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EUROPEAN EMBRYO TRANSFER ASSOCIATION

25ème COLLOQUE SCIENTIFIQUE

25th SCIENTIFIC MEETING

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Dr William Richard Allen

Special Celebration

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POZNAN, Poland, 11th and 12th September 2009

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Dr William Richard Allen

A.E.T.E. Medalist 2009

Dr William Richard Allen
A.E.T.E. Medalist 2009

W.R. 'Twink' Allen: A.E.T.E. Pioneer Award 2009

William Richard Allen was born in August 1940 in Auckland, New Zealand, the second of four children. Soon after his birth he acquired the nickname 'Twink' from his older sibling, and he has been Twink ever since. During his 43 year scientific career, Twink has become arguably the most influential, innovative, charismatic and controversial figure in equine reproduction. He was at the forefront of most of the major breakthroughs that revolutionised the practice of equine reproduction during the last 40 years, was one of the founding fathers of the International Symposia on Equine Reproduction and has been a significant contributor to or the driving force behind the organization and promulgation of countless veterinary and scientific conferences, meetings and societies. Moreover, as a firm adherent to the 'work hard, play hard' ethos, Twink has invested equally boundless reserves of energy and enthusiasm to the meticulous planning of the social events that make such a difference to the success of a scientific gathering. Twink was one of the very first to collect and transfer equid embryos and continued to develop equine ET throughout his career, thereby laying the foundations on which the commercial equine ET industry is now thriving. Arguably, however, his most striking talent was the ability to translate developments in other fields to the science and practice of horse reproduction, and conversely to employ equine embryo technology and transfer as tools to investigate aspects of embryonic, fetal and neonatal development, pregnancy and pregnancy loss with ramifications far beyond the field of horse breeding. Twink is without doubt a deserving recipient of the AETE 'pioneer' award.

Things could, however, have turned out very differently. After completing his schooling at Auckland Grammar School, Twink embarked on a Medical Intermediate Certificate at Auckland University with the aim of studying Medicine. However, a failed physics examination brought the envisaged medical career to an abrupt end and, given the choice of dentistry or veterinary medicine, Twink wisely chose to accept a Veterinary Services Council Scholarship to study at Sydney University, there being no veterinary school in New Zealand at that time. Twink claims to have enjoyed an undistinguished pre-clinical career, but began to excel after embarking on the clinical phase of his training. His first encounter with equine reproduction was via the Sydney University lecturer, Virginia Osborne, an early source of inspiration under whose supervision and on whose Thoroughbred and Standardbred stud-farm Twink learnt to examine a mare *per rectum*.

After graduating in 1965, Twink married his sweetheart Diana Emms, and returned to New Zealand to work at a 'veterinary club' serving the farming community in Kaitaia. A mere 2 months into the five years stipulated under the terms of his Veterinary Services Council Scholarship, Twink's career in farm animal practice was ended by a serious car accident which left him in hospital for 9 months with multiple leg fractures. During the long period of immobilization, Twink applied for and was awarded an International League for the

Protection of Horses scholarship for a one year research project, with the possibility of extension to a higher degree. He proposed to work on the effects of nutrition on reproduction in horses, and was advised to approach Roger Short at the University of Cambridge to act as his supervisor. Twink duly sent a letter to Roger Short explaining that he had received a scholarship and outlining both his study plans and proposed date of arrival. Although his departure was delayed by a further operation on his leg, Twink eventually arrived in Cambridge in 1966, to the considerable consternation of Roger Short who had only recently discovered Twink's letters after returning from a six month sojourn in South Africa. Notwithstanding the uncomfortable introduction, Twink's first year was a success, and he was duly enrolled at Magdelene College to study for a PhD on 'Equine Gonadotrophins'. Twink's outstanding doctoral studies yielded a paper in Nature, established him as a coming force in equine reproductive biology and made him the perfect candidate for a post-doctoral research post organized by the retiring director of the National Stud, Peter Burrell, to develop techniques for freezing stallion semen.

Twink had the great good fortune that his post-doctoral studies were based at the world renowned centre of excellence in reproductive science, the Animal Research Station (ARS) in Cambridge. At the end of his first post-doctoral year Twink's was delighted to be able to return his scientific focus to the mare, albeit largely as a result of the Thoroughbred Breeders Association's horror at learning that he had successfully impregnated pony mares with frozen-thawed semen from the Derby-winning stallion 'Never Say Die'. It was, thus, in the early 1970's, and under the scientific mentorship of Tim Rowson, that Twink entered the world of embryo transfer and, often in concert with two of his most valued scientific colleagues, Bob Moor and Francesca Stewart, embarked on his signature studies on the endometrial cup reaction. Typically, far from playing safe, Twink's first attempts at ET involved transferring donkey, pony or donkey-pony hybrid embryos into recipients of both species. During the coming years, Twink's research progressed at an impressive speed, aided in no small part by the patronage of Colonel Nat Frieze, the new Chairman of the Thoroughbred Breeders Association, and Peter Burrell who had married Connie Mellon, widow of General Richard Mellon of the banking, film and oil family. Frieze and Burrell helped Twink secure generous funding from the RK Mellon Charitable Foundation, and remained firm supporters and friends of Twink's until their deaths.

