within the Fallopian tube; they would also avoid extensive complexing of follicular steroid hormones with binding proteins in the systemic circulation.

Although the classical literature suggested storage of stallion spermatozoa in uterine glands, and more recent work has suggested a storage role for tissues of the utero-tubal junction, there are strong arguments in favour of the caudal (distal) isthmus of the Fallopian tube serving as a functional sperm reservoir during the pre-ovulatory interval. These include siting of the reservoir:

1) beyond gross influences of seminal plasma;
2) largely free of infiltrated polymorphonuclear leucocytes;
3) isolated from metabolic stimulation by uterine and ampullary fluids;
4) in a viscous glycoprotein secretion with suppressed motility; and
5) in a portion of the tract whose epithelium avidly binds viable spermatozoa.

After a pre-ovulatory mating, the sperm suspension in seminal plasma and uterine fluid would bathe the utero-tubal junction for a sufficient period until spermatozoa are established in the Fallopian tube; thereafter the seminal contents of the uterine lumen are expendable. Activation and release of a fertilising spermatozoon arrested by binding in the caudal isthmus could be programmed locally by a Graafian follicle on the verge of ovulation (and thus about to shed an oocyte): hormonal information would be transduced through the mucosa of the Fallopian tube and could reach bound sperm cells *via* endosalpingeal microvilli. Such a system would permit close synchronisation in the final maturation and release of male and female gametes.

Completion of capacitation would be a logical part of this ovarian activation scenario. As a corollary, suppression of full capacitation would be a significant requirement during the pre-ovulatory interval. Although ovarian endocrine control of stallion sperm capacitation appears as the most probable physiological means of coordinating this final phase of maturation, an alternative model would involve sequential waves of capacitation in sub-populations of spermatozoa, ie spontaneous but staggered waves of ripening leading to optimum membrane status, followed by decay and death of groups of spermatozoa in the upper reaches of the female tract. An essential pre-requisite in this less-favoured of the 2 models would be the presence of a sufficiently heterogeneous population of spermatozoa in the

Fallopian tubes to permit such a series of curves of asynchronous ripening and decay. A strong argument against this model is that progression through the utero-tubal junction selects a population of isthmus spermatozoa far more homogeneous than those in the uterus.

Apart from the conventional approaches of serial histology and scanning electron microscopy to establish details of an isthmus sperm reservoir in the mare, and its imposition of a quantitative control of sperm transport to the site of fertilisation, there should be scope for the delicate and demanding surgical approaches already used in sheep, cows and pigs. These involve:

1) transection of the isthmus progressively closer to the ampulla at progressively later post coital intervals to establish the extent of pre-ovulatory progression of viable spermatozoa; this would be monitored by subsequent recovery of the egg and examination for fertilisation and accessory sperm numbers;
2) overcoming the sperm regulatory functions of the isthmus by direct insemination of known numbers of spermatozoa into the ampulla to note the incidence of polyspermic fertilisation and thereby to deduce the extent of the sperm gradient along the Fallopian tube. The stability of the zona block to polyspermy could also be assessed by this method.

The specific nature of sperm head binding to the isthmus epithelium is being examined by a group at Cornell University, as is the nature of sperm activation and release from the epithelium. However, there remains the important question of the passage and fate of follicular fluid released at ovulation. Does it impact in whole or in part on the sperm reservoir and does it contribute in some direct manner to activation of the fertilising spermatozoon? Evidence from extensive studies in sheep, cows, pigs and rabbits indicates that sperm release in tightly controlled numbers from the isthmus reservoir commences shortly *before* ovulation. Experimental introduction of microdroplets of peri-ovulatory follicular fluid into the isthmus lumen of mated animals causes a massive wave of sperm release, this in turn inducing the pathological condition of polyspermic fertilisation. In the physiological situation, by contrast, large numbers of viable spermatozoa are released from the caudal isthmus *after* fertilisation is completed and an irreversible block to polyspermy established. Such spermatozoa do not compromise development of the zygote.

SUCCESSFUL LOW-DOSE INSEMINATION BY HYSTEROSCOPY IN THE MARE

L. H. A. Morris, R. H. F. Hunter* and W. R. Allen

*University of Cambridge, Department of Clinical Veterinary Medicine Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket Suffolk CB8 9BH; *Faculty of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK*

INTRODUCTION

A normal stallion ejaculate contains 2–15 x 10^9 spermatozoa and is deposited directly into the uterus of a mare in oestrous during natural mating. Satisfactory conception rates can be achieved by inseminating mares with 200–500 x10^6 motile spermatozoa into the body of the uterus. Unfortunately, this requirement for relatively high numbers of spermatozoa for conventional insemination limit the potential exploitation of newly available technologies such as the fluorescent activated cell sorting separation of spermatozoa into their X- and Y- chromosome bearing populations, which can currently be undertaken at a rate of only 2–4 x 10^6 cells/h. The present study was undertaken to determine the minimum number of spermatozoa in an intrauterine inseminate needed to achieve high conception rates in mares.

MATERIALS AND METHODS

In 2 successive breeding seasons a hysteroscopic insemination technique was used to deposit doses as low as 10, 5, 1, 0.5, 0.1 or 0.001 x 10^6 motile spermatozoa onto the utero-tubal papilla at the tip of the uterine horn ipsilateral to the ovary containing a dominant >35 mm pre-ovulatory follicle in oestrous mares. Semen was collected from one of 2 identical twin stallions and extended 1:1 with a skim-milk diluent containing glucose and antibiotics. A 1.5 ml aliquot of the extended semen was centrifuged at 200 *g* for 5 min and then at 800 g for 10 min through a discontinuous 90:45% Percoll density gradient to provide a very concentrated fraction of motile spermatozoa. These were resuspended in 30–150 µl Tyrode's medium supplemented with albumin, lactate and pyruvate (TALP), to give the pre-determined dose of spermatozoa. The aliquant was aspirated into an equine gamete intrafallopian tube (GIFT) catheter. This was passed through the working channel of a Pentax EPM 3000 videoendoscope which, in turn, was directed up the ipsilateral uterine horn to deposit the minimal volume of inseminate onto the uterine papilla of the utero-tubal junction. The combination of the TALP medium and the method of its deposition resulted in the formation of a 'clutch' of bubbles, which appeared to be important in retaining the inseminate on the surface of the papilla and hence close to the site of the potential sperm reservoir in the caudal isthmus of the oviduct. Coincidentally with this single uterotubal insemination, each mare was given an ovulation-inducing dose of either human chorionic gonadotrophin (hCG) or gonadotrophin-releasing hormone (GnRH). Daily blood samples for measurement of serum progesterone concentrations and daily ultrasound scanning of the ovaries were carried out to determine the time of ovulation.

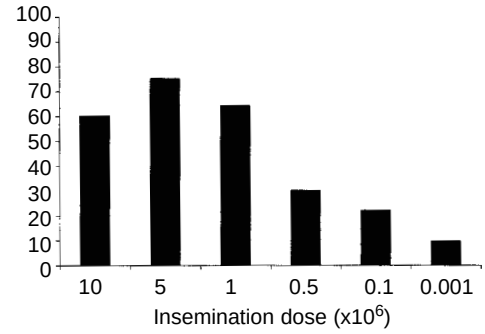

Fig 1: Conception rates from low-dose hysteroscopic insemination.

RESULTS

In total, 79% of the 78 mares used in the study had ovulated within 48 h after the insemination procedure. The total mean progressive motility of the spermatozoa ultimately used for insemination was increased by centrifugation through the Percoll gradient from 58% to 70%. As shown in the graph, hysteroscopic insemination of oestrous mares with 10, 5, 1, 0.5, 0.1 or 0.001 $x10^6$ motile spermatozoa resulted in conception rates of, respectively, 60, 75, 64, 29, 22 and 10%. There were no differences in the conception rates in the mares treated with hCG or GnRH, or in mares that ovulated within 24 or 48 h after administration of these ovulation inducing agents. Indeed, one of 2 mares that ovulated 4 days after the insemination procedure also became pregnant.

SESSION 1I:

Stallion semen - preservation

Chairman: M. Tischner

ASSESSING THE POTENTIAL FERTILITY OF FROZEN STALLION SEMEN

M. S. Boyle

Stallion Reproduction Services, 143 Huntingdon Road, Cambridge CB3 0DH, UK

INTRODUCTION

When semen is frozen for commercial use, there is a duty on the producer to supply a viable product. This can prove difficult as the only guarantee that a stallion's frozen semen has retained the ability to achieve conception is to use thawed samples for a series of successful test inseminations before the semen is offered for sale. In many commercial situations, this will not be a practical option. In addition, because there is often low repeatability between ejaculates from individual stallions (Vidament *et al*. 1997), it may not be valid to make general predictions about the fertility of frozen semen on the evidence of test inseminations using doses from a single ejaculate. Consequently, many producers of frozen semen rely on a post thaw assessment of sperm motility in each ejaculate to indicate whether the sample is still likely to be fully viable. Some authorities claim, however, that post thaw motility correlates poorly with fertility (Squires *et al*. 1987; Samper *et al.* 1991).

To overcome the perceived unreliability of visual motility assessments, various *in vitro* tests have been used in an attempt to improve the accuracy of post thaw semen evaluation. In many cases, there is little evidence to show that these tests are more reliable than visual motility assessments at predicting the fertility of stallion semen. In addition, many are too complicated or expensive to be of use in a busy commercial freezing programme.

In order to re-assess whether post thaw sperm motility could be used to indicate the potential fertility of frozen semen with sufficient consistency to be of use commercially, the results of a large scale commercial semen freezing programme were reviewed.

MATERIALS AND METHODS

Over a 4 year period, semen was collected from 227 stallions in a commercial freezing programme. The number of ejaculates frozen from individual stallions varied between one and 114. Overall, 1,702 ejaculates were processed and frozen.

Evaluation of fresh semen

Within 3 min of collection, gel free volume and sperm concentration of each ejaculate were measured and the semen sample was diluted at least 1:1 with either a skimmed milk/glucose (Kenney *et al.* 1975) or an egg yolk extender (de Vries 1987) in which penicillin and streptomycin were substituted for the sulphanilamide. The percentage of progressively motile spermatozoa was determined by microscopic examination of a standard drop (0.12 µl) of the diluted semen placed under a 22 x 22 mm cover slip on a heated stage. One experienced observer carried out all motility assessments.

Semen freezing

After centrifugation and removal of the supernatant, the sperm pellet was re-suspended in an egg yolk freezing medium (DV11) at a concentration to give a nominal insemination dose of 3 ml. The extended semen was loaded into 0.5 ml straws which were then cooled to -110°C in a programmable freezing machine before being plunged in liquid nitrogen for storage.

Post thaw evaluation

After freezing, at least one straw from each ejaculate was thawed at 37°C for evaluation.

Sperm concentration was determined using a haemocytometer. A 100 µl aliquot of the thawed semen was mixed 1:4 with warmed skimmed milk/glucose extender for microscopic evaluation of progressive sperm motility under similar conditions to those used for the fresh semen. The thawed semen was stored at 5°C and motility was re-assessed (at 37°C) at 24 and 48 h.

Selection of semen samples

Fresh semen: Following a visual assessment of sperm motility in the fresh semen, each ejaculate was allotted to one of the following categories depending on the percentage of progressively motile spermatozoa in the sample: good >65%; acceptable 60%–65%; poor <60%. Ejaculates in which progressive motility was <55% were discarded.

Frozen semen: Each batch of frozen semen was allotted to one of the following categories based on visual assessment of progressive sperm motility in the thawed sample: good >35%; acceptable 30%–35%; no commercial use <30%. A subjective assessment of sperm velocity based on a scale of 1 (unacceptable) to 4 (excellent) was used when allocating samples judged to be on the borderline between categories.

Semen for insemination: After the post thaw evaluation of each ejaculate, the actual number of 0.5 ml straws to use per dose was calculated to ensure a minimum of 300 x 10^6 progressively motile spermatozoa per insemination. Batches in which progressive sperm motility was >30% with satisfactory velocity were cleared for commercial use. Semen in which progressive sperm motility was less than 25% or in which sperm velocity was judged to be less than 2 were discarded. Borderline samples, which did not quite reach the standard for commercial use, were kept for test matings at the discretion of stallion owners.

Comparison of results between fresh and frozen semen characteristics

When analysing the data for either fresh or frozen semen, the results were pooled for each stallion in order to determine overall stallion potential rather than to provide information on individual ejaculates.

Fertility evaluation of frozen semen

To assess the fertility of the frozen semen, the conception rate per cycle achieved at 2 artificial insemination stations over a 4 year period was analysed. In total, frozen semen from 35 stallions was used to inseminate 137 mares in more than 190 cycles. In the majority of cases, insemination was carried out within 8 h following ovulation.

RESULTS

Fresh semen characteristics

Of the stallions, 45% characteristically produced fresh semen that was classified as good (progressive motility >65%), 47% produced semen within the borderline range (60%–65%) and 8% produced poor quality semen (<60%) that was considered unsuitable for freezing. With many stallions, there was often considerable variation between individual ejaculates especially among those that generally fell within the borderline range.

Effect of freezing and thawing on sperm motility

Of stallions with good sperm motility in fresh semen, 71% retained good motility in the frozen sample and of stallions with borderline sperm motility in fresh semen, 75% produced semen with post thaw motility that was acceptable or better. On the other hand, poor quality fresh semen

TABLE 1: Comparison of sperm motility in fresh semen with post thaw sperm motility

		Good	Borderline	Poor	
Fresh semen					
Percentage of stallion in each catagory		45%	47%	8%	
Frozen semen					Percentage of stallion in each catagory
Good	(>35%)	71%	15%	-	(32%)
Acceptable	(30-35%)	22%	60%	-	(44%)
No commercial use	(<30%)	7%	25%	100%	(24%)

TABLE 2: Artificial Insemination Centre 'A'

(Frozen semen from 21 stallions)			
Mares	Cycles	Conceptions	Conceptions/ cycle
108	162	91	56%
(overall conception rate of 84%)			

TABLE 3: Artificial Insemination Centre 'B'

(Frozen semen from 16 stallions)				
Year	Mares	Cycles	Conceptions	Conceptions/ Cycle
1996	1	1	1	100%
1997	7	9	7	78%
1998	9	9	8	89%
1999	12	13	9	77%
Total	29	32	25 (86%)	78%

(<60%) invariably survived poorly following freezing. The effect of freezing on sperm motility is shown in Table 1.

Comparison of semen freezability between breeds

There was considerable variation between breeds in the proportion of stallions that produced semen that froze satisfactorily. Figure 1 compares the post thaw motility characteristics (as shown in Table 1) between the main groups of stallions. In many cases, semen quality also varied widely between ejaculates with individual stallions frequently producing semen in which motility fell outside their characteristic range. The extent of this variability is indicated by differential hatching in the columns.

Conception rates achieved following insemination of frozen semen

Tables 2 and 3 show the results achieved when frozen semen passed as either good or acceptable, from a representative group of stallions, was inseminated under normal commercial conditions at 2 AI centres in the UK.

DISCUSSION

For the majority of stallions in this survey, the initial progressive sperm motility in fresh semen provided a reasonable indication of the results likely to be achieved after freezing. For example, only 7% of stallions with ejaculates in which initial progressive motility was >65% produced frozen semen that was of no commercial use; whereas, for none of the stallions with poor initial sperm motility was it possible to achieve satisfactory post thaw results. The relationship between initial motility and freezability was less clear cut in the borderline category with 25% of these stallions producing semen in which sperm survival following freezing was generally poor.

In 1979, Tischner reported results showing that semen from only 20% of stallions retained good motility after freezing with a further 20% producing semen with poor post thaw motility. In general, these figures are comparable to the findings of the present survey, the only significant difference being an improvement in the proportion of the stallions with semen that retained good post

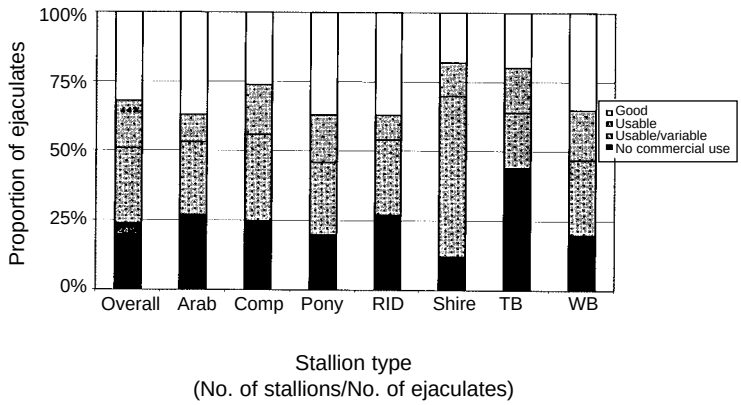

Fig 1: Comparison of semen freezability between breeds. The following abbreviations are used: Comp = British bred sporthorses usually a Thoroughbred cross; RID = Registered Irish Draught; TB = Thoroughbred; WB = Warmblood stallions either recently imported from Continental Europe or 1st or 2nd generation UK bred.

thaw motility. This is doubtless the result of improved freezing techniques that have been developed over the 20 years separating the 2 studies.

