Havemeyer Foundation
Monograph Series No. 6

Proceedings of a Workshop entitled

FROM EPIDIDYMIS TO EMBRYO

18th–21st October 2001
New Orleans, USA

Editors: L. H-A. Morris, L. Foster and J. F. Wade

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EDITORS' FOREWORD

Dorothy Russell Havemeyer, the founder of the Havemeyer Foundation, raced and bred horses in New Jersey and Florida. In 1979 she founded the Foundation and was President until her death in August 1985. During her lifetime the Foundation established a reputation for supporting a wide range of equine research and conducting 'cutting edge' scientific workshops. These workshops are attended by a small number of participants and provide an opportunity for scientists from comparative disciplines to pool their knowledge, thoughts and ideas in an informal and interactive atmosphere.

The Foundation is at the forefront of the equine genome project and has 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 infectious diseases.

The Foundation is justifiably proud of its research activities and its programme of workshops and a series of monographs has been launched to ensure that the information gained is disseminated as widely as possible. This workshop entitled 'From Epididymis to Embryo' was intended to focus on the factors that influence the processes of fertilisation and early embryo development in the horse. The Foundation's support reflects its ongoing interest in reproductive biology. It was enhanced by the presence of research workers who represented non-equine species and this allowed us to shed some light on the problems encountered in the development of *in vitro* embryo production in the horse.

A particular feature of the Havemeyer Workshops has been the instigation of collaborative projects between workers in different disciplines and from multiple geographical locations. This is encouraged by the Foundation and its President, Mr Gene Pranzo, whose support and enthusiasm is always appreciated by the equine research community.

L. H-A. Morris
J. F. Wade

HAVEMEYER SCIENTIFIC WORKSHOPS

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

1995

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

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

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

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

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

1997

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

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

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

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

1998

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

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

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

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

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

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

1999

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

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

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

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

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

2000

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

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

5th International Symposium on Equine Embryo Transfer
July - Saari, Finland
Organiser: Dr T. Katila

2001

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

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

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

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

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

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

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

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

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

2002 **USDA Internationl Plant & Animal Genome Conference**
January - San Diego, California

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

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

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

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

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

HAVEMEYER MONOGRAPH SERIES

The following are monographs available to date at a cost of £9.95 each.

Series No 1
PROCEEDINGS OF THE FIRST MEETING OF THE EUROPEAN EQUINE GAMETE GROUP (EEGG)
Editors: W. R. Allen and J. F. Wade
5th–8th September 1999
Lopuszna, Poland

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PROCEEDINGS OF A WORKSHOP ON FETOMATERNAL CONTROL OF PREGNANCY
Editors: T. A. E. Stout and J. F. Wade
14th–16th November 1999
Barbados, West Indies

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Saari, Finland

Series No 4
PROCEEDINGS OF A WORKSHOP ON EQUINE IMMUNOLOGY IN 2001
Editors: D. P. Lunn and J. F. Wade
24th–28th January 2001
Santa Fe, New Mexico

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Editors: T. A. E. Stout and J. F. Wade
26th–29th September 2001
Loosdrecht, The Netherlands

Series No 6
PROCEEDINGS OF A WORKSHOP ENTITLED 'FROM EPIDIDYMIS TO EMBRYO'
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SESSION I

Chairman:
Dickson Varner

SPERMATOGENESIS AND INFERTILITY

R. G. Saacke

Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA

Clearly, reproductive soundness of the male is critical to reproductive efficiency in any species and therefore warrants close attention. Our ability to evaluate fertility of the male accurately, as well as our influence on that fertility, is contingent upon a more complete understanding of testicular function and the seminal traits which impact upon pregnancy rate. From a broad research base utilising artificial insemination in cattle, we recognise that efficiency of spermatogenesis, quantitatively and qualitatively, is important and that our growing knowledge about the nature of subfertility due to the male is as complex as that of the female. This paper summarises some of the insights and concepts gained by utilising the non-surgically recovered 6-day-old bovine embryo/ovum as a biomonitor of the inseminate.

A number of studies have revealed 2 major components of the inseminate which impact upon pregnancy rates (see Saacke *et al.* 2000 for review). One represents seminal deficiencies that are 'compensable', ie their negative impact (reduced fertility) is overcome by increasing sperm dosage or their negative impact is only apparent when low dosages are employed. The second is considered 'uncompensable' in that subfertility exists regardless of sperm dosage. The former appears to be associated with differences among males in the ability of their sperm to access the ovum, being removed as potential fertilising sperm at specific barriers in the female tract or at the vestments of the ovum. Their restriction at these barriers may be due to limited viability or morphological anomalies of the sperm or due to anatomical barriers in the female tract, the function of which we do not fully appreciate. The second (uncompensable deficiencies) is considered to represent differences among males in the presence of sperm capable of accessing the ovum and initiating the fertilisation process and/or embryogenesis, but unable to sustain either or both events. Such sperm would impact upon pregnancy rate in proportion to their frequency in the ejaculate/inseminate. den Daas *et al.* (1998) showed that, within a male, compensable and uncompensable deficiencies can exist independent of one another or in combinations of variable magnitude. Another way to state their concept is: as sperm dosage to the female is increased for any male, the rate at which the asymptote in fertility is reached (minimum number of sperm required for maximum fertility) appears independent of the final level of fertility that male may achieve. Therefore, in tying events of spermatogenesis to fertility or in judging the impact of semen on pregnancy rate (often referred to as fertility) it is important to differentiate the compensable and uncompensable components in their respective importance to the fertility achieved.

In our laboratory we have utilised the non-surgically recovered 6-day-old embryo/ovum (presumptive morula) as a biomonitor of the inseminate. This embryo/ovum reveals the fertilisation status and embryo quality as well as the quantitative and qualitative accessibility of sperm to the ovum, via the accessory sperm (DeJarnette *et al.* 1992). For cattle, the block to polyspermy is quite stable once established (Hunter *et al.* 1998) providing some confidence that the number of accessory sperm is at least partially reflective of the number competing for fertilisation in the window of time the ovum is receptive. This approach represents a labour-intensive effort to assess the outcome of matings, but has also given us some insights to the complexity of data generated in evaluating males and reproductive strategies (for review, see Saacke *et al.* 2000).

THE 6 DAY-OLD OVUM/EMBRYO AS A BIOMONITOR FOR COMPENSABLE AND UNCOMPENSABLE SEMINAL DEFICIENCIES RELATED TO SPERMATOGENESIS

Historically, spermatozoal morphology has been associated with abnormal spermatogenesis and related to subfertility in a variety of species. Utilising pooled semen from sires in commercial artificial insemination, the 6-day-old ovum/embryo revealed that both fertilisation rate and embryo quality were impaired by semen containing sperm with misshapen heads (DeJarnette *et al.* 1992). To determine the types of misshapen sperm heads potentially contributing to embryonic disturbances, evaluation of abnormal sperm in the inseminate was compared with that comprising the accessory sperm population in the zona pellucida of the ovum/embryo. This would indicate which types of sperm pass the barriers in the female tract as well as undergo capacitation, egg binding and the acrosome reaction in a timely fashion (Saacke *et al.* 1998). This work showed that, morphologically, spermatozoa were excluded from contact with the ovum more by severity than type of head distortion. Thus, subtle forms of misshapen heads and normally shaped heads with nuclear vacuoles (diadem, apical and random vacuoles) gained access to the ovum on an equal basis to their proportion in the ejaculate. Therefore, it would appear that they are the most likely candidates for the uncompensable component in semen.

Through several years of experimentation in our laboratory we recovered more than 1,000 single-ovulating ova/embryos 6-days post artificial insemination. More than 30 bulls, all of which provided acceptable semen, were represented in these data. The mean, median and mode of sperm per embryo/ovum was 12, 2.4 and 0, respectively indicating the skewed nature of sperm distribution and the need to focus on the median sperm per ovum/embryo as the most important value. Surprisingly, median accessory sperm number per ovum was highest for high quality embryos (classified excellent-good = 8 sperm) than for low quality embryos (fair-poor = 5 and degenerate = 1). Unfertilised ova (UFO = 0) were basically devoid of accessory sperm (Saacke *et al.* 2000). The lack of accessory sperm in UFOs was expected; however the positive relationship of sperm numbers and embryo quality was surprising and interpreted to indicate that selection of competent sperm may occur at the zona pellucida, ie competition among sperm favours higher quality embryos. Further evaluation of accessory sperm numbers using only the embryo population from these studies (n=>800) revealed that embryo quality was not optimised until 10 or more sperm were competing per ovum. As more than 60% of all inseminations result in less than 10 sperm competing for fertilisation in the bovine (Saacke *et al.* 2000), it becomes paramount that semen/males with uncompensable deficiencies be identified and efforts be made to increase sperm accessibility to the ovum such that both fertilisation rate and embryo quality are maximised.

PERTURBED SPERMATOGENESIS AND THE NATURE OF POTENTIALLY UNCOMPENSABLE SPERM

As classically misshapen sperm do not compete for fertilisation, recognition of the uncompensable deficiency in semen is not in hand. Research in our laboratory involving the effect of thermal stress on abnormal sperm content of the ejaculate revealed that semen with a variety of specific abnormalities can be produced in a chronological order following a mild scrotal insulation (Vogler *et al.* 1991 and 1993). Some of these abnormalities are quite subtle or without effect on shape (eg nuclear vacuoles). Thus, we chose to use this as a model for assessing the influence of sperm morphology on fertility/embryo quality as well as provide a variety of cells in order to study chromatin

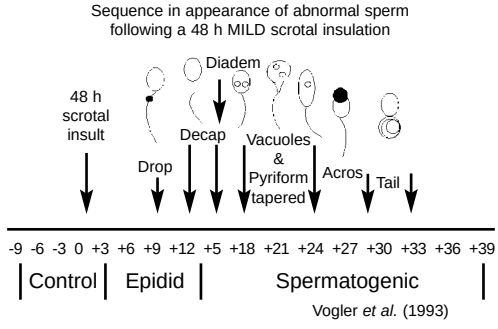

Fig 1: Chronological order of abnormal sperm production following a mild scrotal insulation. Males differ markedly in the production of abnormal sperm following scrotal insulation; however, if present, specific abnormalities occur in a chronological order and at a distinct time, reflecting the location or stage of spermatogenesis the cells were in at the time of the thermal perturbation.

TABLE 1: Effect of semen with sperm having nuclear vacuoles and diadem defects (Fig 1) on embryo quality in superovulated cows (Saacke *et al.* 1994)

Cows	Embryos	[b]Semen	[a]Embryo quality (%)			
			Ex - Gd	Fr - Pr	Deg	Deg/UFO
7	90	Day - 3	74.4	11.1	14.4	0
8	85	Day +21	38.3**	21.2	35.3**	4.7
7	87	Day - 6	66.7	11.5	17.2	4.6
9	141	Day + 9	56.0	22.5	19.9	2.1

[a]Embryo quality, Excellent - Good (Ex - Gd) and Fair - Poor (Fr- Pr) based upon that of Lindner and Wright (1983). Degenerate (Deg) based upon classification of DeJarnette *et al.* (1994). Degenerate/Unfertilised (Deg/UFO), fragmented or cleaved, but without evidence of blastomere nucei.
[a]Source of semen in relation to the 48 h scrotal insemination. Day +21 represented the abnormal content of vacuolated sperm, Days –3 and –6 pre-insulation controls and Day +9, post scrotal insulation, but before abnormal sperm appearance.
**Represent significant shifts in embryo quality from other days (P<0.05).

properties at a later date. The type and chronology of abnormalities for bulls collected at 3-day intervals before and following scrotal insulation is shown in Figure 1.

Because vacuolated sperm (random and diadem) on otherwise normally shaped heads readily access the oocyte *in vivo*, semen from a bull in this study producing such sperm was used to inseminate superovulated cows. Embryos collected 6 days later were evaluated. High levels of vacuolated sperm (38 and 19%, random and diadem, respectively) on Day 21 post thermal insult was compared with pre-insult control semen (Day –3 and –6) and semen ejaculated on Day 9 post insult, but prior to appearance of visible morphological abnormalities in the ejaculate (Table 1). It is clear from these data showing depressed embryo quality to Day 21 semen that vacuolated sperm in the inseminate did signify that sperm incompetent to maintain normal embryogenesis were involved in fertilisation. From the same thermal insult study, males producing semen high in sperm with protoplasmic droplets or decapitated sperm were also judged against controls from the same bull. In both cases, fertility was depressed, but embryonic quality was unaffected suggesting that these traits were most likely compensable in nature (Saacke *et al.* 1994). Thus, ejaculates containing sperm varying in head shape and vacuolar content appear to be associated with the uncompensable deficiencies. In addition, only morphologically normal or near normal sperm with or without nuclear vacuoles access the ovum *in vivo*.

In an effort to better define the uncompensable deficiency, we undertook studies utilising the SCSA (sperm chromatin structure assay, Evenson *et al.* 1980; Ballachey *et al.* 1988) to evaluate chromatin stability to acid denaturation relative to sperm morphology using our scrotal insulation model (Acevedo *et al.* 2001). This work revealed that, as misshapen sperm increased in the ejaculate due to scrotal insulation, vulnerability of sperm DNA to acid denaturation also increased, suggesting concurrent alteration in the sperm chromatin. Reduced substitution of protamines for histones during spermiogenesis is suspected. Interestingly, the perturbed DNA/chromatin was not limited to morphologically abnormal cells. Rather, the chromatin perturbations extended to normally shaped sperm in the abnormal ejaculates indicating that spermatogenic disturbances resulting in morphologically abnormal sperm most likely represents the 'tip of the iceberg' with respect to sperm function. In contrast, semen having only vacuolated sperm in otherwise normal shaped heads, shown to be uncompensable in Table 1, did not express perturbed DNA based upon the SCSA, clearly creating doubt that a single DNA or chromatin analysis will suffice in evaluating spermatogenic flaws not revealed by sperm morphology.

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SPERM TRANSPORT IN THE FEMALE TRACT: COMPARATIVE ASPECTS AND PHYSIOLOGICAL HIGHLIGHTS

R. H. F. Hunter*

Faculty of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK

As will be appreciated by members of this workshop, the processes involved in the transport of spermatozoa within the female genital tract can be analysed from diverse points of view. These would include anatomical, chronological, hormonal and molecular. Such components of sperm transport can be examined after natural mating, artificial insemination, deep hysteroscopic insemination or even after surgical instillation of variable sperm suspensions at different levels within the Fallopian tubes during spontaneous oestrous cycles, or those synchronised by pharmacological approaches. Comparisons between species have not always taken full account of such variables. Nonetheless, useful reviews of sperm transport in mammals have been presented by Drobnis and Overstreet (1992); Harper (1994); Yanagimachi (1994); Hunter (1995) and Mortimer (1997), amongst others.

Defined in simplistic terms, the overall purpose of sperm transport must be seen as progression of a discrete population of competent cells to the site of fertilisation at the ampullary-isthmic junction of the Fallopian tubes. Numerical and chronological features of the transport process are of supreme importance in generating monospermic fertilisation. A steeply-declining gradient in sperm numbers between the site of ejaculation and the ampulla of the Fallopian tube exists before and at the time of ovulation, but changes progressively thereafter. Polyspermic penetration of the vitellus is pathological in mammals, although not so in birds and fish. In recent years, the emphasis in mammalian studies has been less on the site of fertilisation and more on pre-ovulatory establishment of a population of competent spermatozoa in the so-called functional sperm reservoir. This highly-specialised region of the female tract is located in the caudal (distal) region of the Fallopian tube isthmus, a finding demonstrable in a wide range of placental mammals. Indeed, once a functional sperm reservoir has been established in the caudal isthmus, remaining populations of uterine, cervical and vaginal spermatozoa appear to have no direct role in the events of fertilisation. Large numbers of these are evacuated before or during engulfment by polymorphonuclear leucocytes.

Irrespective of the precise site of semen deposition (vagina or uterus), it is essential to distinguish between **sperm transport**, which implies a passive movement of cells in the genital tract, and **sperm migration** which clearly attributes importance to the intrinsic motility of the cell. In many rodents and species such as dogs, pigs and equids, although not in rabbits, ruminants and primates, migration seems to play a minor role in progression of a sperm population to the utero-tubal junction. Passive displacement is paramount, facilitated by: a) the volume of the ejaculate; b) enhanced myometrial contractions; and c) the contribution of uterine fluids. Despite this emphasis, sperm motility may assist in the maintenance of spermatozoa in suspension and prevent adherence to the endometrial surface. Active sperm motility also appears to be essential for passage across the structures of the utero-tubal junction, rendered more formidable before ovulation by their highly oedematous condition. In the absence of pathology, suspensions of dead spermatozoa and polymorphonuclear leucocytes do not traverse the utero-tubal junction at this time, and only do so in a limited manner in the hours following ovulation. The polypoid processes of the utero-tubal junction also prevent passage of bulk

*Contact address: 32, Gilmour Road, Edinburgh, EH16 5NT, Scotland

seminal plasma into the Fallopian tubes. Male accessory secretions contain an ovicidal factor (Chang 1950), so clearly must not reach the site of fertilisation.

Turning to an evolutionary line of thought, there is the related question of why stallion semen is ejaculated into the uterus. The focus here is not so much on the condition of the cervix during oestrus. Rather, it is whether a presumptive advance from vaginal deposition of semen in ancestral species to intra-uterine accumulation in modern equids may have evolved because the utero-tubal junction and adjoining isthmus were such formidable barriers to successful sperm transport. Hence, intra-uterine ejaculation might have arisen, in part at least, to ensure bathing of the junctional papillae by a vigorous population of spermatozoa in suspension in seminal plasma and uterine fluid during and soon after mating. The high titres of prostaglandin E_2 in stallion semen (24.1 ng/ml; Hunter 1973a) could influence the tissues of the utero-tubal junction and adjoining myosalpinx to facilitate passage of a vanguard of spermatozoa into the caudal portion of the isthmus.

As to the functional sperm reservoir, features of this region of the Fallopian tube before ovulation include a viscous glycoprotein secretion, a reduced temperature compared with that of the ampulla, suppressed motility of sperm cells and avid binding by their rostral portion to microvilli or cilia. In addition, the caudal isthmus is strongly influenced by a local counter-current vascular transfer of high concentrations of ovarian follicular hormones to the musculature and mucosa of the Fallopian tube (Hunter *et al.* 1983). An outstanding question is the manner of controlled release of viable spermatozoa from endosalpingeal binding shortly before the time of ovulation. In species such as pigs, sheep and cattle – if not in equids – several thousand spermatozoa may be stored in the caudal isthmus before ovulation and yet initial sperm:egg ratios at the ampullary-isthmic junction are close to unity. This represents an impressive sperm gradient along which the process of capacitation is thought to be completed. Because of increasing concentrations of post ovulatory progesterone secretion, sperm:egg ratios change markedly within an hour or two of fertilisation, but only after establishment of a block to polyspermy that cannot be perturbed by enhanced numbers of motile spermatozoa.

A more detailed understanding of the physiology of sperm transport should enable exploitation of smaller, selected populations of spermatozoa, particularly if introduced deeper into the female tract using endoscopic visualisation or ultrasonic imaging. Such modern techniques have much to offer in the current context, but might reflect to advantage on the components of stallion seminal plasma – such as prostaglandins – that could be influencing the tissues of the female genital tract.

Despite these remarks, experiments that could still be performed to examine sperm passage within the Fallopian tubes of mares might include:

1. Surgical resection of the isthmus to note its regulatory and storage roles (Hunter and Léglise 1971).
2. Instillation of a washed sperm suspension directly into the Fallopian tube ampulla before and after fertilisation to examine the extent of polyspermic fertilisation and stability of the block to polyspermy (see Hunter 1973b).
3. Microinjections of a solution of progesterone in oil under the serosal layer of the caudal isthmus to demonstrate an enhanced sperm release towards the site of fertilisation (Hunter 1972).
4. Introduction of microdroplets of follicular fluid obtained shortly before ovulation directly into the Fallopian tube to examine the influence on sperm release from the isthmus. Similar experiments might also be performed with calcium ionophore (Hunter *et al.* 1999).

Taken together, the results of such experiments should clarify the regulatory role of the isthmus and the extent of pre-ovulatory sperm reserves therein. They would also demonstrate the influence of changing sperm:egg ratios on the normality of fertilisation and on the long-term competence of the zona block to polyspermy.

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SPERM TRANSPORT AND FUNCTION IN THE MARE – A REVIEW

M. A. Scott

Department of Population Health and Reproduction, School of Veterinary Medicine, University of California Davis, 95616, USA

As characterised in other species, the natural history of sperm following insemination involves phases of sperm distribution, pre-ovulatory storage, sperm selection and maturation and, ultimately, fertilisation. The female must tolerate the invasion of these foreign cells and provide an environment that will support this continuum of events to a successful outcome. The establishment of pregnancy following a single insemination up to 6 days prior to ovulation (Burkhardt 1949) or up to 18 h after ovulation (Koskinen *et al.* 1990) indicates that in mares, this continuum can proceed successfully with a variable timetable that is quite different from other domestic species.

Fertility trials that include therapeutic interventions or that manipulate the timing, dose, or site of insemination provide indirect information about sperm transport and function in the mare. Brinsko *et al.* (1990, 1991) found that uterine lavage with 1 litre of a sperm-immobilising solution at 0.5 and 2 h post breeding significantly decreased pregnancy rates in reproductively normal mares, whereas lavage at 4 h did not. The authors concluded that approximately 4 h are required for sufficient numbers of functional sperm to reach the oviducts to assure normal fertility. When mares were inseminated 0–6 h after ovulation had been detected, sperm were found in the oviducts within 2 h, were in highest numbers at 4 h, and numbers had decreased by 6 h after insemination (Bader 1982). The viability of the recovered sperm was not examined. However, Scott *et al*. (1995) confirmed that at 4 h after pre-ovulatory insemination, highly motile sperm are present consistently in the caudal oviductal isthmus of reproductively normal mares. Subfertile mares had fewer sperm in their lower oviducts and motile sperm were never recovered bilaterally (Scott *et al.* 1995). Sperm arrival in the oviducts is delayed if frozen semen is used (Bader and Krause 1980).

Sperm motility is considered to be an expression of functional competence and the ability of that sperm to move within the female genital tract. In one study, mares were inseminated with 7×10^9 sperm and it was found that fewer sperm were recovered from the oviducts 12 h later when semen with a lower percentage of motility was used. In contrast, Scott *et al.* (1995) found that, even though insemination doses were balanced for number of progressively motile sperm, fewer sperm from subfertile stallions reached the oviducts of normal mares 4 h after insemination when compared with the number of fertile stallion sperm. Similarly, at this same timepoint, subfertile mares had fewer sperm in their lower oviducts than fertile mares, despite receiving equal numbers of progressively motile fertile stallion sperm at insemination (Scott *et al.* 1995). Therefore, motility *per se* does not guarantee successful transport to the oviducts. Sperm morphology also appears to reflect functional competence. Not only are a greater proportion of sperm in the oviducts morphologically normal compared with sperm in the inseminate (Scott, unpublished) but also, more than 90% of equine sperm visualised at the utero-tubal junction (UTJ) using scanning electron microscopy (SEM) are morphologically normal even when the inseminate contains high numbers of sperm with major morphological defects (Scott *et al.* 2000). These observations suggest that a functional interaction between competent sperm and the luminal epithelium, particularly at anatomical barriers, ultimately promotes the selection of a physiologically normal population of sperm during transit to the oviducts.

Fig 1: Equine sperm at the utero-tubal junction 4 h after pre-ovulatory inseination.

Normal sperm-epithelium interactions are crucial to the establishment and maintenance of sperm reservoirs, which are needed when insemination precedes ovulation by many hours or days. The existence of a sperm reservoir in the mare is unquestioned, given that a 3–6 day interval between insemination and ovulation can result in pregnancy (Burkhardt 1949). However, the anatomical location(s) of this reservoir has not been confirmed. In the pig, another species with intra-uterine semen deposition at mating, the UTJ and adjacent caudal oviductal isthmus are considered to function as sperm reservoirs because sperm may survive in these locations for as long as 72 h (Hunter 1988). In normal mares, sperm will accumulate in the epithelial pockets and folds of the uterine papilla of the UTJ within 4 h after pre-ovulatory insemination, and their intimate contact and orientation with the epithelium are suggestive of sequestration there (Scott *et al.* 2000; Fig 1). The persistence of sperm with a similar appearance at the UTJ of pre-ovulatory mares 18–26 h after insemination (Scott, unpublished observations) provides stronger evidence of pre-ovulatory storage in that location.