When the Animal Research Station closed in 1986 and its staff moved to the Babraham Institute, Twink and his equine reproduction team initially remained at the Huntington road site with Chris Polge and Phillip Paxman's Animal Biotechnology Cambridge company. However, in 1988 Peter Burrell persuaded the Duke of Sutherland to release a 114 acre corner of his Newmarket estate for Twink to establish the purpose-built facilities and large herd of experimental horses, ponies and donkeys that became the 'Equine Fertility Unit'. After settling at Mertoun Paddocks, Twink and his colleagues, notably Francesca Stewart who tragically died of a brain tumour in 2000, maintained an impressive level of industry and scientific productivity, and the EFU became a world leader in research into equine reproduction and embryo transfer. From his new base, Twink was able to strengthen the links with the Newmarket equine veterinary community that he had first established via Bob Crowhurst and Fred Day; together with Peter Rossdale, Richard Greenwood and others he tested and introduced a range of new reproductive techniques and treatments to clinical practice.

Twink officially retired in 2007 but his appetite for reproductive science (primarily horse, camel and elephant) remains undiminished, albeit now interspersed with an annual

fishing trip back 'home' to New Zealand. Regrettably, the Thoroughbred Breeders Association of Great Britain and Ireland used Twink's retirement to engineer the closure of the EFU. Apparently, a number of powerful Thoroughbred breeders had found Twink's pioneering work in the area of ET and assisted breeding techniques sufficiently disturbing to decide to exact revenge on 'his' unit, conveniently forgetting that many of these very studies had been of huge benefit to modern Thoroughbred breeding practice. While the British horse breeding industry will almost certainly come to regret the untimely loss of a small but remarkably productive scientific centre, Twink will undoubtedly continue to contribute to scientific debate and discussion for years to come.

It is entirely appropriate that Twink should receive the AETE award in Poland. His links with Poland stretch back to 1972 when the then director of the ARS, Thaddeus Mann, asked him to visit the Krakow academy and lecture on horse reproduction for Mann's friend and counterpart in Krakow, Wladyslaw Bielánski. Three years later, to celebrate the 40th anniversary of John Hammond and Arthur Walton's successful transport of ram semen to Poland, Twink returned to Krakow bringing with him six early horse embryos nestled in the oviducts of two female rabbits. Five of these embryo's were transferred to recipient mares synchronised in Krakow, Twink struck up a life-long friendship and scientific collaboration with Bielánski's protégé, Marian Tischner, and the three Welsh pony foals born in 1976 became the mascots for that year's ICAR conference in Krakow.

Twink's sizeable and impressive list of publications, awards and honours is testament to an extraordinary drive and his considerable charisma. His attention to detail and ability to enthuse and inspire via both the spoken and the written word are an object lesson to aspiring scientists. It is therefore a great honour and a pleasure to accept the opportunity to thank Twink on behalf of the many students and fellow scientists who have benefitted from his collaboration, mentorship and friendship over the years. I know I echo the sentiments of many in my admiration not only of his scientific achievements and encyclopaedic knowledge but also of his hospitality and the way in which he maintains contact, friendship and interest in the lives and careers of those with whom he has struck up a bond. We look forward to many more heated scientific or political discussions over a glass of gin.

Tom A.E. Stout, University of Utrecht, The Netherlands

Historical and modern aspects of equine embryo transfer

ALLEN W.R.

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Introduction

After completing his PhD on equine gonadotrophins at Cambridge Veterinary School in early 1970 under the supervision of Professor Roger Short FRS, the author had the great good fortune to land a 2-year post-doctoral research post at the world famous Animal Research Station in Cambridge, under the joint supervision of Tim Rowson OBE, FRS and Professor Thaddeus Mann CBE, FRS. This put him in daily contact with the original "pioneering giants" of embryo transfer and selected technologies in the large farm species, including Tim Rowson FRS, Bob Moor FRS, Chris Polge FRS, Ron Hunter, Ras Lawson, Neil Moore, Ian Wilmut, Ray Newcombe, Bill Christie and many others so it was inevitable that their marvellous enthusiasm and great practical experience and knowhow should rub off onto the small equine reproduction group that, by 1972 and through the generous benefaction of Mr Peter Burrell CBE and the RK Mellon Trust, became formerly constituted as the Equine Fertility Unit. Tim Rowson observed at coffee one morning ……. "You've got experimental ponies and donkeys, Twink. Since they readily interbreed to produce mules and hinnies (Bielanski et al, 1956; Clegg et al, 1962), wouldn't it be fun to transfer embryos between them" That essential sense of fun and broad scientific research for practical animal production experiments underpinned all the work undertaken at the Animal Research Station in those halcyon days so off went the experiment to transfer donkey embryos to horse mares and vice versa, despite the fact that, at that time, normal intraspecies equine embryo transfer had not yet been attempted. No forms had to be filled in and no Home Office or Animal Care Committee permissions sought. Everyone just rallied around and carried on with the "fun experiment".