The breeds producing semen that tended to freeze satisfactorily fell into 2 categories. The first included ponies (native and riding), Irish Draught and Shires where there is usually least human intervention in the breeding process and where the influence of natural selection is, therefore, more pronounced. An alternative beneficial influence could be seen in semen produced by the Warmblood breeds in which fertility evaluation normally plays a part in the selection of stallions intended for breeding. Thoroughbred stallions achieved the least satisfactory results with 44% producing semen that could not be frozen successfully. It is tempting to speculate that the intense commercial pressure to breed from successful Thoroughbred sires, combined with the resources available to enable even subfertile stallions to pass on their genes, has led to a general decline in semen quality in the breed.

It is often difficult to establish the true fertility of frozen semen when it is used commercially. Once semen has been sold, there is little control over standards of mare selection, management, semen handling or insemination technique. In addition, feedback from mare owners is seldom complete and is usually inaccurate. In the present study, in an attempt to overcome the many variables, the results achieved by semen from a representative group of stallions used at 2 artificial insemination centres in the UK were studied. Each centre was under the control of experienced inseminators operating under standard conditions.

The conception rate achieved by Centre 'A' includes a number of experimental inseminations where either the semen used was classified as unsuitable for commercial use or the mares had conception prospects that were less than ideal. Nevertheless, the figure of 56% conceptions compares well with the results reported by other workers. At Centre 'B', the overall conception rate of 78% was comparable to those achieved at well managed studs using natural mating or insemination with fresh semen. In each case, there was no significant evidence to indicate that individual stallions had achieved worse results than would have been expected from post thaw motility assessments.

These figures suggest that, in the absence of a more accurate alternative that is both reliable and simple to use, post thaw motility assessment provides an acceptable way of predicting the fertility of frozen stallion semen provided that it is allied with careful stallion selection and strictly applied quality control over the semen released for use.

REFERENCES

de Vries, P.F. (1987) Evaluation of the use of fresh, extended, transported stallion semen in the Netherlands. *J. Reprod. Fertil. Suppl.* **35**, p 641.

Kenney, R.M., Bergman, R.V., Cooper, W.L. and Morse, G.W. (1975) Minimal contamination techniques for breeding mares: techniques and preliminary findings. *Proc. Am. Ass. equine Pract.* pp 327-335.

Samper, J.C., Hellander, J.C. and Crabbo, B.G. (1991) Relationship between the fertility of fresh and frozen stallion semen and semen quality. *J. Reprod. Fertil. Suppl.* **44**, 107-114.

Squires, E.L., Aman R.P. and Pickett B.W. (1987) Preservation of stallion semen - Colorado experience. In: *Symposium on Equine Reproduction.* Ed: T. Greve: Intervet Scandinavia, Skovlunde, Denmark. 51-56.

Vidament, M., Dupere, A.M., Julienne, P., Evain, A., Noue, P. and Palmer, E. (1997) Equine frozen semen: freezability and fertility field results. *Theriogenol.* **46**, 907-917.

RELATIONSHIP BETWEEN SPERM CHROMATIN STRUCTURE, MORPHOLOGY AND MEMBRANE QUALITY IN STALLION SPERMATOZOA BEFORE AND AFTER CRYOPRESERVATION

H. Torner, C. Warnke* and S. Blottner†

*Research Institute for Biology of Farm Animals, Department of Reproductive Biology, 18196 Dummerstorf, Germany; *University of Rostock, 18069 Rostock, Germany; †Institute for Zoo Biology and Wildlife Research, 10315 Berlin, Germany*

INTRODUCTION

Current methods of evaluating stallion fertility are based on morphological examination of spermatozoa and by calculating the number of normal, progressively motile sperm ejaculated. These methods provide basic information only about sperm quality. Therefore it is very important to develop more sensitive tests for characterisation of semen quality in stallions. The evaluation of cryopreserved semen is particularly complex because good post thaw motility does not always correlate with good fertility, indicating that subcellular damage can affect the fertility without a concurrent impact on motility (Christensen *et al*. 1995). A better understanding of both the physiological and the morphological changes seen in spermatozoa would aid the development of improved techniques for the cryopreservation of stallion spermatozoa. The aim of the present investigation therefore was to describe the changes in sperm chromatin structure, membrane quality, morphology and motility of stallion spermatozoa during routine procedures for cryopreservation. Furthermore the possible correlation between morphological and physiological parameters for stallion sperm evaluation was analysed.

MATERIALS AND METHODS

Semen samples were collected from 6 stallions via an artificial vagina. Three replications were carried out. Each ejaculate was diluted immediately 1:1 in skim milk extender up to the adjustment of sperm concentration to 25×10^6/ml. Extended semen were centrifuged at 900 *g* for 20 min at room temperature. After removing the supernatant, one part was re-suspended in skim milk extender for transport of chilled spermatozoa, the other was re-suspended in egg yolk extender, packaged in 0.5 ml polyvinyl chloride straws and frozen.

After transport of chilled spermatozoa or thawing of frozen spermatozoa from the same ejaculate the samples were diluted, centrifuged and washed with TALP. Forward progression was assessed microscopically in the dark field of a phase contrast microscope. Head and tail abnormalities and condition of the acrosome of spermatozoa were assessed from smears stained by saturated congo-red (2 min), tannin (3 min) and 3% aqueous brilliant cresyl-blue (30–45 s). Damaged plasma membrane (membrane integrity) was detected by fluorescence staining in suspension with propidium iodide. The integrity and resistance of DNA (chromatin integrity) was examined by the microscopic acridine orange test (Tejada *et al*. 1984). To investigate chromatin stability, an artificial sperm decondensation test was used. Before the acridine orange test was carried out, the spermatozoa were treated with 0.32% DTE/Papain (Hingst and Blottner 1993). Percentages were estimated from 200 spermatozoa/sample. To investigate the membrane potential (+inside), the sperm cells were stained with 2.5 µM DIBAC (Molecular Probes) and the part of fluorescence labelled cells were counted by flow cytometry. The intracellular Ca^{2+} concentration was evaluated after pre-treatment of sperm cells with pluronic, followed by staining with Fluo 3 AM and measurement of fluorescent sperm cells by flow cytometry (10.000 counts/ measurement).

RESULTS

Stallion spermatozoa from different donors with the same motility before freezing (about 60%) showed different suitability for cryopreservation (forward

progression after thawing ranged from 9–27.5%). Cryopreservation led to a significant decrease in progressive motility and morphologically normal spermatozoa ($P<0.001$). The main portion of abnormal spermatozoa after freezing was without acrosome (52–69%). Regardless of donor, the freezing process led to a drastic increase of membrane defects (12.5% vs 67%, $P<0.001$). Only 6.5–11.15% of spermatozoa showed chromatin defects after freezing.

After decondensation treatment with 0.32% DTE/Papain the portion of spermatozoa with chromatin defects increased (10.8–20.7%) and varied between donors.

After freezing, the percentage of spermatozoa which were DIBAC-labelled (membrane potential + inside) increased (7.9% vs 26.4%; $P<0.01$). The percentage of spermatozoa with a high level of intracellular Ca^{2+} (Fluo 3AM labelled) decreased after cryopreservation (78.1% vs 58%, $P<0.01$).

Discussion

The data indicate that the freezing process led to drastic changes in physiological and morphological parameters in stallion spermatozoa. The experiment has shown that chromatin integrity and chromatin stability are highly correlated in stallion spermatozoa ($r=0.69$; $P<0.005$). The results suggest that the motility of spermatozoa after cryopreservation depends on the membrane quality and is negatively correlated with the percentage of spermatozoa with membrane damage ($r= -0.71$; $P<0.001$). It seems that the increasing amount of spermatozoa with positive membrane potential after thawing reflects a higher passive diffusion potential in sperm (Cooper *et al.* 1990). Additionally the Ca^{2+} level in spermatozoa after freezing decreases, mainly caused by the increased passive diffusion potential of the spermatozoa membrane. Further investigations are focusing on the characterisation of functional and morphological changes in stallion spermatozoa, in relation to different methods of cryopreservation.

References

Cooper, C.E., Bruce, D. and Nicholls, P. (1990) Use of Oxonal V as a probe of membrane potential in proteoliposomes containing cytochrome oxidase in the submitochondrial orientation. *Biochem.* **29**, 3859-3865.

Christensen, P., Parlevliet, J., Van Butten, A. and Hyttel, P. (1995) Ultrastructure of fresh and frozen-thawed stallion spermatozoa. *Biol. Reprod. Monograph Series* **1,** 769-777.

Hingst, O. and Blottner, S. (1993) Assessment of the chromatin structure of cat sperm before and after cryopreservation. J. *Reprod. Fert.* **12,** 59.

Tejada, R.I., Mitchel, J.C., Norman, A., Marik, J.J. and Friedman, S. (1984) A test for the practical evaluation of male fertility by acridine orange fluorescence. *Fertil. Steril.* **42,** 87-91.

EVALUATION OF FROZEN STALLION SEMEN

T. Katila, P. Kuisma and M. Andersson

University of Helsinki, Department of Clinical Veterinary Sciences, Saari Unit, Pohjoinen Pikatie 800, FIN-04920 Saarentaus, Finland

INTRODUCTION

Some semen evaluation methods work reasonably well for the assessment of fresh equine semen, but not of frozen semen. The best example is motility evaluation. It is still the most commonly used method for evaluation of frozen-thawed stallion semen, both in laboratories and studfarms, because it is the only technique available in practice conditions. There are very few data about the correlation between semen evaluation tests and pregnancy rates after insemination with frozen semen. Although good correlations have been found between different tests, it does not mean necessarily that they would correlate with fertility.

The purpose of this study was to find out which tests used to evaluate stallion semen would correlate with foaling rates of mares inseminated with frozen semen.

MATERIALS AND METHODS

Frozen semen doses were available from 40 commercially used stallions. The semen had been frozen between 1988 and 1997 in 5 different countries: 29 in Sweden; 5 in Finland; 3 in Italy; one in Denmark; one in Germany; and one in the USA. Semen from 2 stallions had been frozen in 5 ml straws, 30 in 2.5 ml straws and 8 in 0.5 ml straws. Twenty stallions were American Standardbreds and the remaining 20 were different kinds of riding horses. Sufficient foaling data – at least 5 mares and 10 cycles – were available from 31 stallions. Foaling data originated from Finland and Sweden from the years 1989 to 1998. The average number of mares/stallion was 33 (range 5–121). The average foaling rate was 58% (range 0–83%). Twelve stallions had a foaling rate >50% and 19 had a foaling rate <50%.

Semen evaluation

Usually 3 straws were needed to perform all the analyses. If only one or 2 straws were available, 2 laboratory technicians worked in the laboratory simultaneously to perform all the tests in a freshly thawed sample. From 14 stallions all tests were conducted on the same batch. For the rest of the stallions, the batches had been frozen within the same month.

The 0.5 ml straws were thawed at +37°C for 30 s in the 2.5 and 5 ml straws at +50°C for 40 or 45 s, respectively. An aliquot of 0.1 ml semen was cultured on a blood agar at +37°C for 48 h. Colony forming units (CFU) were counted after an incubation of 24 and 48 h. Semen concentration was measured in a counting chamber and the total number of spermatozoa/straw calculated. An insemination (AI) dose was one straw when using 2.5 or 5 ml straws and 1–10 of the 0.5 ml straws.

Semen was extended with warm (+30°C) skim milk extender (Kenney *et al*. 1975) to a concentration of 20–30 x 10^6 spermatozoa /ml. Post thaw motility was evaluated subjectively with a light microscope: percentage of progressively motile spermatozoa, percentage of total motility and a velocity score from 1 to 3. Motility was measured also with an automatic sperm analyser (Hamilton Thorn Motility Analyser, HTM-S, version 7.2). A 10 ml semen sample was placed onto a Makler chamber and 2 chambers were prepared from the same sample. Six fields/chamber were video taped for 15 s/field. The video tapes were analysed for total motility, progressive motility and path velocity.

The sample for the longevity test was prepared by placing 0.5 ml of extended semen into a 3 ml vial enclosed with a cap. The sample was kept in a water bath at +37°C for 4 h. The total

and progressive motility and velocity were evaluated by light microscope every hour.

A second straw was thawed for the evaluation of plasma membrane integrity by CFDA/PI staining (carboxyfluoresceindiacetate/propidium iodide). Semen was extended with skim milk extender to a concentration of 50 spermatozoa/ml, of which 950 µl were taken and mixed with 20 µl of formol citrate (1.7 mM). An aliquot of 20 µl CFDA stock solution consisting of 0.46 mg CFDA in 1 ml of DMSO (dimethylsulphoxide) and 10 µl of PI stock solution (0.5 mg PI in 1 ml of 0.9% NaCl-solution) were taken, mixed with semen-formol solution and incubated for 8 min at +30°C. A drop of 5 µl was placed on a slide and overlaid with a cover slip (Harrison and Vickers 1990). The proportion of fluorescent cells was counted in 200 cells in a fluorescence microscope (Olympus BH2 with epifluorescence optics) using oil immersion and a fluorescein filter set.

A third straw was thawed for the plasma membrane viability test by an automatic fluorometer (Fluoroscan Ascent), which reads a 96 well microtitration tray and has an incubation compartment. The interference filter at the exitation path and that of the emission filter had a maximum transmission at 544 nm and 590 nm, respectively. For the fluorometric assay, 20 mg of PI was dissolved in 1:l of BTS (Beltsville Thawing Solution) and dispensed in 3 ml aliquots. Equal aliquots (50 ml) of BTS extended semen sample (80 x 10^6 spermatozoa/ml) and PI solution were dispensed into the well plate, and the well was shaken gently for 2 min. Spermatozoa from the same samples were killed by unprotected rapid freezing-thawing to provide internal control samples consisting of only non-viable cells (100% fluorescence). The control sample was immersed into liquid nitrogen for 1 min and thereafter it was allowed to stand at room temperature for 30 s and then 3 min in a waterbath (37°C). Blanks containing 50 µl of diluted extender and 50 µl of PI were analysed separately for every experiment in 4 replicates. The incubation time was 8 min. The percentage of fluorescence was calculated from the ratio of fluorescence intensities of the rapidly frozen control sample and the sample to be analysed, taking into account the blank values (Juonala *et al.* 1999). For the resazurin reduction test, 400 mg of resazurin were dissolved in 1:1 distilled water. One part of this solution and 9 parts of 0.9% NaCl were mixed. An equal volume of this mixture and extended sperm were combined, shaken for 2 min, incubated for 30 min at 34°C and measured with the fluorometer using the same fluorometer settings as in the plasma membrane viability test (Eriksson *et al*. 1998).

Data analysis

The results were analysed by Statgraphigs using correlation matrix. The level of significance was set at $P<0.05$. Only the results of the 31 stallions with sufficient foaling data were included in the statistical analysis.

RESULTS

Twenty straws showed no bacterial growth, 9 yielded slight growth (<10 CFU), 5 medium growth (10–100 CFU) and 6 straws yielded heavy growth (>100 CFU). There was a wide range of bacteria in the positive samples. The most common organisms were *Enterobacteria, Corynebacteria and coliforms*. The occurrence of bacteria had no correlation with foaling rates.

The total number of spermatozoa in an AI dose was, on average, 655 x 10^6 (ranging from 202 to 1,772 x 10^6). The number of progressively motile sperm was, on average, 254 x 10^6 (ranging from 81 to 612 x 10^6). The dose had no correlation with the foaling rate.

The only parameter that correlated significantly with foaling rate was motility during storage at 37°C, both at 2 h ($P=0.0092$) and at 4 h ($P=0.0286$).

Some of the measured parameters had high correlations with each other. When all parameters were analysed in the same semen batch (14 stallions), very highly significant correlations ($P<0.001$) were detected between initial total and progressive motility measured by HTM-S and by light microscopy. Fluorometric viability measurement had a good correlation with 0 h light microscope motility ($P=0.0006$), CFDA ($P=0.0025$), with HTM-S progressive ($P=0.0165$) and total motility ($P=0.0238$) and between resazurin reduction test ($P=0.0170$). CFDA correlated also with initial motility parameters ($P<0.05$). Progressive motility at 2 h correlated significantly with initial total and progressive motility and with 4 h motility, but motility at 4 h did not correlate with any other parameters.

CONCLUSIONS

None of the parameters measured immediately after thawing correlated significantly with the foaling rate. Only motility assessed after 2 and 4 h of incubation at 37°C correlated significantly with foaling rates. However, no other parameters were evaluated in the longevity trial. It is possible that the stressful condition during storage is the best way of distinguishing between high and low fertility frozen semen. Also other parameters that were only performed immediately after thawing in the present study should be evaluated after storage in future studies.

REFERENCES

Eriksson, B., Juonala, T., Andersson, M. and Rodriguez-Martinez, H. (1998) Viability of frozen-thawed boar semen. *Proc. 50th Anniv. int. Conf. anim. Reprod.* **526**.

Harrison, R.A.P. and Vickers, S.E. (1990) Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J. Reprod. Fert.* **88,** 343-352.

Juonala, T., Salonen, E., Nurttila, T. and Andersson, M. (1999) Three fluorescence methods for assessing boar sperm viablity. *Reprod. dom. Anim.* **34**, 83-87.

Kenney, R.M., Bergman, R.V., Cooper, W.L. and Morse, G.W. (1975) Minimal contamination techniques for breeding mares. *Proc. Am. Ass. equine. Pract.* **21,** 327-336.