It has been established in other species that functional sperm that cross the UTJ prior to ovulation will accumulate in the caudal isthmus and remain there until ovulation is imminent (Hunter 1988). In cattle, sheep and pigs, surgical transection of the oviduct at various intervals between mating and ovulation was used to demonstrate that fertilisation competent sperm do not progress beyond the initial 1.5–2 cm of the caudal isthmus until the peri-ovulatory period (Hunter 1988, for review). It has long been assumed that equine sperm develop a similar reservoir in the caudal isthmus, but this has not been confirmed in any *in vivo* study. Based on examination of flushings from isolated regions of the oviducts, within 4 h after insemination, sperm are regionally distributed in the oviducts of pre-ovulatory normal mares, such that more sperm are located in the caudal isthmus than in the proximal isthmus or ampulla, and motile sperm are located exclusively in the caudal isthmus. It is tempting to interpret these observations as evidence of pre-ovulatory storage. At minimum, they suggest a special role for the caudal isthmus in the process of sperm transport in the mare. In contrast to these results, when Boyle *et al.* (1987) examined equine sperm at the UTJ and caudal isthmus using SEM, they did not find any sperm in the caudal isthmus of pre-ovulatory mares, but did find sperm there after ovulation. These conflicting results may reflect differences in experimental design and methodologies.

The existence of a sperm reservoir in the mare is unquestioned, and yet clinical experience indicates that sperm longevity in the mare is highly variable. How important are the dose or the timing of insemination? During natural cover, the mare receives the entire ejaculate, which often contains billions of sperm. However, an insemination dose of 500 million progressively motile sperm is generally accepted as the industry standard to maximise pregnancy rates following artificial insemination. High conception rates (64%) have been achieved using as few as 1×10^6 motile sperm, by placing the sperm directly on the papilla of the UTJ using hysteroscopy (Morris *et al.* 2000). This approach effectively bypasses the phase of sperm transport through the uterus. An insemination-ovulation interval of 2 days in 79% of the mares receiving hysteroscopic utero-tubal insemination indicates that pre-ovulatory sperm storage occurred with this insemination method.

To examine which insemination results in fertilisation when several are performed before ovulation, Clément *et al.* (2000) determined the

paternity of embryos recovered from mares that had been inseminated every 2 days during oestrus using fresh semen from a different stallion for each insemination. The stallions had demonstrated high per cycle pregnancy rates, and an insemination dose of 2×10^8 progressively motile sperm was used. Those authors reported a mean interval between successful insemination and ovulation of 2.6 ± 1.0 days and found that the insemination performed within 48 h before ovulation was successful only 26% of the time. Results may differ when frozen semen is used. Metcalf (2000) inseminated mares shortly before and then after ovulation (inter-insemination interval of 4–10 h) with 3×10^8 progressively motile frozen-thawed sperm from different stallions. Paternity testing of the resulting foals identified that in 4 of 9 mares, pregnancy resulted from the pre-ovulatory insemination and in 5 of 9 mares pregnancy resulted from the post ovulatory insemination. The success of the post ovulatory insemination for frozen-thawed semen indicates that: 1) sperm function in the second dose was not adversely affected by the physiological inflammatory response that occurred following the first insemination (Metcalf 2000); and 2) that residence in a pre-ovulatory sperm reservoir is not essential for final sperm maturation.

The process of sperm transport in the female genital tract is a complex and dynamic continuum involving a concert of interactions between sperm and the female tract that optimise the likelihood of conception. The female plays a key role in the process by modulating sperm movement, inducing or regulating sperm capacitation, and supporting sperm viability and storage. Sperm with normal structure and function are more likely to survive *in vivo*. Some features of the normal process can be bypassed using specialised insemination methods. For a better understanding of the normal biology of sperm transport, careful selection of experimental animals is imperative.

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SESSION 2

Chairman:
Lee Morris

THE PHYSICOCHEMICAL PROPERTIES OF EQUINE OESTROUS FLUID

C. A. Tiplady, S. Butler*, W. R. Allen and M. R. Mackley*

*University of Cambridge, Department of Clinical Veterinary Medicine, TBA Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH; *University of Cambridge, Department of Chemical Engineering, Pembroke Street, Cambridge CB2 3RA, UK*

INTRODUCTION

Fluid is known to accumulate in the lumen of the uterus in the oestrous mare. However, unlike other species, little is known about the properties and function of such fluid. The increased contractility of the equine uterus during oestrus, particularly in response to teasing (Stecco *et al.* 2001), generates increased pressure between the oedematous endometrial folds and subjects the intra-uterine fluid to shear forces and stresses that could be sufficient to propel the fluid, and consequently the inseminate, up the uterus to the utero-tubal junction. In the rat, it has been shown that uterine fluid actively facilitates the transport of spermatozoa up the genital tract to the site of fertilisation (Warren 1938), and it clearly contains substances essential for the capacitation of spermatozoa and for gamete longevity (Aitken 1979). Reduction in the volume of uterine fluid in the rat significantly compromised contraction frequency and transuterine migration of spermatozoa (Warren 1938; Toner and Adler 1985). Studies in the rat, pig, dog and ewe have all shown that spermatozoa can penetrate oestrous uterine fluid easily so that their transport from the cervix to the tip of the uterine horns occurs in a matter of seconds or minutes, rather than hours (Evans 1933; Warren 1938). Studies in the mare have indicated that the transport of spermatozoa up the uterus and into the oviduct is just as rapid as in other species. Spermatozoa have been found in the oviducts of mares killed 4 h (Scott 2000) and 2 h (Bader 1982) post insemination, and may even arrive at the site of fertilisation within minutes (Katila 2000).

The physicochemical properties of uterine fluid and the effects of the physical environment within the uterus must be taken into account when studying the migration of spermatozoa up the uterus towards the utero-tubal papilla. The present experiment was conducted to characterise the rheological properties of oestrous uterine fluid from healthy mares and to determine if a correlation may exist between fluid dynamics and the passage of spermatozoa within the uterus.

MATERIALS AND METHODS

Uterine fluid was visualised in oestrous mares by transrectal ultrasonography. It was then aspirated by means of a wide bore catheter passed through the working channel of a videoendoscope. Each sample was analysed in a Rheometrics RDSII controlled strain rheometer with a 100 g/cm transducer. A 1–2 ml aliquot of fluid was compressed to a gap of 0.5 ± 0.25 mm between a 50 mm static top-plate coupled to a force rebalance transducer and a parallel dynamic bottom plate mounted to a high resolution stepper motor. The rheometer applied well-defined stress and strain to the sample whilst simultaneously measuring the bulk behaviour of the fluid in response to controlled deformation. Each sample was subjected to a strain sweep, a frequency sweep and a steady shear test. Strain sweeps were carried out at a constant frequency of 10 rads/s with steadily increasing strain amplitudes from 0.1 to 2,000% strain. Frequency sweeps were performed at a constant strain of 10% with steadily increasing frequencies and the steady shear characterised the relaxation spectrum under a steadily increasing rate of shear stress from 0.1 to 2,000/s.

Elasticity (G'), viscosity (G") and complex viscosity (Eta*) were calculated for each test.

RESULTS

All the uterine fluid samples were non-Newtonian and they exhibited significant complex viscosity

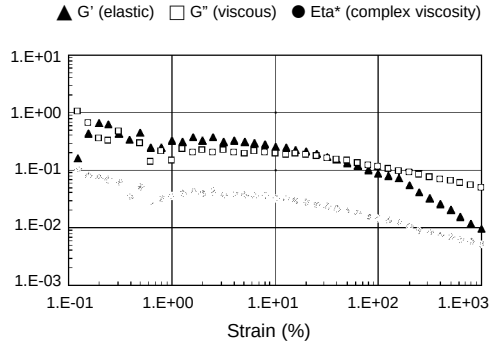

Fig 1: Strain sweep at a frequency of 10 rads/s.

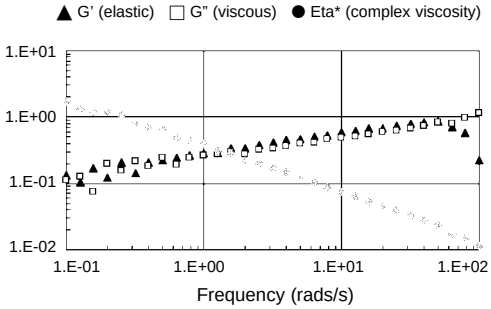

Fig 2: Frequency sweep at 10% strain.

(Eta*) enhancement. They displayed a linear viscoelastic strain region between 5 and 20% strain with the elastic moduli (G') dominating the viscous moduli (G") as shown in Figure 1. Within the linear viscoelastic strain region the relaxation processes making up the internal structure indicated that the uterine fluid displayed the fingerprint of a gel (Fig 2).

Under increasing rates of shear stress all the fluid samples displayed strong shear thinning behaviour spanning 2–3 orders of magnitude. At low shear the fluid retained a high viscosity (0.5 ± 0.2 Pa/s), or gel-like qualities, whereas at high shear the viscosity decreased greatly to become close to that of water ($4.3 \times 10^{-3} \pm 2.3 \times 10^{-3}$ Pa/s; where water is 1×10^{-3}, Fig 3).

Discussion

Uterine fluid has a complex composition derived from endometrial gland secretions and a transudate of serum (Tunon 1999). In the mare the possible significance of uterine-specific proteins, compared to those originating from serum, on the preservation and migration of spermatozoa,

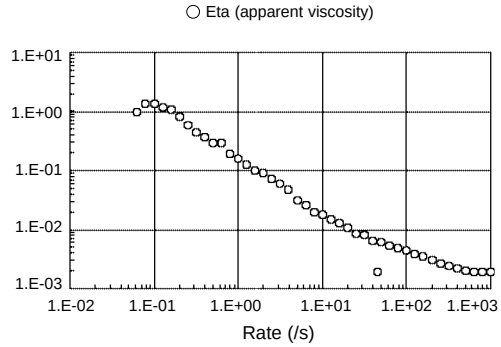

Fig 3: Steady shear stress with increasing rate.

fertilising capacity and early embryonic development is not understood.

As shown in other species (Warren 1938; Toner and Adler 1985; Lopez-Gatius *et al.* 1994, 1996), both the rheological and the biological properties of uterine fluid greatly assist the migration of spermatozoa. From the findings in this preliminary study, equine oestrous uterine fluid appears to display similar properties. It is non-Newtonian, exhibits both elastic and viscous moduli and it shows significant complex viscosity enhancement when subjected to increasing rates of shear stress. Theoretically, the increased contractility of the mare's uterus in oestrus applies similar shear forces and stresses to the fluid as those applied in the rheometer. This allows the speculation that, *in vivo*, the forces generated by the oedematous endometrial folds would exact enough pressure to alter the viscosity of the uterine fluid significantly, from a gel-like state into a watery solution, which would then flow much more readily through the tracts and channels between the folds. Spermatozoa inseminated into this complex fluid would inadvertently 'catch-a-ride' as the fluid is squeezed through the channels and would, therefore, be delivered to the utero-tubal papilla at the tip of the uterine horn. Under non-contractile conditions, where little or no shear force is applied, the fluid would adhere to the surface of the endometrium. Such reduction in viscosity would also allow the spermatozoa to penetrate into the uterine fluid as the mechanical resistance of its internal composition is broken down (Lopez-Gatius *et al.* 1996). Such penetration could be the catalyst for a whole host of events, including epithelial interaction, capacitation and, ultimately, fertilisation.

Clearly, further studies are required to gain a better understanding of the physicochemical and

biological properties of equine uterine fluid and the relationship of these factors to the transport and modification of spermatozoa in preparation for fertilisation.

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CELL-CELL INTERACTIONS BETWEEN SPERMATOZOA AND THE OVIDUCTAL EPITHELIUM IN THE HORSE – INFORMATION FROM *IN VITRO* STUDIES

B. A. Ball

Department of Population Health and Reproduction, University of California, Davis, California 95616, USA

Equine spermatozoa can survive for an extended period of time in the mare's reproductive tract before fertilisation. Current information indicates that spermatozoa are likely to be stored in the region of the isthmic oviduct adjacent to, and perhaps including, the utero-tubal junction. This 'sperm reservoir' is best characterised on a functional rather than a morphologic basis in most domestic animals. The cell-cell interaction between spermatozoa and the oviductal epithelium appears to be important in establishing this sperm reservoir, and this cell-cell interaction modulates sperm function prior to fertilisation.

Current evidence suggests that the cell-cell interaction between equine spermatozoa and oviductal epithelium modifies both sperm and oviductal epithelial cell (OEC) function. The interaction with OEC preferentially selects morphologically normal sperm with intact plasma and acrosomal membranes, which may be important in the process of enriching the population of sperm that will ultimately participate in fertilisation (Thomas *et al.* 1994a). As a consequence, spermatozoa that have been damaged during cryopreservation have a reduced ability to adhere to OEC and may be less efficient in establishing a sperm reservoir in the oviduct (Dobrinski *et al.* 1995).

Sperm adhesion to OEC prolongs the viability of sperm (Ellington *et al.* 1993a; Dobrinski *et al* 1996a). Several lines of evidence suggest that adhesion of spermatozoa prevents capacitation and prolongs sperm viability until near the time of ovulation (Ellington *et al.* 1993a; Dobrinski *et al* 1996a, 1997). Adhesion of equine sperm to oviductal epithelium prevents a premature rise in intra-cellular calcium concentration $[Ca^{2+}]_i$ in sperm, thereby preventing capacitation. Release of sperm from oviductal epithelium is associated with a rise in $[Ca^{2+}]_i$, capacitation and the ability to bind the zona pellucida and initiate acrosomal exocytosis (Ellington *et al.* 1993b). The effect of adhesion on maintenance of low $[Ca^{2+}]_i$ and prevention of capacitation appears to be tissue specific and does not depend upon secreted factors from the oviductal epithelium. Although the mechanism for maintenance of low $[Ca^{2+}]_i$ as a function of cell adhesion is unknown in this system, it is hypothesised that it may be related to activation of a calcium-ATPase in spermatozoa or a suppression of tyrosine phosphorylation.

Adhesion of sperm to oviductal epithelium also alters oviductal cell function. Sperm adhesion to oviductal cells increased $[Ca^{2+}]_i$ in oviductal cells (Ellington *et al.* 1993c), and altered the synthesis and secretion of a number of oviductal proteins (Thomas *et al.* 1995). Although the identity of these proteins has not been determined, some of these proteins associate with the sperm membrane and are likely to alter sperm function (Ellington *et al.* 1993d). Although an oestrous-associated oviductal glycoprotein has not been identified in the horse (Battut *et al.* 1995), work in other mammalian species suggests that oviductal glycoproteins may associate with spermatozoa and alter sperm adhesion to the extra-cellular matrix known as the zona pellucida (Verhage *et al.* 1998). Together, these studies indicate that there is a dynamic interaction between spermatozoa and oviductal epithelial cells, which alters function of both cell types as part of events preceding fertilisation.

The molecular basis for adhesion of sperm to oviductal epithelium appears to be mediated, at least in part, by a calcium-dependent lectin. The carbohydrate moiety recognised by the sperm lectin varies with species and has been characterised as fucosyl, or galactosyl residues for

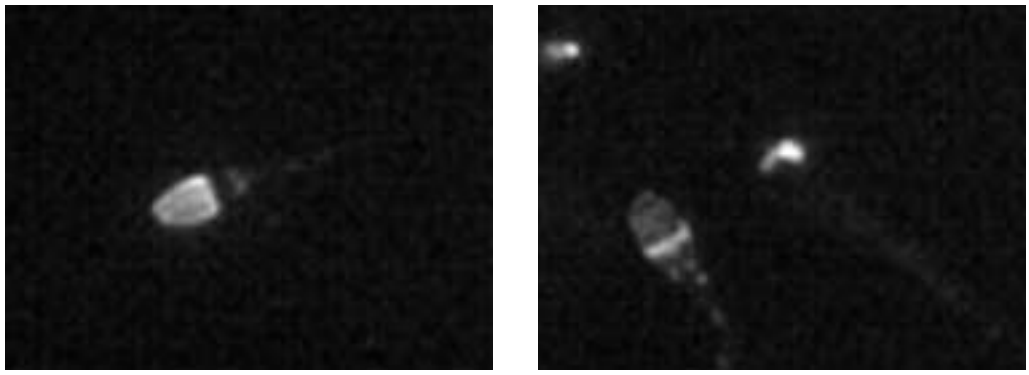

Fig 1: Immunocytochemistry of equine sperm using antibody directed against galactose-binding protein. Uncapacitated sperm (top) demonstrate label over the entire rostral periacrosomal membrane. Capacitated sperm demonstrate label in the equatorial segment.

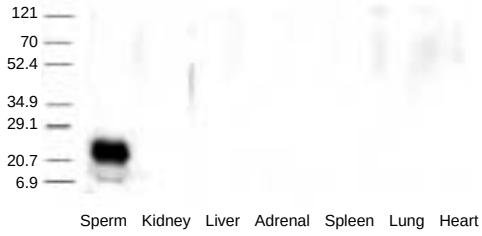

Fig 2: Immunoblot of equine sperm membrane and other somatic tissues. Antibody directed against purified galactose-binding protein from equine sperm membrane.

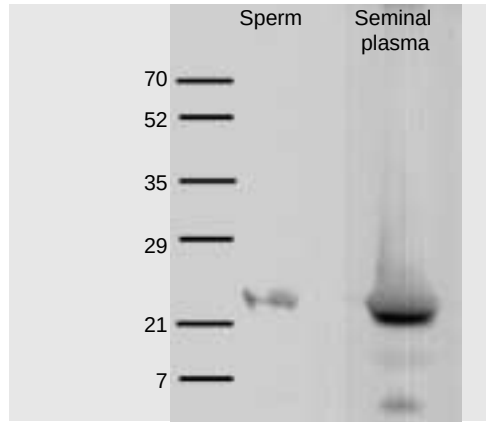

Fig 3: Immunoblot of equine sperm membrane and seminal plasma. Antibody directed against purified galactose-binding protein from equine sperm membrane.

bovine or equine sperm, respectively (Dobrinski *et al.* 1996b; Suarez *et al.* 1998). Galactosyl residues appear to be among the most abundant present in the isthmic oviduct of the mare, based upon lectin histochemistry, and may represent the ligand for sperm adhesion in the equine oviduct (Ball *et al.* 1997). The expression of these galactosyl residues varied with cycle stage in the mare, and expression of galactosyl glycoconjugates was greater in the isthmus compared to the ampulla of the equine oviduct (Ball *et al.* 1997). Therefore, putative sperm binding sites were more readily available in the isthmic oviduct during oestrous, a finding that is consistent with enhanced sperm adhesion to isthmic oviductal explants during oestrous (Thomas *et al.* 1994b). Consistent with this observation is that both galactose, and the glycoprotein asialofetuin - which expresses terminal galactose residues - inhibit adhesion of equine sperm to OEC (Dobrinski *et al.* 1996b).

Recent studies in the author's laboratory have attempted to characterise possible galactose-binding proteins on equine spermatozoa as possible candidates for sperm adhesion to OEC. Equine sperm membrane proteins were subjected to galactose-affinity chromatography, and bound proteins were eluted with excess galactose in a calcium-free buffer. Major proteins of 30, 25 and 18 kD, recovered after galactose-affinity chromatography, were used for generation of a polyclonal antibody directed against galactose-binding proteins. Immunocytochemistry with this antisera demonstrated labelling primarily over the rostral periacrosomal sperm head in uncapacitated sperm (Fig 1). Labelling was limited to the equatorial region in capacitated spermatozoa. The 25-kD protein was not expressed in other somatic tissues but was present in both ejaculated spermatozoa and seminal plasma (Figs 2 and 3).

Recent reports have provided new information regarding the possible mechanism for sperm adhesion to oviductal epithelia in cattle. Suarez *et al.* (1998) reported that bovine sperm adhesion was mediated via a calcium-dependent lectin that recognises fucosylated ligands similar to the Lewisa trisaccharide. Further characterisation of this fucose-binding protein indicated that the protein identified was bovine seminal plasma protein A1/A2 (BSP A1/A2), which is a secretory protein of the vesicular gland (Ignotz *et al.* 2001). This protein anchors to choline phospholipids of the sperm head and is lost after capacitation. Purified BSP A1/A2 blocks sperm adhesion to oviductal epithelium, and the reintroduction of the protein to capacitated sperm facilitates their adhesion to oviductal cells (Ignotz *et al.* 2001).

Structurally related proteins have been isolated and characterised from equine seminal plasma as horse seminal plasma proteins one and 2 (HSP-1 HSP-2, Calvete *et al.* 1997). These proteins are also heparin- and phosphorylcholine–binding proteins that constitute the major proteins of equine seminal plasma. Although HSP-1/2 appear to form a hetero-oligomer in seminal plasma, individually their molecular masses (reported as 28 and 22 kDa, Calvete *et al.* 1997) appear similar to that of the galactose-binding protein isolated from equine sperm and seminal plasma in the author's laboratory. Further work is required to determine the identity of these proteins and their possible role in sperm-oviductal adhesion in the horse.

In conclusion, adhesion of equine sperm to OEC is important in establishing a sperm reservoir within the equine oviduct. The cell-cell adhesion of sperm acts to select a population of normal, motile and membrane-intact sperm that will ultimately be available for fertilisation. Sperm adhesion to OEC appears to prevent sperm capacitation and prolong the viability of sperm by preventing an increase in tyrosine phosphorylation and preventing an increase in intra-cellular calcium. The cell-cell adhesion between sperm and OEC also affects oviductal cell function as reflected by alteration in protein synthesis and secretion from OEC.

The basis for sperm adhesion to OEC appears to be mediated via a lectin-like interaction between sperm and OEC. In the horse, the abundant galactosyl glycoconjugates present in the isthmic oviduct may provide the ligand for sperm adhesion. It appears likely that a seminal plasma protein, perhaps HSP1/2, binds to choline phospholipids in sperm membrane at ejaculation and promotes the adhesion of sperm to the oviductal epithelium. Further work is necessary to confirm the role of these proteins in sperm adhesion within the oviduct of the mare.

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CAPACITATION OF MAMMALIAN SPERMATOZOA: A PHYSIOLOGICAL PERSPECTIVE SET AGAINST PRESUMPTIVE MATURATION *IN VITRO*

R. H. F. Hunter*

Faculty of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK

Capacitation of mammalian spermatozoa was first recognised as an essential preliminary to fertilisation 50 years ago. Two physiologists working independently on different species in separate continents concluded that, over and above the time needed for spermatozoa to reach the site of fertilisation, a period of final maturation in the female tract was required before spermatozoa could penetrate the egg investments. C.R. Austin worked with rats in Sydney, Australia, and M.C. Chang in Massachusetts, USA, used rabbits. They found that final maturation took 2–6 h, and the phenomenon was soon endorsed in other species. Despite numerous studies in the intervening years, and a series of major reviews since 1970, this fundamental process is still not fully understood.

There have been essays on the physiology of capacitation (Bedford 1970; Chang and Hunter 1975; Yanagimachi 1994) and key treatments of sperm biochemistry (Harrison 1996; Flesch and Gadella 2000), but an integrated appreciation of cellular dynamics in the female reproductive tract is scarcely on the horizon. Nonetheless, it is accepted that capacitation represents an essential pre-fertilisation event during sperm progression towards the ampullary-isthmic junction of the Fallopian tube and requiring a species-specific minimum time. It involves a major molecular reorganisation and acts to destabilise membranes around the anterior portion of the sperm head, ultimately enabling a series of point fusions between the outer acrosomal membrane and the overlying plasma membrane. This modification is termed the acrosome reaction. Such vesiculation of membranes permits enzyme release from the acrosomal sac, ideally when a sperm cell is in the vicinity of an egg. A distinction is drawn between capacitation and the subsequent acrosome reaction (Chang and Hunter 1975). In viable cells *in vivo*, completion of the former precedes initiation of the latter.

Other speakers will address sperm cell biochemistry, so this paper concentrates on physiological observations. The first concerns an involvement of seminal plasma, for male secretions coat ejaculated spermatozoa and must be largely removed in the early stages of capacitation. Indeed, Chang showed that addition of cell-free seminal plasma to spermatozoa presumed to be capacitated returned them to their former state; hence the notion of a decapacitation factor in seminal plasma (Chang 1957). A further period of incubation in the female tract was required before fertilising ability could be demonstrated. Much of the decapacitating activity may reside in epididymal secretions, for addition of cell-free plasma from the cauda epididymidis significantly increased the time required for capacitation of boar spermatozoa from the upper corpus epididymidis (Hunter *et al.* 1978). In other words, sperm cells are progressively stabilised during epididymal transit, and secretions from this Wolffian duct derivative require neutralisation or removal in the female tract. Such experiments could usefully be repeated with stallion tissues. It is worth emphasising that epididymal secretions represent only a small proportion of total seminal volume, but spermatozoa are in contact with epididymal fluids for longer (days) than other components of seminal plasma (minutes or hours).