The initial results were disappointing, and bordered on disheartening, for two simple, practical reasons. First, following the procedures developed and now running smoothly in sheep and cattle (Moore and Shelton, 1964; Rowson and Moor, 1966, Rowson et al, 1969), donor mares and donkeys were flushed non-surgically on days 4, 5 or 6 after ovulation and, to save on flushing medium, a purpose-built rigid flushing device, with an inflatable cuff at its anterior tip, was passed, by transrectal guidance, into the base of the uterine horn ipsilateral to the side of the previous ovulation (Figure 1). Embryo recovery rates from this single horn flushing method were very low at around 40-50% both in the author's laboratory (Allen and Rowson, 1972, 1975, Allen, 1982) and in Japan (Oguri and Tsutsumi, 1974). This compared poorly to recovery rates of 70-90% from cattle using the same method (Rowson and Moor, 1964; Rowson et al, 1969; Lawson et al, 1975) and it wasn't until it was realised that the abnormally slow passage of the equine embryo through the oviduct meant it wasn't even entering the uterus until 144-168 hours after ovulation (i.e. 6-6.5 days; Battut et al, 1997) and its continual movement throughout the uterine lumen, from then until day 16-17 (van Niekerk, 1965; Ginther, 1983) meant that the uterine flushing should be delayed until day 7 or 8 after ovulation and the whole uterus, not just the ipsilateral horn, flushed with a much larger volume (1-2 litres) of flushing medium using a simple 2-way flexible flushing catheter, that

embryos began to be recovered. It was a salutary lesson that taught the author to ever afterwards regard the mare as reproductively very different from ruminants and pigs.