SESSION III:

Stallion semen - commercial aspects

Chairman: B. Colenbrander

EFFECT OF GLUCOCORTICOID TREATMENT ON FRESH AND DEEP FROZEN SEMEN AND ON ENDOCRINE PARAMETERS IN THE STALLION

J. Juhasz, M. Vidament*, P. Nagy, M. Kulcsar, D. Guillaume*, M. Magistrini* and Gy. Huszenicza

*University of Veterinary Science, Budapest, Hungary; *INRA, Equine Reproduction, Nouzilly, France*

The aim of the study was to determine the possible effects of a glucocorticoid treatment on the reproductive function of breeding stallions. Nine stallions of various breeds, aged 3–17 years were included. They were divided into 2 groups according to their age and semen quality (5 treated and 4 control stallions). Treated stallions were given dexamethasone-sodium phosphate (30 mg/stallion/day, Dexadreson, Intervet, The Netherlands) for 7 days in mid-November. Semen was collected and frozen twice a week from mid-September until the end of January. For freezing, lactose-EDTA-egg-yolk freezing extender was used. Freezing was carried out in 0.5 ml straws in 200 x 10^6 spermatozoa/ml final concentration above N_2 vapour.

The following parameters were evaluated from fresh semen: total and gel-free volume, pH, progressive motility (under light microscope), concentration, total sperm number, morphology (fixed in formol solution, determined under phase interference microscope), acrosome integrity (stained with FITC-PSA). After thawing the following parameters were evaluated: post thaw motility (under light microscope), membrane functional integrity (HOS test), acrosome integrity (stained with FITC-PSA) and morphology (fixed in formol solution, determined under phase interference microscope).

GnRH (40 mg Receptal, Hoescht, Germany) and hCG (10,000 iu Chorulon, Intervet, The Netherlands) challenge tests were carried out before, immediately after, one month and 2 months after the end of the treatment. During the treatment period, daily blood collections were carried out. From the plasma samples LH, FSH and testosterone were determined. TRH- (Sigma, USA) and ACTH-stimulations (60 mg Cortrosyn, Organon, The Netherlands) were carried out before, one day and 16 days after treatment. From the plasma samples T3, T4 and cortisol were determined.

In fresh semen, total sperm number per ejaculate was significantly ($P<0.001$) higher in the treated group on the sixth week after treatment. Midpiece abnormalities tended to be increased in 2 treated stallions between Days 10 and 40, proximal protoplasmic droplet tended to be higher in one treated stallion between Days 14 and 55 after treatment. No other parameters differed significantly or tended to be lower or higher between the pre-treatment and post treatment periods or between the treated and control groups. Post thaw motility tended to be decreased in 2 of 4 treated stallions beginning on Day 10 after treatment and returned to pre-treatment level around Day 60. Results of HOS tests showed a similar pattern and tendency to post thaw motility in all stallions. No effect of treatment could be observed on morphology or acrosome integrity.

During treatment, plasma testosterone levels were significantly lower and LH levels significantly higher in treated stallions ($P<0.05$). Plasma FSH concentration was not affected by the treatment. LH response to GnRH-stimulation was significantly higher immediately after the end of the treatment in the treated group ($P<0.05$). Testosterone response to GnRH-stimulation was significantly higher in the treated group one month after the end of the treatment ($P<0.05$). FSH response to GnRH-stimulation was not altered by the treatment. Testosterone response to hCG-stimulation was the same in the treated and control groups in all challenge periods. Basal cortisol concentrations and maximal response to ACTH were significantly decreased one day after treatment in treated stallions ($P<0.001$). This difference disappeared by Day 16. Basal T4-levels

and maximal response to TRH were significantly lower ($P<0.05$) in treated stallions by Day 16. A similar but not significant tendency was also observed for T3 in the same group.

It is well known and accepted that corticosteroids in general suppress the function of the hypothalamus-pituitary-gonadal axis (Moberg 1987). They can act at the level of hypothalamus, pituitary and the gonads altering their hormone synthesis and/or release function. The site of their effect depends on dose and length of exposure. Most studies have investigated the effect of stress or various adrenal diseases (Cushing's syndrome) on reproductive function (McKenna *et al.* 1979). Our experiment did not aim to model the stress, but to investigate the side effect of dexamethasone treatment on the reproductive function of stallions.

Dexamethasone treatment with the applied dose primarily altered the testosterone production of the testes in the short term. There are glucocorticoid binding receptors in the testes which can alter the testosterone producing function of Leydig-cells after binding has occurred (Welsh *et al.* 1979; Sapolsky 1985; Moberg 1987). This could be why the testosterone levels were significantly lower in the treated stallions during the treatment period. During treatment, LH levels were significantly higher in the treated group which could be caused by the decreased testosterone level or by the direct effect of dexamethasone on hypothalamus or pituitary.

This elevated LH level and storage could be the reason for the significantly higher LH response to GnRH-stimulation immediately after the treatment. Unchanged testosterone response to hCG-stimulation after treatment indicates that dexamethasone at the applied dose did not have a negative influence on the testosterone producing capacity of the testes. There were no significant or marked alterations in semen parameters in the treated stallions although, in some cases, slight differences were observed which could be caused by individual sensitivity to the drug. Decreased testosterone level during treatment is probably sufficient to maintain normal spermatogenesis.

In conclusion, dexamethasone applied at the recommended therapeutic dose does not cause long term alterations in the endocrine profiles or significant changes in semen parameters in breeding stallions.

REFERENCES

Moberg, G. P. (1987) Influence of the adrenal axis upon the gonads. *Oxford Rev. Reprod. Biol.* **9**, 456-496.

McKenna, T. J., Lorber, D., Lacroix, A. and Rabin, D. (1979) Testicular activity in Cushing's disease. *Acta endocr.* **91**, 501-510.

Sapolsky, R. M. (1985) Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids. *Endocrinology* **116**, 2273-2278.

Welsh, T. H., McGraw, R. L. and Johnson, B.H. (1979) Influence of corticosteroids on testosterone production in the bull. *Biol. Reprod.* **21**, 755-763.

FERTILITY DATA IN THE HORSE

K. Darenius

Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, 8-750 07 Uppsala, Sweden

INTRODUCTION

The aim of the present work was to study the occurrence of pregnancy and neonatal loss with regard to differences between stallions as well as between various categories of mares and breeding techniques, using field data from one carefully controlled studfarm.

MATERIALS AND METHODS

Fertility data from 1,206 mares (Standardbred trotters [SB], Swedish Warmbloods [SWB] and Thoroughbreds [TB]) were collected during 7 breeding seasons. The mares were mated/inseminated at one stud, under the same management conditions. The stud is in central Sweden and most mares were mated during May to July. Different breeding techniques were used, natural breeding as well as artificial insemination (Al) with fresh, chilled or frozen semen.

During oestrus, gynaecological examinations were generally performed on alternate days to monitor follicular and endometrial activity, to detect any pathological conditions before and after mating/insemination and to confirm ovulation. In the case of natural breeding and Al with fresh and chilled semen, matings/inseminations were performed every second day. Most of the mares were inseminated only before ovulation.

For frozen semen, mares were examined daily. Close to ovulation they were examined 2–4 times a day to determine the approximate time of ovulation. They were inseminated within 12 h pre-ovulation (39% of the mares), within 6–8 h post ovulation (37%) or both pre- and post ovulation (24%).

Ultrasound scanning for pregnancy diagnosis was performed around Day 14–17 and, in most of the mares, also Day 40–45 post ovulation. Additional examinations were carried out in mares with twins or where early fetal death was suspected. The conception rates are based on the results from examinations performed at the earliest convenient opportunity.

The variations among stallions, breeding methods and categories of mares, as regards the various fertility measures used, was analysed using normal X2 tests in order to prove statistically any heterogeneity among classes. Levels of significance are given as follows: ns=not significant, *=P<0.05, **=P<0.01,***=P<0.001.

DEFINITIONS

The following terms are used in this paper:

Early fetal death (EFD): embryonic and fetal death up to 150 days gestation, except visible abortions;

Abortion: visibly expelled fetus at any time of gestation;

Live foal: a foal that is alive 10 days post foaling;

Dead foal: a foal that is born dead or dies within 10 days post foaling;

Miscellanous loss: various reasons for pregnancy loss, such as induced abortion or death of mare during pregnancy;

Total loss: EFD + abortions + dead foals.

RESULTS

Fertility data are expressed on a seasonal basis with regard to total conception and live foal rates. The results are also expressed on a per cycle basis for conception, twinning, early fetal death, abortion, dead foal, miscellanous loss and live foal rates.

The average per cycle conception rates were: Al with fresh semen - 61.6%; frozen semen - 43.5%; chilled semen - 63.7%; and natural breeding - 59.9%.

Stallions with a high conception rate per cycle produced significantly ($P<0.01$) more twins than those with a low conception rate per cycle. Of all the breeding techniques, the lowest twinning rate was recorded from frozen semen (1.9% per cycle), compared to 4.2%, 4.8% and 4.7% for fresh semen, chilled semen and natural breeding, respectively.

For individual stallions, regardless of breeding technique, the EFD rate varied from 0–10.4%, the abortion rate from 0–11.1% and the dead foal rate from 0–5.6% per cycle. On a seasonal basis, the gross percentages for mares resorbing, aborting and producing dead foals were 7.4%, 3.8% and 4.3%, respectively, of the pregnant mares.

'Nearly significant' differences ($P<0.10$) were found in the per cycle EFD rates between SWB stallions, and they were significant when the EFD and abortion rates were combined ($P<0.05$).

Early pregnancy loss was observed in 74 mares, 37 of which were re-mated/re-inseminated within the same season. Conception rates per cycle for these mares could be compared with the total figures for all breeds and techniques. However, the resorbing mares had a lower per season live foal rate ($P<0.01$), due to a considerably higher rate of repeated EFD (12.8%, $P<0.01$), and higher rates of abortions and dead foals. The total loss per cycle for resorbing mares was 21.4%, compared with 9.0% for all mares ($P<0.01$).

DISCUSSION

Estimations of fertility in the horse are influenced greatly by the conditions of the study. For instance, different breeds, timing and frequency of breeding, insemination dose, selection of mares and management make comparisons between studies difficult. Dowsett and Pattie (1982) found the most sensitive method for evaluating stallion fertility to be percentage of pregnancies per service and the second best to be percentage of pregnancies per cycle. The number of services may vary greatly due to different management strategies, and thus it seems reasonable to postulate that the most applicable parameter is currently conception rate/cycle. For individually presented stallions, conception rate per cycle varied from 23.0–79.2%, which is in accordance with the maximum of 80% per cycle reported by Dowsett and Pattie (1982). Provided management conditions are acceptable, the conception rate/cycle is a reliable measure of stallion fertility. It is a more informative indicator of fertility in a particular stallion than, for instance, seasonal conception rate.

The difference in twinning rate between individual stallions and different breeding techniques seem to reflect the longevity of the sperm cells. Stallions with a high conception rate/cycle produce more twins than those with a low rate. The risk of twinning after using frozen semen is also lower than when other breeding techniques are used. In the mare, double ovulations increase the likelihood of conception but also increase the risk of resorption of one or both conceptuses (Ginther *et al.* 1982; Woods and Ball 1987). The twinning rate in resorbing mares was 10.9%/cycle, compared with 3.8%/cycle overall.

The resorbing mares had a high incidence of renewed EFD if mated/inseminated within the same breeding season; 12.8% per cycle or 21.4% of the pregnant mares. This is in accordance with the repeated pregnancy loss rate of 19% reported by Woods *et al*. (1987). Still, almost 50% of the mares produced a foal, if the EFD was detected within the season, which is encouraging for renewed breeding.

Of the 37 mares re-inseminated/re-mated, 30% lost their embryos before Day 20, 35% before Day 30 and 27% before Day 45. In total, if re-examination was performed, most resorptions were detected before Day 45, which shows the importance of repeated ultrasound examinations for pregnancy diagnosis.

REFERENCES

Dowsett, K.F. and Pattie, W.A. (1982) Characteristics and fertility of stallion semen. *J. Reprod. Fert., Suppl.* **32**,1-8.

Ginther, O.J., Douglas, R.H. and Woods, G.L.(1982) A biological embryo-reduction mechanism for the elimination of excess embryos in mares. *Theriogenology*, **18**, 475485.

Woods, G.L., Baker, C.B., Baldwin, J.L., Ball, B.A., Bilinski, J., Cooper, W.L., Ley, W.B., Mank, E.C. and Erb, H.N. (1987) Early pregnancy loss in brood mares. *J. Reprod. Fert., Suppl.* **35**, 455-459.

Woods, G.L. and Ball, B.A. (1987) Pregnancy loss in the mare. Proc. Am. *Ass. equine.* **32**, 29-34.

PREGNANCY RATES FOLLOWING INSEMINATION WITH FRESH VERSUS FROZEN SEMEN

Z. Müller and J. Müller

Equine Reproduction Centre, 530 02 Pardubice - Mnetice 126, Czech Republic

INTRODUCTION

Artificial insemination in horses is a well established technique which is now considered to be a routine breeding procedure. Notably the influence of insemination with frozen semen on gene migration is very apparent in the international breeding of top sport horses.

Despite this, insemination methods using both cooled and frozen semen are still being developed. There are several variations in methods of semen collection, freezing and the use of extenders. To date, no single method has been shown to be the most advantageous. The aim of our work has been to identify factors that influence the success or otherwise of specific strategies.

MATERIALS AND METHODS

Semen collection

For the purpose of shipping of cooled semen, different types of artificial vagina were used for collection. Semen collection for freezing of insemination doses was carried out using the Krakow model of an open artificial vagina, in the presence of a mare. Semen was collected 4 days per week for cooled semen or 3 days per week for frozen semen.

Semen processing and shipping

Cooled semen: Kenney's (instant non-fat dry milk 2.4 g, glucose monohydrate 4.9 g, sodium bicarbonate 7.5% sol 2.0 ml, gentamicin sulphate 50 mg/ml 2.0 ml, distilled water 92 ml) or LC (lactose 11 g, disodium EDTA 0.1 g, sodium citrate dihydrate 0.089 g, sodium bicarbonate 0.008 g, deionised water 100 ml, egg yolk 1.6 g) extenders were used for diluting raw semen. A minimal dilution ratio of 1:2 (raw semen:extender) was used and one insemination dose contained at least 200 million progressively motile sperms. Diluted insemination doses presenting 10–20 ml were cooled to 4°C and shipped. On collection days, semen was sent by train around the Czech Republic. Following telephone orders prior to 9.00 am, the insemination doses were usually at their destination by 7.00 pm.

Frozen semen: LC extender containing 3.5% glycerol was used to dilute raw semen yielded by fractionated collection. Therefore the procedure did not include centrifugation. The ratio of dilution corresponded to a minimum of 200 million progressively motile sperms per insemination dose after thawing. Semen was frozen in 6 ml aluminium tubes.

Insemination: Insemination was carried out by veterinarians and insemination technicians. In 1998 breeders could, following training at a workshop, carry out insemination of their own mares with fresh semen only. Insemination with frozen semen was carried out by 21 workers and with cooled semen by 48 workers.

Palpation and ultrasonography of the ovaries and uterus were used to determine the optimal time of insemination (approaching ovulation). In sporadic cases when using cooled semen, the insemination was timed on the basis of positive teasing and viscosity of cervical gel. Insemination was always repeated after 48 h up to the detected ovulation or the end of heat.

Timing of insemination with frozen semen was based on regular ultrasonographic examination of mares during oestrus. Examination frequency varied according to practical conditions from once

or twice a day to, approaching ovulation, every 3-–6 h. If necessary, insemination was repeated after 24 h up to detected ovulation. Mares inseminated post ovulation were inseminated up to 3 or maximally 6 h after ovulation.

Animals: Cooled semen from 17 stallions and frozen semen from 28 stallions was used for insemination of mainly Warmblood mares aged 3–21 years. In the period 1994–1998 1,146 mares were inseminated (262 with frozen and 884 with cooled semen) in a total of 1,586 heat cycles. Frozen semen was used in 344 and cooled semen in 1,243 cycles. Some of the frozen semen was imported in 0.5 ml straws from France or Germany.

Evaluation: Results were assessed on the basis of:

- competence of individual technicians;
- individuality of stallions;
- semen extender (cooled semen);
- packaging system (frozen semen);
- different practical conditions;
- pre- *vs* post ovulation insemination.

RESULTS AND DISCUSSION

Total average per cycle pregnancy rates of 36% with frozen semen and 44% with cooled semen were achieved.

Competence of individual technicians

Results achieved by individual insemination specialists varied from 32%–64% using fresh and from 25%–56% using frozen semen (Fig 1).

There were obvious differences between frozen and cooled semen. The variations reflect mainly the skill level of individual specialists and their experience in that area.

Notably, in frozen semen, it is necessary to synchronize the time of insemination and ovulation. When using cooled semen, the shipping interval of 48 h must be considered.

Individuality of stallions

Results of insemination with cooled semen varies from 29%–57% according to individual stallion. For this programme, stallions were selected on the basis of breeding value rather than semen quality. However, when using frozen semen, stallions were selected according to the freezeability of the ejaculate and insemination doses were chosen according to motility after thawing. These selection processes probably reduced the variation of results achieved with frozen semen (26–39%; Fig 2). The influence of stallion individuality on pregnancy rate is significant but can be limited by selection of the insemination doses.