Another key observation concerns sequential actions of the female reproductive tract on the timing of capacitation. The uterus and Fallopian tubes act synergistically in preparing spermatozoa for fertilisation. If a suspension of spermatozoa is introduced directly into the Fallopian tubes, it requires appreciably longer for capacitation than one

**Contact address: 32, Gilmour Road, Edinburgh, EH16 5NT, Scotland*

first exposed to the uterus. Again, this was demonstrated in rabbits (Adams and Chang 1962), pigs (Hunter and Hall 1974) and hamsters, so it would be worth confirming in mares. Such a coordinated action of the female tract suggests subtle influences on sperm cell biochemistry, with the bulk of the capacitation process occurring in the Fallopian tubes - at least when viewed chronologically.

Follicular fluid assists induction of capacitation *in vitro* (see Yanagimachi 1994), so the role of this fluid soon after ovulation requires further study *in vivo.* Although much of it may enter the Fallopian tube at ovulation, most is soon refluxed into the peritoneal cavity in species without an intact ovarian bursa. Even so, a sufficient microvolume may remain in association with the egg investments and liberated granulosa cells to contribute importantly to the completion of capacitation. The last phrase is used advisedly for, in large farm species with their long pre-ovulatory intervals, it is now appreciated that capacitation is completed close to the time of ovulation (Hunter 1987). It needs to be suppressed during most of the pre-ovulatory interval, for fully-capacitated spermatozoa are unstable and short-lived. The strategy for suppression of completion of capacitation is imposed within the Fallopian tubes, and seemingly involves specific contact with the epithelium of the caudal isthmus. The mare with her long pre-ovulatory interval may provide an ideal model in which to examine suppression of full capacitation in the female tract, and the nature of binding reactions with the endosalpinx. An involvement of Ca^{2+} ions in release from binding has been demonstrated *in vivo* (Hunter *et al.* 1999).

If the timing of completion of capacitation is a function of the time of ovulation, as noted above, then this suggests strongly that sperm maturational changes in the female tract come under the influence of ovarian follicular hormones, mediated via the mucosa and endosalpinx. In this regard, there is clearly scope for experimental study in the mare. First, however, it would be valuable to assess whether there is a counter-current transfer between the ovarian vein and tubal branch of the ovarian artery of potent concentrations of follicular hormones. Such a means of local transfer to the vascular arcade bordering the Fallopian tube isthmus has been demonstrated in other species (Hunter *et al.* 1983), and would enable incisive programming of sperm-epithelial interactions.

Finally, the manner in which capacitation is achieved *in vitro* may not have close parallels with the process *in vivo.* An *in vitro* system may enable gross modification of the sperm cell surface but only appears successful for fertilisation because vast numbers of spermatozoa are used. It does not bear comparison with the very small numbers of spermatozoa initially completing peri-ovulatory capacitation in the Fallopian tubes and, within that caution, may not provide a suitable or sensitive approach to the subtleties of sperm cell biochemistry. Interpretation of capacitation requires clarification of the dynamic interactions between sperm cell and female tract, not least during the pre-ovulatory phase of sperm-epithelial binding in the caudal portion of the isthmus.

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CAPACITATION RELATED CHANGES IN STALLION SPERMATOZOA

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

*Departments of *Equine Sciences, †Farm Animal Health and **Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

Capacitation is a collective term for the changes that a spermatozoon undergoes when it comes into contact with the female reproductive tract. These changes include reorganisation of membrane proteins, metabolism of membrane phospholipids, scrambling of phospholipids, a reduction in cholesterol levels and hyperactivation. These changes, together with the subsequent acrosome reaction (AR), are essential for fertilisation. The chlortetracycline (CTC) stain is used traditionally for identifying capacitated sperm. The molecular basis of the interaction between CTC and the sperm plasma membrane is far from clear. In addition, the technique is laborious as it cannot be used for analysis using a flow cytometer (FACS). In contrast, merocyanine 450 can be used as a reporter probe for capacitation related changes in the plasma membrane. These changes can be evaluated both by fluorescence microscopy, confocal laser microscopy or by FACS analysis in a large number of sperm cells. The latter technique has proved to be more accurate for analysing sperm samples for capacitation related changes. In contrast, the percentage of hyperactivated sperm cells of the motile cells in a specific sperm sample did not change when capacitation was induced and thus could not be used for evaluation of capacitation.

There are several physiological inducers of capacitation and AR of which ZP3, a glycoprotein present in the extracellular matrix of the oocyte, and progesterone (P4), present in cumulus oophorus cells and the follicular fluid, are the most important. In addition, bicarbonate/CO2 plays a major role in the induction of capacitation *in vitro*. However, the intracellular signalling cascade induced by these vectors is far from clear. We demonstrated, by using protein kinase A (PKA) stimulators/inhibitors, that bicarbonate specifically activates a PKA pathway for induction of capacitation and subsequent AR. In contrast, by using protein kinase C (PKC) and protein tyrosine kinase (PTK) inhibitors, we revealed that P4 specifically mediates its effect via a PKC and a PTK pathway in stallion sperm. Although progesterone and bicarbonate have separate signalling pathways, they induce AR synergistically.

SESSION 3

Chairman:
Katrin Hinrichs

PROCESSING FACTORS WHICH INFLUENCE VIABILITY AND FERTILITY OF CRYOPRESERVED EQUINE SPERMATOZOA

M. L. Macpherson, M. D. Shore, M. H. Fernandez, C. D. Miller, J. A. Thompson, T. L. Blanchard and D. D. Varner

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

INTRODUCTION

Stallion semen is generally centrifuged prior to cryopreservation so that it may be placed in small packages for freezing, while retaining a threshold number of spermatozoa required for maximum fertility. The objective of centrifugation is to achieve a high spermatozoal recovery rate, while minimising cellular damage. Centrifugation force and time, and composition of centrifugation extender, vary considerably among laboratories which process stallion semen for freezing (Samper and Morris 1998).

A wide variety of freezing extenders have been used with stallion semen. No single extender has been identified as superior for this purpose Most extenders contain milk products, egg yolk, or a combination of these 2 ingredients. Various sugars, electrolytes, and detergents are also used in different extenders, as are glycerol or other cryoprotectants. This study was conducted to evaluate the effects of centrifugation protocol and composition of freezing extender on motility and fertility of cryopreserved equine spermatozoa.

MATERIALS AND METHODS

In Experiment 1, 3 ejaculates each from 2 stallions were used to evaluate the effect of centrifugation extender on spermatozoal motility. Semen was diluted to 25 x 10^6 sperm/ml in: 1) non-fat milk-glucose (Kenney); 2) non-fat milk-sucrose; 3) non-fat milk-glucose-sucrose (TAMU); 4) Merck I; or 5) Merck III extender. Spermatozoal motility was evaluated following storage at 37°C for 30 min. In Experiment 2, 3 replicates of pooled semen from 5 stallions were used to evaluate the effect of centrifugation force and time on spermatozoal recovery rate. Semen was diluted to 50 x 10^6 sperm/ml in TAMU extender and subjected to one of 5 centrifugation forces: 1) 400 x g for 10 min; 2) 400 x g for 15 min; 3) 500 x g for 10 min; 4) 500 x g for 15 min; or 5) 1,000 x g for 5 min. Spermatozoal numbers in resuspended spermatozoal pellets and aspirated supernatant were enumerated using a hemacytometer. In Experiment 3, 4 ejaculates each from 2 stallions were used to evaluate the effects of 2 centrifugation techniques on spermatozoal motility. Semen was diluted in TAMU extender and subjected to: 1) 400 x g for 15 min; or 2) 500 x g for 10 min. The motility of resuspended spermatozoa was determined. In Experiment 4, one stallion and 47 mares were used to evaluate the effect of centrifugation extender on fertility of post thaw spermatozoa. Semen was centrifuged using TAMU or Merck I extender, then pellets were resuspended in a lactose-EDTA-egg yolk freezing extender (Martin *et al*. 1979). Semen was packaged at 120 x 10^6 sperm per ml in 5 ml straws and immediately frozen by placement at 1 cm above the surface of liquid nitrogen for 20 min. In Experiment 5, one stallion and 32 mares were used to evaluate the effect of freezing extender on fertility of post thaw spermatozoa. Semen was centrifuged using TAMU extender, then spermatozoal pellets were resuspended in lactose-EDTA-egg yolk or milk-egg yolk freezing extender and frozen as described above. In Experiments 4 and 5, mares were bred by crossover design. All breeding outcomes were determined at Day 16 post ovulation, using transrectal ultrasonography.

Spermatozoal motion characteristics were evaluated using a computerised spermatozoal-motility analyser equipped with a heated stage (HTM IVOS; Version 10.8; Hamilton Thorne Research, Massachusetts, USA). A minimum of 7

TABLE 1: Effect of centrifugation extender on motility of stallion spermatozoa (0 ± sem)

Extender type	MOT	PMOT	VCL
Non-fat milk-glucose (Kenney)	91.0^{a} ± 1.5	79.1^{a} ± 1.6	119.5^{a} ± 7.3
Non-fat milk-glucose-sucrose (TAMU)	93.0^{a} ± 1.0	79.3^{a} ± 2.6	133.1^{a} ± 9.7
Non-fat milk-sucrose,	78.6^{b} ± 2.8	48.6^{b} ± 4.9	83.7^{b} ± 8.5
Merck I	65.2^{c} ± 10.4	33.8^{c} ± 10.9	74.7^{b} ± 14.8
Merck III	$73.3^{b.c}$ ± 4.9	56.0^{b} ± 4.3	75.7^{b} ± 8.0

^{a,b,c}Within rows, means with different superscripts differ (P<0.05)

TABLE 2: Effect of centrifugation force and time on recovery rate of stallion spermatozoa in resulting pellets (0 ± sem)

Centrifugation force and time (min)	Recovery rate (%)
400 x g for 10 min	54.7^{d} ± 1.8
400 x g for 15 min	75.0^{c} ± 1.0
500 x g for 10 min	87.0^{b} ± 0.6
500 x g for 15 min	96.2^{a} ± 0.4
1000 x g for 5 min	75.1^{c} ± 3.9

^{a,b,c,d} Within rows, means with different superscripts differ (P<0.05)

TABLE 3: Effect of centrifugation force and time on motility of stallion spermatozoa (0 ± sem)

Centrifugation force and time (min)	PMOT	VCL
400 x g for 15 min	65.5^{a} ± 3.4	91.4^{a} ± 3.7
500 x g for 10 min	61.4^{a} ± 4.8	88.3^{a} ± 4.3

^{a} Within rows, means did not differ (P>0.05)

fields and 500 spermatozoa were evaluated for each sample. Three motility variables were evaluated: 1) percentage of motile spermatozoa (% MOT); 2) percentage of progressively motile spermatozoa (% PMOT); and 3) mean curvilinear velocity (VCL) of spermatozoa (µm/s).

Results

Milk-based centrifugation extender containing glucose or glucose-sucrose optimised (P<0.05) all spermatozoal motion characteristics following incubation at 37°C for 30 min (Table 1). Spermatozoa exposed to Merck I extender yielded lower mean PMOT than did spermatozoa exposed to any other extender (P<0.05). Spermatozoa exposed to non-fat milk-sucrose yielded lower values for all motility parameters than that for spermatozoa exposed to Kenney or TAMU extender (P<0.05).

Centrifugation data are presented in Table 2. A centrifugation g-force of 500 x g for 15 min yielded the highest spermatozoal recovery rate of all treatment groups (P<0.05), but spermatozoa in resuspended pellets were heavily damaged, as determined by subjective visual examination of spermatozoal motility. Centrifugation of spermatozoa at 500 x g for 10 min yielded a higher spermaotozoal recovery rate than that of the remaining treatment groups (P<0.05). Similar spermatozoal recovery rates were detected for centrifugations of 400 x g for 15 min and 1000 x g for 5 min; however, the coefficent of variation was substantially lower in the former (2.2 vs. 9.1).

Centrifugation forces of 500 x g for 10 min and 400 x g for 15 min were selected for more critical evaluation of motility parameters in recovered spermatozoa because these 2 treatments appeared to yield the best combination of spermatozoal harvest and viability. These treatments resulted in similar values for motility variables (P>0.05; Table 3).

One-cycle pregnancy rates of 32% (15/47) and 19% (9/47) were not different (P>0.05) for semen centrifuged in TAMU or Merck I extender prior to cryopreservation, respectively. One-cycle pregnancy rates for semen cryopreserved in milk-egg yolk extender (37%; 12/32) were not different (P>0.05) than that for semen cryopreserved in lactose-EDTA-egg yolk extender (28%; 9/32).

Discussion

Information available to date suggests that cryopreserved semen is most dramatically affected by individual stallion variation (Samper 1995; Samper and Morris 1998), but processing techniques are likely to impact the final outcome. This study suggests that centrifugation extenders

consisting of non-fat milk and sugar are more suitable than commercial extenders that contain an assortment of electrolytes, buffers, and sugars, but no milk products. Data in this study also revealed that exclusion of glucose from centrifugation extender (ie use of milk extender containing only sucrose as a sugar) was detrimental to sperm motility.

This study suggests that a centrifugation force of 500 x g for 10 min maximises spermatozoal harvest without producing a substantial detrimental effect on spermatozoal motility. Milk-based extenders may be more advantageous for centrifugation of spermatozoa than Merck-style extenders, based on spermatozoal motility parameters; however, the type of centrifugation or cryopreservation extender used did not significantly impact upon pregnancy rates in this study. Others have reported that a lengthy pre-freeze cooling period enhances post thaw motility of spermatozoa exposed to a milk-based extender. This procedure was not followed in the current study. However, pregnancy rates for semen cryopreserved in milk-egg yolk extender were no different than for semen cryopreserved in lactose-EDTA-egg yolk extender (28%; 9/32).

Without question, a greater understanding of the injurious effects that cryopreservation-associated techniques exert on spermatozoa is required, so that repeatable methods for obtaining a commercially valuable product can be developed. The intensified interest of the horse industry in frozen semen may be the impetus required for development of reliable and successful cryopreservation techniques. The challenge for investigators will be to clarify the mechanisms of cryoinjury, and devise strategies to minimise these impairments, so that a uniformly-applicable cryopreservation technique will be effective for a large percentage of breeding stallions.

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LOW NUMBERS OF SPERMATOZOA IN THE EQUINE OVIDUCT ARE COMPATIBLE WITH FERTILISATION

L. H-A. Morris, R. H. F. Hunter, C. A. Tiplady and W. R. Allen

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

INTRODUCTION

The reported success of the hysteroscopic low-dose utero-tubal insemination technique (Morris *et al.* 2000; Morris and Allen 2003; Squires *et al.* 2003) highlights many questions about the interactions of spermatozoa with the reproductive tract in the oestrous mare. Outstanding among these are the factors that govern the progression of spermatozoa from the crypts on the uterine side of the utero-tubal junction to the site of fertilisation within the oviduct. Deposition of a suspension of spermatozoa directly onto the utero-tubal papilla may overwhelm its normal regulatory functions and allow higher than normal numbers of spermatozoa to reach the sperm reservoir and beyond with a consequential risk of polyspermic fertilisation. However, this seems unlikely as high conception rates were achieved when as many as 10×10^6 spermatozoa were deposited onto the ipsilateral utero-tubal papilla.

In this study, the number of spermatozoa recoverable from different regions of the mare's oviduct, after hysteroscopic deposition of 10×10^6 spermatozoa directly onto the papillae of both utero-tubal junctions was investigated at different times after insemination.

MATERIALS AND METHODS

Pony and Thoroughbred mares were allocated randomly into 5 groups. Group 1 mares (n=5) were killed 4 h after insemination, Group 2 mares (n=5) were killed 8 h after insemination, Group 3 mares (n=12) were monitored ultrasonographically for the occurrence ovulation and, 14 days later for pregnancy, Group 4 mares (n=2) were inseminated conventionally into the uterine body with 1×10^9 spermatozoa and killed 4 h later and Group 5 mares (n=3) were similarly inseminated with 1×10^9 killed spermatozoa and killed 4 h later.

The reproductive tracts were recovered from the mares within 15 min after death. The oviduct was dissected free from surrounding stromal tissues and the cranial tip of each uterine horn was trimmed away to leave a perimeter of approximately 0.5 cm width around the utero-tubal papilla. The length of the dissected oviduct was measured and it was then sectioned into infundibulum, ampulla, isthmus and caudal isthmus-utero-tubal junction and each section was flushed in both directions with 5 ml phosphate buffered saline (PBS) supplemented with 1% Triton X (Sigma, Aldrich). The flushings from each section were centrifuged at 400 g for 10 min, 9.9 ml of the supernatant was decanted, and the pellet was resuspended in the remaining 0.1 ml of fluid and all the recovered sperm were counted. The utero-tubal junction and the caudal isthmus of

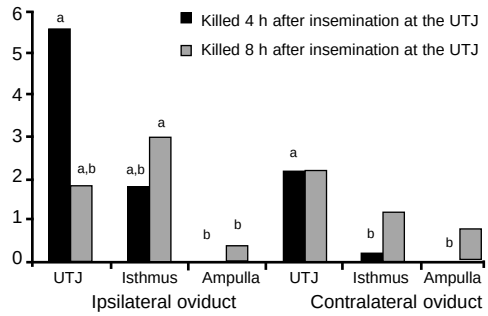

Fig 1: Mean numbers of spermatozoa recovered from different regions of the oviducts ipsilateral and contralateral to impending ovulation.

[a,b]Different superscripts denote significant differences (one-tailed t-test, $P<0.1$) in the numbers of spermatozoa recovered from the different regions of the same oviduct at either 4 h or 8 h after insemination.

TABLE 1: Mean (± sd) numbers of spermatozoa flushed from each region of the oviduct at autopsy

Method of insemination	No. of samples	No. spermatozoa in inseminate	Time of death after insemination	Mean ± sd numbers of spermatozoa recovered from:		
				UTJ	Isthmus	Ampulla
Conventional A.I.	4	1.0×10^9	4 h	$15.8 \pm 19.2^{a,b}$	3.3 ± 2.2^{a}	0^{b}
UTJ*	10	10×10^6	4 h	3.9 ± 5.8^{a}	1 ± 2.0^{b}	0^{c}
UTJ	10	10×10^6	8 h	2 ± 2.4^{a}	2.1 ± 2.6^{a}	0.6 ± 0.8^{b}

^{a,b,c} Values within each row with different superscripts are considered significantly different for a one-tailed t-test when $P<0.1$; *UTJ = utero-tubal junction

the oviduct were prepared for scanning electron microscopic counting of any remaining spermatozoa as described by Fléchon and Hunter (1981).

RESULTS AND DISCUSSION

A high conception rate (67%) was achieved in Group 3 mares despite recovering very few spermatozoa (<20) from the oviducts of Groups 1, 2 and 4 mares. Sperm gradients existed between the utero-tubal junction, isthmus and the ampulla (Table 1). These changed with time but did not result in any more spermatozoa entering the oviduct (Fig 1).

The most important finding in this study was that the deposition of 10×10^6 spermatozoa directly onto the uterine side of the utero-tubal junction did not overwhelm its regulatory function so that remarkably low numbers of spermatozoa entered the oviduct to form a sperm reservoir capable of promoting high pregnancy rates. Nevertheless, despite the low numbers of spermatozoa recovered from the oviduct, sperm gradients were established between the utero-tubal junction, the isthmus and the ampulla which changed over time, seemingly without allowing more spermatozoa to enter the oviduct. Furthermore, there was evidence of paracrine control from the preovulatory follicle influencing the 'shifts' in the sperm populations in different regions of the ipsilateral oviduct. These 'shifts' in the small populations of spermatozoa within the oviduct enabled an initial sperm:oocyte ratio close to one to be etsablished. Such a mechanism would clearly be important to avoid polyspermic fertilisation.

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SESSION 4

Chairman:
Ron Hunter

SEX SELECTION OF STALLION SPERMATOZOA

E. L. Squires, A. C. Lindsey* and J. E. Bruemmer

*Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523; *XY, Inc., 1108 N. Lemay Avenue, Fort Collins, Colorado 80524, USA*

A safe and reliable method for pre-conceptual sex selection of offspring has been sought for decades in humans, livestock and companion animals. Johnson *et al.* (1989) were the first to report a reliable method to predetermine sex using DNA as a quantitative marker for X- and Y-chromosome-bearing spermatozoa and sorting spermatozoa via flow cytometry. X-chromosome-bearing stallion spermatozoa contain approximately 3.8% more DNA than Y-chromosome-bearing spermatozoa. Sperm are stained with a Hoechst dye and placed through the flow cytometer. When passing through the flow cytometer, the sperm are excited with lasers and the difference in DNA content is determined. A charge is then put on the sperm, which provides the ability to separate X- and Y-chromosome-bearing spermatozoa. This technique has been used in several experiments to sort stallion spermatozoa and produce foals of predetermined sex. At the current rate at which stallion sperm can be sorted, only a few million spermatozoa can be obtained within a few hours. Thus, techniques of low-dose insemination are needed in order to utilise sex-sorted spermatozoa (Seidel *et al.* 1999b).

Three methods of low-dose insemination have been attempted: 1) insemination into the oviduct by surgical flank incision (McCue *et al.* 2000); 2) deep uterine insemination (Buchanan *et al.* 2000; Rigby *et al.* 2003); and 3) hysteroscopic insemination (Morris *et al.* 2000). Although the accuracy of sorting spermatozoa by flow cytometry is quite high (>90%), this technique is somewhat impractical. The very high cost of equipment and labour limits the availability of flow cytometry for separation of stallion sperm. Ideally, the stallion would come to a central facility that contained the equipment and personnel. Alternatively, the mare would come to the facility and the semen would be shipped for subsequent sorting and insemination, or the stallion would come to the facility for collection of semen, sorting and subsequent freezing of the sorted spermatozoa.

This paper contains the results of several experiments which were designed to maximise fertility with sex-sorted stallion spermatozoa. In the first experiment (Buchanan *et al.* 2000), mares were inseminated with 500×10^6 progressively motile spermatozoa into the uterine body (controls) or inseminated into the tip of the uterine horn ipsilateral to the pre-ovulatory follicle with 25 and 5×10^6 progressively motile spermatozoa. Mares in all three groups were inseminated either 40 or 34 h after GnRH administration. More mares became pregnant when inseminated with 500 than with 25×10^6 spermatozoa (90% vs 57%) but pregnancy rates were similar for mares inseminated with 5×10^6 progressively motile spermatozoa (35%).

In a preliminary trial (Buchanan *et al.* 2000), 21 mares were inseminated with 25×10^6 spermatozoa sorted into X- and Y-chromosome-bearing populations. Eight of 20 mares became pregnant.

The objectives of Experiment 3 were to compare pregnancy rates from 2 methods of insemination using low sperm numbers and to compare pregnancy rates resulting from hysteroscopic insemination of 5×10^6 non-sorted and 5×10^6 spermatozoa sorted for X- and Y-chromosome-bearing populations. Mares were randomly assigned to one of 3 treatments. Mares in Treatment 1 were inseminated with 5×10^6 spermatozoa deposited deep into the uterine horn with the aid of ultrasonography. Mares in treatment 2 were inseminated with 5×10^6 spermatozoa deposited onto the utero-tubal

junction via hysteroscopy. Mares in treatment 3 were inseminated with the hysteroscopic technique using 5 x 10^6 flow-sorted spermatozoa. Hysteroscopic insemination resulted in more pregnancies (5 of 10) than did the ultrasound-guided technique (0 of 10) when non-sorted sperm were inseminated. Pregnancy rates were not significantly lower when hysteroscopic insemination was used for sorted (5 of 20, 25%) and non-sorted spermatozoa (5 of 10, 50%).

The ability to cryopreserve sorted spermatozoa would increase greatly the practicality of using flow-sorted spermatozoa in the horse industry. Stallion sperm do not survive for a long time after the sorting process. Therefore, mares must be inseminated immediately after sperm sorting. However, if the sperm could be frozen following sorting, they could be used at any future time or location. Seidel *et al.* (1999a) reported that pregnancy rates for heifers inseminated with flow-sorted, frozen-thawed spermatozoa were similar to those for frozen-thawed non-sorted spermatozoa. Thus, the objective of Experiment 4 (Lindsey *et al.* 2002) was to determine if pregnancies could be achieved when low numbers of flow-sorted, frozen-thawed stallion spermatozoa were inseminated into mares.