Figure 1

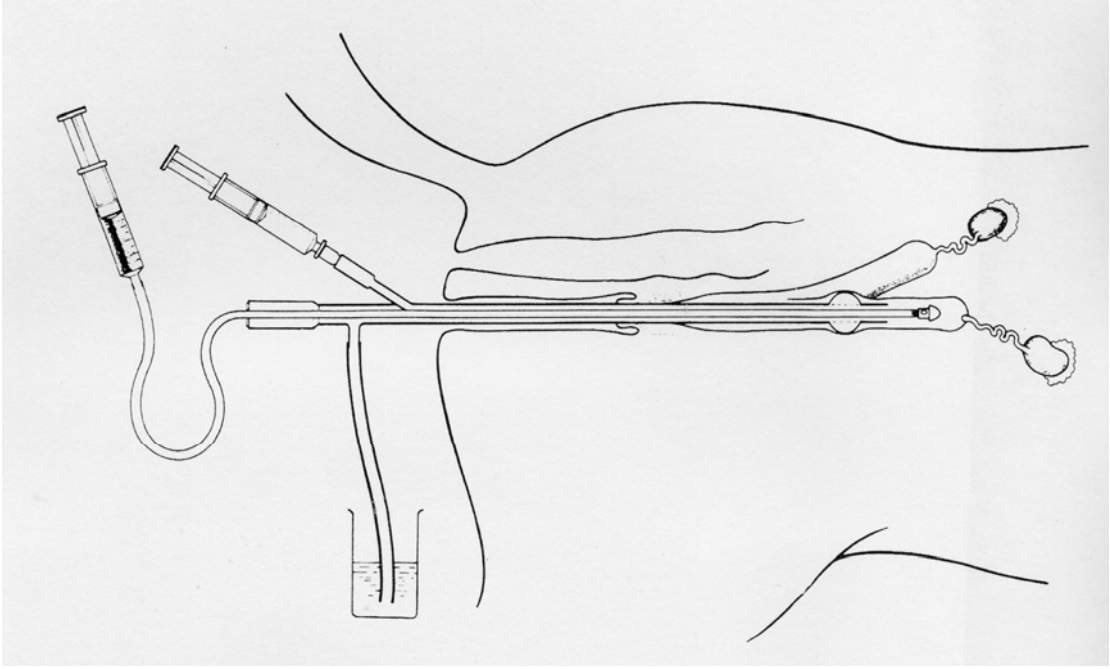


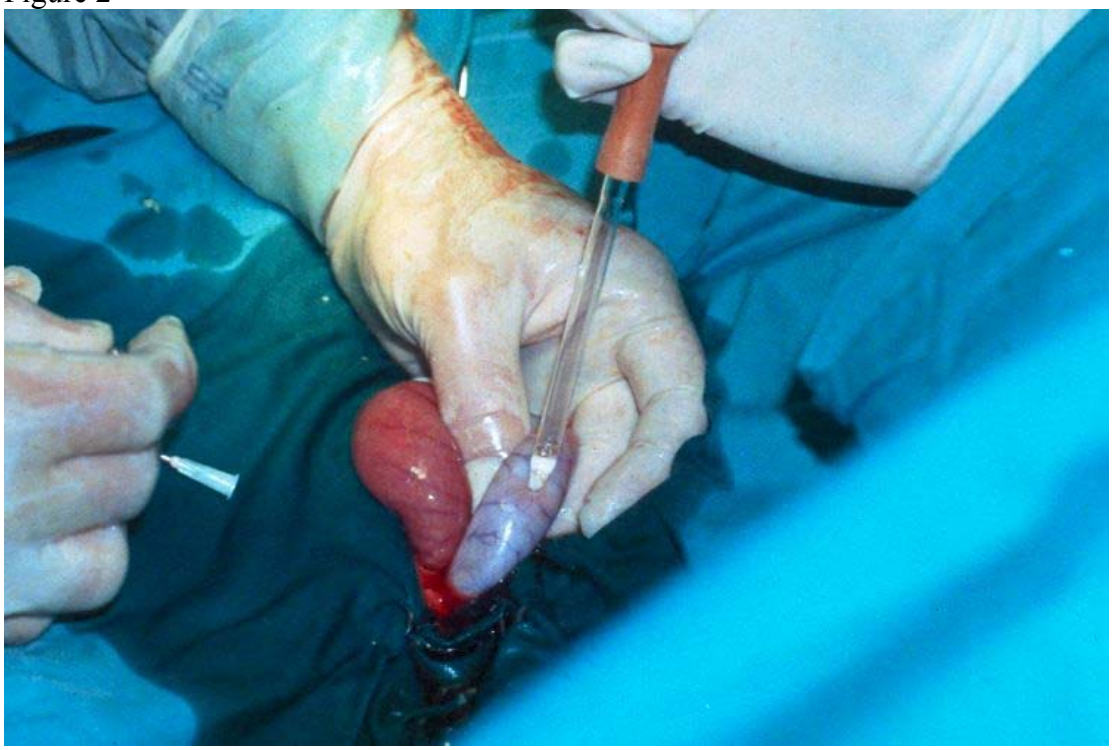
Diagram showing the original, rigid apparatus used to flush just the ipsilateral uterine horn of donor mares (From Allen 1982a)

Again, mimicking the work in cattle, embryo transfer in those early days in Cambridge was carried out surgically to bypass the perceived difficulty of passing the rigid transfer pipette through the tight and tortuous cervix of maiden heifers, the preferred and most fertile recipients, and because adequate surgical facilities were available at the Animal Research Station. Hence, as many as 6-8 heifers or cows, followed by 3 or 4 mares, would be anaesthetised during a single morning session, each by an intravenous "slug" of 5g sodium pentobarbitone followed by intubation and maintenance on a gaseous mixture of halothane and oxygen. The cow or mare was rolled onto its back and a mid-ventral laparotomy incision made through which the corpus-luteum bearing ovary and ipsilateral uterine horn were exteriorised. A blunted, solder-filled, 18 gauge needle was then used to punch a hole in the uterine wall through which a flame-tipped Pasteur pipette containing the embryo in a minimum (0.2-0.3 ml) of medium was passed to inject the embryo into the uterine lumen (Figure 2).

This early surgical method for the transfer of cattle, sheep and horse embryos worked well and gave good pregnancy rates of 75-90%. But the expense and relative impracticability of the surgical approach was always going to seriously limit full, commercial, on-farm development and exploitation of embryo transfer in cattle so Tim Rowson, aided particularly by Ray Newcombe, Ari Brand from Holland, Alan Trounson from Australia and Bill Christie from Scotland, spent the next 15 years during the 1970s and early 1980s developing and honing methods for successful non-surgical recovery and transfer of cattle embryos, efficient hormonal synchronisation of ovulation in donors and recipients, reliable and repeatable induction of superovulation in the donors and simple, practical on-farm methods for deep freezing the embryos in liquid nitrogen. They made huge advances in all these areas and

readily passed on their findings to the scientific and farming communities via a series of scientific and lay publications and a number of practical teaching sessions. The work is well documented, (Rowson, 1971; Newcomb, 1979, 1980).