Semen extender (cooled semen)

Our results show no significant effect on pregnancy rate by the 2 extenders used for diluting cooled semen (43% after using Kenney's and 45% after LC extender).

Packaging system (frozen semen)

In comparison with French straws, higher pregnancy rates were achieved after freezing the semen in aluminium tubes (32% *vs* 39%). This

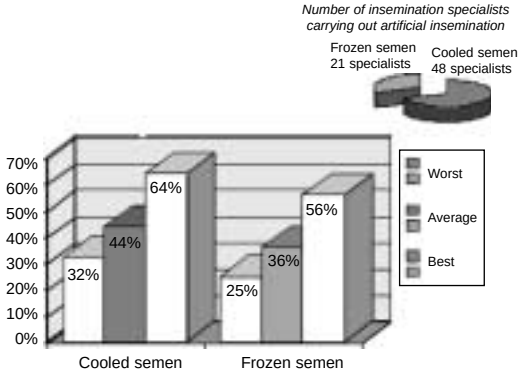

Fig 1: Variation of per cycle pregnancy rates acheived by individual insemination specialists.

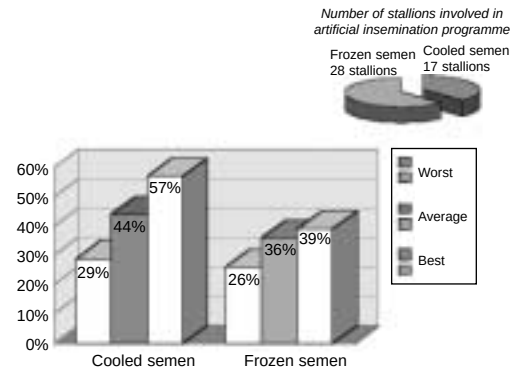

Fig 2: Variation of per cycle pregnancy rates based on individuality of stallions.

difference may relate to the number of straws used or damage arising from transport and storage, rather than to different fertilising capacities.

Different practical conditions

Significantly better results (54% for frozen and 39% for cooled semen) were achieved by the specialised clinic compared to inseminations carried out at the breeders' operations (42% and 33% per cycle pregnancy rate).

This may be, in part, because of the opportunity to monitor oestrus and predict ovulation. Also, the post insemination treatment of a mare can be an important factor, especially after using frozen semen.

Pre- and post ovulation insemination

Pregnancy rates of 37% and 33% were achieved before and after ovulation, respectively. Therefore, although the rate decreases, insemination carried out up to 6 h after ovulation can still produce satisfactory results and has the advantage of reducing the number of insemination doses used.

COMPARISON OF FROZEN AND COOLED SEMEN

Cooled versus frozen semen

- higher fertility rate;
- tolerance of a less precise insemination procedure - based on the presumption that cooled semen maintains its fertilising capacity for 48 h after insemination;
- permanent breeding occupation of the stallion - a stallion must be available for semen collection any time during the breeding season;
- problems relating to transport for long distances
- dependence of insemination on semen distribution (mare's insemination management) - partially it can be solved by daily semen collection.

Frozen versus cooled semen

- precise timing of insemination - frozen semen can be possible thawed any time to inseminate a mare just before or after ovulation;
- transport is simple and can be planned before long term semen storage;
- production is possible at any time during the year - a stallion can be used while competing;
- lower fertility rate;
- necessity of precise insemination technique;
- more expensive - semen processing and storage.

CONCLUSIONS

Insemination with cooled semen is a method used successfully at present. It is suitable for a wide and quick extension of selected stallions.

Insemination with frozen semen is a method which will become increasingly popular and may, as in cattle breeding, replace cooled semen eventually. At present it is used mainly for breeding top sport horses.

Much work is still required in the field of optimising insemination technology.

LARGE SCALE COMMERCIAL APPLICATION OF FRESH, COOLED AND DEEP FROZEN STALLION SEMEN

H. Sieme, E. Klug* and E. Töpfer-Petersen†

*Celle National Stud; *Clinic for Horses, Veterinary School, Hannover; †Institute for Reproductive Medicine, Veterinary School, Hannover, Germany*

In the last decade, equine artificial insemination (AI) has become the main zootechnique of equine breeding practice in the Warmblood and Standardbred breeding industry in Germany, France, The Netherlands, Belgium, Denmark and Sweden.

In Germany in 1999, approximately 180 AI centres were registered; 60 licensed by the European Government (92/65EG modified by 95/172EG).

Among riding horses in 1998, 48,395 matings were registered (riding horse mare population in 1997 was 83,349). A total of 3,460 registered private stallions mated 28,193 mares (58.2%) and 623 state-owned stallions covered 20,202 mares (41.8%). AI was used for 64.6% of all coverings. In 1998, 30,552 live foals (61.6%) were registered out of 49,494 matings in 1997.

During the pre-breeding season, pubertal and all other sires are subjected to a full physical examination, including monitoring of sexual behaviour and bacteriological examination of the genital tract. Spermiograms are conducted on all pubertal stallions and those which achieved below average conception rates in the previous season. In addition, all stallions older than 18 years and all stallions used for AI are examined for semen quality.

Stallions are not allowed to serve simultaneously in artificial and natural breeding programmes. Semen collection is confined to one daily collection per stallion to adjust daily sperm output to a predictable level.

Factors influencing the success of AI in the horse include stallion fertility and management, semen preservation techniques and management of insemination and mare. Regardless of technical factors like extender, dilution ratio, preparation technique (dilution, centrifugation, alternatives), cooling rate, storage conditions and insemination volume, it is obvious that sperm dose and frequency of pre-ovulatory inseminations are important limiting factors.

Effect of time of insemination, relative to ovulation, is under study. Published fertility data from different countries are available but the basis of fertility calculation differs between countries (fertility per cycle, end of season results, foaling rate), which complicates a comparative estimate of system efficacy.

There is even variation in stud fee strategies between countries. In the German riding horse population, the stud fee is paid per mare and season and prices differ between stallions. Barren mares normally receive a discount in the following season. Price fixing for exported frozen semen is usually calculated per insemination dose. Another strategy would be a stud fee split into: 1) an initial fe for AI/service per mare and season combined with 2) a secondary fee per pregnancy evaluated by veterinary examination in the autumn.

SESSION 1V:

Oocytes - recovery and maturation

Chairman: R. H. F. Hunter

ASSESSMENT OF EQUINE FOLLICLES AND OOCYTES COLLECTED AT A SLAUGHTER HOUSE

A. Okólski and W. Mlodawska

Department of Animal Reproduction, University of Agriculture, 30-059 Cracow, Al. Mickiewicza 24/28, Poland

INTRODUCTION

One of the cheapest and most simple ways to collect horse oocytes is to obtain mare ovaries from a slaughter house. The aim of this study is to present the results of our investigation on different methods of oocyte collection from ovarian follicles after slaughter. Also, we determined the chronology and stage of maturity of equine oocyte nuclei and the degree of atresia in the ovarian follicles from which some of the oocytes were collected.

MATERIALS AND METHODS

The ovaries of mares of unknown reproductive history were collected from a local slaughter house once a week for a period of 3 years. Immediately after slaughter the ovaries were transported in a thermos flask at room temperature to the laboratory. Here, they were washed 3 times in fresh Dulbecco's phosphate buffered saline medium supplemented with decreasing doses of penicillin and streptomycin

Two different methods of oocyte recovery were used. In the first one the follicular fluid containing oocytes was aspirated with a syringe from the ovaries of 900 mares. Then, under a stereomicroscope, the oocytes were separated from the follicular fluid.

In the second method, follicles were isolated from the ovaries of 319 mares, placed separately on a Petri dish, measured and cut. The cumulus oocyte complex (COC) was located on the follicle wall or floating in the follicular fluid. The COCs were scraped from the wall using a scalpel blade. The morphology of the COCs was assessed using the system of Leibfried and First (1979) and Hinrichs *et al.* (1993), modified by our own observations. Two hundred and forty four oocytes were cultured *in vitro* in TCM-199 medium, with FSH, LH and E2 from 3–48 h to evaluate the chronology of mare oocyte maturation *in vitro*.

Data were analysed by chi-square test and $P<0.05$, was taken as the level of statistical significance.

RESULTS AND DISCUSSION

As a result of aspiration of 7,798 follicles, a total of 3,158 (40.5%) oocytes per mare were collected, of which only 1,125 (35.6%) qualified for culture. By cutting 2,043 isolated follicles, a total of 1,872 (91.6%) oocytes were obtained (5.9 per mare) of which 1,235 (66.0%) were accepted for culture. Thus, by cutting it was possible to obtain a significantly higher percentage of oocytes suitable for culture than by aspiration ($P<0.01$). The percentage of oocytes accepted for culture increased with increasing follicle diameter, being 30%, 54%, 61% and 78% ($P<0.01$) for follicles with diameters of 4–10, 11–15, 16–20 and 21-–35 mm, respectively.

When 564 follicles and oocytes were evaluated for the macroscopic degree of atresia, the percentage of oocytes with compact cumuli increased significantly with increasing diameter of the follicles being 23.3% for the follicles <10 mm and 64.2% for the follicles > 21 mm ($P<0.01$). The percentage of oocytes floating in the follicular fluid decreased from 52.0% in the smallest follicles to 9.9% in the largest ones. However, Hinrichs (1997) found that oocytes having expanded cumulus mature *in vitro* more quickly and in higher proportion than do cumulus compact oocytes, and the period of protein synthesis necessary for maturation is shorter for expanded

cumulus than for the compact one. Also Torner *et al*. (1997) found that only expanded cumulus oocyte complex and cumulus oocytes complex with few cumulus layers at time of recovery were capable of developing into zygotes (2 pronuclei) and into 2-cell stage embryos.

When we estimated chronology of events of oocyte maturation *in vitro* in TCM-199 medium, we found that before culture 77.3% of oocytes were in germinal vesicle stage. After 9 h culture the stage of germinal vesicle breakdown was attained by 36.4% of oocytes. Most oocytes ie 57.1%, 55.6%, 75.0% and 66.7% reached the metaphase II stage of development after *in vitro* culture for 30, 36, 42. Extending the time of culture from 42–48 h did not increase the number of oocytes in metaphase II.

Conclusions

The method of cutting, as opposed to aspiration, resulted in a significantly greater number of morphologically normal COCs. With the growth of follicles, the probability of obtaining morphologically normal oocytes increases considerably. The majority of mares' oocytes reach metaphase II after 30 h of culture.

Acknowledgements

This research in supported by KBN Grant No. P06D 019 11.

References

Hinrichs, K., Schmidt, A.L., Friedman, P.P., Selgrath, J.P. and Martin, M.G. (1993) *In vitro* maturation of horse oocytes: characterisation of chromatin configuration using fluorescence microscopy. *Biol. Reprod*. **48**, 363-370.

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

Leibfried L. and First, N.L. (1979) Characterisation of bovine follicular oocytes and their ability to mature *in vitro*. *J. anim. Sci*. **48**, 76-86.

Torner H., Blottner S., Alm H., Becker F. and Kanitz W. (1997) *In vitro* fertilisation of horse oocytes depending on type of cumulus and sperm treatment. *Proc. 4th int. Symp. equine Embryo Transfer and other advanced Techniques, Reims*.

ZONA PELLUCIDA HARDENING AND SPERM BINDING ASSAY FOR EQUINE OOCYTES INCUBATED *IN VITRO*

W. Mlodawska, J. Bezard*, M. Walul and A. Okólski

*Department of Animal Reproduction, Agriculture University, Al. Mickiewicza 24/28, 30-059 Kraków, Poland, *INRA Reproductive Physiology and National Studs, 37380 Nouzilly, France*

INTRODUCTION

Zona pellucida (ZP) hardening expressed as an increase in its resistance to enzymatic digestion occurs following fertilisation, parthenogenetic activation and *in vivo* and *in vitro* ageing of the egg-cell. Spontaneous ZP hardening can also be induced during *in vitro* oocyte incubation (De Felici and Siracusa 1982). One consequence of ZP hardening is a considerable decrease of oocyte capacity for *in vitro* fertilisation and embryonic development (Gianfortoni and Gulyas 1985; Katska *et al*. 1989). It is supposed that spontaneous ZP hardening of oocytes may be one reason for low efficiency of *in vitro* fertilisation in horses. The aim of the study was to determine whether exposure of equine oocytes to culture medium supplemented with various sera would affect ZP hardening and its capacity to bind spermatozoa.

MATERIALS AND METHODS

The study was performed on oocytes collected by aspiration of individual follicles (≥ 5 mm) from the ovaries of 28 mares, within 2–4 h after slaughter (Mlodawska *et al*. 1999). The age and reproductive history of mares were unknown.

Estimation of ZP hardening was performed directly after oocyte collection, following 45–48 h *in vitro* maturation (IVM) or after IVM + additional co-culture with stallion spermatozoa. Oocytes (3–5) were matured in 100 ml TCM 199 with Earle's salts, 2.2 g/ml NaHCO3 and L-glutamine (Gibco) supplemented with CEG (9.5 µg/ml eFSH, 15 µg/ml eLH), oestradiol-17ß (1 µg/ml; Sigma), antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone; Gibco) and 20% of inactivated FCS (fetal calf serum) or EMS (estrus mare serum), under paraffin oil at 38.5°C in 5 % CO_2 in air.

For oocyte insemination frozen semen from the same ejaculate of a fertile stallion was thawed, washed twice in HGLLBSA and then in HHBSA (Mlodawska *et al*. 1999). Before insemination each oocyte was mechanically denuded of all cumulus and corona cells by aspiration through a fine bore pipette and incubated individually with 2.5 x 10^5 spermatozoa/ml in 100 µl HHBSA for 20 h. After co-culture each oocyte was washed in 9 sequential droplets of PBS to remove the loosely bound spermatozoa. The stage of oocyte maturity was estimated and the number of spermatozoa binding ZP counted under phase-contrast microscopy. Time necessary for complete dissolution of ZP of each oocyte was measured individually in 10 ml of Ringer-Krebs solution containing 1 mg/ml protease (Sigma).

Data were analysed by ANOVA using SAS.

RESULTS AND DISCUSSION

ZP hardening was assessed on a total of 122 oocytes, 25 of which were estimated directly after recovery from immature ovarian follicles. The mean (± se) digestion time of ZP of these oocytes was 255 ± 14 s.

After 45–48 h of IVM in the presence of FCS or EMS, 62.5% (15/24) and 54% (13/24) oocytes, respectively, possessed polar body (PB) clearly visible under ZP, thus reaching the metaphase II stage (Table 1).

The presence of various sera in culture medium did not significantly affect oocyte maturation to metaphase II stage. The average digestion time of ZP of oocytes matured with FCS or EMS was 252±17 s and 239±13 s, respectively. The applied sera had no significant effect on ZP

TABLE 1: Equine ZP hardening and sperm binding after oocytes IVM with various sera

Maturation medium	After IVM (45–48 h)						After IVM + 20 h incubation with spermatazoa							
	Total		without PB		with PB		Total		without PB			with PB		
	n	t(s)	n	t(s)	n	t(s)	n	t(s)	n	t(s)	spz/ooc	n	t(s)	spz/ooc
TCM+FCS	24	252±17	9	291±36	15	228±13	23	273±16	8	316±41	9±1.8	15	250±9	10±1.5
TCM+EMS	24	239±13	11	245±25	13	234±14	26	302±17	11	300±25	10±2.4	15	303±25	15±2.3

n - number of oocytes; PB - polar body visible under ZP; t (s) - digestion time (mean ± se) of ZP; spz/ooc - number of spermatazoa (mean ± se) bound with ZP of oocyte

This research is supported by KBN Grant No 5 P06D 019 11

solubility. On the contrary, Dell'Aquila *et al.* (1998) observed a longer ZP digestion time for the oocytes matured with EMS or BSA, than with FCS, fetal equine serum or follicular fluid. However, increased ZP resistance to enzymatic digestion, connected with seasonal reproductive function and cycle stage of donor mares, was observed by Fusco *et al*. (1998) only for oocytes maturing with BSA. Oestrus in mares lasts 6–7 days, so anti-hardening properties of EMS may depend both on blood sampling day and individual hormonal condition of donor mare.

Studies of human, monkey and mare oocytes have shown that oocyte maturation stage may affect ZP capacity to bind spermatozoa (Oehninger *et al*. 1991; Mlodawska *et al*. 1999). Therefore, assessment of ZP hardening should allow for stage of oocyte maturation.

After IVM, the average digestion time of ZP of oocytes with visible PB was 228±13 s and 234±14 s for oocytes matured in the presence of FCS or EMS, respectively (Table 1). That time was not significantly shorter than that for oocytes without visible PB, ie degenerated or immature oocytes. In the mouse, ZP of immature oocytes in dictyate stage is less soluble, than that of *in vivo* matured oocytes tested directly after ovulation (De Felice and Siracusa 1982). In cattle, however, ZP of post ovulatory oocytes is more resistant to enzymatic digestion than ZP of immature pre-ovulatory ones (Katska *et al*. 1989).