Mares were randomly assigned to one of 4 treatment groups. Treatment 1 consisted of fresh, non-sorted spermatozoa. Treatment 2 consisted of fresh, flow-sorted spermatozoa. Treatment 3 consisted of frozen-thawed, non-sorted spermatozoa and Treatment 4 consisted of flow-sorted, frozen-thawed spermatozoa. Mares were synchronised in the months of July and August and, upon obtaining a 35 mm follicle, were induced to ovulate by administering 3,000 IU of hCG either 6 h (fresh spermatozoa) or 30 h (frozen-thawed spermatozoa) prior to insemination. No difference was found in the pregnancy rate for mares inseminated with fresh, non-sorted (4 of 10, 40%); fresh, flow-sorted (6 of 16, 37.5%); frozen-thawed, non-sorted (6 of 16, 37.5%) and flow-sorted, frozen-thawed spermatozoa (2 of 15, 13%). Pregnancy rates tended to be lower following insemination of frozen-thawed, flow-sorted spermatozoa. Further studies are needed to improve the viability of spermatozoa after sorting, freezing and thawing.

Since the use of flow-sorted, frozen-thawed spermatozoa resulted in relatively poor pregnancy rates. Subsequent studies focused on pregnancy rates of mares inseminated with sperm that had been stored for 18 h, then sorted and inseminated. This would simulate the conditions in which the semen is collected on the farm and shipped to a central facility that had the equipment and personnel necessary for sorting spermatozoa. The mare would be at a central facility and, upon sorting of the spermatozoa, mares would be inseminated immediately. Two studies have been conducted to evaluate the fertility of stored, sorted stallion spermatozoa. In the first study, mares were assigned to one of 2 treatments: 1) insemination with 20 x 10^6 spermatozoa that had been flow-sorted immediately following collection, and 2) insemination with 20 x 10^6 spermatozoa that had been stored for 18 h, then sorted by flow cytometry and inseminated immediately after sorting. Sperm for Treatment 2 was stored at a concentration of 25 x 10^6 spermatozoa/ml at 20°C for 18 h. Pregnancy rates were similar for those mares inseminated with fresh, flow-sorted spermatozoa (6 of 20, 30%) and those inseminated with stored, flow-sorted spermatozoa (7 of 20, 35%).

A study conducted during the 2001 breeding season, determined the fertility of spermatozoa stored at 2 temperatures, 5 and 15°C and inseminated either hysteroscopically or by rectally guided, deep-uterine insemination. Semen from 3 stallions was available for this study. Upon collection of semen, the spermatozoa was diluted to 25 x 10^6 spermatozoa/ml and stored at either 5 or 15°C for 18 h. After storage, the spermatozoa were separated into X- and Y-chromosome-bearing populations. For spermatozoa stored at 5°C, 22 mares were inseminated by a hysteroscopic technique and 24 mares by deep-uterine, rectally guided insemination. Although not significantly different, more mares inseminated with the hysteroscope (12 of 22, 54%) became pregnant compared to those inseminated with the rectally guided technique (9 of 24, 37.5%). The highest pregnancy rate was obtained for mares inseminated with spermatozoa that had been stored at 15°C, sorted and then inseminated hysteroscopically; 18 of 25 mares (72%) became pregnant with this treatment. It would appear that storage at 15°C was better than at 5°C and perhaps hysteroscope insemination was better than rectally guided insemination.

In summary, acceptable pregnancy rates can be obtained with spermatozoa that has been sorted into X- and Y-chromosome-bearing populations. Furthermore, the ability to store semen prior to sorting and insemination does not appear to

decrease the viability. This provides the opportunity for semen to be collected on the farm and sent to a central facility for subsequent sorting and insemination. However, the technology that is badly needed in order to enhance the use of sexed semen is the ability to sort stallion spermatozoa then freeze. Currently the pregnancy rates with this procedure are quite low.

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HYSTEROSCOPIC INSEMINATION OF MARES WITH FROZEN-THAWED EPIDIDYMAL SPERMATOZOA

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

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

The first reported pregnancy from the use of frozen-thawed stallion spermatozoa was in a mare inseminated with epididymal spermatozoa. However, the *in vivo* fertility of cauda epididymal spermatozoa tends to be lower than ejaculated spermatozoa in cattle and in sheep. Reasons for these differences may include variations in the cell surface characteristics and the lower motility of epididymal spermatozoa. Furthermore, the *in vivo* fertility of cauda epididymal ram spermatozoa only achieved that of ejaculated spermatozoa when it was deposited surgically in the region of the utero-tubal junction (Fournier-Delpech *et al.* 1979). In mares, deposition of low numbers of fresh or frozen-thawed ejaculated spermatozoa onto the papillae of the utero-tubal junction by hysteroscopy has resulted in conception rates in excess of 50% (Morris *et al.* 2000, 2003; Squires *et al.* 2003). The present study investigated the fertility of cauda epididymal spermatozoa after deposition onto the papillae of the utero-tubal junction by hysteroscopic insemination of mares.

In the preliminary study, the plasma membrane integrity and the calcium distribution of cauda epididymal spermatozoa were evaluated before and after freezing. Spermatozoa were flushed from the cauda epididymes of nine colts after castration under general anaesthetic. The recovered spermatozoa were suspended in a skim milk-glucose diluent, either with or without, 50% (v/v) seminal plasma. For comparison, semen was collected from either of 2 identical twin Pony stallions and extended (1:1, v/v) with the same skim milk-glucose diluent. Both the epididymal and ejaculated spermatozoa were frozen in a lactose-egg yolk – glycerol diluent in liquid N2 vapour and samples were taken for staining with chlortetracycline (CTC) before dilution, after dilution and after thawing. The spermatozoa were then examined by fluorescent microscopy using a 380–420 nm filter and were classified into one of the following 4 categories: CTC I, non-capacitated; CTC II intermediate stage; CTC III capacitated; or CTC IV acrosome reacted. The plasma membrane integrity of the epididymal spermatozoa was also determined using the fluorescent PSI stain.

Epididymal spermatozoa were then flushed from the epididymes of an additional 13, 2- to 3-year-old colts after routine standing castration and then processed as described above. When required, the frozen spermatozoa were thawed at 37°C for 30 s and hysteroscopic inseminations were performed as described previously (Morris *et al.* 2000). A total of 83 mares were randomly allocated to one of the following 7 insemination groups: 1) hysteroscopic insemination with 200 x 10^6 fresh epididymal spermatozoa; 2) hysteroscopic insemination with 200 x 10^6 fresh epididymal spermatozoa which had been exposed to 50% seminal plasma during recovery from the epididymes; 3) conventional insemination with 115 ± 18 x 10^6 frozen-thawed spermatozoa; 4) hysteroscopic insemination with 113 ± 23 x 10^6 frozen-thawed spermatozoa; 5) hysteroscopic insemination with 107 ± 4 x 10^6 frozen-thawed spermatozoa which had been exposed to seminal plasma during flushing from the epididymis; 6) hysteroscopic insemination with 6 ± 5 x 10^6 frozen-thawed spermatozoa which had been centrifuged at 400 g for 10 min through a 50:100% density gradient and resuspended in 100 µl sperm TALP; 7) hysteroscopic insemination with 9 ± 6 x 10^6 frozen-thawed spermatozoa which had been simply resuspended in 100 µl sperm TALP.

Evaluation of the CTC staining patterns of fresh and frozen-thawed epididymal spermatozoa revealed that there was a predominance of

TABLE 1: Chlortetracycline capacitation status of epididymal spermatozoa (%)

Sample		n	CTC I	CTC II	CTC III	CTC IV	% viable
Epididymal	Raw	8	1[a]	90	7	2[a]	75[a]
	Extended	9	2[a]	79	5	14[a]	56[a]
	Thawed	9	0.2[a]	66	2	36[b]	30[b]
Epididymal + SP	Extended	9	7[b]	72	6	17[a]	60[a]
	Thawed	8	0 [a]	60	0	40[b]	29 [b]

TABLE 2: Chlortetracycline capacitation status of ejaculated spermatozoa

Sample	n	CTC I	CTC II	CTC III	CTC IV	% progressively motile
Raw	6	62[a]	28[a]	9	1	64
Extended	6	54[a]	39[a]	5	2	64
Thawed	6	0[b]	88[b]	5	7	51

[a,b] Different superscripts denote differences between values within a column (Chi-square multiple comparisons and the Bonferroni adjustment P<0.005)

TABLE 3: Pregnancy results from insemination of mares with epididymal spermatozoa

Type of spermatozoa	Exposure to seminal plasma	Volume of inseminate (100 µl)	Total number of spermatozoa (x 10^6)	Proportion of mares pregnant (%)
Epididymal (fresh)	-	100	150 – 200	4 / 11 (36)
	+	100	150 – 200	5 / 9 (56)
Epididymal (frozen - thawed)	-	500	113 ± 23	2 / 12 (17)
	+	500	107 ± 4	3 / 14 (21)
Epididymal (frozen-thawed) Processed though density gradient	-	150	6 ± 5	4 / 14 (29)
No density gradient	-	150	9 ± 6	3 / 10 (30)

spermatozoa in the CTC II category. After thawing, there was a significant increase in the acrosome reacted forms (CTC IV) of the epididymal spermatozoa. These observations are in contrast to those of frozen-thawed ejaculated spermatozoa (Tables 1 and 2), where the populations of spermatozoa shifted from the CTC I category prior to freezing to the CTC II category after thawing.

Regardless of exposure to seminal plasma or processing through the density gradient, there were no significant differences in the mean (± sd) percentage motilities (30 ± 3.5%) of the epididymal spermatozoa in each group observed after thawing. When 200 x 10^6 fresh epididymal spermatozoa were deposited directly onto the utero-tubal papilla, 9/20 (45%) mares conceived (Table 3). These conception rates (Table 3) were substantially reduced when mares were inseminated either hysteroscopically (9 / 51, 18%) or conventionally (1/13, 8%) with 200 x 10^6 frozen-thawed epididymal spermatozoa at 32 h after administration of human Chorionic Gonadotropin (hCG). When the insemination dose was reduced to 5–10 x 10^6 motile frozen-thawed epididymal spermatozoa, there was an apparent beneficial effect of exposure to sperm TALP prior to insemination, such that no pregnancies were obtained in the 20 mares inseminated with spermatozoa not exposed to sperm TALP and 29% (7/24) of mares conceived after insemination with epididymal spermatozoa exposed to sperm TALP after thawing (Table 3). In an additional group, no mares (0/9) conceived when inseminated hysteroscopically after ovulation at 41 h following administration of hCG. Moreover, there were no

differences in the conception rates in the groups of mares inseminated with fresh or frozen-thawed epididymal spermatozoa that had been either exposed or not exposed to seminal plasma prior to insemination or freezing (Table 3).

The poor fertility of epididymal spermatozoa observed in these experiments may reflect the differences between stallions in their intrinsic fertility and their response to sperm freezing, as well as the variable fragility of the plasma membranes of spermatozoa which have accumulated for varying periods of time during storage within the caudae epididymes. It is also speculated that there may be a negative local effect of the local anaesthetic which was infused into the testicle and spermatic cord during the routine standing castration. Further studies are required to investigate the differences in fertility observed between epididymal and ejaculated spermatozoa in horses.

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A COMPARISON BETWEEN *IN VIVO* AND *IN VITRO* MATURED OOCYTES IN THE MARE

G. Goudet

Equipe Reproduction Equine, PRC, INRA, 37380 Nouzilly, France

Profound changes occur in mammalian oocytes of pre-ovulatory follicles as a consequence of acute gonadotrophic stimulation. The processes driving these changes are generally divided into 2 categories, those involved in the progression of meiosis to Metaphase II, and those occurring in the cytoplasm that promote monospermic fertilisation and preparation for pre-implantation development. These processes are referred to as nuclear and cytoplasmic maturation, and are usually highly co-ordinated.

In the equine, research and knowledge on nuclear and cytoplasmic maturation of the oocyte lag behind other mammalian species. The low availability of oocytes for study is the greatest barrier. As a consequence, scientists used to transpose to the mare the knowledge gained in the other mammalian species. However, although the mechanisms of equine oocyte maturation do not seem to be fundamentally different from the other mammals, some differences can be underlined. The most obvious particularity in the mare is the hormonal environment during the resumption and completion of meiosis in the pre-ovulatory follicle. The mare does not present any short pre-ovulatory LH surge, but a slow increase lasting many days, with maximum values observed one day after ovulation (Whitmore *et al.* 1973). Based on the *in vivo* conditions, several laboratories have attempted to define the appropriate culture methods for equine oocytes. However, the *in vitro* maturation rate of equine oocytes remains lower than those observed in the bovine and porcine species (Squires 1996; Hinrichs and Williams 1997). Moreover, although the steps of oocyte maturation *in vivo* and *in vitro* do not seem to be fundamentally different, some differences can be underlined.

The mechanism of oocyte maturation includes morphological and biochemical modifications. In the equine, most studies are histological studies and focus on the nuclear maturation. Very few studies deal with the biochemical level.

The steps of the nuclear maturation of the equine oocyte *in vivo* as well as *in vitro* conditions are the breakdown of the nuclear membrane, the formation of the metaphase plate of the first meiotic division, the extrusion of the first polar

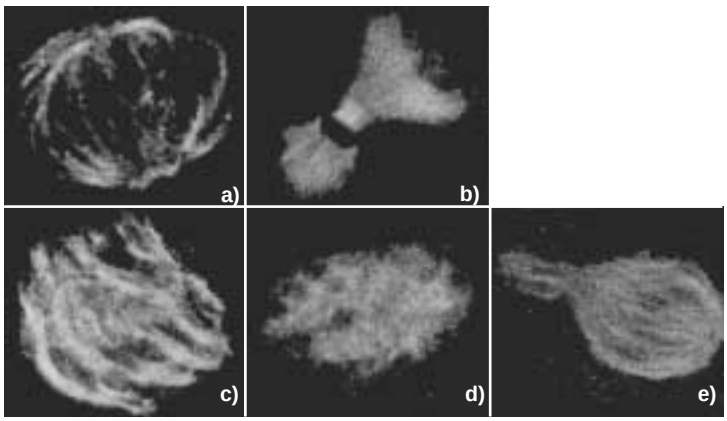

Fig 1: Meiotic spindle morphology in equine oocytes. a) Normal spindle: barrel-shaped with 2 poles and distinct microtubules between poles. b) Telophase I spindle. c) Prometaphase spindle: barrel shaped with 2 poles and disorganised microtubules between poles. d) Scattered tubulin: a dense network but no poles and no distinct microtubules. e) Multipolar spindle: 3 poles and distinct microtubules between poles. The polar body is visualised at another focal plane. X2750.

TABLE 1: Meiotic spindle size

	In vivo maturation	*In vitro* maturation	
Width	13.9 ± 2.9 µm	17.9 ± 5.1 µm	P<0.001
Length	12.0 ± 3.8 µm	18.0 ± 4.9 µm	P<0.001

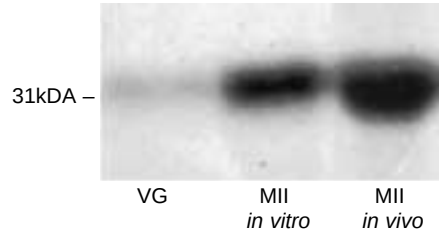

Fig 2: Histone H1 kinase activity in equine oocytes before meiosis resumption and after meiosis completion in vitro *and* in vivo. *Lane 1: One immature oocyte. Lane 2: One oocyte in Metaphase II after* in vitro *culture. Lane 3: One preovulatory oocyte that reached Metaphase II* in vivo.

body and the subsequent formation of the metaphase plate of the second meiotic division. Bézard *et al.* (1997) studied the chronology of the *in vivo* maturation of the equine oocyte, after induction of ovulation. They showed that the equine oocyte reaches Metaphase I and Metaphase II 24 and 35 h, respectively, after induction of ovulation using exogenous gonadotrophin. During *in vitro* maturation, the percentage of oocytes in Metaphase I and in Metaphase II did not increase further after 20 and 30 h in culture, respectively (Bézard and Palmer 1992; Squires 1996).

Goudet *et al.* (1997) evaluated the meiotic spindle morphology in equine oocytes that reached Metaphase I, Telophase I or Metaphase II after *in vivo* or *in vitro* maturation. The spindles were classified as normal metaphase spindle (barrel-shaped with 2 poles and distinct microtubules between poles), telophase I spindle, prometaphase spindle (barrel-shaped with 2 poles and disorganised microtubules between poles), scattered tubulin (a dense network but no pole and no distinct microtubules) and multipolar spindle (Fig 1). The first 3 classes were considered as normal spindles.

After *in vivo* maturation, all the Metaphase I oocytes, and 86% of the Metaphase II oocytes had a normal spindle. After *in vitro* culture, 65% of the Metaphase I oocytes and 77% of the Metaphase II oocytes had a normal spindle (Goudet *et al.* 1997). Moreover, the metaphase spindles from oocytes cultured *in vitro* were significantly wider and longer than spindles from oocytes analysed after *in vivo* maturation (Goudet *et al.* 1997; Table 1).

As a change in the critical concentration of tubulin in the cytoplasm may induce an alteration in the size of the spindle. It was hypothesised that *in vitro* culture may induce a change in the concentration of cytoplasmic proteins, such as tubulin.

During *in vivo* maturation of equine oocytes, Grøndahl *et al.* (1995) observed the migration of the cortical granules beneath the oolemma. After *in vivo* maturation, migration of cortical granules was achieved or in progress in 100% of the Metaphase II oocytes (Goudet *et al.* 1997). After *in vitro* culture, migration of cortical granules was achieved or in progress in 83% of the Metaphase II oocytes (Goudet *et al.* 1997). *In vitro* maturation may not induce full cytoplasmic maturation.

During *in vivo* as well as *in vitro* maturation of equine oocytes, the cells from the cumulus oophorus expand. *In vivo*, 83% and 100% of the oocytes have an expanded cumulus 12 h and 35 h, respectively, after induction of ovulation (Bézard *et al.* 1997). After *in vitro* culture, most oocytes have an expanded cumulus, depending on the culture medium.

The meiotic maturation is accompanied, and probably regulated, by changes in the phosphorylation patterns of various cellular proteins. Two important components of this activity are the maturation promoting factor (MPF) and the mitogen-activated protein kinase (MAPK). Very few data are available in the equine on the biochemical aspect of oocyte maturation. Goudet *et al.* (1998a) investigated the expression of MPF components (p34cdc2 and cyclin B) and MAPK after *in vitro* or *in vivo* maturation. An assay system for MPF activity in equine oocytes was established and changes in activity in relation to the nuclear stage after *in vivo* or *in vitro* maturation were investigated (Goudet *et al.* 1998b). It was demonstrated that the MAPK and the 2 subunits of MPF, p34cdc2 and cyclinB, were present in equine oocytes after *in vivo* or *in vitro* maturation. However, MPF activity, as measured by phosphorylation of histone H1, was significantly higher in pre-ovulatory oocytes that reached Metaphase II *in vivo* than in the oocytes that reached Metaphase II after *in vitro* culture (Fig 2).

In conclusion, the steps and mechanisms of *in vivo* and *in vitro* maturation are not fundamentally different. However, some morphological and biochemical differences between *in vivo* and *in vitro* conditions were observed. This underlines the need of more studies to improve the *in vitro* culture conditions for equine oocytes. Moreover, the Metaphase II stage is only part of the maturation process. It is necessary to take into account some other morphological and biochemical criteria to evaluate oocyte maturation.

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EVALUATING *IN VITRO* MATURATION OF HORSE OOCYTES

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

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

INTRODUCTION

During oocyte maturation, the oocyte must undergo complex and highly integrated nuclear (ie the resumption of meiosis and progression from the germinal vesicle stage to metaphase of the second meiotic division) and cytoplasmic changes to become a viable, fertilisable and developmentally competent ovum. Most studies of horse oocyte maturation have focussed on describing changes in the chromatin configuration and very little attention has been paid to cytoplasmic events. The majority of studies have been conducted with cumulus oocyte complexes (COCs) recovered from the ovaries of mares killed at the slaughterhouse and there have been great between-laboratory differences in the time from slaughter to COC collection and the temperature at which the ovaries were maintained in the interim. There are further between-laboratory differences in the methods of collection, selection and handling of COCs during maturation, and these differences undoubtedly affect the acquisition of developmental competence by *in vitro* matured (IVM) oocytes (Hinrichs and Schmidt 2000). However, whether the competence of oocytes matured *in vitro* is lower than that of oocytes matured *in vivo* - which would suggest that current IVM procedures for horse oocytes are suboptimal - remains to be definitively tested. The ultimate criterion for the competence of an IVM oocyte is its ability to be fertilised and to develop into a viable embryo. Unfortunately, the lack of an efficient equine *in vitro* fertilisation (IVF) system has prevented us from using embryo development as an endpoint. For this reason, most research on equine oocyte IVM has been based either on comparison with *in vivo* matured oocytes, or on improving the proportion of oocytes reaching Metaphase II. It is questionable whether the control of oocyte maturation *in vitro* closely ressembles that *in vivo*. Firstly, COCs removed from their follicle appear to be less dependent on their hormonal environment (Bevers *et al.* 1997) and secondly, *in vitro* studies are most often performed with COCs aspirated from small to medium sized follicles, which differ from large dominant follicles selected during the reproductive cycle of a mare. Furthermore, oocyte maturation is a complex process during which cytoplasmic rearrangement and maturation is probably as critical as chromatin reorganisation. Therefore, assessing oocytes entirely on their nuclear development is unlikely to give a reliable estimate of their developmental competence.

The recent application and success of alternative IVF techniques such as ICSI (Li *et al.* 2001) offers a means of assessing the developmental competence of oocytes, while transfering oocytes into the oviduct of inseminated mares (GIFT) may also prove a very useful way of comparing fertilisability and developmental capacity of *in vivo* and *in vitro* matured oocytes (Scott *et al.* 2001). While the above techniques will certainly improve the ability to assess oocyte quality, a fuller description of the nuclear and cytoplasmic events that occur during oocyte maturation will help further to pinpoint factors affecting the acquisition of developmental competence.

This paper reviews recent developments in the techniques available for evaluating IVM of equine oocytes, with an emphasis on the cytoskeletal changes that accompany chromatin reorganisation during meiosis. The use of hormones during equine IVM will be discussed in relation to the need for a reliable test for assessing oocyte competence. The problems encountered during

IVF of equine oocytes will be discussed in relation to the development of a test for oocyte quality and competence.

Assessing cytoplasmic and nuclear changes during IVM

Although there have been numerous reports of the nuclear changes that occur during IVM of horse oocytes (see Hinrichs *et al.* 1993), little is known about the changes in the cytoplasm during maturation. Studies on this topic have been limited to preliminary observations of the migration of specific organelles (eg cortical granules) and of the morphology of the meiotic spindle (Goudet *et al.* 1997). However, it is clear that, as in other species a series of complex cytoplasmic changes must accompany nuclear maturation if a developmentally competent oocyte is to result and that these events are largely dependent on a complete reorganisation of the oocyte cytoskeleton.

Recently, confocal laser scanning (CLSM) and epifluorescence microscopy were used to examine changes in the cytoskeletal architecture of equine oocytes (n=466) during IVM. Oocytes were cultured for 0, 12, 24 or 36 h at 39ºC in M199 supplemented with pFSH and eLH before being fixed and stained with labels for microtubules (monoclonal anti-_-tubulin), microfilaments (AlexaFluor 488 Phalloidin) and DNA (TO-PRO_3 or DAPI). At 0 h, 74% of the oocytes (77/104) were in the germinal vesicle stage, with a diffuse chromatin configuration and with their microfilaments and microtubules distributed throughout the ooplasm. After 12 h of culture, 43% of oocytes had reached prometaphase (53/125) and the microtubules were now concentrated around the chromosomes, while the microfilaments had largely accumulated near the oocyte cortex. At 24 h, most of the oocytes had reached Metaphase I (MI: 28/111= 25%) or Metaphase II (MII: 28/111= 25%) and, by 36 h, 42% of oocytes were in MII (52/124). In MI oocytes, microtubules were found exclusively in the meiotic spindle surrounding the aligned chromosomes and, in MII oocytes, the spindle was a symmetrical barrel-shaped structure with 2 poles and with the chromosomes aligned along the metaphase plate. While the microfilaments remained concentrated within the oocyte cortex, further accumulations were visible over the spindle and the polar body. The changes observed supported strongly a role for both microtubules and microfilaments in chromosomal reassortment and in polar body formation (Tremoleda *et al.* 2001). These findings emphasised the probable importance of cytoplasmic, in particular the cytoskeleton, elements in maturational changes that are likely to influence subsequent developmental competence. The techniques used may prove very useful when evaluating other aspects of cytoplasmic maturation in equine oocytes and in highlighting abnormalities resulting from IVM.