Figure 2



Ventral midline surgical transfer of horse embryos by means of a flame polished Pasteur pipette inserted through a hole punched in the wall of the uterine horn using a blunted, solder-filled 18G hyperdermic needle.

Commercial equine embryo transfer followed behind cattle, but initially at a rather slower pace. This was due, in part, to the resistance of most breed registration authorities to register foals begot by embryo transfer, the inability to induce genuine superovulation in donor mares and the relatively poor pregnancy rates being achieved from non-surgical transcervical transfer of the embryos (Oguri and Tsutsumi, 1974; Douglas et al, 1974; Allen and Rowson, 1975; Squires et al, 1982; Iuliano et al, 1985; Lagneaux and Palmer, 1989)

However, the turning point was reached in the early 1990s when Robert Pashen, a former PhD student in the Equine Fertility Unit, and Francisco Lascombes collaborated in Argentina to establish the first of what nowadays has become a veritable growth industry of highly efficient, commercial, equine embryo transfer centres throughout Argentina, all dedicated to producing multiple offspring from elite polo ponies over a 3-month period at the end of the polo playing season (Pashen, et al 1993; Riera and McDonough, 1993; Lascombes and Pashen, 2000; Meadows et al, 2000; Lisa and Meadows, 2008; Lascombes and Pashen, 2008). A second upsurge has occurred in Quarterhorses in North America and Canada during the past decade with the courtroom imposed lifting of the ban allowing only a single foal per year by embryo transfer from individual donor mares (Squires et al, 1999; Jasko, 2002; IETS, 2006) and in Europe, South America and Australasia, significant numbers of embryos are being recovered and transferred successfully from Sporthorse mares (eventers, showjumpers, dressage horses, etc) while still in active competition (Stout, 2006). In addition to relaxation of breed society restrictions, these increases in the use of embryo transfer have been driven

also by steady increases in non-surgical transfer rates arising from operator experience (Squires et al, 1999; Jasko et al, 2002) and improvements in transfer techniques (Wilsher and Allen, 2004) and with the application of more thought and care to the handling of the embryos during the recovery and transfer phases (Squires, 1999; Lisa and Meadows, 2008). Furthermore, it has now become a practical proposition to double normal embryo recovery rates by twice-daily treatment of donor mares for 6-8 days with partially purified extracts of equine FSH extracted from horse pituitary glands post mortem (Squires et al, 1996; Alvarenga et al, 2001) or generated by recombinant gene technology (Roser et al, 2008).

Ovulation synchronisation and superovulation

The advent of injectable prostaglandin F analogues in the 1970s (Douglas and Ginther, 1972; Allen and Rowson, 1973; Palmer and Jousset, 1975), injectable progesterone preparations and the orally active progestagen, altrenogest, in the 1980s (Palmer, 1979; Allen et al, 1980), gonadotrophin-releasing hormone (GnRH) in various implant and injectable forms in the 1990s (Allen et al, 1987; Johnson et al, 2002; Fleury et al, 2004) and then progesterone alone and progesterone and oestrogen mixed, and injectable altrenogest, all in micronized, long-acting injectable formulations, together with a very efficient injectable GnRH analogue formulation for induction of ovulation of mature follicles (Burns et al, 1993; Morrow and Burns, 2007; Burns et al, 2008) have, together, supplied a very complete, efficient and cost-effective armoury for simple and sufficiently accurate synchronisation of oestrus and ovulation in groups of donor and recipient mares. Added to this extensive battery of synchronisation-effective hormones is the apparently equine-unique relative lack of stringency in the need for close synchrony of ovulation between donor and recipient animals to achieve maximum embryo transfer rates. For example, whereas the original studies of Moore et al (1960) and Moore and Shelton (1964) in the sheep, and Rowson and Moor (1966) and Rowson et al (1969) in the cow established limits of +1 to -1 days asynchrony for maximum embryo transfer success in cattle and only a slight loss of fertility at +2 to -2 days in sheep, a range of +1 to -3 days has long been accepted to maintain maximum transfer rates in the mare (Allen and Rowson, 1975; Allen, 1982a, Squires et al, 1985) . And, very recently in the author's laboratory, that range has been shown to be able to be widened even further to an amazing 8 days, i.e. from +2 to -6 days, when transferring large, day 10 embryos non-surgically without hormone supplementation of the recipient mares (Wilsher and Allen, 2009). Furthermore, a range of 5 days (+1 to -4 days) is achievable when transferring smaller day 7 or 8 embryos by administering the prostaglandin synthetase inhibitor, meclofenamic acid (Arquel V; Pharmacia Animal Health, Northants, UK) to the recipient for 8 days, between days 9 and 17 of dioestrus (Wilsher et al, 2006).