After 20 h of additional co-culture of oocytes with stallion spermatozoa a non-significant elongation of ZP dissolution time was observed. Mean number of spermatozoa bound with ZP of oocytes inseminated after IVM in the presence of FCS was 9±1.8 without visible PB and 10±1.5 with visible PB. This means from 10±2.4 to 15±2.3 were found on ZP of oocytes inseminated after IVM in the presence of EMS (Table 1). Oocytes with visible PB bound slightly more spermatozoa than oocytes without PB, but this was not statistically significant.

CONCLUSIONS

Our study suggested that *in vitro* culture of mares'oocytes in TCM supplemented with FCS or EMS did not significantly affect ZP digestion time or capacity to bind spermatozoa.

REFERENCES

De Felici, M. and Siracusa, G. (1982) 'Spontaneous' hardening of the zona pellucida of mouse oocytes during *in vitro* culture. *Gamete Research* **6**, 107-113.

Dell'Aquila, M.E., De Felici, M., Maritato, F., Lacalandra, G. and Minoia, P. (1998) Effects of culture conditions on zona pellucida hardening of *in vitro* matured equine oocytes *Proc. 14th Symp. A.E.T.E., Venice,* 140.

Fusco, S., Dell'Aquilla, M.E., De Felici, M., Lacalandra, G.M. and Minoia, P. (1998) Zona pellucida hardening of *in vitro* matured equine oocytes as influenced by season and stage of estrous cycle. *Proc. 14th Symposium A.E.T.E., Venice,* 158.

Gianfortoni, J.G. and Gulyas, B.J. (1985) The effects of short-term incubation (ageing) of mouse oocytes on *in vitro* fertilisation, zona solubility and embryonic development. *Gamete Research* **11**, 59-68.

Katska, L., Kauffold, P., Smorag, Z., Duschinski, U., Torner, H. and Kanitz, W. (1989) Influence of hardening of the zona pellucida on *in vitro* fertilisation of bovine oocytes. *Theriogenol.* **32**, 767-777.

Mlodawska, W., Palmer, E., Duchamp, G., Okólski, A. and Bézard, J. (1999) Zona pellucida - sperm binding assay for equine oocytes, *J. Reprod. Fert. Suppl. (in press).*

Oehninger, S., Veeck, L., Franken, D., Kruger, T.F., Acosta, A.A. and Hodgen, G.D. (1991) Human preovulatory oocytes have a higher sperm-binding ability than immature oocytes under hemizona assay conditions: evidence supporting the concept of 'zona maturation'. *Fertil. Steril.* **55**, 1165-1170.

IN VIVO OOCYTE ASPIRATION FROM MARES BEFORE AND AFTER TREATMENT WITH CRUDE EQUINE GONADOTROPHINS (CEG)

I. Brück, J. Bézard*, M. Baltsen†, B. Synnestvedt, I. Couty*, T. Greve, and G. Duchamp*

*Department of Clinical Studies, Section for Reproduction, Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark; *INRA, PRMD 37380 Nouzilly, France; Laboratory of Reproductive Biology, Center for Children; Women and Reproduction, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark*

INTRODUCTION

In the mare, the shortage of oocytes and the variability in nuclear maturation at a certain stage of the oestrous cycle hinders the optimisation of methods for *in vitro* maturation and *in vitro* fertilisation. Increasing the number of small to medium sized follicles available for *in vivo* aspiration could possibly increase the overall oocyte yield. Equine pituitary extract has a super-stimulatory effect on follicular growth when treatment is commenced after temporary suppression of follicular development and before the selection of the pre-ovulatory follicle (Pierson and Ginther 1990; Dippert *et al.* 1992). Thus, administration of gonadotrophins during early dioestrus, in the absence of a dominant follicle, might stimulate the growth of small follicles.

Two meiosis-activating sterols isolated from pre-ovulatory follicular fluid of women and mares (FF-MAS) and testes of bulls (T-MAS) are able to induce resumption of meiosis in hypoxanthine-arrested mouse oocytes (Byskov *et al.* 1995; Baltsen *et al.* 1997). Furthermore, FSH stimulates the production of FF-MAS in cultured murine cumulus cells (Byskov *et al.* 1997). Gonadotrophin administration might therefore be reflected in an increase in the follicular concentration of FF-MAS.

The aims of the present study were to investigate whether initial follicular removal during oestrus synchronises *in vivo* oocyte maturation and whether administration of crude equine gonadotrophins (CEG) affects follicular development, oocyte recovery rate, *in vivo* oocyte maturation and follicular concentrations of meiosis-activating sterols (MAS).

MATERIALS AND METHODS

At the beginning of September, the oestrous cycles of 19 Welsh pony mares (3–16 years old) were synchronised by the intra vaginal insertion of a sponge containing 0.5 *g* altrenogest (Regumate, Roussel UCLAF, Romainville, France) and 50 mg oestradiol benzoate (ß-oestradiol 3-benzoate; Sigma, La Verpillère, France) for 7 days. On the day of sponge removal, the mares received 150 µg cloprostenol (Estrumate, Pitman-Moore, Meaux, France) im to induce luteolysis. During oestrus and when the largest follicle measured 33 mm in diameter, each mare was treated once with 25 mg CEG in 5 ml physiological saline, iv (Duchamp *et al.* 1987).

Between 22 and 30 h after CEG administration, all follicles measuring 4 mm or more in diameter were evacuated by transvaginal ultrasound-guided follicular aspiration (first aspiration=A1) as previously described by Duchamp *et al.* (1995). The day of first aspiration (A1) was characterised as Day 0. On Day 8 (early dioestrus), all follicles 4 mm in diameter were aspirated again (second aspiration=A2). Between Days 1 and 8, follicular number and diameter (5 mm) was monitored daily in all mares by ultrasonography. For each day, follicles from both ovaries were pooled.

The ponies were separated randomly into 2 groups: CEG group (n=10) and control group (n=9). From Day 0 to Day 7, mares in the CEG group received daily 25 mg CEG in 5 ml physiological saline im; control mares were injected im with 5 ml physiological saline. The batch used for the present experiment contained 0.5 mg eFSH (2%) and 1.5 mg eLH (6%).

All follicular fluid samples were examined individually for the presence of COCs. Upon

recovery, the cumulus aspect of each COC was classified under light microscopy as follows: CC = the oocyte partly or entirely enclosed by several layers of compact cumulus cells; CR = the oocyte enclosed by corona radiata cells only; EX = the oocyte entirely enclosed by an expanded cumulus investment; DE = the oocyte denuded. Immediately after classification, the COCs were placed in 500 µl PBS supplemented with 525 iu/ml hyaluronidase (type III, 875 iu/mg; Sigma, La Verpillère, France) at 37°C for 2 min. Thereafter, the COCs were completely denuded with small glass pipettes. All oocytes were fixed in acetic acid:methanol (1:3) for approximately 2 days, and stained with 1% w/v orcein.

At A2, the follicular fluid samples from follicles >15 mm were centrifuged at 2000 *g* for 10 min to remove cell debris and the supernatants were stored at - 20°C for subsequent analysis. FF-MAS and T-MAS in follicular fluid were determined quantitatively by HPLC and photo diode array detection as described by Baltsen and Byskov (2000).

For statistical analysis of the follicular development, the follicles were grouped according to their diameter: 10 mm, 11–15 mm, 16–20 mm, 21–25 mm and >25 mm. All results are presented as mean ± se. Differences in number of follicles, number of recovered oocytes, oocyte recovery rate, cumulus expansion and nuclear maturation were analysed using independence in 2-dimensional contingency tables applying a Yates corrected chi-square model. Student-*t*-test was used to compare mean number of follicles on a certain day after A1, follicular size, sum of follicular diameters on Day 8, follicular FF-MAS and T-MAS concentrations. In all analyses the probability $P<0.05$ was considered to express statistical significance.

Results

There was no significant difference in the number of follicles within the follicular size groups on any of the 8 days between control and CEG treated mares. On Day 8, the mean follicular size was 10.2 ± 0.6 mm and 10.9 ± 0.9 mm for the control and the CEG group, respectively. The largest follicle on Day 8 measured 18.3 ± 0.5 and 18.9 ± 0.7 mm, respectively for the control and the CEG group.

The oocyte recovery rates at A1 (94/257) and A2 (80/184) were similar. At A2, oocyte recovery rate and *in vivo* oocyte maturation were not affected by CEG treatment.

The number of expanded COCs recovered from follicles ⩽29 mm was significantly higher at A1 than at A2. The number of oocytes in germinal vesicle was significantly higher in A2 (42.5%) than in A1 (17.8%) and the number of oocytes in metaphase (I+II) was significantly higher in A1 (27.4%) than in A2 (3.1%; $P<0.005$).

FF-MAS and T-MAS were identified in follicular samples of control and CEG treated mares. CEG treatment did not affect follicular FF-MAS or T-MAS concentrations. Mean follicular FF-MAS concentrations were higher than mean T-MAS concentrations. The FF-MAS and T-MAS concentrations were similar in follicular fluid containing oocytes in germinal vesicle, germinal vesicle breakdown and MII stage.

Conclusions

The present study demonstrates that follicular aspiration during oestrus allowed a new follicular population to develop and resulted in a higher degree of synchronisation of oocyte development in respect to cumulus expansion and nuclear maturation. The availability of a more homogeneous population of oocytes might facilitate a better optimisation of *in vitro* maturation and *in vitro* fertilisation techniques in the mare. Exogenous CEG administered during early dioestrus did not affect the growth of small follicles, the oocyte yield after *in vivo* aspiration or *in vivo* oocyte maturation.

Acknowledgements

We wish to thank Monique Ottogalli for the preparation of the sponges and the pituitary extract, Christine Briant for analysis of the CEG and the technical staff of the equine stables at INRA for assistance with the mares. This study was supported by the Danish Agricultural and Veterinary Research Council, The Danish Environmental Research Program and The Danish Research Councils.

References

Baltsen, M., Brück Bøgh, I. and Byskov, A.G. (1997) Assay for meiosis activating sterol (MAS) in follicular fluids: presence of MAS is correlated to preovulatory follicular maturation. *Biol.Reprod.* **56,** Suppl 1, 95.

Baltsen, M. and Byskov, A.G. (2000) Quantitation of meiosis activating sterols, MAS, in human follicular fluid using HPLC and photo diode array detection. *Biomedical Chromatography (in press).*

Byskov, A.G., Andersen, C.A., Hossaini, A. and Guoliang, X. (1997) Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. *Molecular Reprod. Develop.* **46,** 296-305.

Byskov, A.G., Andersen, C.Y., Nordholm, L., Thøgersen, H., Guoliang, X., Wassman, O., Andersen, J.V., Guddal, E. and Roed, T. (1995) Chemical structure of sterols that activate oocyte meiosis. *Nature* **374,** 559-562.

Dippert, K.D., Hofferer, S., Palmer, E., Jasko, D.J. and Squires, E.L. (1992) Initiation of superovulation in mares 5 or 12 days after ovulation using equine pituitary extract with or without GnRH analogue. *Theriogenol.* **38,** 695-710.

Duchamp, G., Bézard, J. and Palmer, E. (1995) Oocyte yield and the consequences of puncture of all follicles larger than 8 millimeters in mares. *Biol. Reprod. Monograph Series* **1,** 233-241.

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

Pierson, R.A. and Ginther, O.J. (1990) Ovarian follicular response of mares to an equine pituitary extract after suppression of follicular development. *Anim. Reprod. Sci.* **22,** 131-144.

COMPARISON OF DIFFERENT STAINING METHODS TO EVALUATE HORSE OOCYTES

H. Alm, H. Torner and W. Kanitz

Research Institute for the Biology of Farm Animals, Dummerstorf-Rostock 18196, Germany

INTRODUCTION

After recovery of cumulus oocyte complexes, the proportion of immature oocytes differs between investigators. For example, reports of germinal vesicle stages found at the time of recovery have ranged from 6% (King *et al*. 1990), 37% (Torner and Alm 1995) and 28% (Zhang *et al*. 1989) to 60% (Del Campo *et al*. 1992), 75% (Hinrichs *et al*. 1993) and 80% (Willis *et al*. 1994). This wide variation could relate to different staining methods and/or different interpretation of meiotic configuration. Few descriptions of oocyte morphology and stages of meiotic configuration in horse oocytes are available.

The aim of this study was to compare different staining methods and assess whether these affect chromatin configuration.

MATERIALS AND METHODS

Recovery of cumulus oocyte complexes

Oocytes were recovered either by ultrasound guided follicle aspiration or by follicle scraping of ovaries post mortem. After recovery, the oocytes were classified according to cumulus morphology, and oocytes with compact and expanded cumulus were used for investigations.

Staining procedure and evaluation

For chromatin evaluation, the cumulus cells were removed by pipetting in a solution of trypsin/EDTA. Oocytes were stained with Hoechst 33258, orcein or lacmoid and evaluated with fluorescence microscopy or phase contrast microscopy. In one group the oocytes were fluorescence labelled with Hoechst 33254, then fixed and stained with orcein or lacmoid to assess whether the meiotic configurations were comparable between the 2 methods.

RESULTS

Fixation with acetic acid and staining with orcein or lacmoid identified clear meiotic configuration. The portion of immature oocytes with diplotene chromatin and germinal vesicle membrane was 11.9%. The portion of oocytes with visible nuclear membrane, but condensed chromatin was 43.1%. Using Hoechst 33254, the clear diplotene chromatin was visible in 6.9% of the oocytes. Condensed chromatin in different configuration was observed in 52.8% of the oocytes.

A direct comparison was made between Hoechst 33254 and orcein on the same oocytes (n=35). Of these 35 oocytes, 7 showed diplotene chromatin both after Hoechst and orcein. In all 7 oocytes, a good visible membrane was observed. Twenty six oocytes showed condensed chromatin after Hoechst and, in 19 of these oocytes (73.1%), a membrane could be observed after orcein staining only.

It is still unclear whether this condensed chromatin represents a healthy stage or signs of degeneration. Maybe the presence of the membrane can help to distinguish between intact and degenerated condensed chromatin.

CONCLUSION

As the DNA-specific fluorochrome Hoechst 33258 is described to be non-toxic, and given the hypothesis that diffuse fluorescent nucleus is an early stage of immature oocytes, and the condensed chromatin is a progress in the meiotic competence of immature oocytes (Hinrichs 1997),

it would be good to find a culture system which allows mature oocytes after staining to show that these oocytes are really meiotic competent.

In summary, the reason for the different results between studies may be the method of staining and interpretation of condensed chromatin.

REFERENCES

Del Campo, M.R., Denose, M.X. and Parrish, J.J. (1992) *In vitro* maturation of equine oocytes. *Theriogenol.* **37**, 200.

Hinrichs, K., Schmidt, A.L., Friedman, P.P., Selgrath, J.P. and Martin, M.G. (1993) *In vitro* maturation of horse oocytes: characterisation of chromatin configuration using fluorescence microscopy. *Biol. Reprod.* **48**, 363-370.

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

King, W.A., Desjardins, M., Xu, K.P. and Bousquet D. (1990) Chromosome analysis of horse oocytes cultured *in vitro*. *Genet. Sel. Evol*. **22**, 151-160.

Torner, H. and Alm, H. (1995) Meiotic configuration of horse oocytes in relation to the morphology of the cumulus-oocyte-complex. *Biol. Reprod. Monograph Series* **1,** 253-259.

Willis, P., Caudle, A.B. and Fayrer-Hosken, R.A. (1994) Fine structure of equine oocytes matured *in vitro* for 15 hours. *Mol. reprod. Dev.* **37**, 87-92.

Zhang, J.J., Boyle, M.S., Allen, W.R. and Galli, C. (1989) Recent studies on *in vitro* fertilisation of i*n vitro* matured horse oocytes. *Equine vet. J. Suppl.* **8**, 101-104.

DIALYSIS FOR THE STUDY OF FOLLICULAR DEVELOPMENT IN MARES

W. Kanitz, F. Becker, J. Kurth and F. Schneider

Research Institute for Biology of Farm Animals, Unit of Reproductive Biology, 18196 Dummerstorf, W-Stahl-Allee 2, Germany

INTRODUCTION

Knowledge about changes in local steroid concentrations in follicular fluid and early corpora lutea are important for the study of follicular maturation and corpora lutea formation *in vivo*. The method of microdialysis offers the possibility of recovering samples from the extracellular space or from body fluids. The method is based on processes of diffusion. In farm animals, microdialysis was originally used for the study of corpus luteum function in pigs (Jarry *et al*. 1990). Liebermann and Schams (1994) and Khandawood *et al*. (1994) applied the method *in vitro*. The authors investigated the function of luteal tissue from the bovine/ovine species.

The aims of our study were to design a catheter for microdialysis in horse follicles, to calibrate the probe and to develop a technique for implantation of the catheter without interrupting maturation of the follicle.

MATERIALS AND METHODS

Construction and calibration of the probe

We used hollow fibres (Althin Medical Inc., Philadelphia) for the construction of the probe. The diameter of the probe was 200 µm and the length of the probe was 1 cm. The probe was connected via a catheter with an electrical pump, which ensures a volume of 1–2 µl NaCl per min. Because results of microdialysis depend on the area of the probe, the environmental temperature, the volume per time and the gradient between the concentrations of the substances in the probe and the follicular fluid, a calibration of the system is necessary before it can be used *in vivo.* Therefore we used a standard solution with 1,000 pg oestradiol and 500 pg progesterone per 0.1 ml 0.9% NaCL and native follicular fluid for calibration. Progesterone and oestradiol concentrations were determined by RIA.