Culture conditions that influence oocyte competence

Most equine IVM protocols include combinations of LH, FSH and oestradiol added to the medium to enhance maturation. The effect of these hormones and their influence on maturation and subsequent fertilisability is not clear. The rationale for their inclusion is their postulated physiological influence on oocyte maturation *in vivo*. However, there are marked differences between *in vivo* and *in vitro* conditions, not least of which is that although COCs retain an attachment to granulosa cells, they lose their intimate contact with the follicle wall and follicular environment. Indeed, maturation is suppressed in horse oocytes incubated while still attached to the follicle wall (Hinrichs *et al.* 1995), whereas granulosa cells and the cumulus hillock attachment appear to support meiosis and the acquisition of developmental competence (Dell'Aquilla *et al.* 2001).

The influence of the various hormones included in culture media for equine oocytes is not yet clearly defined. The diversity in hormone preparations and concentrations used and the addition of serum, which contains undefined and varying concentrations of various hormones, further complicates analysis. More convincing data is available for the influence of the various hormones on cattle oocytes during IVM and these indicate that FSH but not LH promotes maturation. This finding is explained by the fact that the cumulus cells of bovine COCs contain mRNA for FSH but not LH receptor (Van Tol *et al.* 1996). Similarly, while oestradiol is added regularly to culture media for equine IVM, there is no convincing evidence that it has a positive influence on oocyte maturation. Some studies have demonstrated a negative effect of oestradiol on spindle formation during IVM of bovine oocytes.

Recently, the influence of factors such as growth hormone (GH: Goudet *et al.* 2000) and the insulin-like growth factors (eg IGF-I: Carneiro *et al.* 2001) on the IVM of equine oocytes has been studied. Both GH and IGF-I were reported to have a beneficial effect on nuclear maturation rates and, in addition, IGF-I had a positive effect on cytoplasmic maturation, as measured by the rate of parthenogenic cleavage of the matured oocytes. Compared to other species, the proportion of equine oocytes reaching MII after IVM is low (average 50–70%), while the proportion that are degenerate at the onset (~20%), or become so during maturation (43%: Tremoleda *et al.* 2001), as observed by their aberrant chromatin and cytoskeletal patterns, is very high. The need to improve IVM conditions for horse oocytes thus remains, but this will require targeted studies to determine which factors actually promote maturation and developmental competence. In addition, techniques for assessing oocyte competence *in vitro* must be developed and refined, at least until IVF becomes a reliable proposition.

Effect of IVM on IVF

The failure of conventional IVF in horses has often been ascribed to inadequate or aberrant oocyte maturation *in vitro*. Indeed, the only two foals born after conventional IVF were derived from oocytes matured *in vivo*. However, it has yet to be definitely proven that aberrant development of oocytes *in vitro* is the limiting factor for equine IVF. Recently, confocal laser scanning microscopy was used to analyse closely sperm-oocyte interaction during IVF and, in particular, to determine whether sperm would penetrate the zona pellucida (ZP) of oocytes matured *in vivo* (n=15) or *in vitro* (n=67). COCs for IVM were recovered from the ovaries of slaughtered mares and cultured for 36 h, while *in vivo* matured oocytes were collected by transvaginal ultrasound-guided aspiration of pre-ovulatory follicles from oestrus mares injected 32 h previously with hCG. Mature COCs were then incubated with percoll-treated, frozen-thawed stallion sperm in fertil-TALP (Parrish *et al.* 1988) containing 150 ng/ml progesterone. After 6 h of IVF, oocytes were permeabilised, fixed and stained with FITC-PNA and EthD-1 to visualise the acrosomal membranes and DNA, respectively. Irrespective of how oocytes were matured, sperm did not penetrate the ZP. However, sperm did bind to the ZP and did so in greater numbers to the ZP of *in vivo* (≈33 sperm/oocyte), than *in vitro* (≈0–1 sperm/oocyte), matured oocytes. Unexpectedly, bound sperm did not show clear signs of having acrosome reacted. Most displayed a mottled, swollen acrosomal cap while many more 'free' acrosomal caps were also found attached to the ZPs.

In summary, in the IVF system used, sperm were unable to penetrate the ZP of either *in vivo* or *in vitro* matured oocytes. While more sperm bound to the ZP of oocytes matured *in vivo*, it appeared that the primary problem was inadequate activation of the sperm acrosome reaction. This barrier needs to be overcome before IVF can be used to determine quality and developmental competence of IVM oocytes. Recent studies using GIFT and ICSI suggest that while IVM oocytes are fertilisable, they have a decreased developmental competence. Evaluating structural and molecular changes in the oocyte may help to pinpoint the basis of any oocyte-related inadequacies.

In conclusion, there is still much work to do before IVM and IVF become reliable techniques for producing horse embryos and offspring. An important part of refining these processes will undoubtedly be the development of suitable techniques for monitoring their success. Nuclear and cytoplasmic maturation are highly interdependent, structural and molecular changes occurring within the cytoplasm during IVM that undoubtedly affect the acquisition of developmental competence and, therefore, warrant further investigation.

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RELATIONSHIPS BETWEEN OOCYTE CHARACTERISTICS AND DEVELOPMENTAL POTENTIAL

K. Hinrichs, Y. H. Choi, C. C. Love, D. D. Varner, S. P. Brinsko and L. B. Love

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

The reported rates of *in vitro* maturation of horse oocytes vary among laboratories. A major factor in this variability is the selection of oocytes for culture. Laboratories differ in the type of follicles from which oocytes are collected, the method of collection, and the criteria used to categorise oocytes. These parameters have significant effects on the apparent meiotic competence of cultured oocytes.

Meiotic competence increases with increasing follicle size (Hinrichs and Schmidt 2000), so selection of larger follicles (eg selection of only those follicles visible at the surface of the ovary) is related to an apparent increase in maturation rate. Oocytes from atretic follicles have greater meiotic competence than do those from viable follicles (Hinrichs and Williams 1997). Follicle atresia is associated with expansion of the cumulus. The increase in meiotic competence with follicle size applies to both oocytes with compact (Cp) and expanded (Ex) cumuli. However, at each follicle size under 20 mm, the maturation rate for Ex oocytes is higher than that for Cp oocytes (Hinrichs and Schmidt 2000). Follicles in early atresia contain oocytes with the highest meiotic competence, and these oocytes may have either Cp or Ex cumuli (Hinrichs and Williams 1997). This probably accounts for a large portion of the confusion among laboratories in oocyte cumulus classification and its relationship to meiotic competence. Approximately 20% of collected oocytes are from follicles in early atresia, and because over 85% of oocytes in this category mature (Hinrichs and Williams 1997), the group into which they are placed (ie Cp or Ex) will appear to have a higher maturation rate.

Cumulus appearance differs between oocytes aspirated from the follicle and oocytes scraped from the follicle wall, as aspiration tends to strip the majority of the cumulus from the oocyte (Alm *et al.* 1997; Dell'Aquila *et al.* 2001). When only the corona radiata is present, it is not possible to determine whether the oocyte originally had a compact or an expanded cumulus. In addition, aspiration may preferentially collect oocytes from less viable follicles, in which the cumulus is not strongly attached to the follicle wall (Dell'Aquila *et al.* 2001). Even when oocytes are recovered using a standardised technique such as follicle scraping, the criteria used for placement of oocytes in different categories, and the category of oocytes selected for culture, vary among laboratories. In the authors' laboratory, oocytes are collected from all visible follicles (as small as 2–3 mm diameter), after serial slicing of ovaries. Only those oocytes having both compact mural granulosa and a compact cumulus are considered to be Cp. In recent studies, use of these stringent criteria for cumulus classification resulted in 28–38% of oocytes being classified as Cp, 57–65% as Ex and 4–8% as degenerating. The maturation rate for Cp oocytes selected under these conditions ranged from 14–25% and that for Ex oocytes from 66–78%. In contrast, other laboratories have reported up to 85% of collected oocytes as having compact cumuli (Choi *et al.* 1993), and this percentage is commonly reported as over 50%.

Many of the above factors are interrelated, eg the difference in proportion of Cp oocytes and their maturation rate observed among laboratories may be due not only to classification criteria, but also to the follicles selected for collection, as both the proportion of oocytes which are Cp, and the maturation rate of Cp oocytes, increase with follicle size (Hinrichs and Schmidt 2000). The authors have also found recently that immediate removal of oocytes from the ovary (within 1 h of ovary collection from the mare) is associated with

higher maturation rates *in vitro* (K. Hinrichs, unpublished data). Thus, a laboratory located close to an abattoir, which uses aspiration (reducing the time needed for oocyte collection and favouring collection from atretic follicles) of only follicles visible on the surface of the ovary (ie larger follicles) and which has non-stringent criteria for classification as Cp, will report a much higher rate of maturation for 'Cp' oocytes than will a laboratory located a distance from an abattoir that collects oocytes from all follicles on the ovary (or only those under a given size) by follicle scraping and uses stringent criteria for classification of an oocyte as Cp.

It is only recently that it has been possible to evaluate developmental potential of horse oocytes. Conventional *in vitro* fertilisation has not been repeatably successful. However, intracytoplasmic sperm injection (ICSI) has become a useful means for achieving high rates of fertilisation. The authors used ICSI to determine the developmental potential of different classes of oocytes after maturation. The activation rate of Ex horse oocytes after ICSI was significantly higher that that for Cp oocytes (78% and 57%, respectively; $P<0.05$). In addition, the percentage of ICSI-fertilised oocytes cleaving with normal nuclei was signficantly higher in Ex than in Cp oocytes (66% and 39% respectively; $P<0.05$). Thus, it appears that even those Cp oocytes that have meiotic competence (reach MII in culture) have deficits in cytoplasmic competence, which impair their ability to activate and cleave after fertilisation.

The effect of duration of maturation on fertilisation rate and embryo development after ICSI in the 2 oocyte types was evaluated. For Cp oocytes, there was no significant effect of duration of maturation (24 or 42 h) on rates of fertilisation/activation, normal cleavage, or embryo development. However, because of the low proportion of oocytes in this classification, and the low maturation rate, there were only 19–25 ICSI-Cp oocytes per time group. For Ex oocytes, there was no difference in fertilisation/activation or cleavage rates between oocytes matured for 24 h or 42 h before ICSI. However, a significantly higher proportion of resulting embryos had 4–8 nuclei at 48 h after ICSI in the 24 h maturation group than in the 42 h maturation group (24/42, 59% vs. 15/42, 36%, $P<0.05$).

Because Ex oocytes originate from atretic follicles, it is possible that their developmental competence (eg ability to continue embryo development and form blastocysts) is impaired. It is difficult to assess developmental competence *in vitro,* because culture systems are currently inadequate. Therefore, the developmental competence of Ex oocytes after transfer to the oviducts of mares *in vivo* was evaluated. Transfer of presumptive zygotes resulting from ICSI of Ex oocytes resulted in 75% normal cleavage, with embryos having an average of 16 nuclei at 4 days after transfer (Choi *et al.* 2003a). This is comparable to the expected stage of development for embryos originating *in vivo.* To evaluate the capacity for Ex-ICSI embryos to form blastocysts, 40 ICSI embryos were transferred into the oviducts of one recipient mare and then flushed the mare's uterus and oviducts for embryo recovery on Day 7. Of 9 embryos/oocytes collected from the uterus, 8 were blastocysts. Two of the blastocysts were expanded, 5 blastocysts were just beginning expansion, and one blastocyst was degenerated. Of 11 embryos/oocytes collected from the oviducts, 6 were well-developed morulae having more than 50 nuclei, 4 were morulae with fewer than 50 nuclei, and one had degenerated nuclei. Thus, ICSI embryos produced from IVM Ex horse oocytes may produce normal blastocysts at a reasonable rate (up to 40%) after transfer to the oviduct *in vivo.*

Differences between Ex and Cp oocytes after nuclear transfer were also evaluated (Choi *et al.* 2003b). Rates of successful enucleation and fusion were equivalent between Ex and Cp oocytes. The activation and cleavage rates in Ex oocytes tended to be higher than those for Cp oocytes (29% vs. 14% for activation and 9 and 0% for cleavage for Ex and Cp oocytes, respectively). However, these differences were not significant. Three embryos were produced, all from Ex oocytes.

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FOLLICLE GROWTH IN MARES FOR OOCYTE PRODUCTION

R. Cochran, M. Meintjes, D. Hylan, M. Sansinena, R. S. Denniston, D. Paccamonti* and R. A. Godke

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

The mare has presented a unique problem to those scientists involved in assisted reproductive technology research. Embryo transfer (ET) was the first realistic procedure available to farm animals that could help to enhance the reproductive potential of the female. With the standard ET procedure, donor females are exposed to gonadotropin-stimulation to produce multiple ova for *in vivo* fertilisation. Embryo collection and transfer procedures are considered relatively simple in the mare, producing acceptable pregnancy rates with good quality recipient females. Unfortunately attempts to superovulate donor horses generally produce poor results, making equine ET less acceptable to the horse industry. This may be due to the unique anatomical structure of the equine ovary and/or other unknown factors.

The advent of *in vitro* fertilisation (IVF) techniques offered new opportunities for researchers in animal reproductive management. This *in vitro* technique was successfully used to produce the first offspring in farm animals, a healthy bull calf, reported by Brackett *et al.* (1982). Oocyte collection from live donors and IVF procedures became commercially available to dairy and beef cattle producers in the USA in the early 1990s. With thousands of bovine offspring produced worldwide, IVF is now used routinely in commercial cattle embryo transplant units. IVF in the mare has not developed to the level for in-field use as expected. Although several offspring have been reported in France using IVF in the mare in the early 1990s, repeatable IVF protocols are currently not available. As oocyte maturation and *in vitro* sperm zona penetration are primary problems hindering IVF development in horses, other assisted reproductive technologies, such as intracytoplasmic sperm injection (ICSI) and nuclear transfer are now being investigated.

The primary limiting factor in developing these new technologies for the mare is the lack of sufficient numbers of good quality equine oocytes. Although only one follicle normally ovulates during oestrus, mares have one or 2 waves of multiple follicles during an oestrous cycle (Ginther and Pierson 1989). It was proposed that a transvaginal ultrasound-guided aspiration (TUGA) approach could take advantage of these developing follicle populations, harvesting oocytes to develop further the IVF procedure for the horse. Researchers began to evaluate TUGA procedures to collect oocytes from horse and pony mares (Brück *et al.* 1992; Cook *et al.* 1992; Bracher *et al.* 1993; Meintjes *et al.* 1995a). The basic oocyte aspiration method is similar to that used in cattle, but with minor modifications. Briefly, mares require sedation instead of an epidural block, and most often a 12-gauge needle is used to puncture the follicles. Extra rinsing of the follicle is necessary in the horse, since the oocyte is usually well embedded into the granulosa cells of the follicle wall. Using this approach, oocytes have been successfully recovered by members from this laboratory from mixed-breed cyclic mares and ponies (Meintjes *et al.* 1995a), Altrenogest-treated mares (Cochran *et al.* 1999b) and free-ranging zebras in South Africa (Meintjes *et al.* 1997a). Oocyte recovery rate from this transvaginal approach usually ranges between 35 and 70% of follicles punctured per mare.

With TUGA now available, various research groups began to develop ICSI for horses, where individual sperm cells are injected into the oocytes to trigger the fertilisation process (Meintjes *et al.* 1996; Squires *et al.* 1996). California State University produced the first foal from abattoir-derived oocytes. The first pregnancies and live foals produced by ICSI with oocytes aspirated

TABLE 1: Oocyte production from fresh and chronically hCG-treated and aspirated mixed breed, cyclic mares of various ages

Group	Aspirations (n)	Response* at ~24 h (%)	Oocytes recovered (%)
Chronic	51	6 (11.8)[a]	8 (15.7)[a]
Fresh	59	58 (98.3)[b]	41 (69.5)[b]
Total	**110**	**64 (58.2)**	**49 (44.5)**

* Response to exogenous hCG as determined by granulose cell expansion and recovery.
[a-b] Values within columns with different superscripts are different (P<0.05).

from live mares were reported the same year in the United States and in Australia (Cochran *et al.* 1998a,b; McKinnon *et al.* 1998). After ICSI, embryos are most often surgically transferred at the 2- to 4-cell stage into the oviducts of suitable recipients (Cochran *et al.* 2000), as *in vitro* culture has still not been perfected for IVF-derived equine embryos. Shortly thereafter, a twin ICSI pregnancy was produced at Louisiana State University from the transfer of 2 sperm-injected equine oocytes harvested via TUGA from Altrenogest-treated mares (Cochran *et al.* 1999b). One of the fetuses was manually eliminated and the remaining ICSI pregnancy produced a healthy live foal.

One of the limiting factors when using the TUGA technique is the operator's ability to recover the oocyte from the follicle. To obtain *in vivo*-matured oocytes from the mare, exogenous hCG is generally used to induce the ovulatory process, with oocyte aspiration occurring ~24 h post hCG administration. Using hCG to enhance ovulation has been a standard veterinary practice and research procedure for years. The question then arises, how many times can a mare be subjected to this hCG/oocyte collection procedure, both within a season and over a period of years. In a recent study at this station (Hylan, D. *et al.* unpublished data), the oocyte recovery rate was 45.5% from TUGA for a group of mixed breed cyclic mares (n=20) that were treated with hCG once they had a pre-ovulatory follicle >33 mm in diameter. The hCG and TUGA regimen was executed on each mare during each oestrous cycle during the summer and early autumn months. When the data from the chronically treated donor mares (hCG given multiple times a season for 4 or more consecutive years) were separated from the mares that had not been chronically exposed to multiple hCG treatments, the oocyte recovery rates were then 15.7% compared with 69.5%, respectively (Table 1). The results indicate that oocyte production is reduced markedly in mares repeatedly subjected to this treatment procedure for consecutive breeding seasons. These findings suggest that chronically hCG-treated mares should not be the first choice for oocyte collection using follicle aspiration. It remains unclear if this response is due to mares becoming refractory to hCG or whether the aspiration procedure itself contributes to this decline.

The use of TUGA of oocytes and IVF procedure does offer an alternative to horse owners who have genetically valuable mares that for some reason are unable to produce viable embryos through standard embryo collection procedures. This technology can be used on oocytes harvested from older ovulating or non-ovulating mares, females with physical injuries (eg leg injury) and problem mares which have uterine anomalies. At this station, oocytes are being harvested from early post partum (<15 days) beef and dairy cattle, before the female begins cyclic activity (Perez *et al.* 2001). The approach allows the opportunity to produce one or more extra calves from the cow before she is mated to produce a natural pregnancy. Correspondingly, the combination of TUGA and ICSI procedures should not be overlooked to collect oocytes from early post partum mares (<15 days post foaling) and produce extra ICSI embryos for recipient transfer.

One problem in harvesting oocytes for *in vitro* embryo production is the seasonality of mares. A second problem with the larger farm animal donor females is that their gestation intervals are lengthy in comparison with those of smaller domestic mammals, and that these donor females are out of *in vivo* embryo production during their gestation. However, it has been reported that in both cows and mares follicle wave development continues during early to mid-gestation. With this in mind, it was deemed logical to take advantage of having these developing follicles available for oocyte

harvest during gestation and thus, an attempt was made to collect oocytes from these females using TUGA during early pregnancy (Meintjes *et al.* 1994, 1995a,1995b, 1996).

The main concern was whether the oocyte aspiration procedure would affect the ongoing pregnancies. TUGA was successfully executed during the first trimester in both pregnant cows (Meintjes *et al.* 1995b) and pregnant mares (Meintjes *et al.* 1994, 1995a, 1996, 1997b), and this aspiration procedure produced no conceptus loss during gestation. The first offspring produced from oocytes collected by TUGA from pregnant donor animals resulted from cows (Meintjes *et al.* 1995b) and horses (Cochran *et al.* 1998a) at this station. This procedure has produced live ICSI foals from both cyclic and pregnant mares at this station (Cochran *et al.* 1998a,1998b, 2000). Generally, pregnant donors were found consistently to produce more oocytes per collection (≥35%) than similar cyclic females. When using TUGA for follicle reduction in pregnant mares, synchronised follicle growth resulted in more uniform oocytes at collection (Meintjes *et al.* 1997b).

When using pregnant mares as oocyte donors, there is often a lack of cyclic females to use as embryo recipients. It should not be overlooked, however, that hormone-supplemented mares have been used successfully as embryo recipients. Recent reports from CSU (Carnevale *et al.* 1999) and Texas A&M University (Hinrichs *et al.* 2000) verify that non-cyclic mares can be used successfully for oocyte transfer and gamete intrafallopian transfer (GIFT). At this station, daily administration of equine somatotropin (eST) has been used on cyclic mares to increase the number of small to medium-size follicles for TUGA procedures (Cochran *et al.* 1999a). In subsequent trials, the administration of eST to seasonally anovulatory mares increased the development of small to medium-size follicles in anoestrous mares (Cochran *et al.* 1999c). When seasonally anoestrous mares were administered daily with eST followed by administration of a GnRH agonist, the mares developed small to medium-size follicles of which at least one follicle progressed to an ovulatory size. Based on recent studies at this station, equine oocytes harvested by TUGA from both medium-sized and ovulatory-sized follicles of cyclic and pregnant mares are potentially viable for *in vitro* embryo production.

SUMMARY

Currently, transvaginal ultrasound-guided oocyte aspiration is being used to harvest valuable oocytes from domestic females representing rare bloodlines, clinically infertile females and reproductively senescent mares. Research continues to find applications for this technology, including harvesting oocytes from prepubertal fillies and early post partum mares for *in vitro* embryo production. The use of ultrasound-guided oocyte aspiration should not be overlooked to obtain oocytes for *in vitro* embryo production. Also, more oocytes are often harvested from early pregnant mares (up to 150 days of gestation) than from cyclic mares. Although only a small number of test tube foals have been produced to date, the ICSI procedure appears to be the method of choice to produce IVF horse embryos at the present time.

There is still much to be studied and learned in the use of assisted reproductive technologies to maximise reproductive potential in genetically valuable animals. Now that repeatable oocyte retrieval methods are being fine-tuned, it is likely these procedures will become used routinely to obtain oocytes for further gamete and embryo research and also by seedstock producers for *in vitro* embryo production from horses in the commercial sector.

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SESSION 5

Chairman:
Jonathan Hill

FACTORS ASSOCIATED WITH THE OOCYTE, SPERM AND OVIDUCT AFFECTING SUCCESS OF OOCYTE TRANSFER

E. M. Carnevale, L. J. Maclellan, M. A. Coutinho da Silva and E. L. Squires

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

Use of oocyte transfer has expanded our understanding about sperm and oocyte interactions within the mare's oviduct. These findings are based on our ability to manipulate factors affecting recipients, oocytes and sperm when using assisted reproductive techniques versus natural ovulations. Objectives were to review recent results in commercial and experimental oocyte transfer and to examine factors that affected success.

Because oocytes are transferred into the recipient's oviduct, mare factors associated with fertilisation and embryo development can be studied. Oocytes can be transferred into different types of recipients. Transfer of oocytes into ovulating recipients allows exposure to natural changes in hormonal concentrations. When donor oocytes were transferred into the contralateral oviducts of ovulating recipients, embryo development rates were not different for the recipients' own oocytes versus transferred oocytes (11/16, 69% and 28/39, 72%, respectively). Results demonstrated that procedures and techniques used for oocyte transfer are effective, with embryo development rates being the same for ovulated versus transferred oocytes. Aspiration of a recipient's pre-ovulatory follicle results in the removal of the recipient's oocyte, preventing fertilisation of her oocyte. However, during aspiration of a follicle, follicular fluid and numerous granulosa cells are also removed. Loss of granulosa cells may delay or reduce CL function; therefore, progesterone supplementation is used. Transfer of oocytes into recipients allowed to ovulate their own follicle versus mares in which the pre-ovulatory follicle was aspirated resulted in similar embryo development rates (28/39, 72% versus 18/26, 69%, respectively). In the commercial oocyte transfer programme at CSU, non-cyclic and cyclic recipients have been used. Non-cyclic recipients were treated with exogenous steroids; estrogen for approximately 7 days before transfer and progesterone after transfer; and cyclic recipients had their oocyte removed by follicular aspiration. Use of non-cyclic versus cyclic recipients resulted in similar pregnancy rates (72/194, 37% and 19/63, 30%, respectively). Results suggest that presence of a pre-ovulatory follicle or follicular fluid is not required for fertilisation, that fertilisation occurs in the oviduct contralateral or ipsilateral to the pre-ovulatory follicle, and that the hormonal requirements for fertilisation and early embryo development can be replicated in non-cyclic mares.