With these remarkably wide asynchrony limits permitted by the mare, donor-recipient synchrony is achieved simply in cycling mares, either by a single i.m. injection of a prostaglandin analogue given to the donor 1 or 2 days ahead of the same treatment applied to the recipient when both are between days 5 and 14 of dioestrus and therefore have a corpus luteum in their ovaries which is sensitive to the luteolytic actions of the prostaglandin (Palmer and Jousset, 1975; Allen, 1982a). Or alternatively, when the stages of the oestrous cycles are unknown, an 8-10 day course of oral or vaginal altrenogest (Regumate, Intervet Laboratories, Milton Keynes, UK) followed by an i.m. injection of prostaglandin analogue on the last day of Regumate administration to induce luteolysis of any persisting corpus luteum in the ovaries (Palmer, 1979; Voss et al, 1979; Hyland and Bristol, 1979). Follicular development in the donor and recipient is monitored by daily or alternate-day ultrasound scanning of the ovaries and an ovulation-inducing drug, such as human Chorionic Gonadotrophin (hCG; Chlorulon, Intervet Laboratories), a slow-release subcutaneous implant formulation of GnRH analogue

(Ovuplant; Peptech Laboratories, Sydney, Australia), or an i.m. injection of the GnRH analogue, buserelin (Biopharm Laboratories, Lexington, Kentucky) is given to the donor 1 or 2 days before the recipient when each exhibits a dominant preovulatory follicle of >35mm diameter (Bruyas et al, 2000; Gastel et al, 2006; Losinno et al, 2008). This causes ovulation 33-42 hours later in >85% of treated animals thereby achieving the required degree of synchrony of -1 or -2 days between donor and recipient. The desire for a slightly negative asynchrony in the mare is thought to accommodate the temporary slowdown in the rate of development of the embryo caused by the disturbances of the embryo flushing, in vitro washing and embryo transfer procedures at 7 or 8 days of age (Allen, 2005).

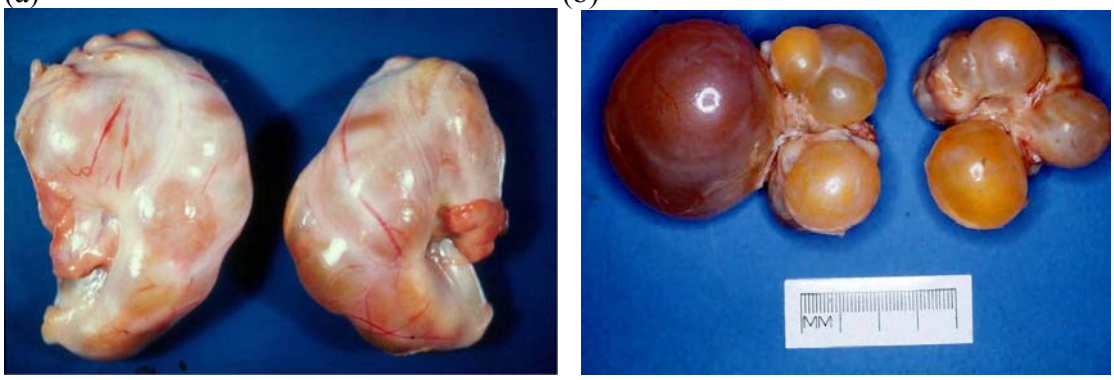
Very often nowadays in large commercial equine embryo transfer centres the recipient mare is scanned rectally just prior to transfer to confirm she shows a well developed, echogenic corpus luteum in her ovaries and she exhibits good tone in her uterus as a reflection of adequate levels of progesterone in her blood; a lessening of either of these two parameters usually means replacement of that particular recipient with another one from the pool.. Such pre-transfer clinical evaluation of recipients is held generally to improve transfer success rates in these commercial enterprises (Lisa and Meadows, 2008) and the same degree of selectivity could be achieved by using modern, AELIA assays to measure progesterone concentrations in the peripheral blood of the recipients (Allen and Sanderson, 1987; Allen, 2005).