Implantation of the probe

After trials of endoscopically guided implantation of the probe, and after several attempts at laparotomy, we developed an ultrasound guided implantation technique. The probe was placed in 7 pre-ovulatory follicles. Fixation of the probe inside the follicles was achieved by umbrella-like fibres on the surface of the catheter. The electrical pump was connected with the catheter and both were fixed on the mares. The samples were recovered in plastic tubes at 12 h intervals and stored until determination of progesterone and oestradiol at - 20°C.

RESULTS

After 1 h of dialysis of the standard solution the recovered fluid contained 303 pg oestradiol per 0.1 ml and 142 pg progesterone per 0.1 ml. The mean recovery rate was 30.0±3.7% for oestradiol and 28.4±3.2% for progesterone. Calibration of the probe with native follicular fluid containing known concentrations of oestradiol and progesterone gave recovery rates of 15.5±6.8% for oestradiol and 16.2±6.5% for progesterone. The mean duration of dialysis *in vivo* was 3.5 days (range: 0.5–7 days). Five of 7 follicles (71%) ovulated at the expected time after hCG injection. In 4 cases, microdialysis could be performed in the developing corpus luteum. In this first step it was possible to measure oestradiol and

progesterone in the samples. Oestradiol concentrations varied between 5,230 and 251 ng/ml whereas progesterone concentrations varied from 14 to 144 ng/ml. Changes in oestradiol concentrations were not uniform between mares after induction of ovulation but progesterone concentrations increased in all cases after ovulation.

Conclusion

The results of this first report indicate that microdialysis can become a valuable tool for the investigation of equine follicular maturation and corpus luteum formation.

References

Jarry, H., Einspanier, A., Kanngiesser, L., Dietrich, M., Pitzel, L., Holtz, W. and Wuttke, W. (1990) Release and effects of oxytocin on estradiol and progesterone secretion in porcine corpora lutea as measured by an *in vivo* microdialysis system. *Endocrinol.* **126,** 2350-2358

Khandawood, F.S., Gargialo, A.R. and Dawood, M.Y. (1994) *In vitro* microdialysis of the ovine corpus luteum of pregnancy: Effects of insulin-like growth factors on progesterone secretion. *Biol. Reprod.* **51** 1299-1306

Liebermann, J. and Schams, D. (1994) Actions of somatotropin on oxytocin and progesterone release from microdialysed bovine corpus luteum *in vitro*. *J. Endocrinol*. **143**, 243-250

SESSION V:

Oocytes - fertilisation *in vitro*

Chairman: P. Daels

EQUINE EMBRYO PRODUCTION USING TRANSFER OF OOCYTES INTO A PRE-OVULATORY FOLLICLE: A RETROSPECTIVE STUDY

J. Bézard, G. Duchamp, I. Couty, N. Morais, J-M Yvon, P. Daels and E. Palmer

Reproductive Physiology in Domestic Mammals, National Institute of Agricultural Research (INRA), 37380 Nouzilly, France

INTRODUCTION

The success rate of *in vitro* production of equine embryos is still disappointingly low, probably because of suboptimal *in vitro* conditions used for maturation of oocytes and capacitation of spermatozoa. In addition, the lack of an efficient method for induction of superovulation and the low embryo recovery rate in aged, subfertile mares have accentuated the need for alternative assisted reproductive techniques. The objective of this study was to develop a method for transfer of oocytes collected from the pre-ovulatory follicle of donor mares to the pre-ovulatory follicle of recipient mares. Oocyte collection and transfer was performed either by blind puncture through the flank or by the ultrasound-guided transvaginal puncture of the pre-ovulatory follicle.

MATERIALS AND METHODS

Experimental protocol

Welsh pony mares and mules were used from March to July. Ovarian activity was assessed by routine rectal ultrasound scanning. When the largest follicle measured 33 mm in diameter, each mare was treated to induce ovulation by an iv injection of 25 mg crude equine gonadotropin (CEG; Duchamp *et al.* 1987). They were inseminated the day of, and the day after, CEG injection. Oocyte collection and transfer were performed 24 or 35 h after induction. Detection of ovulation was performed by ultrasonography and pregnancy detection by embryo collection on Day 7–10 or by scanning.

Blind punctures (Palmer et al. *1987)*

Fifty four pony mares were used as donors of oocytes (n=31) and recipients (n=23) at 35 h after CEG injection. The pre-ovulatory follicle (PF) of the donors was aspirated using a needle (length 200 mm; od 1.8 mm) connected to a silastic catheter of 80 mm length and a 50 ml syringe to collect the follicular fluid (FF) and to wash 2–5 times with 20 ml PBS + heparin (50 iu/ml). The collected oocytes were placed in 2 ml of PBS or FF in a small catheter at 38.5°C until the transfer. The same technique was used for the transfer in recipients. The catheter with donor oocytes was connected to the long needle for injection of oocytes into the recipient PF: additional transfers by injection of 2 or 3 oocytes into the recipient PF keeping its own oocyte (n=8 and 1, respectively) and non-additional transfers by injection of one oocyte into a recipient PF from which the proper oocyte was removed just before transfer (n=14).

Transvaginal punctures (Bézard et al. *1995)*

A total of 112 pony mares and 9 mules (M) were used at 24 h or 35 h after induction of ovulation for oocyte collection from donors (n=10 and 36, respectively) and for oocyte transfer in recipients (n=10 and 21 + 9 M, respectively). Two and 33 pony mares ovulated before puncture at 24 h and 35 h, respectively. The PF of the donors was aspirated using a single lumen needle (length 600 mm; od 1.8 mm) fixed on the probe. After collection of the FF, the follicle was flushed 5 times with PBS + heparin (50 iu/ml). Oocytes were placed immediately in 0.7 ml of PBS in a small transfer system at 38.5°C until the transfer. Additional transfers in recipients were achieved using the same long needle as for the oocyte

TABLE 1: Embryo recovery after transfer of oocytes to pre-ovulatory follicles

Method	Time of collection post induction	Time of transfer post induction	No of transferred oocytes/ transfer	Transfer with (+) or without (-) recipient oocyte	No of transfers in pony (PM) or mule (M)	No of mares ovulating after transfer	No of pregnant mares	No of mares with one embryo	No of mares with >1 embryo
Blind	35 h	35 h	1	- (nad.T)	14 (PM)	14	2	2	
	35 h	35 h	2 to 3	+ (ad.T)	9 (PM)	9	4	2	2
Transvaginal	24 h	24 h	1	+ (ad.T)	10 (PM)	8	4	2	2
	35 h	35 h	1	+ (ad.T)	21 (PM)	20	12	9	3
	35 h	35 h	1	+ (infecond)	9 (M)	9	1	1	

These studies were conducted with the financial support of the French National Stud (Haras Nationaux)

collection but connected to a catheter and a 20 ml syringe. First, a slow aspiration of part of FF was performed to avoid the collection of the recipient oocyte. Secondly, the syringe was removed from the catheter to adapt immediately the transfer system for injection of one donor oocyte. In this experiment, parentage testing of all embryos was performed to determine the dam origin (Guérand *et al* 1997).

RESULTS

Blind punctures (Table 1)

After an additional transfer, 44% of recipients (n=9) were pregnant, 50% had a multiple pregnancy including an embryo originating from the donor mare. In this experiment, 25% of the total oocytes (19 from donors + 9 from recipients) gave one embryo. After a non-additional transfer, 14% of recipients (n=14) had a single embryo indicating that 14% of the 14 donor oocytes transferred gave one embryo.

Transvaginal punctures (Table 1)

After an additional transfer, no significant difference was found in pregnancy rates between the 2 times of collection and transfers 24 and 35 h after induction with 50% (n=8) and 60% (n=20) respectively of the recipient pony mares, 50% and 25%, respectively carrying 2 embryos. In this case, 38% of the total oocytes (28 from donors + 28 from recipients) gave one embryo. From the 16 pregnancies, the dam origin detection showed that 50% of the donor oocytes (n=16) and 81% of the recipient oocytes (n=16) gave one embryo. In 27% of gestations with one embryo, the embryo originated from the donor. Eleven per cent of recipient mules (n=9) had one embryo of the donor pony mare.

CONCLUSIONS

The additional transfer of oocytes increases embryo production. The blind transfer of more than one oocyte resulted in a 44% multiple gestation and 1.75 embryos per pregnancy. The ultrasound transfer of one oocyte produced 31% double gestations and 1.31 embryos per pregnant mare. Pregnancy rate following a non-additional oocyte transfer remains too low for routine use. Oocyte collection and transfer 24 h post induction did not appear to damage the donor oocyte which remains capable of achieving final maturation and producing an embryo. Mules can be used as recipients. This method could be a useful tool for testing the quality of *in vitro* matured oocytes.

REFERENCES

Bézard, J., Goudet, G., Duchamp, G. and Palmer, E. (1995) Preovulatory maturation of ovarian follicles and oocytes in unstimulated and superovulated mares. *Biol. Reprod. Monograph Series* **1**, 261-271.

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

Guérand, M., Mahla, R., Lagneaux, D., Amigues, Y., Palmer, E. and Bézard, J. (1997) Parentage testing of day 10 equine embryos by amplified PCR analysis of microsatellites. *Equine vet. J. Suppl* **25**, 69-71.

Palmer, E., Duchamp, G., Bezard, J., Magistrini, M., King, W.A., Bousquet, D. and Betteridge, K.J. (1987) Non-surgical recovery of follicular fluid and oocytes of mares. *J. Reprod. Fertil. Suppl*. **35,** 689-690.

INTRACYTOPLASMIC SPERM INJECTION (ICSI) OF HORSE OOCYTES

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

University of Cambridge, Department of Clinical Veterinary Medicine; Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK

INTRODUCTION

The difficulty of inducing decondensation of the stallion spermatozoon during ICSI of horse oocytes may relate to the maturation status of the oocyte and/or the activation treatment after sperm injection. Oocytes matured *in vivo* become fertilisable because of normal, synchronised maturation of the nucleus and ooplasm. Oocytes collected from ovarian follicles *in vivo*, or ovaries obtained at a slaughter house and cultured i*n vitro*, can release the first polar body of metaphase II (MII) during 24–40 h of *in vitro* maturation (Zhang *et al*.1989; Goudet *et al*.1997). However, although these MII oocytes have completed nuclear maturation, it remains difficult to fertilise them by ICSI and induce their development during subsequent culture, either *in vitro* or *in vivo* (Hamano *et al*. 1999; Li *et al*.1999). This suggests that MII oocytes obtained from *in vitro* maturation may be deficient in 'fertilisation and/or early development factors', which prevents maturation of the ooplasm (Sutovsky and Schatten 1997). In this study, the actions of glutathione (GSH), buthionine sulfoximine (BSO) and 4-dimethylamino-pyridine (DMAP) on ooplasm maturation were assessed by adding them individually to IVM medium and noting the response of the matured oocytes to ICSI.

MATERIALS AND METHODS

Oocyte collection and in vitro *maturation*

Cumulus-oocyte complexes (COCs) were recovered from slaughter house ovaries by scraping the follicle wall, and aspirated from ovarian follicles *in vivo* by transvaginal ultrasound guided follicle puncture. The basic medium for *in vitro* maturation consisted of TCM199 with 20% FBS, 10 mg/ml FSH, 5 mg/ml LH and 1 mg/ml oestradiol-17ß; culture was carried out at 38°C in 5% CO_2-in-air. Four groups of COCs were treated by adding: a) 15 mM GSH to the basic medium for 24 h followed by further culture in basic medium for 12–16 h; b) 10 mM BSO to the basic medium for 24 h followed by further culture in basic medium for 12–16 h; c) 1.9 mM DMAP to the basic medium for 24 h followed by further culture in basic medium for 12–16 h; and d) continuous culture in basic medium for 36–40 h. At the end of these maturation periods the cumulus cells were separated from the COCs by pipetting the latter in medium containing hyaluronidase. The proportion of oocytes that had reached MII stage was recorded before selection for ICSI.

Preparation of spermatozoa for ICSI

Frozen semen in 0.25 ml plastic straws was thawed at 35°C and washed twice with STALP solution containing 0.3 mg/ml BSA by centrifugation at 300 *g* for 5 min. The spermatozoa were then kept in EBSS+20% FBS at 38°C until used for ICSI.

ICSI and activation treatment of the oocytes

ICSI was carried out in 20 mM Hepes buffered EBSS-20% FBS medium at 30°C. Between 5 and 10 MII oocytes were placed in the ICSI medium and a spermatozoon injected into the cytoplasm of each one. The spermatozoa were exposed to the EBSS-20% FBS containing 10% PVP during the operation and the oocytes were activated by immersion in STALP solution containing 50-400 µM thimerosal for 5 min before injection of the spermatozoon. After activation, they were transferred to G1.2 medium for development culture.

TABLE 1: Nuclear maturation in oocytes derived from living mares versus abattoir ovaries*

Oocytes**	No. treated	MII(%)	≠MII	Degenerate
Living mares	5	4	(80)	0 1
Abattoir ovaries†	104	48	(46)	50 6

* Basic culture medium (TCM199 with 20% v:v FBS; 10 µg/ml FSH; 5 µg/ml LH; and 1 µg/ml oestradiol-17ß) used for both types of oocytes

** Nuclear classification of cultured oocytes, MII = metaphase II; ≠MII = includes the germinal vesicle (GV) and is still at metaphase I; Degenerate = oocytes with a heterologous cytoplasm or showing vacuolisation)

†The ovaries were transported from the abattoir at 12–26°C

TABLE 2: Nuclear maturation of oocytes cultured in medium added with GSH, BSO or DMAP

Treatment*	Oocytes			
	No. treated	MII(%)	≠MII	Degenerate
15 mM GSG	10	4 (40)	4	2
10 mM BSO	16	7 (43)	5	4
1 mM DMAP	8	2 (25)	4	2

*After culture for 24 h in TCM199 medium with added 15 mM GSH, 10 mM BSO or 2.0 mM DMAP, the oocytes were cultured for a further 12–16 h in basic medium

The results were assessed, either after 18–20 h by staining the oocytes with Orcein to observe activation and sperm transformation status, or after 24–30 h by staining them with Hoechst 33342 to observe cleavage and normality of development

CONCLUSIONS

The rate of nuclear maturation was higher in horse oocytes derived from living mares than from slaughter house ovaries (80% vs 46%), and it was not improved by adding GSH (40%), BSO (44%) or DMAP (25%) to the culture medium. Adding BSO to the maturation medium blocked decondensation of the spermatozoon injected into the cytoplasm, which may relate to GSH synthesised by oocytes cultured *in vitro*. Further, oocytes exposed to BSO in the maturation medium showed a lower activation rate than the other groups (43% vs 83%). Finally, cleavage rates in oocytes subjected to ICSI increased when the thimerosal concentration in the activation medium was increased from 50 to 200 µM (17% to 75%).

TABLE 3: Oocyte activation and sperm decondensation after injection

		Culture medium			
		Basic medium	+GSH	+BSO	+DMAP
Oocyte	No. treated	6	8	7	2
	MII	1	3	4	2
	AII-TII	3	4	3	0
	FPN	2	1	0	0
Spermatozoon	Condensed	4	3	7	2
	Decondensed	2	5	0	0
	MPN	0	0	0	0

AII-TII = anaphase II-telophase II; FPM - female pronocleus; MPN = male pronucleus

TABLE 4: Fertilisation of oocytes following ICSI and thimerosal treatment for oocyte activation

Oocyte development	Concentration of thimerosal (µm)			
	50	100	200	400
No. oocytes	12	23	1	8
No. oocytes cleaved (%)	2 (17)	11 (48)	12 (75)	0
No (%) cleaved oocytes showing				
Normal fertlisation	0	7 (64)	10 (83)	0
Parthenogenesis	2 (100)	3 (27)	2 (17)	0
Abnormal cleavage	0	1 (9)	0	0

* Oocytes were harvested from abattoir ovaries and cultured in TCM199 with 20% v.v FBS for 36-40 h to induce maturation.

REFERENCES

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

Hamano, K., Li, X., Qian, X., Funauchi, K., Furudate, M. and Minato, Y. (1999) Gender preselection in cattle with intracytoplasmically injected, flow cytometrically sorted sperm heads *Biol. Reprod.* **60,** 1194-1197.

Li, X., Hamano, K., Qian, X, Funauchi, K, Furudate, M and Minato, Y. (1999) Oocyte activation and parthenogenetic development of bovine oocytes following intracytoplasmic sperm injection *Zygote* **7,** 233-237.

Sutovsky, P. and Schatten, G. (1997) Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization *Biol. Reprod*. **56,** 1503-1512.

Zhang, J.J., Boyle, M.S. and Allen, W.R. (1989) Recent studies on *in vitro* fertilisation of *in vitro* matured horse oocytes *Equine vet. J. Suppl.* **8,** 101-104.