Oocyte transfer has provided a means to examine more closely the oocyte and factors associated with oocyte viability and fertility. Most oocytes are collected from pre-ovulatory follicles 0 – 16 h before expected ovulation or 20 – 36 h after administration of hCG to the donor. Oocytes, collected within a few hours pre-ovulation (36 h), were transferred in less than 2 h into a recipient's oviduct. Oocytes collected at approximately 24 h after hCG were cultured *in vitro* for about 14 h before transfer or transferred into recipients' oviducts for completion of maturation. Embryo development rates, combined from different experiments, were 78% (18/23), 66% (27/41) and 43% (6/14) for oocytes collected at 36 h or at 24 h and matured *in vitro* or within the oviduct, respectively. Results demonstrated that collection of oocytes 24 – 36 h after hCG administration to donors can lead to successful embryo development after oocyte transfer and that the final stage of oocyte maturation can occur within the oviduct.

Insemination of oocyte recipients with an adequate number of semen from a fertile stallion is essential to maximise success. Recipients are often inseminated approximately 12 h before and 2 h after

transfer. Insemination before and after transfer ensures that adequate numbers of sperm are present to maximise fertilisation rates. In recent studies, recipients inseminated with fresh semen from fertile stallions only before or after transfer had good embryo development rates (82%, 9/11 and 57%, 8/14). In ongoing research, recipients were inseminated before and after oocyte transfer with cooled semen from different stallions. Resulting embryos were collected and submitted for parentage testing. Of 16 collected embryos, 15 resulted from insemination before transfer, and one from insemination after transfer. This confirms that more oocytes are fertilised from insemination before than after transfer and suggests that a second insemination is probably not required when good semen from fertile stallions is used. In the commercial oocyte transfer programme, oocytes were obtained from older, subfertile mares; and semen was obtained from different stallions of variable fertility. Most semen was cooled and transported. Pregnancy rates were different ($P<0.05$) for recipients inseminated before or before and after transfer versus insemination only after transfer (18/45, 40%; 27/53, 51% versus 0/12, respectively) during the breeding season in 2000. Results in 2001 were similar, although no recipients were inseminated only after transfer. Pregnancy rates for insemination before transfer versus before and after transfer were not significantly different (15/55, 27% and 13/34, 38%). Although pregnancy rates were not significantly different when recipients were inseminated before versus before and after oocyte transfers, those inseminated before and after transfer had an 11% increase in pregnancy rates, suggesting insemination before and after transfer could optimise commercial results when using variable semen with less than optimal fertility.

During gamete intrafallopian transfer (GIFT), oocytes and sperm are transferred into the oviduct. The first successful oocyte transfer occurred approximately 10 years earlier, but GIFT was not successful in the mare until 1998. After intrauterine insemination, the mare's reproductive tract influences sperm transport, selection and storage. During GIFT, these factors are modified when sperm are placed directly into the oviduct. In our laboratory, oocytes and sperm are transferred into the oviduct contralateral to the recipient's ovulation. Therefore, The developing corpus luteum can provide hormonal support without fertilisation of the recipient's oocyte. Embryonic vesicles resulting from GIFT have been parentage tested, and no vesicles from 15 recipients resulted from the recipient's ovulation. Therefore, movement of sperm to the contralateral oviduct did not occur. In previous research, fresh sperm were used, and embryo development rates after intraoviductal insemination with 500,000 sperm were compared with intrauterine insemination after oocyte transfer. Embryo development rates were not different between groups (3/11, 27% and 57%, 8/14). In a subsequent study using fresh semen, insemination within the oviduct with 200,000 fresh sperm versus intrauterine insemination before and after oocyte transfer resulted in similar embryo development rates (12/22, 55% and 17/26, 65%, respectively). However, in recent results demonstrated that the use of treated sperm had a deleterious effect on the success of GIFT. Embryo development rates after GIFT using fresh semen were significantly higher than after GIFT using cooled or frozen semen (12/18, 67%, 4/31, 13% and 1/12, 8%). Results of GIFT confirm that capacitation of sperm can occur within the equine oviduct. The transfer of fresh sperm into the oviduct consistently resulted in good embryo development rates. However, the transfer of cooled or frozen sperm into the oviduct resulted in low embryo development rates. Therefore, some factor or factors associated with the cooling or freezing of sperm had a deleterious effect on the ability for fertilisation to occur. Sperm viability or longevity could have been affected by sperm treatments. However, components of sperm extenders potentially could bind with sperm and influence results after transfer within the oviduct. Further research is needed to determine the specific factors affecting reduced embryo development rates when cooled or frozen sperm are placed within the oviduct.

Assisted reproductive techniques, such as oocyte transfer, have provided new information about requirements for successful interactions of the oocyte, sperm and oviduct. Results of studies demonstrated that the presence of a pre-ovulatory follicle and follicular fluid were not required for fertilisation, and fertilisation of oocytes was as likely to occur in the oviduct contralateral as ipsilateral to the pre-ovulatory follicle. When semen from fertile stallions was used, intrauterine insemination of recipients only before oocyte transfer resulted in high embryo development rates. Intraoviductal insemination of recipients was successful when fresh semen was used; however, the use of cooled or frozen semen resulted in reduced embryo development rates.

EMBRYO METABOLISM AND IMPLICATIONS FOR CULTURE REQUIREMENTS

D. Rieger

Animal Biotechnology Embryo Laboratory, Department of Biomedical Science, University of Guelph, Guelph, Ontario, Canada, N1G 2WI

Early embryonic development is marked by relatively rapid cell division, differentiation, and changes in cell function, all of which require ATP, reducing equivalents, and precursors for synthesis of macromolecules. For example, in cattle and horse embryos, total nuclear DNA increases 100 fold or more during the 7 days of development from the zygote to the blastocyst stage.

PATHWAYS OF CARBOHYDRATE METABOLISM

As shown in Figure 1, 3 major biochemical pathways are involved in carbohydrate metabolism; the Embden-Meyerhof pathway (EMP) of anaerobic glycolysis, the pentose-phosphate pathway (PPP), and the mitochondrial Krebs cycle of oxidative metabolism. Glucose is first phosphorylated to glucose-6-phosphate and then enters either the PPP or the EMP. In the PPP, carbon-1 of glucose is removed as CO_2 to yield ribose-5-phosphate for nucleotide synthesis, and

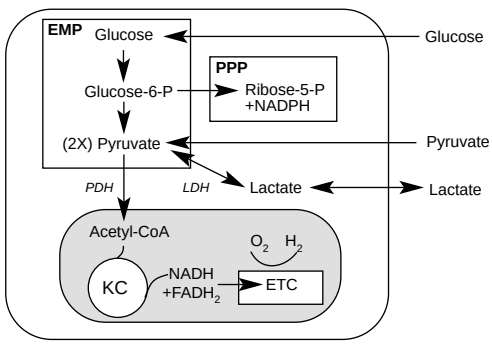

Fig 1: A highly simplified schematic of the pathways of carbohydrate metabolism. Arrows indicate one or more reactions. The heavy line represents the cell membrane and the shaded area represents the mitochondrion.

reducing equivalents (hydrogen atoms) are transferred to NADP to produce NADPH for the synthesis of lipids and other macromolecules. Complete metabolism of glucose-6-phosphate through the EMP yields two pyruvate molecules per molecule of glucose, but EMP intermediates are also used as precursors for synthetic processes. Examples are fructose-6-phosphate for glycosylation of proteins and lipids, and glycerol-3-phosphate for triglyceride synthesis. Pyruvate can be used for amino acid synthesis, decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH), or reduced to lactate by lactate dehydrogenase (LDH). Acetyl-CoA can be metabolised through the Krebs cycle or used for the synthesis of lipids and amino acids. Within the Krebs cycle, the carbon atoms of acetyl-CoA are released as CO_2 and hydrogen atoms are transferred to NAD and FAD for the reduction of O_2 to H_2O by the electron transport chain. Anaerobic metabolism of glucose through the EMP produces only 2 ATP per molecule of glucose, compared with 38 ATP per molecule of glucose produced by the complete oxidative metabolism. However, EMP metabolism occurs in the complete absence of oxygen, and is capable of both rapid increase and high throughput compared to oxidative metabolism.

PATTERNS OF EMBRYO METABOLISM

Studies of embryo metabolism though the 1960s and 1970s showed that mouse and rabbit embryos could metabolise glucose, pyruvate, lactate and other substrates, that the PPP was very high relative to most somatic tissues, and that the culture medium could affect metabolic patterns. Most notably, it was found that glucose alone could not support early embryonic development,

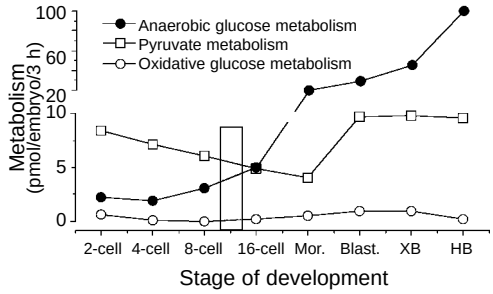

Fig 2: Glucose and pyruvate metabolism by the early cattle embryo. The shaded vertical bar indicates the time of major activation of the embryonic genome. (From Rieger et al. *1992b)*

which led to the concept of a block to glycolysis, possibly due to citrate inhibition of phosphofructokinase, a key regulatory enzyme of the EMP (Rieger 1984). In a landmark paper, Leese and Barton (1984) described a microfluorometric technique for the measurement of glucose and pyruvate uptake by individual mouse embryos, and showed that pyruvate uptake was much greater than glucose uptake until the 8-16 cell stage and then decreased, while glucose uptake increased markedly from the morula to blastocyst stage. This led to the concept of a metabolic switch from pyruvate to glucose metabolism at the morula stage

More recently, using radiolabelled substrates, a similar pattern was shown in cattle embryos in which the oxidative metabolism of pyruvate and glutamine predominated during the early cleavage stages and then declined to a minimum at the morula stage (Fig 2; Rieger *et al.* 1992a,b). Metabolism of glucose through the EMP and the PPP was low and then increased significantly at the 8-16 cell stage, the time of major activation of the embryonic genome. In contrast to the observations of Leese and Barton (1984) in the mouse embryo, it was found that pyruvate and glutamine metabolism, as well as glucose, increased markedly through development, expansion, and hatching of the blastocyst. This is likely to be related to the high energy demands of the maximally-expanding cattle blastocyst.

Interestingly, it was found that little or no glucose carbon was metabolised through the Krebs cycle by the cattle embryo, at any stage. Latter studies showed that most or all the glucose taken up by mouse, sheep and cattle embryos is converted to lactate and released into the surrounding medium (Gardner *et al.* 2000). The reasons for this apparently inefficient waste of glucose carbon are unclear. Gardner *et al.* (2000) suggest that it may serve to regenerate NAD^+ for subsequent use in anaerobic glycolysis. At the same time, the removal of lactate (and hence pyruvate) would prevent product inhibition of the EMP and allow it to operate maximally.

The metabolism of the oocyte is coupled to the surrounding cumulus cells. Pyruvate can support the maturation of denuded oocytes, while glucose can support maturation only if the cumulus cells are present (Biggers *et al.* 1967). *In situ*, glucose is metabolised to pyruvate by the surrounding cumulus cells, and the pyruvate is passed to the oocyte through gap junctions between processes of the cumulus cells and the oolemma (de Loos *et al.* 1991). Pyruvate metabolism by denuded cattle oocytes increases significantly during maturation, while anaerobic glucose metabolism is low and constant (Rieger and Loskutoff 1994).

Embryo development can be affected by direct or indirect modification of energy metabolism. The energy substrate composition of oocyte maturation medium affects subsequent development of cattle embryos (Rose-Hellekant *et al.* 1998). Exposure of early embryos of many species to high levels of glucose can block development, usually at the time of major activation of the embryonic genome (see Rieger 1992). Co-culture with bovine oviductal epithelial cells improves the development of cattle embryos, most likely by depleting the culture medium of glucose and increasing the concentration of lactate (Rieger *et al.* 1995; Edwards *et al.* 1997). The inhibition of 3-phosphoglycerate kinase, and hence anaerobic glucose metabolism, permits development of mouse zygotes through the 2-cell block (Lane and Gardner 2001). Inhibition or uncoupling of oxidative metabolism from Day 5 to Day 7 improves the development of cattle embryos (Thompson *et al.* 2000).

METABOLIC ACTIVITY OF THE HORSE EMBRYO

Glucose metabolism by the horse embryo increases 55 fold from Day 4.5 to Day 7.5, more than doubling when corrected for embryo volume (Brück and Hyland 1991). During the rapid expansion of the horse blastocyst on Day 6.5 to 7.5, there is no significant change in pyruvate uptake but glucose uptake increases 6 fold (Lane *et al.* 2001) and glucose metabolism increases by as

much as 30 fold (Rieger *et al.* 1987). Glucose uptake and metabolism by the horse blastocyst is approximately 5 to 10 times greater than that of a cattle blastocyst of the same size. In contrast to the embryos of cattle and other species, glucose carbon is metabolised through the Krebs cycle by the horse blastocyst (Rieger *et al.* 1987). This probably reflects the energy requirements of Na^+-K^+ ATPase necessary for expansion of the blastocoelic cavity, as well as the requirements for cell division. The high rate of glucose uptake by the horse blastocyst may also be required for conversion to fructose, which is sequestered in blastocoelic fluid (Ruddock *et al.* 2000)

Conclusions

In general, embryo development is improved by culture in media that reflect their metabolic potential. Glucose concentrations should be low compared to pyruvate and lactate for the culture of the early cleavage stages, but increased concentrations of glucose are required during blastocyst development and expansion. The very high rate of glucose uptake and metabolism by the horse blastocyst suggests that higher concentrations of glucose may be required than are used for embryos of other species. Studies of the metabolism of cleavage stage horse embryos are required to determine the optimal substrates and concentrations for development from the oocyte to the morula stage.

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VITRIFICATION OF EQUINE OOCYTES COLLECTED FROM SLAUGHTERHOUSE OVARIES OR BY TRANSVAGINAL ASPIRATION OF MARES

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

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

The ability to cryopreserve oocytes is critical to widespread application of reproductive technologies, and for preservation and maintenance of genetic resources. Establishing efficient methods of oocyte cryopreservation poses difficulties because of specific features of oocytes, including the high volume:surface ratio, the surrounding zona pellucida and the layers of cumulus and granulosa cells that form the cumulus oocyte complex (Le Gal and Massip 1999). Despite increased efforts in cryopreservation of oocytes, results are far from acceptable for commercial purposes. Traditionally, cryopreservation techniques involved low concentrations of cryoprotectants and slow freezing techniques. Alternative methods using vitrification have focussed on a variety of cryoprotectants, incubation times and stages of oocyte maturation (Vatja 2000).

Specific problems associated with cryopreservation of oocytes at different meiotic stages of maturation have been reported in the literature. In ovulated oocytes, scattering of chromosomes due to microtubule clumping or loss (Sathananthan *et al.* 1988), spindle disorganisation (Martino *et al.* 1996), and decreases in fertilisation rates (Fuku *et al.* 1995) have all been reported. Oocytes cryopreserved at the germinal vesicle (GV) stage have shown ultrastructural changes including modifications to microvilli, ooplasm, mitochondria and vesicle formation (Fuku *et al.* 1995). Cryopreservation of oocytes at different stages of maturation *in vitro* merits further studies to overcome problems associated with chilling oocytes, including disruption of metaphase spindle integrity.

Based on these data 2 experiments were designed for the vitrification of equine oocytes collected from slaughterhouse ovaries or by transvaginal aspiration of mares.

In the first study, sucrose or trehalose were added to a vitrification solution containing ethylene glycol (EG) and dimethylsulphoxide (DMSO). The addition of macromolecules, such as sugars, to the cryoprotectant solution was an attempt to alleviate the toxicity of the high concentrations of cryoprotectant within the vitrification solution.

Oocytes were vitrified at 2 stages of nuclear maturation. Oocytes collected April to July from slaughter house ovaries were matured in Equine Maturation I (EMMI), a modification of G2 (Gardner and Lane 1999) with: 0.32 mM Na pyruvate, 10.5 mM Na lactate, 0.4 mM cystine, 0.1 mM taurine, 0.4 mM cysteamine, 10 ng/ml IGF, 100 ng/ml EGF, MEM vitamins, 8 mg/ml BSA, 10 ug/ml streptomycin, 1 µg/mL LH, 15 ng/mL FSH, 1 µg/ml E_2 and 500 ng/mL P_4 without HSA or phenol red. Oocytes were matured for 12 h or 24 h before being partially denuded of cumulus and vitrified. Cryoprotectants were loaded in 3 steps: 5% DMSO and 5% EG for 30 s, 10% DMSO and 10% EG for 30 s, and 20% DMSO and 20% EG with either 0.65 M sucrose or 0.65 M trehalose for ~20 s, before being loaded onto a nylon cryoloop (Lane *et al.* 1999). Oocytes were thawed using 3-step dilution with 0.250 M, 0.188 M and 0.125 M sucrose or trehalose. Oocytes in 12 h and 24 h groups were cultured in EMMI for 14 h and 2 h post thaw, respectively, before undergoing ICSI.

Frozen semen was prepared over a discontinuous Percoll gradient. Denuded oocytes were injected with a single spermatozoon. Non-vitrified oocytes matured for 26 h were used as controls. All oocytes were activated with 10 µM ionomycin for 5 min and cultured for 20 h before being fixed. Zygotes were stained with aceto-orcein to assess pronucleus formation.

TABLE 1: Effects of sugar and time after onset of maturation on zygote development

	No. vitrif	No. injected	No. polar bodies	No. 2PN	No. 1PN	No. dead	No. >2PN	Un-readable
Control	0	34	25[a]	18[a]	8	4	2	2
12 h sucrose	50	32	17	16[a]	4[a]	8	1	3
12 h trehalose	47	30	11[b]	13[a]	7	5	2	3
24 h sucrose	54	34	21	14[a]	13[b]	4	0	3
24 h trehalose	49	36	26	15[a]	7[a]	9	0	5
Shams	0	15	11[a]	0[b]	9	4	0	2

Values without common superscripts differ ($P < 0.05$, χ^2)

Oocytes vitrified in trehalose at 12 h had lower rates of PB formation than the controls. Fertilisation rates, assessed by formation of 2 pronuclei, were similar for control and vitrified oocytes. Oocytes vitrified in sucrose at 24 h had more single pronucleus formation than in trehalose at 24 h and sucrose at 12 h, but no treatment differed from controls. There was no evidence of fertilisation from the activation of non-injected oocytes. Equine oocytes vitrified in either sucrose or trehalose at 12 or 24 h evaluated by ICSI appear viable, but transfer of zygotes will be needed for accurate assessment.

In Experiment 2, vitrified and thawed or control oocytes were transferred into inseminated recipient mares. Light-horse mares (n=33) between 3 and 15 years of age were used for the study. Ovarian follicular activity was monitored by transrectal ultrasonography. When mares demonstrated relaxed uterine and cervical tone, uterine oedema and a follicle ≥ 35 mm in diameter, 2,000 iu hCG were injected iv to initiate follicular and oocyte maturation. Oocytes were collected by transvaginal, ultrasound-guided follicular aspiration 24 to 26 h after administration of hCG. Oocytes were cultured in equine maturation medium (EMM1) for 2 to 4 h before vitrification. Cryoprotectants were loaded as per Experiment 1. Upon thawing, oocytes were cultured for 10–12 h in EMM1 before being transferred to inseminated recipient mares. Non-vitrified oocytes cultured for 14–16 h in EMM1 were used as controls.

Of the 38 oocytes collected from mares, 26 were vitrified and 12 were transferred as controls. After thawing, 19 of the 26 oocytes were morphologically intact and transferred into recipients resulting in 3 embryonic vesicles on Day 16. After reduction of one of the twin vesicles, the recipient maintained an ongoing pregnancy from the vitrified oocyte. With control transfers, 10 of the 12 oocytes resulted in embryonic vesicles on Day 16. Two healthy foals were born from the transfer of vitrified oocytes after 326 and 328 days of gestation, respectively.

This study resulted in the first foals to be produced from the transfer of vitrified and thawed equine oocytes. Although viability of vitrified oocytes was significantly lower than control oocytes, the establishment of pregnancies and birth of foals from cryopreserved oocytes is a significant advance in developing methods to preserve equine gametes.

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TECHNIQUES FOR ACHIEVING FERTILISATION *IN VITRO* IN THE HORSE

Y. H. Choi, C. C. Love, L. B. Love, D. D. Varner and K. Hinrichs

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

Research on conventional *in vitro* fertilisation (IVF) in the horse has been conducted throughout the last decade, with low success (4–33%). Only 2 foals have been produced by conventional IVF, both from fertilisation of *in vivo* matured oocytes (Palmer *et al.* 1991; Bezard 1992). Many methods of sperm capacitation for IVF have been tested in the horse. Essentially, the only capacitation methods that have been successful have been the use of calcium ionophore, or, in frozen sperm, heparin (Dell'Aquila *et al.* 1997). Though these treatments have been reported to allow moderate fertilisation rates (< 40%), they have not been repeatable by other laboratories, or even within laboratories. The problem with fertilisation is overcome *in vivo*, as fertilisation rates are high (70%) when *in vitro*-matured oocytes are transferred to the oviducts of inseminated mares for fertilisation and recovered 40–44 h after transfer (Love *et al.* 2000).

The major barrier to fertilisation *in vitro* appears to be the penetration of sperm through the zona pellucida. When the zona was dissected, drilled or completely removed, excellent IVF rates (52–86%) were obtained (Choi *et al.* 1994; Li *et al.* 1995). Both zona dissection and zona drilling require micromanipulation, so these are labour-intensive techniques. Unfortunately, the higher fertilisation rate associated with partial or total zona removal was accompanied by a high rate of polyspermy (22–57%). Methods for sperm capacitation affected fertilisation rates even when the zona was artificially breached. The concentration of calcium ionophore used to capacitate sperm significantly affected cleavage rates in zona-drilled oocytes (0–79%; Li *et al.* 1995). Furthermore, sperm treatment with different macromolecules (polyvinylalcohol vs. bovine serum albumin) during capacitation and fertilisation affected fertilisation rates of partially zona-removed oocytes (53 vs. 11%; Choi *et al.* 2000). Fertilised embryos produced from partially zona removed or drilled oocytes could develop to the blastocyst stage in *in vitro* culture (Li *et al.* 1995; Choi *et al.* 2001).

To circumvent the need for sperm to penetrate the zona, several researchers have worked with intracytoplasmic sperm injection (ICSI). With this technique, one sperm is injected through the zona into the cytoplasm of the oocyte. ICSI has proven to be a much more successful method of fertilisation than is IVF in the horse with 21–68% fertilisation and 20–65% cleavage without polyspermy. Subzonal injection of sperm (SUZI) has been reported in the horse, but with significantly lower rates of embryo formation than was achieved with ICSI (6% vs. 39%; Meintjes *et al.* 1996). Embryos produced from ICSI have developed to blastocysts during *in vitro* culture (Dell'Aquila *et al.* 1997; Maclellan *et al.* 2000; Li *et al.* 2001), and foals have been produced both by transfer of ICSI zygotes to the oviduct (Squires *et al.*, 1996; Cochran *et al.*, 1998; McKinnon *et al.*, 2000) and by transcervical transfer of *in vitro*-cultured blastocyts to recipients (Li *et al.* 2001). There is some disagreement on the necessity for chemical activation after ICSI. Some workers have found low activation rates after ICSI unless oocytes receive additional activation stimulus, while others have found equivalent rates without additional stimulus. The requirement for activation appeared to be related to the sperm source used for ICSI, as laboratories using frozen-thawed sperm were more likely to report good fertilisation without additional activation stimulus than were laboratories using fresh sperm.

The authors hypothesised that frozen-thawed sperm may activate oocytes more readily because

the membrane damage induced during freezing potentiates the release of activation factors from the sperm. Fertilisation rates were compared after ICSI using fresh and frozen-thawed sperm from the same stallion. A Piezo drill was used to penetrate the zona, immobilise sperm before injection and to break the oocyte plasma membrane. There was no significant difference in normal fertilisation (71% and 60%) or cleavage rates (72% and 55%) for oocytes injected with fresh or frozen-thawed sperm, respectively. However, it is possible that the release of sperm activating factors was equalised among treatments by the use of the Piezo drill. The Piezo technique may break the sperm plasma membrane more efficiently before injection than does the conventional ICSI procedure of scoring the sperm tail with the injection pipette or rolling the sperm against the bottom of dish before injection. While normal fertilisation rates were high in this study, there were an additional 20–33% of incompletely activated oocytes which had metaphase III, telophase, condensed or pronuclear oocyte chromatin with individualised sperm chromosomes or a decondensing sperm head. For these incompletely activated oocytes, additional activation treatment may have allowed the oocyte to complete full activation and develop.