The large size and unusual bean-shape of the equine ovary, together with its envelopment by a remarkably tough and fibrous tunica albuginea, means that ovulation and release of the oocyte can only occur through the relatively small ovulation fossa situated deep within the concavity of the "bean" (Figure 3). This unique architectural feature, combined with remarkably low levels of receptors for equine Chorionic Gonadotrophin (eCG), in equine gonadal tissue (Stewart et al, 1976, 1977), have bedevilled many attempts over the years to induce genuine superovulation in donor mares, along the lines of the 10-20 blastocysts recoverable frequently from the uteri of superovulated donor cows or sheep following a single injection of a relatively low dose of eCG or pituitary gonadotrophin extract given in mid-dioestrus 1 or 2 days before a luteolytic dose of PGF analogue (Rowson, 1971; Betteridge, 1977. Considerable progress has been made in recent years in doubling the mean embryo recovery rate per flushing attempt in valuable donor mares, from around 0.85 in untreated animals to 1.5-1.8 in those treated in mid-dioestrus for 5-6 days with twice daily injections of partially purified preparations of equine pituitary gonadotrophins (Squires et al, 1986; Alvarenga et al, 2001; Niswender et al, 2003) and more recently, with equivalent results, using recombinant derived preparations of equine FSH (Niswender et al, 2008; Roser et al, 2008), followed by an ovulation-inducing hormone such as hCG or equine pituitary LH when the majority of the developing follicles, monitored closely by daily scanning of the ovaries, reach +35mm in diameter. Apart from the considerable purchase and labour costs associated with the multiple gonadotrophin injections, the operator experiences the frustration of ultrasonographically viewing the "apparent ovulation" (i.e. the replacement of non-echogenic follicular fluid with an echogenic luteal structure) of as many as 6-10 large, follicles only to recover 0-3 blastocysts when flushing the mare's uterus 7 or 8 days later. This big discrepancy between apparent ovulation rate and actual embryo recovery rate in gonadotrophin-stimulated mares is very likely the result of simple, physical competition between the large follicles to simultaneously track through the ovarian stroma to reach the ovulation fossa, causing the majority of them to luteinise before they can ovulate and release their oocyte through the fossa (Figure 4). Or alternatively, but much less likely, the increased size of the hyperstimulated ovary disturbs the normal ability of the fimbria of the oviduct to "pick up" the multiple oocytes as they are released from different areas within the ovulation

fossa. Whatever the precise cause of this marked reduction in embryo recovery rate from superovulated mares, the problem is clearly physical, rather than biochemical or biological, in nature and is therefore unlikely to be overcome in the future.

Figure 3

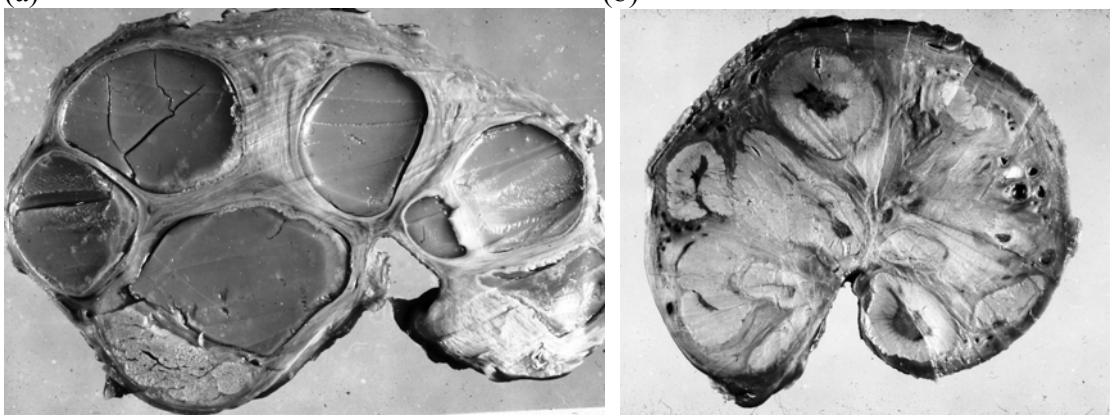
(a) (b)



Ovaries recovered from an early oestrous mare before and after removal of the tunica albuginica showing the ovulation fossa within the concave surface of the ovary.

Figure 4

(a) (b)



Sections of mares' ovaries illustrating how multiple large follicles, perhaps generated by exogenous gondadotrophin treatment would complete with one another and luteinise before reaching the ovulation fossa.

Despite these limitations, the much increased use of exogenous gonadotrophin preparations in commercial equine embryo transfer centres in recent years, particularly in Quarterhorses in America, to cost effectively double embryo collection rates without apparently causing gonadotrophic desensitisation or down-regulation in repeatedly treated animals (McCue, 1996; Squires, 1999, Alvarenga et al, 2001; Scoggin et al 2002; Niswender et al, 2003) is a significant step forwards. Furthermore, the advent of recombinant-derived equine FSH and LH (Roser et al, 2004, 2008) to replace the rapid decline in availability of pituitary-derived equine gonadotrophins arising from the absurd, politically driven closure of horse abattoirs throughout America, is indeed a timely saviour for the whole equine embryo transfer industry in that country.

Nevertheless, it would be of considerable interest to compare the overall productivity and efficiency, in terms of the numbers of elite embryo transfer pregnancies created per donor mare per season, between, say, the American system using repeated gonadotrophic

stimulation of the ovaries donor mares prior to each embryo flush, with the well-honed Argentinian system involving intensive management of the donor and recipient mares to enable repeated flushings of non-gonadotrophin treated donors over a shorter period or "season", with the great advantage of the "flushing effect" experienced by the polo pony donor mares in their rapid transition from peak fitness, greyhound-like playing condition to the sleek, grass-fed broodmare condition at the embryo transfer centre, plus the attendant increase in natural twinning rate that accompanies such a dramatic change (Niswender et al, 2008; Lisa and Meadows, 2008; Lascombes and Pashen, 2008).