SESSION VI:

Embryo preservation

Chairman: T. Katila

CONFOCAL MICROSCOPIC ANALYSIS OF MICROFILAMENT DAMAGE INDUCED BY CRYOPRESERVATION OF EQUINE EMBRYOS

M. Huhtinen and J. Paranko

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

INTRODUCTION

Cleavage of the embryonic cells involves 2 cytoskeleton dependent processes. Karyokinesis, the mitotic division of the nucleus, is coordinated by the mitotic microtubule spindle formation. The second process is cytokinesis. The mechanical force driving the cell division is provided by the contractile ring of actin microfilaments. If cytokinesis is disrupted, embryonic development ceases.

Cryopreservation has been shown to have an adverse effect upon the cellular actin cortex and cytoplasmic network of human embryos. The actin cortex was totally disrupted and microfilaments showed widespread ooplasmic dispersion. Major variation was seen within a cohort of embryos, but differential temperature sensitivity appeared to be limited to the cytoskeleton (Levy *et al*. 1998).

Confocal fluorescence microscopy is an ideal tool for analysis of difficult biological specimens such as fluorescently labelled multicellular embryos. The aim of the present work was to study the applicability of confocal microscopy for the analysis of cryodamage in the early uterine stage equine embryos. The number of dead cells was recorded by DAPI-labelling under epifluorescence microscopy. Double labelling for nuclear chromatin and microfilamentous cytoskeleton was analysed by confocal microscopy.

MATERIALS AND METHODS

Thirty-seven embryos, 140 to 190 mm in diameter, were allocated into 2 different freezing protocols as follows:

1) Embryos (n=18) selected for freezing in glycerol were washed 3 times in PBS + 10% FCS. Glycerol and 50 mM glutamine were then added in 5 min steps in RT to give glycerol concentrations of 2.5%, 5%, 7.5%, and finally 10%. The embryos were loaded into 0.25 ml plastic straws and frozen conventionally in a programmable freezer.
2) Embryos (n=19) to be frozen in ethylene glycol were washed 3 times in Emcare holding medium. They were then placed into 1.5 M ethylene glycol in Emcare for 10 min in RT. Within the next 10 min, the embryos were loaded into straws and frozen like embryos in the glycerol group.

All embryos were thawed by holding the straws in the air for 10 sec and then in a water bath at 30°C for 20 s. In the glycerol group, cryoprotectant was removed in 6 steps with sucrose (Huhtinen *et al*. 1997). Half of the embryos in each treatment group were stained with DAPI and transferred, half the remaining were fixed for confocal microscopic analysis.

Embryos (n=9) in the glycerol group were stained with DAPI (1 µg/ml) in PBS + 10% FCS for 15 min in RT and then washed in PBS. Embryos (n=10) in the ethylene glycol were placed for 15 min into Emcare holding medium containing DAPI. They were then washed in fresh Emcare holding medium. Embryos were evaluated under epifluorescence microscopy for <10 s, and the image was recorded on a videotape. After DAPI-staining, the embryos were transferred non-surgically into the uteri of recipient mares that had ovulated 4 days before transfer.

Five additional fresh embryos were fixed for confocal microscopy to serve as controls for cryopreservation.

For confocal microscopy, embryos were fixed in 3.7% formaldehyde for 15 min at RT. After

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

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

* = The embryo continued its development after transfer

3 washes in PBS, they were extracted with the solution 0.1% Triton X-100 in PBS for 5 min, washed 3 times in PBS and treated with RNAse (1 mg/ml) in PBS for 15 min. After short washes in PBS, the embryos were incubated in PBS containing 1% BSA for 15 min. Thereafter they were incubated for actin labelling in the presence of Oregon Green 514 phalloidin and for nuclei staining in the presence of propidium iodide (0.5 mg/ml) for 30 min at RT. After 3 washes in PBS, the embryos were mounted and analysed by Leica TCS NT confocal microscope.

RESULTS

Four of 9 embryos frozen with glycerol continued their development after transfer to recipient mares. In the ethylene glycol group, 3 of 10 transfers were successful. No statistical difference in pregnancy rate was noted between the 2 freezing protocols.

The number/percentage of DAPI-stained cells is presented in Table 1. No statistical differences were noted between the 2 freezing protocols when the amounts of stained cells were compared.

Actin and nuclei labelling indicated well preserved cytoskeleton in the outer embryo cell layers. Diffuse staining in the inner parts of the embryo may be indicative of improper cryopreservation.

The results obtained so far indicate that this double-staining technique and confocal microscopy is promising for analysing the cellular damage induced by cryopreservation of equine embryos. However, the damage seen in the cytoskeletal structures after thawing is not necessarily severe enough to prevent further embryo development. Only a large number of pregnancies after transfer would prove the practical applicability of each freezing protocol. When a small number of embryos are available for research purposes, as is the case with equine embryos, new analytical developments are required for a more accurate analysis and understanding of embryonic defects caused by different freezing regimens.

REFERENCES

Huhtinen, M., Lagneaux, D., Koskinen, E. and Palmer, E. (1997) The effect of sucrose in the thawing solution on the morphology and mobility of frozen equine embryos. *Equine. vet. J.* **25,** 94-97.

Levy, R., Benchaib, M., Cordonier, H., Soucier, C. and Guerin, J-F. (1998) Laser scanning confocal imaging of abnormal and arrested human preimplantation embryos. *J. assist. Reprod. Genet*. **15**, 485-495.

SESSION VII:

Commercial development in equine embryo transfer

Chairman: M. S. Boyle

COMMERCIAL DEVELOPMENT OF AN EQUINE EMBRYO TRANSFER CENTRE IN SPAIN

G. Formiguera

CREA, Hípica El Montanyà, Seva, Spain

This paper describes the development of a centre which is intended to provide financial and practical advantages for the clients, the providers of recipient mares and ourselves.

Throughout the paper, donor owners are referred to as 'clients' and recipient owners as 'breeders'.

Two years ago we decided to set up the first equine embryo transfer centre in Spain. The aim was to develop and promote this technique and felt that there was potentially a large market. The intention was to provide a low-cost quality product to encourage clients to participate.

The major economic factor encountered was that of the recipient mares. This is because:

a) The centre needed a large number of recipient mares before the breeding season started. Because the exact number of embryos to transfer is not known in advance, more mares are bought than are likely to be required.
b) Once bought, they have to be kept by the centre until they are pregnant.
c) Once pregnant, the mares have to be bought by the client.

Other problems included:

a) finding suitable brood mares;
b) clients being saddled with mares they have no use for after weaning; or
c) clients sending these mares back to the centre for further embryo transfer treatment using their donor mare embryos. This leads to the additional difficulty of synchronising the cycles of these 2 particular mares.

The centre is in Seva - 50 km north of Barcelona and 50 km south of the Catalan Pyrenees. Barcelona provides the technological and transport support for the programme and, in the Pyrenees, we found the solution to some of our problems.

For centuries Catalan hill-farmers have bred horses to work on the land and as a meat supply. The predominant system is one of common grazing land. Over the years, the mares of these strong, warm-blooded Hispanic-Breton horses have been selected, above all, to be good mothers, producing a large foal every year and raising it without human intervention. Costs for the farmer are therefore minimal - both in terms of upkeep of the herd and raising of new livestock.

This represented a potential source of good, reliable mares which could be used as recipients in our breeding programme. They would have been relatively cheap to buy, but this would still leave us with some of the problems outlined above. Therefore, we considered a system of renting mares from the farmers, which would drastically reduce the pre-breeding season costs. In addition, if we left the mares *in situ* during pregnancy and lactation, we would reduce upkeep costs. The client, in turn, would not need to buy a recipient mare.

We approached the farmers in November 1997. Given their traditional approach to farming, it was no surprise that, initially, they had no faith in the artificial nature of an embryo transfer programme and, following the first meeting, only 2 breeders requested further information. The major attraction of the scheme for them was financial - by renting out their mares they could earn almost double the amount that they would earn from breeding and selling the foals in the traditional way.

A further meeting was held with these 2 farmers to discuss and clarify terms and conditions. The initial contract, which was verbal, included the following:

i) The breeder (farmer) is paid by the client (through the Centre) in 2 installments; the first when the mare is 45 days pregnant and the second when the foal is weaned. The first payment is non-refundable;

ii) Breeders do no more or less than they already do in terms of the care and upkeep of mare and foal;

iii) The breeders cannot sell the mare or foal during the rental period;

iv) They contact the Centre immediately if any health or other problem with the mare and/or foal is detected, such as spontaneous abortion, accident, illness or death;

v) They relinquish responsibility of care of mare or foal should there be a problem or suspected problem;

vi) The client pays all extra costs (eg vaccines);

vii) In case of the death of mare or foal, the client is released from the second payment.

In May 1998, we had our first pregnancy - a 9-year-old show-jumper mare was inseminated with fresh semen from a young show-jumping stallion. A week later, the embryo was transferred to a 2-year-old Hispanic-Breton filly, rented by the client.

By the end of the 1998 breeding season we had 5 more pregnancies by embryo transfer. Of these, 3 donor mares were show-jumpers and the other 2 competed in endurance riding. Further interest from other owners suggested that the technique is worth exploiting. This programme shares the advantages of other embryo transfers with the added benefit that clients do not have to buy a recipient mare nor are they left with it after weaning. This suits mare owners who have no land or who keep their mares in livery.

As an indication of the saving that can be achieved by renting, rather than buying the mares, in Spain it would cost about 1,000 euros to buy an acceptable brood mare. If the mare was later sold, it would be very difficult to recover the cost. There are also minimal upkeep costs of about 800 euros per year. Conversely, renting an Hispanic-Breton mare living in the Pyrenees costs, in total, about 650 euros. If there is no foal or the foal has any kind of defect, the client only loses 50% of this as the first payment to the breeder.

Since its inception, we have changed and refined many aspects of the initial programme. For example, the contracts are written and are becoming more detailed and specific.

The initially unconvinced breeders are now wanting to become involved in the programme which means that a ready and reliable source of good recipient mares is available. Given that clients are not tied to using a particular recipient mare, we can match recipient and donor mares according to their ovulation which makes the process easier, quicker and cheaper. Planning for future breeding programmes can be flexible with no need to balance the number of recipient and donor mares.

In summary, the system works as follows:

i) Pyrenean farmers who wish to rent out their mares contact the centre;

ii) The centre takes a reproductive history of each mare and conducts a physical and reproductive examination;

iii) The mares are transported to the centre one month before the breeding season;

iv) Clients contact the centre with breeding requirements;

v) Donor mares are transported to the centre;

vi) Recipient and donor mares are synchronised;

vii) Embryo transfer is carried out;

vii) Pregnant recipient mares stay at the centre until at least Day 45 of pregnancy;

viii) The recipient mares are returned to either the Pyrenees or the clients' premises;

ix) After successful birth and weaning of foals, the rental agreement comes to an end.

This programme suits the needs of all parties, relies on the abundance of good breeding mares in close proximity to the centre and makes viable the commercial development of an equine embryo transfer centre.

FACTORS AFFECTING EMBRYO RECOVERY, EMBRYO DEVELOPMENT AND PREGNANCY RATE IN A COMMERCIAL EMBRYO TRANSFER PROGRAMME

S. Meadows, H. Lisa and C. Welsh*

*Irish Sport Horse Developments, Castle Irvine Demesne, Irvinestown, Co. Fermanagh, Northern Ireland; *Erne Veterinary Group, New Street, Enniskillen, Co. Fermanagh, Northern Ireland*

INTRODUCTION

The Equiova programme is a breed improvement scheme, devised to identify Irish mares with high genetic merit for show jumping and cross them with proven, top quality show jumping stallions from Europe, using embryo transfer, in an effort to improve the genetic standard of the Irish horse. Each season 15 donor mares are selected and their numbers are swelled by mares from private clients. The fact that a single group of mares is followed closely over several cycles annually has permitted close observation of the effects of mare age, reproductive condition and semen type on embryo recovery and development and on pregnancy rates.

MATERIALS AND METHODS

Mares

The group consisted of 50 mares, 19 of which were donors for 2 or more years. The observation period lasted for 4 consecutive breeding seasons (1996–1999). The average age was 11.4 years with a range of 2 to 25 years. Most donor mares were Irish Sport Horses, part bred mares, usually 3/4 Thoroughbred and 1/4 Irish Draught.

Reproductive management

Donors and recipients were examined daily by transrectal ultrasonography at the start of oestrus. For donor mares destined for AI with frozen semen this was increased to up to 5 times per day around the time of ovulation. If chilled or fresh semen had been selected, a maximum of 3 examinations per day were performed. The aim was to perform one well timed insemination per cycle rather than repeated inseminations. The mares were inseminated with one of 3 semen types, fresh, chilled or frozen from one of over 30 different stallions. Chilled semen was transported to the centre in either an Equitainer or a polystyrene box with a cool pack. If frozen semen was used the mare was inseminated after ovulation had been observed, except in a few cases. Semen was thawed according to the stud's instructions, usually in a water bath at 37–38°C for 30 s and the mare inseminated within 120 s of semen thawing. Embryo recovery was carried out 7–9 days after ovulation was detected, by non-surgical uterine lavage with 3 litres of sterile Dulbeccos Modified Phosphate Buffered Saline (DPBS) supplemented with 1% v/v Fetal Calf Serum (FCS). Non-surgical embryo transfer was performed within 2 h of embryo recovery to part bred recipients which had ovulated one day before to 2 days after the donor mare.

Pregnancy confirmation

Pregnancy was confirmed by transrectal ultrasonography 7–10 days after transfer. The pregnant recipients were monitored by repeated scanning at 3-weekly intervals until Day 60 of gestation, when they left the embryo transfer centre.

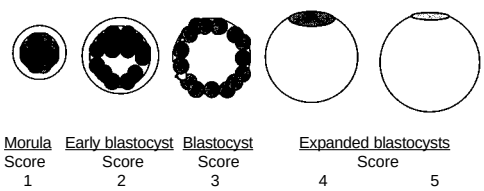

Fig 1: The basis of the scoring system used to assess embryo development.

TABLE 1: Embryo recovery rates for all mares and mares split into 2 age groups

Mare category	Overall	Fresh semen	Chilled semen	Frozen semen
All mares	59.6% n = 171	87.6% n = 78	47.4% n = 45	46.6% n = 48
<14 yo	67% n = 134	98% n = 69	48% n =25	51% n = 40
⩾14 yo	43% n = 37	47% n = 9	47% n = 20	32% n = 8

TABLE 2: Pregnancy rates at 14 days for all mares and mares split into 2 age groups

Mare category	Overall	Fresh semen	Chilled semen	Frozen semen
All mares	80% n = 136	87% n = 68	73% n = 33	73% n = 35
<14 yo	80% n = 108	86% n = 59	76% n =19	75% n = 30
⩾14 yo	76% n = 28	100% n = 9	70% n = 14	63% n = 5

Interpretation of results

Details of the total number of AI cycles, number of embryos recovered and number of pregnancies established were pooled to allow the calculation of embryo recovery rates and pregnancy rates. Mares of 14 years or more were considered as aged.

A scoring system was devised whereby varying stages of embryo development were given a numerical value, in order to allow assessment of the size and stage of development of embryos recovered. See Figure 1 for allocation of scores.

RESULTS

Embryo recovery

Semen type had a marked effect on embryo recovery. Embryo recovery rates from mares inseminated with chilled or frozen semen were just over half those of mares inseminated with fresh semen. The number of embryos recovered from aged mares was generally lower than for the younger counterparts. Recovery rates were exceptionally poor when frozen semen was used on older mares (Table 1).

Pregnancy rates

Pregnancy rates at 14 days did not vary greatly between semen type or age group, although the number of pregnancies established after insemination with fresh semen was consistently higher than with chilled or frozen semen. As well as lower recovery rates, the combination of frozen semen and old mares resulted in a lower pregnancy rate at 14 days (Table 2).

Mare age did not only affect embryo recovery rates, fewer embryos derived from older mares survived to 60 days gestation, despite being transferred to younger recipient mares. Only 1 of 108 pregnancies established after embryo recovery from young mares was lost between 35 and 60 days, whereas 3 of 28 were lost in the older age group. This gave a loss rate of <1% for the young group compared to 9% for the aged mares.

TABLE 3: Time taken for transport of embryos and embryo score in mares according to age group

Mare category	Mean interval from AI to ovulation to flush (days)	Mean interval from ovulation	Mean embryo score
<14 yo	18.1	7.7	3.7
⩾14 yo	19.6	7.9	2.5

Embryo size and stage of development

Embryo transport along the oviducts and differentiation of the embryo was delayed in older mares. Embryos took longer to reach the uterus and were consistently smaller and less developed than would be expected at recovery, indicated by a lower mean embryo score (Table 3).

CONCLUSIONS

Semen type had a marked effect on embryo recovery rates. Recovery rates declined with increasing donor age. Although pregnancy rates did not differ greatly at 14 days, a higher pregnancy loss rate between days 35 and 60 was observed in the aged mare group. Mare age also affected oviductal transport and development of embryos. Embryos recovered from older donors were consistently smaller and less advanced in terms of development.