While work on answering the poor fertilisation rates associated with conventional IVF in the horse should continue, establishment of ICSI as a repeatable and efficient method for fertilisation of horse oocytes now offers a method to evaluate developmental competence of horse oocytes. In addition, ICSI can provide large numbers of horse embryos for study of requirements for equine embryo development *in vitro.*

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SESSION 6

Chairman:
Ed Squires

DEVELOPING THE INTRACYTOPLASMIC SPERM INJECTION (ICSI) PROCEDURE FOR THE HORSE

M. Sansinena, D. Paccamonti*, R. Cochran, M. Meintjes, B. Reggio, R. S. Denniston, C. Pinto*, B. Eilts* and R. A. Godke

*Department of Animal Science, Louisiana State University Agricultural Center and *Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, Louisiana 70803, USA*

In vitro fertilisation (IVF) of mammalian oocytes has been of interest to scientists for over a century. Successful IVF in humans was first reported in 1978 (Steptoe and Edwards 1978) and use of assisted reproductive technology for treatment of human infertility has increased dramatically since then (Fig 1). Successful IVF in horses was not reported until 1991 (Palmer *et al.* 1991). Attempts by many others to produce IVF foals have not been successful, making it clear that more research was needed before progress could be made in horses.

The reasons for the low success rate of equine IVF remain unclear. Equine oocytes have a thick zona pellucida compared with other species and *in vitro* maturation of these oocytes takes longer than other domestic farm animal species (Hinrichs *et al.* 1993). The thick zona pellucida of *in vitro*-matured equine oocytes may act as a barrier to sperm cells prepared *in vitro* (Li *et al.* 1995). Zona pellucida hardening, defined as an increased resistance to protease digestion when cultured in serum-free medium, has previously been described in mouse and rat oocytes (De Felici and Siracusa 1982; Zhang *et al.* 1991), and more recently has been reported to occur in horse oocytes (Fusco *et al.* 1998). The potentially altered zona pellucida of *in vitro*-matured oocytes (Chan 1987; Cohen *et al.* 1990), in addition to less than adequate sperm cell preparation, is likely to contribute to poorer than expected IVF rates in the horse. Thus, interest turned to other approaches to assist fertilisation (eg zona drilling, subzonal sperm injection, intracytoplasmic sperm injection) that had been developed for laboratory animals.

Live offspring have been produced in laboratory animals (Gordon and Talansky 1986) and humans (Cohen *et al.* 1991, 1992) by using pre-fertilisation zona drilling procedures. Perforating the zona pellucida (eg renting, drilling) to enhance fertilisation was first used on equine oocytes by Choi *et al.* (1994a) but the size of the opening, the concentration of sperm cells and the time of exposure of the zona-drilled oocytes were found to alter expected fertilisation rates (Choi *et al.* 1994b).

In an attempt to reduce the incidence of polyspermy in humans, subzonal sperm injection (SUZI) and intracytoplasmic sperm injection (ICSI) procedures were evaluated in human infertility units. However, because of its increased

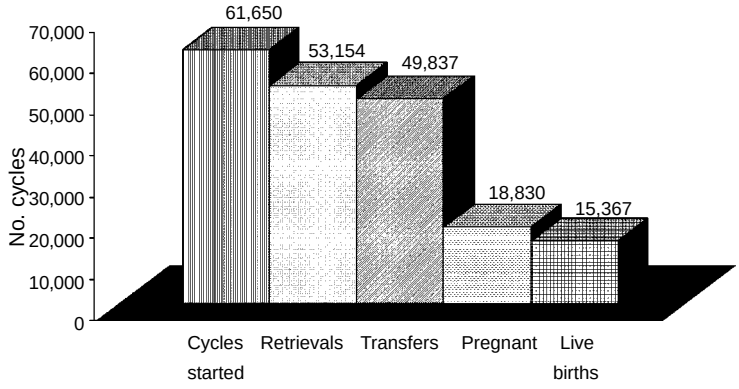

Fig 1: Outcome of human ART cycles using fresh, non-donor oocytes or embryos (Centers for Disease Control [CDC] data from 1998).

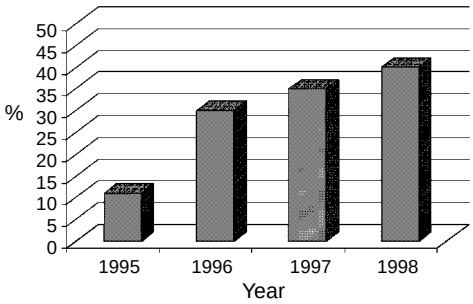

Fig 2: Percent of sperm insertion procedures in humans performed by intracytoplasmic sperm injection (CDC data).

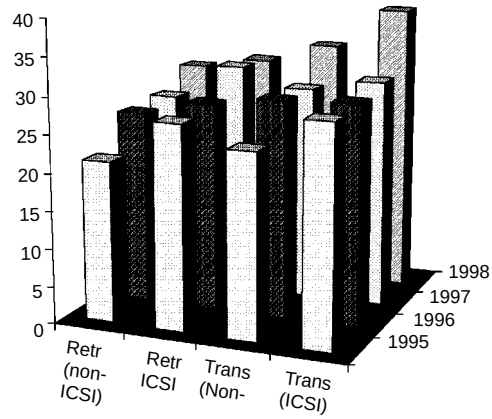

Fig 3: Success rates as measured in live births per retrieval (Retr) and live births per transfer (Trans) for fresh, non-donor ART cycles among human couples with male factor infertility (1995 – 1998 CDC data).

efficiency in eliminating polyspermic fertilisation, ICSI has become preferred over the SUZI procedure (Palermo *et al.* 1993). Direct injection of sperm into the ooplasm has been used extensively in sea urchin (Hiramoto 1962) and frog *(Xenopus laevis)* research (Brun 1974). The first experiments in mammals were conducted in rodent species, although with variable success. Formation of the male pronuclei and subsequent early cleavage was first achieved in the hamster (Uehara and Yanagimachi 1976). Because the hamster oocyte allows for pronuclear formation with sperm from a multitude of different species, it has been a useful research tool for the development of ICSI techniques for other animals. Live births from microinjected oocytes were first reported by Hosoi *et al.* (1988) in the rabbit, followed with oocytes from cows (Goto *et al.* 1990), mice (Roknabadi *et al.* 1994) and sheep (Catt *et al.* 1996), The advent of ICSI has increased the potential of assisted reproductive technologies to propagate mammalian species, with one of the best examples being man. In humans, ICSI was first used as a method for treating male factor infertility (Palermo *et al.* 1992; Ng *et al.* 1993). Other methods of human sperm insertion have largely been abandoned in favour of ICSI, which has increased from 11% of the IVF procedures in 1995 to 40% of the cases in 1998, and to >70% in some infertility units today (Fig 2).

When ICSI and SUZI procedures were evaluated on oocytes from mares, cleavage rates were found to be higher for ICSI (Meintjes *et al.* 1996). In a relatively short period, pregnancies produced with ICSI using oocytes from abattoir ovaries (Squires *et al.* 1996), non-pregnant mares (Meintjes *et al.* 1996; McKinnon *et al.* 1998) and

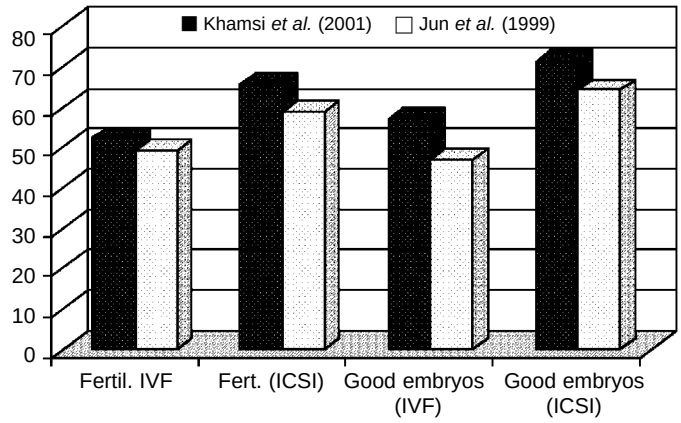

Fig 4: Fertilisation rate and percent of embryos classified as 'good' after ICSI or IVF in humans in cases not due to male factor infertility (from Jun et al. *1999; Khamsi* et al. *2001).*

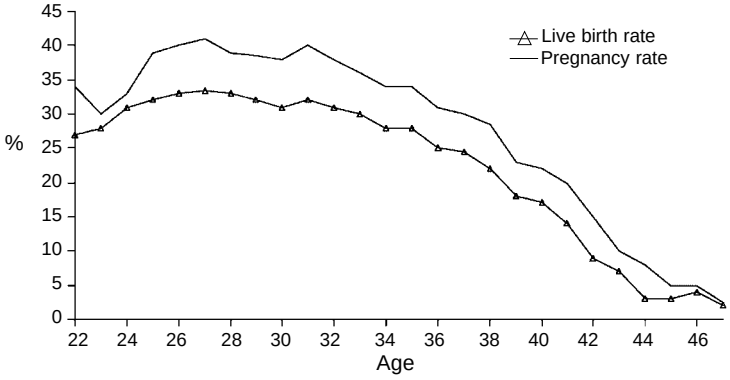

Fig 5: Pregnancy and live birth rates for fresh, non-donor ART cycles by age of woman (1998 CDC data).

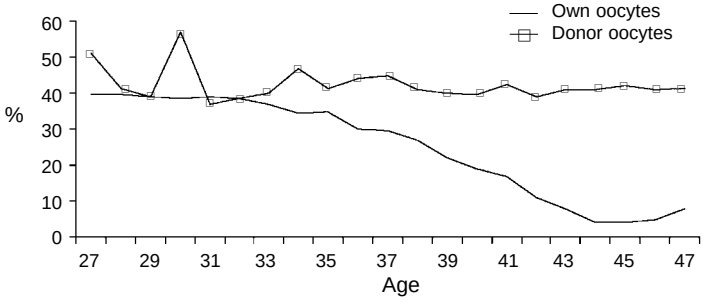

Fig 6: Live births per transfer for fresh embryos from own and donor oocytes, by age of (human) recipient (1998 CDC data).

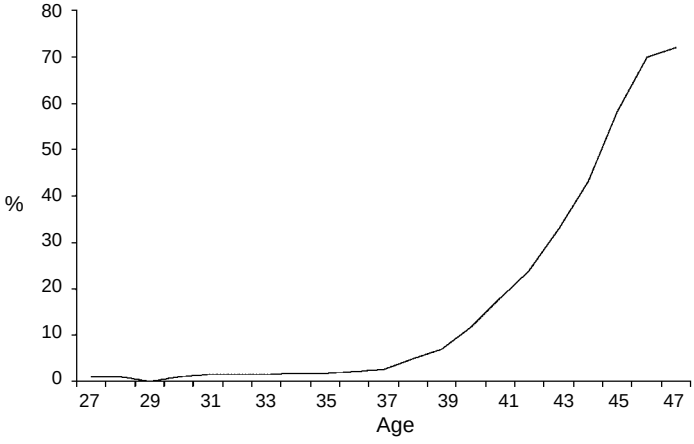

Fig 7: Percentage of ART cycles using donor oocytes, by age of (human) recipient (1998 CDC data).

pregnant mares (Cochran *et al.* 1998a,b) were announced. The following year, multiple ICSI embryos were transferred to the oviducts of surrogate females in an effort to conserve recipient mares, and in one of these females a twin-ICSI pregnancy was produced (Cochran *et al.* 1999). More recently, offspring from other mammals have been produced by ICSI with pig (Kolbe *et al.* 2000), cat (Pope *et al.* 1998; Gomez *et al.* 2000) and rhesus monkey (Nusser *et al.* 2001) oocytes.

In humans, ICSI is most often used to help overcome male factor infertility (Fig 3), but it has been considered to be generally unsuccessful when infertility is due to poor oocyte quality, which often occurs in older women. However, recent studies (Jun *et al.* 1999; Khamsi *et al.* 2001) have reported that ICSI can increase fertilisation rates and produce good quality embryos in cases of non-male infertility (Fig 4). Similar to the situation in women, a complicating factor in horse reproduction is that older mares often produce poorer quality oocytes (Fig 5). In humans, the goal

is to carry a pregnancy to term, even if the conceptus is not genetically related to the gestating female. Consequently, donor oocytes from younger fertile women are often used in ICSI procedures to produce embryos for older infertile women (Figs 6 and 7). In horses, it is most often the goal is to produce genetically related offspring from a valuable mare, regardless of whether that mare or a surrogate mare carries the pregnancy to term.

Non-surgical blastocyst transfer in humans has become the preferred procedure in some of the more progressive IVF programmes, which gives the opportunity to evaluate developing ICSI-derived embryos during *in vitro* culture. Thus, only the embryos with the best quality scores/grades are selected and transferred, reducing the chance of multiple pregnancies in surrogate females. It has been reported that implantation rates for human embryos transferred at the blastocyst stage are higher than those for earlier stage (8-cell) embryos (Gardner *et al.* 1998). Until recently, equine ICSI pregnancies were produced by surgical transfer of early stage embryos (Cochran *et al.* 2000). However, several groups (Galli *et al.* 2000; Li *et al.* 2000) have reported methods to improve ICSI blastocyst production, so that the embryos could be transferred non-surgically to recipient mares. This would reduce the need for surgery and allow repeated use of recipient females. With human ICSI fertilisation rates ranging from 60–85%, it has become clear that much remains to be learned about this sperm injection technology in the horse.

The quality of the oocytes used for ICSI studies has been the subject of much discussion. Cytoplasmic oocyte maturation involves the synthesis of proteins from nuclear and mitochondrial transcripts used by the oocyte during early embryonic development. Oocytes collected for *in vitro* maturation (IVM) are normally obtained at the germinal vesicle (GV) stage. Protein synthesis *in vitro* may differ from that which occurs *in vivo*, thus affecting fertilisation outcome and subsequent embryo development. Therefore, the differences in oocyte competence will be likely to affect the outcome after sperm injection (Gomez *et al.* 2000). Other studies have addressed the issue of oocyte competence using both *in vivo* and *in vitro* matured oocytes in ICSI studies in various farm animal species, including the cow (Heuwieser *et al.* 1992), horse (Grøndahl *et al.* 1997) and pig (Kolbe and Holtz 2000).

Key events such as meiotic maturation, fertilisation and the activation of the embryonic genome involve both nuclear and cytoplasmic processes. In humans, women suffering from idiopathic infertility continually experience poor embryo development, high rates of embryo fragmentation and implantation failure after their IVF treatment procedure. In this group of patients, attempts to improve embryo development have included altering ovarian stimulation protocols, ICSI, co-culture, assisted hatching and blastomere fragment removal. However, the success is still limited, leaving the use of donor oocytes as the primary option. Alternatively, an ooplasm donation from another female may overcome this problem.

Ooplasmic (or cytoplasmic) donation techniques have been developed with laboratory animals and are now used to help solve difficult human infertility cases (Cohen *et al.* 1997). With this approach, a portion of the cytoplasm from a donor oocyte (5–15%) is microinjected into the recipient oocyte either prior to sperm injection or along with the sperm during the ICSI procedure (Cohen *et al.* 1998).

The mechanisms by which ooplasmic transplantation can restore growth and viability of developmentally compromised embryos still remain unclear. Cellular components that might benefit development include the pool of stored mRNA sequences and proteins regulating oocyte function and embryo development, as well as mitochondria and other organelles. Cytoplasmic transfer has become the subject of debate because of the possibility of heteroplasmy of mitochondrial DNA. Recently, Barritt *et al.* (2001) demonstrated that active mitochondria were transferred from human donor oocytes to recipient oocytes during cytoplasmic transfer. Recipient oocytes contain a heteroplasmic population of mitochondria after cytoplasmic transfer and these mitochondria persisted in pre-implantation embryos.

The advent of ICSI has increased the potential of assisted reproductive technologies to propagate mammalian species, and has provided a tool for research in cell cycle control and the mechanisms involved in sperm-induced oocyte activation. The potential application of ICSI and ooplasmic transfer still remains to be explored in the horse, but it may be an alternative to circumvent *in vivo* fertilisation and/or implantation failures due to compromised oocyte quality.

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FETAL DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI) IN THE HORSE

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

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

The influence of co-culture with either oviduct epithelial cells or fetal fibroblast cells on the *in vitro* maturation of equine oocytes and their potential for development to blastocysts and fetuses following intracytoplasmic sperm injection (ICSI) was investigated. The development of the fetuses obtained after blastocyst transfer was monitored throughout gestation.

The oocytes were obtained by slicing abattoir ovaries and they were then matured *in vitro* for 28–30 h in TCM199 alone or in co-culture with oviduct epithelial cells or fetal fibroblasts. Metaphase II oocytes were selected after removing the cumulus cells and an ionomycin-treated spermatozoon was immobilised and injected into the ooplasm of each oocyte (ICSI). The injected oocytes were then cultured for 7–9 days in DMEM, alone or in co-culture with a monolayer of cumulus cells. At the end of the development period *in vitro*, morphologically normal early blastocysts were transferred, surgically or non-surgically to the uteri of recipient mares.

Nuclear maturation rates were similar in the groups of oocytes that were matured alone in TCM199 (107/220, 49%), in co-culture with oviduct epithelial cells (133/250, 53%) or in co-culture with fetal fibroblasts (37/72, 51%). Likewise, there were no differences in the cleavage rates obtained after ICSI among the groups of metaphase II oocytes that had been matured alone in TCM199 (58/92, 63%) or in co-cultures of either oviduct epithelial cells (52/80, 65%) or fetal fibroblasts (17/30, 57%).

The proportions of blastocysts that developed from the 2-cell embryos derived from oocytes matured by co-culture with either oviduct epithelial cells (10/33, 30%) or fetal fibroblasts (2/12, 17%), and subsequently cultured for 7–9 days on a cumulus cell monolayer, were not significantly different. However, both of these co-culture groups produced significantly higher proportions of blastocysts than were obtained from the groups of oocytes that were matured alone in TCM199, regardless of whether subsequent development culture was with (0/20, 0%) or without (0/16, 0%) the cumulus cell monolayer. These results demonstrate a beneficial influence of co-culture with either oviduct epithelial cells or fetal fibroblasts when maturing oocytes *in vitro*.

Six blastocysts obtained from oocytes co-cultured with oviduct epithelial cells were transferred into recipient mares and 4 pregnancies resulted (Table 1). All pregnancies were monitored ultrasonographically for normal fetal development

TABLE 1: Fetal development from *in vitro* produced equine embryos transferred into the uterus of recipients

Transfer	Embryo stage/number recipient	Development results pregnancy (%)	fetus (%)
Surgical	Early blastocyst / 4	3 (75)	3* (75)
Non-surgical	Early blastocyst / 2	1 (50)	1** (50)

*A single fetus was observed in each recipient, and all were morphologically normal when assessed by ultrasound

**Monozygotic twin fetuses were obtained in the pregnant recipient after the non-surgical transfer of one embryo (76 days at 15/05/2000)

TABLE 2: Prolonged gestation and post partum evaluation of an ICSI derived foal

Foal characteristics	ICSI foal	Normal foal
Gestation length (days)	413	230–360
Birthweight (kg)	18.6	20–35
% of maternal weight	5.65	9–11
Allantochorion (kg)	0.653	17–23
Placental area (cm^2)	6000	8000-9500

at 15, 25, 35, 60 and 210 days after ovulation. Two mares carried normal male foals to term.

In one mare, a singleton conceptus was diagnosed at 15 days after ovulation and subsequently twins were diagnosed in the same mare at 25 days after ovulation. At this time each twin fetus had a separate developing allantois but they appeared to share a common yolk sac. These twins were monitored until Day 76 after ovulation when they were removed surgically from the uterus. Both male fetuses appeared to have normal gross morphological development and yet they shared a common umbilicus. PCR analysis of the fetuses confirmed that they were monozygotic.

The fourth mare exhibited prolonged gestation and experienced minimal udder development, fetal dystocia and had a Caesarian section at 413 days of gestation (Table 2). This mare had a normal pre-partum rise in progesterone. Examination of her placenta revealed its site of implantation at the bifurcation of the uterine horns and there was poor development of the microcotyledons.

NUCLEAR TRANSFER IN DOMESTIC SPECIES

J. R. Hill

Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853-6401, USA

Hundreds of animals have been cloned from body cells since the first clone, Dolly, was displayed to a stunned world press in February 1997. Although the concept of cloning (or nuclear transfer) had been practiced in amphibians in the 1950s and again in cattle, sheep and pigs in the 1980s, Dolly was the first mammal to be cloned from a cell taken from an adult animal (somatic cell cloning). After Dolly, came mice cloned by a Hawaiian group, then cattle from Massachusetts and Japan (Cibelli *et al.* 1998; Wakayama *et al.* 1998). Over the next few years goats and pigs were cloned (Baguisi *et al.* 1999; Onishi *et al.* 2000; Polejaeva *et al.* 2000) while the numbers of cloned mice and cattle continued to increase. The most lucrative commercial application for cloning is through combination with genetic modification (Schnieke *et al.* 1997). This is a quantum leap in making genetically modified animals for production of human therapeutic proteins and organs for transplant. The copying of elite livestock has already been commercialised, and the application to endangered animal conservation is actively being investigated.

As numbers of cloned animals have increased, trends in both failed and successful development have emerged. The initial publication by Wilmut *et al.* (1997) showed lower pregnancy rates, higher gestational losses and lower neonatal survival when compared with either *in vitro* or *in vivo* produced embryos. More subtle defects have also been reported such as mitochondria derived exclusively from the recipient oocyte, variation in gene expression and telomere length.

In each of the species where somatic cell cloning has been successful, it has also been very inefficient (Wilmut *et al.* 1997; Cibelli *et al.* 1998; Wakayama *et al.* 1998; Onishi *et al.* 2000; Polejaeva *et al.* 2000). Early first trimester pregnancy rates are less than those normally expected. Immediately following initial positive diagnosis of pregnancy, extraordinarily high rates of embryonic loss occur, where up to 80% of pregnancies miscarry by the second trimester. In late gestation, placental and fetal abnormalities occur at a much higher than normal rate and, finally, lowered viability at birth is common.

The overall success rates for cloning, as measured by the proportion of live offspring that result from each reconstructed embryo, are low – around 1%. Although many cloned embryos may develop to pre-implantation stage, the vast majority will not result in a viable pregnancy. Initially, a high proportion of cloned embryos divide normally and reach the blastocyst stage at the expected time. These embryos show similar development and morphology to *in vitro* fertilised embryos cultured under the same conditions. This observation of apparently normal early development provokes a unique feeling of amazement and awe at the capacity of the oocyte to redirect development of a somatic cell nucleus. However, the initial excitement at producing an apparently normal embryo without using germ cell nuclei, is tempered by the subsequent unpredictable development of the nuclear transfer embryos following implantation into surrogate uteri. As pregnancy progresses, placental and fetal abnormalities dramatically reduce the numbers of cloned fetuses that survive to term. It is clear that the apparently normal appearance of cloned embryos does not correlate with their subsequent viability.

Incomplete first trimester placental development has been documented in bovine and ovine NT embryos where a lack of placental vascularisation and attachment sites are apparent (Hill *et al.* 2000; De Sousa *et al.* 2001). It is

presumed that this inadequate placental development is the major cause of the high rate of first trimester death in cloned fetuses. In third trimester cloned bovine pregnancies, placental abnormalities such as oedema and hydroallantois, have occurred in up to 50% of cows pregnant with cloned fetuses (Hill *et al.* 1999; Wells *et al.* 1999; Chavatte-Palmer *et al.* 2000). The number of placental attachment sites in the bovine (placentomes) may be reduced from normal by as much as 80%, which suggests that the completeness of placental development varies widely in cloned animals. It appears that placental gas exchange capacity is significantly reduced as late gestation cloned fetuses have been found to be hypoxic (Garry *et al.* 1998). In mice, over-developed placentae have been reported in cloned mice with placentae up to twice normal weight (Wakayama and Yanagimachi 1999; Ogura *et al.* 2000; Wakayama and Yanagimachi 2001).