A FAILED ATTEMPT TO COLLECT, TRANSPORT AND TRANSFER EMBRYOS FROM COMPETING MARES

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

University of Cambridge, Department of Clinical Veterinary Medicine, Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK

INTRODUCTION

The breeding of non-racehorse competition or sport horses in Britain is severely hampered by a shortage of intact stallions and fertile young mares selected on the basis of their own superior performance within the competitive discipline for which they were bred. This situation could be helped greatly by the judicious application of one or more of the modern technologies to the national breeding programme. Such technology includes repeated non-surgical recovery of embryos from fillies in active training and competition, for deep freezing or direct transfer to synchronised recipient mares, or repeated transvaginal ultrasound guided recovery of oocytes from competing fillies for fertilisation by intracytoplasmic injection (ICSI) of previously sex-sorted stallion spermatozoa. The present study was undertaken to determine the practicality and efficacy of the first of these 2 options in the field.

MATERIALS AND METHODS

Nine superior sport horse mares, 7 of which were still in active competition, were recruited to the project as embryo donors. They ranged in age from 7 to 19 years and included 4 eventers, 3 showjumpers, one dressage horse and one high goal polo pony. They were all maintained at their owners' training establishments scattered through the south of England at road distances of between 94 and 253 miles from the farm on which the main group of 12 Irish Draught or Irish Draught X Thoroughbred recipient mares were kept. These recipients had been ‘loaned’ for the duration of the experiment and they ranged in status from maiden to multiparous and in age from 3 to 16 years. Five other Thoroughbred recipient mares, aged 3–15 years, were maintained on the same establishment as the single dressage donor mare in the programme was to receive embryos from that animal only.

The monitoring of follicular development and ovulation in, and the blood sampling, treatment or insemination of, the donor mares was carried out on an alternate day basis by one or both of the authors, with occasional help from local veterinary practitioners. Similarly, cycle stage, follicular development and ovulation in the main herd of recipient mares was monitored and manipulated to suit donor animals by regular alternate day visits to the recipient mare farm.

Embryos were recovered non-surgically from the donor mares on Day 7 or 8 after ovulation (Day 0) by transcervical uterine lavage with Dulbecco's phosphate buffered saline (PBS) containing 1% w:v bovine serum albumin (BSA), using a closed system with an in-line embryo filter. The embryos were assessed microscopically, placed in Ham’s F10 medium in a 15 ml conical tube gassed with CO_2 and maintained at 30°C in a pre-warmed incubator, and transported by road to the recipient mare farm. They were then re-assessed in a makeshift embryo laboratory in a stable, placed in ovum culture medium containing 10% v:v fetal calf serum (FCS) and transferred transcervically to the uterus of the sedated recipient mare that had ovulated 0–3 days after the donor, using a 0.5 ml straw in a sheathed plastic disposable equine embryo transfer gun.

RESULTS

Twenty-nine embryo recovery attempts carried out during a 16-week period between April and mid-July, 1998 yielded 24 embryos (80%; Table 1). However, these included 5 sets of twin embryos

TABLE 1: Outcome of embryo transfer following collection from sport horse mares and transport by road

Donor mare	Discipline	Age (years)	Distance from recipient farm (miles)	No. embryo recovery attempts	No. embryos recovered (%)	No. embryos transferred	No. recipients pregnant (%)	Pregnancy outcome
1	Dressage	8	0	4	6 (150)	5	2	Live foals
2	Showjumper	7	112	3	2 (67)	2	0	-
3	Showjumper	19	204	5	1 (20)	1	1	Resorbed (d40-65)
4	Showjumper	9	133	4	1 (25)	1	0	-
5	Eventer	11	94	3	0 (0)	-	-	-
6	Eventer	10	136	4	9 (225)	8	1	Resorbed (d20-34)
7	Eventer	14	187	1	1 (100)	1	1	Live foal
8	Eventer	9	116	2	1 (50)	1	0	-
9	Polo pony	9	253	4	3 (75)	2	1	Resorbed (d16-26)
	Totals			**30**	**24 (80%)**	**20 (67%)**	**6 (29%)**	**3 (14%)**

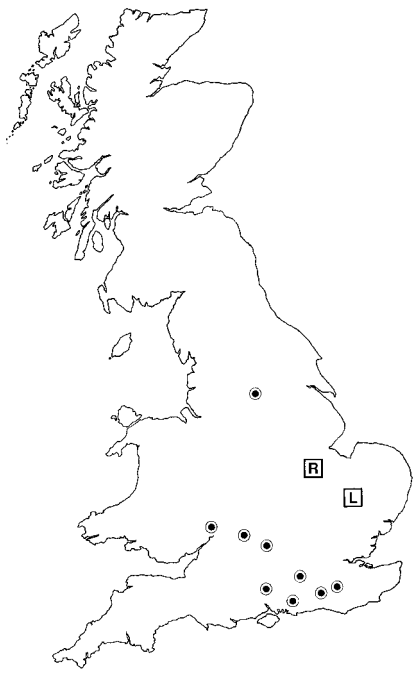

Fig. 1: Distribution of the home bases of the 9 donor mares (Θ), the authors' laboratory (L) and the main herd of recipient mares (R) throughout the south of England.

and 2 sets of triplet embryos so that the proportional recovery rate fell to 52%. This compares unfavourably with the recovery rate of 70–80% achieved in the authors' laboratory when flushing healthy, fertile experimental mares that have been supervised closely during oestrus and inseminated optimally with semen from stallions of known high fertility.

Twenty-two early, normal or expanding blastocysts were transferred non-surgically to recipient mares. Most of those transfers occurred between 9.00 pm and midnight after a car journey of 2.5–6 h, and when the recipient required sedation with a cocktail of dometidine hydrochloride and butorphanol tartrate. Only 6 pregnancies resulted, 2 of which were in the 5 on-site recipient mares supervised regularly by a competent and experienced local veterinary clinician. Embryonic or fetal death occurred subsequently in 3 of the recipients which meant that the final live foal birthrate from the 22 transfers was a disappointingly low 14% (Table 1).

DISCUSSION

A number of factors are considered to have contributed cumulatively to these poor results, including:

i) An abnormally long and cold spring which delayed the onset of the breeding season. This meant that time and effort were wasted monitoring slow growing and non-ovulatory follicles at the start of the trial.

ii) Variation in the fertility and reproductive responses of the widely dispersed donor mares, and insufficiently frequent or expert monitoring of ovarian changes in some of them.

iii) Difficulties relating to the fertility and/or handling of the semen used to inseminate the donor mares.

iv) Selection of the recipient mares for size, rather than temperament and reproductive parameters, so that many of the multiparous animals were difficult to handle and had tortuous and difficult cervices for non-surgical transfer.

v) Transfer facilities that were not ideal. Most transfers were performed at night to sedated mares and most of the embryos had been transported at 30°C for 3–6 h before transfer.

Although the results of the study were disappointing, some valuable lessons were learned:

i) Donor mares should be temporarily resident at a well organised equine reproduction facility around the time of insemination and ovulation.

ii) Stallions should be selected on the basis of their fertility, as well as their genetic excellence and/or athletic prowess.

iii) Recipient mares with any suspicion of subfertility, behaviour problems or difficult cervices must be eliminated at the outset.

WORKSHOP SUMMARY

WORKSHOP SUMMARY

R. H. F. Hunter

Faculty of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

First, if I may, I am going to take this opportunity on behalf of all the speakers to reiterate our thanks to the organisers of the meeting, not only to Professors Twink Allen and Marian Tischner for initiating and focusing our days of discussion, but to all of the Tischner family and to their colleagues for the special arrangements and wonderful hospitality here in Lopuszna. Particular thanks are extended to Jan Wade for being so effective and helpful an organiser and coordinator behind the scenes, and for producing the excellent record of matters so far – the book containing the programme, the list of participants and a complete record of abstracts.

Now to the speakers themselves: throughout the Workshop, we have had well-prepared, consistently good lectures with splendid modern slides, and everyone has been agreeably concise – a point worth emphasising. No-one has droned on and on, thank goodness. So, if and when the question arises as to whether another Workshop would be appropriate in due course, the response must surely be 'yes' if it meets the high standards of the present one. Perhaps you remember the initial letter from Twink along the lines that "… he and Marian Tischner being the autocrats that they are, have decided to arrange …". Well, if these stimulating days together here in Lopuszna have been the fruits of autocracy, then long live benevolent dictatorships. We need more of them.

Now for 10 minutes or so of scientific comment without mentioning names or indeed specifically covering every topic or presentation. My own impression is that the Workshop has dealt very largely with applied research and I am not sure just how much of the underlying science has been clarified – or remains to be clarified – in your chosen species of animal. Research with an application in view is doubtless of special appeal to the Havemeyer Foundation, but the techniques and therapies to be used in the future, say 10 years from now, must stem in part from fundamental studies. Reproductive physiology is still a relatively young discipline, so wisdom would caution not too heavy an emphasis on applied research with the prospect of immediate application.

Of course, the opening paper from Utrecht did deal with fundamental changes – changes in the membranes of stallion spermatozoa as an approach to gaining a more complete understanding of the process of capacitation. Certainly excellent work which started the Workshop off at an impressive intellectual level. However, one of the problems remains to define which of the 2 currently mooted models of capacitation applies to stallion spermatozoa. Is there a continuous availability of sub-populations of capacitated sperm in the female tract after mating, ie. a series of curves of ripening and decay, or do stallion spermatozoa respond to the ovarian control model, only achieving full capacitation at the time of ovulation? I suggest that we still do not have an answer, but would propose that the second of the 2 models operates in this species with a prolonged pre-ovulatory interval.

A step back from sperm cell interactions with the female tract concerns their interactions with the epididymal duct. How much is known about sperm surface modifications within the epididymis and the transfer of macromolecules from the epithelium in particular portions of the duct? Indeed, how much do we know about androgen binding protein and its target tissues in the stallion? Almost certainly not nearly as much as in the ram, bull, boar, rabbit or rat. If correct, one feels strongly that such fundamental studies need to proceed simultaneously with attempts to exploit suspensions of epididymal spermatozoa obtained at autopsy or during elective castration.

Keeping on the subject of sperm cells a little longer, does everyone here working with cryopreserved stallion spermatozoa recall that studies with frozen-thawed boar spermatozoa indicated that sperm cells from many – perhaps a majority – of boars under test were extremely vulnerable to the uterine environment when thawed and extended in insemination medium? Expressed in another way, introduction of frozen-thawed boar spermatozoa into the uterine lumen damaged a high proportion of such gametes, and the greater the extent of exposure to the uterine tissues (eg intra-cervical insemination), the greater the resultant damage and the lower the conception rate. By contrast, insemination at the utero-tubal junction or just into the Fallopian tube isthmus was found to be highly beneficial. If we accept for a moment that this may also be the case for frozen-thawed stallion spermatozoa (and bearing in mind that both species have intra-uterine accumulation of semen at mating), then endoscopic techniques of deep insemination close to or onto the utero-tubal junction, already shown to be valuable in another context, assume an even greater importance. Moreover, although technically challenging, a means of insemination into the isthmus would be a very specific advance towards exploiting low numbers of cryopreserved stallion spermatozoa. Coincident with such applied studies, further work on the relationships between structural modifications in preserved spermatozoa and subsequent fertility of the inseminate is definitely to be encouraged.

A few more words concerning spermatozoa. There is preliminary evidence from cattle that re-suspending the spermatozoa of low fertility bulls in seminal plasma derived from high fertility bulls may act to improve the conception rates of inseminations from poor bulls. These are early days: this work is recent and the results are confounded from several points of view, but it does seem important enough for practitioners of equine insemination to be alert to future possibilities. The cellular physiology underlying a seemingly crude approach may have a rational explanation: substitution of beneficial male duct proteins on and in the sperm plasma membrane may facilitate both sperm survival in the female tract and subsequent sperm-egg interactions. Perhaps a more timely acrosome reaction or a more coordinated vesiculation of plasma and outer acrosomal membranes is a consequence of this approach.

Finally, the interactions between stress, season of the year and sperm quality came to light in several presentations and made a strong impression on this reviewer. Without any exaggeration, such interactions seem of immense importance and yet will be difficult to unravel without extensive multi-centre collaborations. Here, the experiments of the Hungarian-French teams offered one example of an inspired step forward, focusing especially on a putative role for perturbed glucocorticoid secretion in syndromes of stress leading to low fertility. Presumably a variety of mechanisms of action will need to be invoked, and the scope for further studies is therefore considerable.

Turning now to ovarian follicles and to oocytes, and as one with a research background in other large domestic species, I was struck forcibly by the limited number of oocytes that can be obtained by means of follicular aspiration, puncture or maceration techniques, frequently only 4–5 oocytes per ovary or per mare. This is dramatically different from the situation in sheep, cows or pigs. Why should it be so? You must have reflected on the problem. Assuming that the explanation is not primarily due to the initial complement of oogonia and oocytes in the fetal gonad, nor to massive waves of follicular atresia before puberty, then there would seem to be scope here too for imaginative new approaches. Not least, these could involve a combination of enzymatic treatment and mild sonication of ovarian tissues. If the problem resides primarily in the nature of attachment of the cumulus-oocyte complex to the wall of the Graafian follicle, then such approaches might be beneficial. If the problem is associated with the number of developing vesicular follicles, then there may be room for a form of controlled and predictable ovarian stimulation. The special morphology of the equine ovary does not appear as an overwhelming block to progress in this field. So let us not be discouraged but rather more inventive.

Related points to which some members of the Workshop may have devoted serious thought are: (1) whether morphologically good Graafian follicles can shed defective oocytes at ovulation and (2) whether presumptively poor follicles can yield viable oocytes? Do the latest staining techniques really enable us to make such vital distinctions? Remaining with the theme of oocytes and especially of ovulated eggs, do we actually know what the viable lifespan of the mare's egg is in the Fallopian tube? Indeed, is this concept meaningful in the sense

of a viable lifespan of say 6–8 hours, or 10–12 hours after ovulation, essentially constant within and between animals and season, or is there substantial variation? Bearing in mind that we are dealing with only one egg liberated per cycle in contrast to millions of spermatozoa introduced, the egg is clearly more vulnerable from a quantitative point of view and possibly also from a structural angle with its large cytoplasmic component. In any event, it seems a research topic of considerable significance to clarify this lifespan and to see if it is open to modification. This point is made even though the population of spermatozoa should ideally be established in the Fallopian tube(s) before ovulation – and would be so under biological conditions. However, procedures of artificial insemination may alter these relationships and the shortened lifespan of frozen-thawed spermatozoa might dictate a peri- or post ovulatory insemination. In brief, gamete lifespans cannot be taken for granted and could definitely benefit from a further bout of well focused research in meaningful numbers of animals.

As to the zona pellucida, we heard of excellent studies on this envelope around the mare's oocyte. In view of the recent advances in understanding glycoproteins of the zona, especially those referred to as ZP3 and ZP2, this was extremely topical work. Even so, one must question what the phenomenon of so-called zona hardening actually tells us about oocyte or embryo quality and ask what is, approximately, the maximum number of spermatozoa that can bind to the zona pellucida before completion of hardening. Do we know? With regard to the second of these questions, one has the impression that it is rather low and also that the incidence of polyspermy – multiple sperm penetration of the vitellus – is again low in the horse egg. But impressions are not enough. Careful experiments are needed that yield answers *in vivo*, not conveniently in a culture dish.

Moving from gametes to embryos, and in relation to the detailed reports on transplantation trials, presumably the only rigorous test of viability is generation of a full-term fetus. Of course, useful indicators of potential viability may be developed but it is not easy to suppose that we can ever, or would ever wish to, avoid the ultimate test of competence. Predictive tests of embryonic viability may be refined in due course but their accuracy will almost certainly be for populations (ie large numbers) of embryos rather than for an individual embryo about to be transplanted. Nonetheless, the reports on transplantation studies and indeed on neonatal loss have been invaluable to all members of the Workshop.

Various new techniques were described, and these seem to have potential in more ways than highlighted. The work on protocols for *in vivo* oocyte recovery or *in vivo* replacement or *de novo* introduction of oocytes into follicles are exciting approaches in their own right. In reality, their scope may extend from studies of oocyte and follicular atresia through aspects of the actual process of ovulation to developing a model for the early stages of ectopic pregnancy. And the microsampling approach to the composition of follicular fluid, somewhat reminiscent of the Göttingen technique for microsampling secretions of the corpus luteum, although a far more difficult surgical preparation, should not be limited to withdrawal of microdroplets of follicular fluid. It would also enable accurate local testing of a number of gonadotrophin, prostaglandin and peptide growth factor treatments, and might enable some dissection of the interplay between thecal and granulosa cell synthesis and secretion of steroid molecules. So here is scope for the very best sort of fundamental research, and it is much to be encouraged.

Intra-cytoplasmic sperm injection (ICSI), confocal microscopy and multiple embryo transfer could all be commented on in similar detail. They are approaches that offer much mileage! Undoubtedly, we will be hearing stimulating reports on their extensive application at future meetings, so I shall discipline myself not to speculate on their potential.

There is a suspicion that I have spoken for almost too long. If so, my sincere apologies and similarly so should anyone feel that I have not made sufficient reference to the work of their laboratory. There remains the hope that in some manner, perhaps almost cryptic, it has been embraced by the above remarks. As noted at the outset, everyone has done good work, everyone has participated actively and in an open spirit, and together we have had a stimulating and superb Workshop. We must hope for another. Such an investment would be more than justified both intellectually and in a practical context of equine breeding programmes and procedures. My own thanks go to all members of the Workshop and our joint thanks go to the Havemeyer Foundation for their imaginative and generous support.

R.H.F. Hunter
September 1999

AUTHOR INDEX