Postnatal viability is markedly lower for many cloned animals (Wilmut *et al.* 1997; Kato *et al.* 1998; Hill *et al.* 1999; Renard *et al.* 1999; Kato *et al.* 2000; Kubota *et al.* 2000; Eggan *et al.* 2001). It is apparent that fetal viability in cloned animals varies between experiments and between species, with cloned mice and goats displaying better postnatal viability (Wakayama and Yanagimachi 2001; Keefer *et al.* 2001; Reggio *et al.* 2001). Whether these differences are due to technique, animal strain or to placental type remains to be determined. Neonatal viability has been shown to be compromised due to pulmonary immaturity (neonatal respiratory distress syndrome), complicated by persistent fetal circulation (pulmonary hypertension, right to left shunt) and disturbed placental function (Hill *et al.* 1999; Chavatte-Palmer *et al.* 2000). It appears that inadequate placental function impairs fetal and neonatal viability in cloned neonates. At birth, cloned calves and lambs commonly show signs of a stressful uterine environment (meconium staining, hypoxia). Placental reserve capacity is most likely limited due to inadequate development. Although cloned fetuses are seldom smaller than average, they share many attributes of fetuses suffering from placental insufficiency. Cloned neonates also appear to be incompletely prepared for the critical transition to breathing room air. Thus, at present, some cloned neonates are unable to survive despite intensive treatment.

These major developmental abnormalities alert us to the inefficiency inherent in cloning mammals. Gestational and neonatal abnormalities are consistent with irregular expression and most likely incomplete reprogramming of imprinted genes (Young and Fairburn 2000; Jaenisch and Wilmut 2001; Rideout *et al.* 2001). It appears that inadequate placental function impairs fetal and neonatal viability throughout gestation and at birth. This results in a lack of normal *in utero* development which has profound consequences on fetal and neonatal viability. This aspect will have to be addressed before cloning can be considered to produce normal offspring reliably.

A casual perusal of the lay and scientific literature may give a confusing picture of the health status of cloned animals. Some well publicised reports give a more negative view of mammalian cloning by focusing attention on abnormalities in telomere length, gene expression or methylation patterns. Others focus on success and proclaim the birth of apparently normal offspring. The practical outcome is that there are many cloned animals that behave and appear normal, while closer investigations have revealed that even some of these apparently normal animals are subtly different from one another and from the naturally produced population. Most of these differences appear to be due to epigenetic abnormalities acquired during nuclear reprogramming (Rideout *et al.* 2001). Whether these observed differences (such as telomere lengths or imprinted gene expression) have any bearing on health in later life remains to be seen.

The ability to clone animals from somatic cells is a tremendous advance for biomedical research and for agriculture. The majority of cloned animals alive today are normal in appearance and behaviour. Indeed, a significant number of cloned cattle, sheep and goats have produced normal offspring, with many cloned transgenic animals now producing valuable proteins in their milk for pharmaceutical use.

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IN VITRO DEVELOPMENT OF HORSE OOCYTES RECONSTRUCTED WITH THE NUCLEI OF FETAL AND ADULT CELLS

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

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

Since the development of embryo transfer techniques in the horse (Allen and Rowson 1975), a variety of new breeding technologies such as *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and nuclear transfer (NT) have been applied to horse breeding, both in the laboratory and on the studfarm (Palmer *et al.* 1991; Li *et al.* 2000a,b; 2001). However, the low efficiency of *in vitro* embryo production in the horse has limited its commercial application in this species. The reasons for this are numerous and include the restricted availability of oocytes, practical problems with *in vitro* oocyte maturation and fertilisation, and development of an effective *in vitro* culture system for early embryonic development. In the present study the authors investigated the basic conditions required for NT in the horse using both fetal and adult fibroblasts as donor cells.

MATERIALS AND METHODS

TCM199 (Gibco BRL, #22340-012, New York, USA) and DMEM (Gibco BRL, #22320-014) were used as the basic media for oocyte maturation and culture of the reconstructed oocytes after NT. A monolayer of oviduct epithelial cells (OEC) from a frozen horse OEC culture line was used in co-culture with the oocytes during their maturation and during development of the reconstructed oocytes.

Horse ovaries were obtained from 2 abattoirs and transported to the laboratory over 20 h in PBS containing 125 iu/ml penicillin and 35 iu/ml streptomycin while being maintained at a temperature of 10–20ºC. Cumulus oocyte complexes (COCs) were recovered from the ovaries by scraping the walls of follicles 0.5–3.0 cm in diameter and groups of 20–30 COCs were matured *in vitro* by co-culture with OEC for 28–30 h at 38ºC in 5% CO_2-in-air. Fetal fibroblast cells (FFC) and adult skin fibroblast cells (AFC) were used as donor cells after they had undergone 3–7 passages. FFC were derived from a 32-day-old Thoroughbred x Pony fetus and AFC were obtained from subdermal biopsies recovered from a 4-year-old female Pony.

MII oocytes were enucleated in EBSS-20% v:v FBS with 5 µg/ml cytochalasin B (CCB) and a selected donor cell was injected into the perivitelline space of the recipient enucleated oocyte, alone or in combination with a small volume of inactivated Sendai virus. The fibroblast-cytoplasm couplets produced from FFC and AFC were aligned manually in 0.28 M mannitol fusion buffer and subjected to 2 DC pulses of 220–250 kv/cm, each for 30 µs. Fibroblast-cytoplasm couplets that fused were then activated chemically by transfer to PBS containing 5 µm ionomycin for 5 min, followed by culture for 2, 4 or 6 h in TCM199 medium containing 5 µg/ml CCB and 10 µg/ml cycloheximide.

Groups of 5–10 reconstructed oocytes were co-cultured with a monolayer of OEC in 500 µl drops of development medium for 6–7 days at 38ºC in an atmosphere of 5% CO_2-in-air. Nuclear reprogramming (nucleus 2–3 times bigger than that of the original donor cell with an obvious nucleolus or some condensed chromatin) in the reconstructed oocytes was analysed 12–18 h after fusion treatment by staining with 5 µg/ml Hoechst 33342.

RESULTS AND DISCUSSION

Fusion of fetal and adult fibroblast cells with recipient cytoplasm

The effects of method of fusion of the FFC and AFC to recipient oocytes after NT are presented in

TABLE 1: Fusion of horse fetal and adult fibroblast cells with recipient cytoplasm followed by treatment with DC pulses alone or in combination with Sendai virus

Fusion treatment			Fusion results	
Donor cells	DC pulses	Sendai virus	Total number of oocytes	No. fused (%)
FFC	+	-	44	25 (57[b])
FFC	+	+	51	42 (82[c])
AFC	+	-	41	20 (49[b])
AFC	-	+	32	5 (16[a])
AFC	+	+	121	98 (81[c])
AFC Control*	-	-	15	0

DC = direct current; FFC = fetal fibroblast cells; AFC = adult skin fibroblast cells; Values with different superscripts are significantly different (P<0.05); *Neither DC pulses nor Sendai virus were used for attempted fusion treatment.

TABLE 2: Nuclear reprogramming and cleavage of reconstructed horse oocytes following nuclear transfer using fetal and adult fibroblast cells

Donor cells	Culture time in CCB + cycloheximide (h)	Number fused oocytes	No. reprogrammed (%)	No. 2-cell (%)
FFC	2	16	10 (63[a])	2 (13[a])
	4	18	18 (100[b])	6 (33[ab])
	6	34	30 (88[b])	18 (53[b])
AFC	6	81	68 (84[b])	28 (35[ab])

CCB = cytochalasin B; FFC = fetal fibroblast cells; AFC = adult skin fibroblast cells; Values with different superscripts are significantly different (P<0.05)

TABLE 3: *In vitro* development of 2-cell horse embryos obtained from oocytes reconstructed by nuclear transfer of fetal or adult fibroblast cells

Donor cell	No. 2-cell embryos	No. (%) 2-cell embryos that developed to			
		<8-cell	>8-cell	>16-cell/morula	blastocyst
FFC	26	10 (38)	8 (31)	7 (27)	1 (4)
AFC	28	14 (50)	8 (29)	4 (14)	2 (7)

FFC = fetal fibroblast cells; AFC = adult skin fibroblast cells.

Table 1. The fusion rate of FFC induced by DC pulses alone was significantly lower than that obtained when DC pulses were combined with Sendai virus (57% vs 82%, P<0.05). Similarly, rates of fusion with SFC were lower when the cell-couplets were stimulated by DC pulses alone compared to the combination of DC pulses and Sendai virus (49% vs 81%, P<0.05). On the other hand, Sendai virus alone induced fusion of only 16% (5/32) of the cell-couplets in the SFC group. The results therefore suggest that, at least in the horse, there exists a useful synergy between electrical stimulation and treatment with Sendai virus for the induction of cell fusion.

Nuclear reprogramming and cleavage of the reconstructed oocytes

The rates of nuclear reprogramming and cleavage of the reconstructed oocytes in the FFC and AFC groups are compared in Table 2. When using FFC as donor nuclei the rate of reprogramming was increased by lengthening the culture time in CCB and cycloheximide from 2 h to 4 or 6 h (63%, 100% and 88%, respectively; 2 h vs 4–6 h, P<0.05). Furthermore, higher cleavage rates were obtained after 4–6 h culture (33–53%). Similar nuclear reprogramming and cleavage rates were obtained when AFC were used as donor cells

following 6 h culture in DMEM supplemented with CCB and cycloheximide (84% and 35%, respectively).

In vitro development of 2-cell stage embryos

The development of 2-cell stage embryos reconstructed from the transfer of either FFC or AFC is summarised in Table 3. There were no significant differences in development rates of 2-cell stage embryos obtained from FFC and AFC ($P>0.05$). Only a very low proportion (4–7%) of 2-cell embryos derived from both donor cell types developed to blastocysts after 6–7 days co-culture *in vitro* with a monolayer of OEC. No differences in morphology were observed in the blastocysts derived from either fetal or adult cell types. This deficiency of developmental competence in the cloned horse embryos might be related to such factors as damage to the cytoskeleton during enucleation or exposure to ultraviolet light, or it could be due to failure of formation of the 'equine blastocyst capsule' *in vitro.* This capsule is a tough, elastic investment which develops uniquely between the zona pellucida and the trophectoderm in the Day 6.5 late morula/early blastocyst stage equine embryo (Betteridge 1989; Oriol *et al.* 1993). It is considered to be essential for survival of the young embryo in the potentially hostile maternal uterus during the period of embryonic mobility between Days 6 and 17 after ovulation (Ginther 1985), by providing structural protection from the strong myometrial contractions and to aid the accumulation and imbibition of essential nutrient components in the exocrine endometrial gland secretions (Allen 2001).

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WORKSHOP SUMMARY

WORKSHOP SUMMARY

R. H. F. Hunter*

Faculty of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK

Friends and colleagues,

Being asked to make some final remarks at this Workshop is certainly a privilege, but also a serious commitment. One must strive to be fair and balanced and not to indulge too many personal preferences or fly too many kites. In being invited to perform in the present role, the hope must be that one can use long years of laboratory work, of experimental surgery, of time in the library and attending other Workshops as background from which to try to say something useful. You would expect nothing less. However, it is not the easiest of tasks for there has been a battle with the clock, trying to balance professional duty against the powerful social attractions of mixing with friends old and young, of meeting new faces, of exploring an exciting city. So, hiding away in a hotel room composing these few sentences has been a somewhat strange activity and not altogether welcome for the reasons given.

What has been welcome has been the prospect of expressing warmest and best thanks – and I know yours too – to Mr Gene Pranzo and his Havemeyer Foundation for sponsoring this wonderful Workshop and for providing agreeable and generous hospitality. And thank you too, Gene, for the initiative of publishing your Workshop proceedings in these handsome volumes. They represent a highly valued academic resource and one which will also be a permanent tribute to your Presidency of the Havemeyer Foundation.

Let me next extend special thanks to Lee Morris for arranging the programme and for creating what has proved to be a most fruitful and interactive Workshop. White-haired old boys tend to have thoracic palpitations when receiving long-distance 'phone calls from Lee, but she works her friends hard. Even so, it is especially good of you, Lee, to have invited a non-equine contributor to participate at least twice and to round off the meeting. As an aside, there can be no doubt that you were an excellent graduate student in Guelph but, in reality, it has been your time with Twink Allen that has enabled you to flower into a mature researcher on the international stage, so I feel sure you would wish me to extend thanks to Twink – an éminence grise behind so many of these workshops.

Finally, in these opening remarks, we turn to Jan Wade, the splendid organiser behind the scenes. Jan is so efficient, so helpful and so thoughtful in setting up these meetings that they run like Rolls Royces or should we say Cadillacs. Always appearing relaxed, always extremely friendly, Jan's eagle eye doesn't miss a trick, and all aspects of the Workshop have come under her supervision. So thank you so much, Jan, and also for being the key pair of hands in designing and producing these elegant volumes. Already they have been much appreciated, not least by many of my veterinary colleagues in continental Europe.

It is perhaps none of my business as a mere speaker at the end of our two and a half days of deliberations, but I feel I must comment on the title of the Workshop or of the anticipated volume. Yes, 'From Epididymis to Embryo' is clear and crisp, indeed it has a certain resonance. But if I were a medical or veterinary publisher, I might wish to see some allusion to female involvement, and would prefer a title along the lines – 'From Epididymis to Embryo via the Female Tract' or 'From Epididymis to Embryo via the Oocyte'. As of writing, there is absolutely no meaningful route from epididymis to embryo without some component of female involvement.

**Contact address: 32, Gilmour Road, Edinburgh, EH16 5NT, Scotland*

Now, down to business and no more mention of names of contributors to the present Workshop. Please forgive me if you are unhappy with this, but I feel it is more appropriate to be impersonal in the remarks that follow and not to appear to favour one particular presentation over another. In any event, I fully enjoyed each and every one of them. As you will appreciate all too clearly, none of us could hope to summarise the extensive contributions of the Workshop – both the formal papers and the subsequent discussions – in the 20 minutes that remain. So what I am hoping you may accept will be straightforward comments based on my own interests and understanding, on a reasonably extensive background in large farm animal and laboratory rodent research, and an enthusiasm for all topics reproductive. The comments will not necessarily be in chronological order vis à vis the programme and cannot be all inclusive.

First, a few remarks concerning the zona pellucida. This accessory investment around the egg proper was mentioned frequently during the Workshop, commencing in the very first presentation with extensive reference to its content of accessory spermatozoa. A suggestion here might be that comparisons of numbers of accessory sperm in the zona will only be strictly meaningful if made at similar times after ovulation. Other factors being equal, a bovine egg recovered from the oviduct of a mated or inseminated animal one day after ovulation would be expected to contain fewer accessory sperm in the zona than if recovered two days after ovulation. So, unless the post ovulatory interval is standardised, the meaning of accessory sperm counts would need to be interpreted with great caution.

In such studies, there is also a presumption that the quality of the zona pellucida is comparable between oocytes. We should question whether this is so, indeed even between oocytes shed in subsequent cycles in the same animal. You may ask what thoughts prompt such a suggestion. Please bear in mind that the zona pellucida of an ovulated egg is not only a product of the oocyte, uniquely so as claimed in the mouse, but in other species also of its investing somatic cells – granulosa cells of the cumulus – shortly before and after ovulation, and also of oviduct secretions including glycoproteins such as oviductin in the hamster and primates. These products – vitelline molecules, granulosa cell molecules and ampullary epithelial molecules – will together generate zonae of variable composition and, one would imagine, of variable responsiveness to sperm penetration. Let us, therefore, not regard the zona as a reasonably inert structure of consistent composition in either unfertilised or fertilised eggs. Subtle variations could be expressed in several ways, for example, in the:

1. initial penetrability by a fertilising spermatozoon;
2. rate of instigation of the zona block to polyspermy in response to cortical granule exocytosis;
3. ability of accessory spermatozoa both to enter and reach a given distance within the substance of the zona pellucida.

One more paragraph concerning the zona pellucida. There was that perplexing question as to whether a sperm that had undergone an acrosome reaction shortly before reaching the zona could then penetrate into the zona substance. I propose that we keep an open mind on this. The easy answer is of course 'No', especially if we extrapolate from current dogma. However, if we bear in mind that the zona does not have a smooth surface – as we tend to portray it in diagrams – but rather a highly uneven and pitted surface, then might it not be possible for an actively motile sperm to be trapped by the zona surface, physically, that is, instead of chemically bonding, and then to commence penetration under the influence of hyperactive motility? If you subscribe to an involvement of lytic enzymes in zona penetration, then please recall that part of the acrosomal content of lytic enzymes is bound to the inner acrosomal membrane and might still be able to function. So, in answer to that excellent question, perhaps an acrosome-reacted sperm could indeed penetrate a zona, or make a good stab at it if it happened to be in the vicinity of the egg at the time of undergoing the acrosome reaction.

As to the curves of fertility predicted under insemination conditions of different numbers of spermatozoa and variable quality of bull, these were reminiscent of the exciting studies and indeed modelling by Arthur Walton in the late 1940s and 1950s. Some participants in the Workshop will recall that Dr Walton (an Edinburgh graduate) was Sir John Hammond's colleague at the Animal Research Station, Cambridge, and together with L.E.A. (Tim) Rowson was instrumental in developing bovine artificial insemination programmes in the United Kingdom.

If we now broaden our discussion to the duct systems, what do we know of the nature of the fluid

– or plasma – in the caudal portion of the stallion's epididymis? Does it contain high viscosity proteins such as immobilin described in other species, and are there any parallels with secretions in the caudal portion of the oviduct? During our discussions, there was mention of an absence of viscous secretion in the caudal portion of the mare's isthmus. Is this certain, for it would be a cyclically-dependent secretion, and would be anticipated in the pre-ovulatory interval. If it could be demonstrated, then this would constitute valuable circumstantial evidence for the isthmus portion of the duct acting as a sperm storage site before ovulation. Such secretions – a kind of mucus – are found in the pre-ovulatory interval in a range of other mammals.

There were extensive discussions of sperm transport within the female, beautiful scanning electron micrographs of potential interactions between the sperm head and epithelial surfaces, but no specific details of possible junctions and ion transfer channels between endosalpingeal or endometrial organelles – microvilli – and an intimate sperm cell. Attention was drawn to components of the lectin binding system, but the physiology of sperm release from storage sites – be they utero-tubal or in the caudal isthmus – still requires clarification. Whether transfer of oocytes into the ampullary portion of the oviduct can prompt a release of sperm cells from the isthmus was clearly raised, yet there remained questions as to the role of follicular fluid or its constituents in phases of sperm release. If spermatozoa are stored on the uterine side of the utero-tubal junction, then how do they escape engulfment by polymorphonuclear leucocytes? Are they protected by special secretions or simply by being sequestered deep in crypts and slit-like crevices? Would uterine fluids not have a stimulating influence on their metabolism?

Finally, is there local programming of sperm storage and release by the neighbouring gonad – more particularly by its pre-ovulatory follicle? Even if this proves to be so in mares, we need to take note of the fact that oocyte transfer to the contralateral duct can be successful. What is the explanation? In all situations, please note that any local transfer mechanism would be supplemented by an influence of the systemic circulation.

There is another somewhat critical point concerning analysis of cellular events within a sperm population, excellent though techniques in the laboratory may be. Most ejaculated spermatozoa will not penetrate an egg and initiate the events of fertilisation. In fact, for a variety of reasons concerning cellular competence, a significant proportion of ejaculated spermatozoa would never fertilise an egg under physiological circumstances. Accordingly, when one is working with populations of spermatozoa *in vitro*, be they epididymal or ejaculated, how can one be absolutely certain of analysing membranous and molecular events in a fertilising spermatozoon? I suggest one cannot and that, once again, extreme caution is called for in the interpretation of data generated *in vitro.* At the very least, and if techniques could be sufficiently sensitive, then corroboration of in vitro findings on populations of spermatozoa recovered from the oviducts would add confidence. However, such populations of cells would themselves be vulnerable, and great care would be needed to avoid artefacts.

As to sperm processing for storage under a variety of conditions, one feels that there must be an influence of subtle lesions, variable in extent between samples, but acting ultimately to compromise fertility. Could we not reason in the following manner? If a brief exposure to the physiological fluids of the female tract leads to membrane modifications and eventually to capacitation (a fragile short-lived state), then surely exposure to diluents, extenders or buffers combined with trajectories across time and temperature gradients could also result in more fragile spermatozoa with shorter lifespans. To a simple biologist, it seems remarkable that damage cannot be demonstrated by transmission electron microscopy, but this is said to be the case. Even so, molecules such as cholesterol almost certainly must be leached from the plasma membrane during *in vitro* procedures with a resultant influence on viable lifespan of a cell. Improved techniques should enable us to pin-point more of the changes and then to begin to think of approaches to compensate for them.

What does seem clear is that minimising the extent of uterine exposure of a stored sperm suspension is beneficial in various ways, not least in reducing the vulnerability of stored spermatozoa to attack by polymorphonuclear leucocytes. The exciting work describing the nature of uterine fluids is undoubtedly of relevance here, perhaps in due course suggesting physical approaches for reducing the vulnerability of stored sperm cells during passage into the oviducts. Meanwhile, we must remain grateful for techniques of deep insemination

close to or on to the utero-tubal junction.

We heard much about oocytes and embryos in various contexts, all presentations being accompanied by beautiful images. Unfortunately, we remain seriously perplexed – by which I mean ignorant – on so many aspects of ovarian physiology. We have no idea why the initial complement of oocytes is so large, nor the extent to which incompetence is already present in many oocytes at the time of birth. We all appreciate that there must be multiple conversations between the oocyte and surrounding somatic cells, yet we have little idea of the nature of those conversations at any given time. To this reviewer, it is amazing that so much can be achieved in vitro and apparently normal fertilisation be obtained after major cellular insults – as for example during ICSI or after procedures of freezing and thawing, or after sex separation of spermatozoa. Please be cautious in your interpretation of these recent exciting results. Fertilisation and development up to the stage of blastocyst formation are impressive features, but organogenesis, placentation and development to term of viable fetuses – quite another matter. Please don't be seduced by agreeable levels of success in producing blastocysts. Such embryos must be shown to have full developmental potential.

Work on oocyte and embryonic metabolism and metabolic pathways would seem of the greatest significance to the *in vitro* enterprise, and sadly has not received sufficient emphasis or sufficient support, although it was extremely well addressed and covered in this Workshop. Lots of major points were made, but also the critical one that we know all too little about equine oocytes and embryos. Developmental success will be linked to our knowledge of metabolic requirements and thus to provision of suitable substrates and conditions of culture. Such studies deserve high priority.

There is one little anecdote with which to regale you before drawing these remarks to a close. In the very first presentation of the Workshop, we heard about the influences of scrotal insulation on sperm quality and on the seminiferous epithelium of bulls. Here is a variation on the theme of modifying temperatures in scrotal testes.

In 1968 when I went to France as a keen young man to the Institute of Professor Charles Thibault at Jouy-en-Josas, just south-west of Paris near Versailles, Britain was still endeavouring to join the so-called Common Market. We had been vetoed twice by President Charles de Gaulle, a troublesome old chap if ever there was one, despite his enormous talents. In those days, an Englishman based in Sydney had just been on sabbatical with Professor Thibault – a physiologist by the name of G.M.H. Waites. Dr Waites was also interested in the influence of temperature on the testes, but his approach was to look at differential temperature effects within the same male. In brief, his technique was to insulate one half of the pendulous scrotum of a ram to warm up the testis therein and, by contrast, to chill the other half of the scrotal sac in a bucket of ice to cool that particular testis. As you may imagine, the apparatus for these experiments was weird and wonderful, and attracted large numbers of veterinarians, farmers and characters of quite another type to the research institute. On learning the objectives of the work, the comment was widely and vociferously made that if such were the sorts of experiments that Englishmen were going to perform on French farm animals, then we were certainly not ready to join the Common Market! We were kept out until 1972, by which time such benchmark studies were largely forgotten.

I have spoken for too long. It is a Sunday morning and I wish to close with a text – or rather a classical quotation. It is inscribed as the Frontispiece of the most famous of books in a certain category – in fact on alpine mountaineering. I should be curious to know if any of you highly-educated people could name the book or the author. Here is the quotation.

'TOIL AND PLEASURE,
IN THEIR NATURES OPPOSITE,
ARE YET LINKED TOGETHER IN
A KIND OF NECESSARY CONNECTION.'

Well, we have toiled a little and we have enjoyed ourselves very considerably. The latter seems a useful definition of pleasure.

My personal thanks to all of you, safe homeward journeys for each and everyone, and here's hoping for further meetings before too long.

R.H.F. Hunter
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LIST OF PARTICIPANTS

Twink Allen
United Kingdom

Barry Ball
USA

Elaine Carnevale
USA

Young Ho Choi
USA

Ben Colenbrander
The Netherlands

Richard Fayrer-Hosken
USA

Robert Godke
USA

Ghylene Goudet
France

Jonathan Hill
USA

Katrin Hinrichs
USA

Ron Hunter
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Lee Morris
Australia

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USA

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Canada

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USA

Mary Scott
USA

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USA

Claire Tiplady
United Kingdom

Jordi Tremoleda
The Netherlands

Dickson Varner
USA

Jan Wade
United Kingdom

AUTHOR INDEX