Embryo recovery and transfer

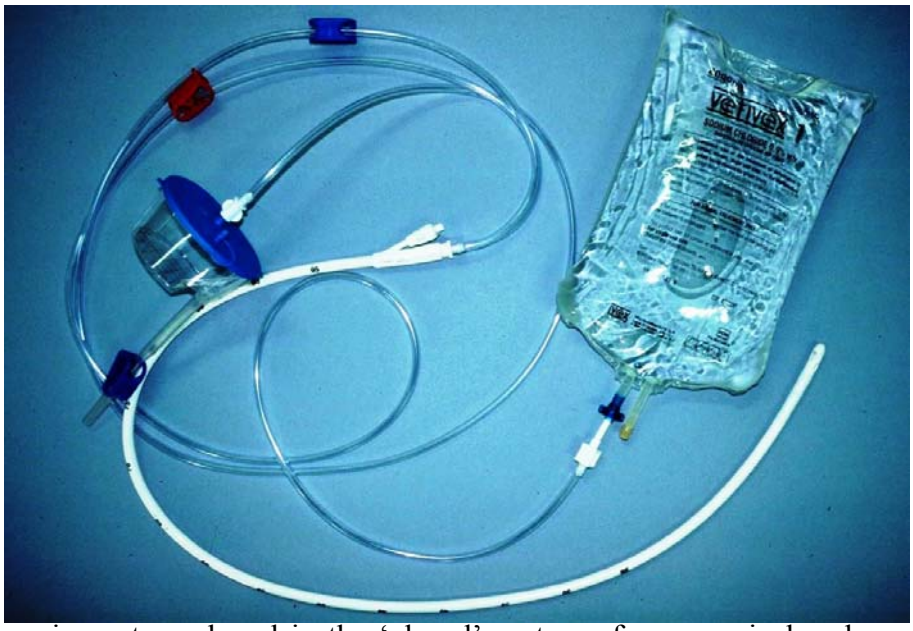
As mentioned previously, the realisation of the long oviducal transport time of the equine embryo and the need to flush the whole uterus, not just the ipsilateral uterine horn, of donor mares caused a major jump in embryo recovery rates in the 1970s. Since that time, however, little has changed in the basic methods employed for embryo recovery. The relatively straight and easily distensible cervix of the mare allows passage of a simple, flexible, 2-way flushing catheter into the uterus for irrigation, 2 or 3 times, with 1-2 litres of a simple, cheap, saline-based flushing medium containing a small quantity (0.5% w:v) of an innocuous protein (such as fetal calf serum or oestrous mare serum) to prevent the embryo from "sticking" to the sides of the flushing apparatus. The inclusion of an in-line embryo filter and the commercial availability of bags of sterile flushing medium to create a truly "closed" flushing system (Figure 5) have greatly improved the vital sterility of the flushing process. Furthermore, the frequent use nowadays of intravenous oxytocin (20i.u.) administered to the donor mare during the last of the 2 or 3 flushes to aid in the complete expulsion of all the flushing medium from the uterus, has combined to push embryo recovery rates up to the maximum per cycle conception rates for the breed of horse. In short, present day embryo recovery efficiency is as good as it is likely to ever get.

Embryo transfer, on the other hand, has seen some major and significant changes and improvements over the past 30 years. In the 1970s and 1980s the majority of horse embryos were transferred surgically, either by a mid-line ventral laparotomy under general anaesthesia as described previously (Figure 2, Allen and Rowson, 1975; Allen, 1982a) or via flank laparotomy under local anaesthesia (Squires et al, 1982, 1985). A number of published comparisons between surgical and non-surgical transfer rates in the 1980s highlighted the benefit of the surgical approach (Imel, 1981; Juliano et al, 1985; MacKinnon et al, 1988; Squires, 1993) and it wasn't until the commercial polo pony embryo transfer centres in Argentina blossomed in the 1990s that non-surgical, transcervical transfer began to take over. In the highly competitive, but extensive, system of horse management in South America, surgical transfer was simply not a cost-effective option and it quickly became apparent that operator skill in manipulating the transfers gun or pipette through the dioestrous mare's cervix with minimal physical disturbance or trauma (Figure 6), combined with increased sterility achieved by protecting the transfer pipette in a plastic chemice during passage through the vagina, plus the ability to discard any recipient mare with a history of infertility, an atonic uterus or, especially, a cervical abnormality that might impede passage of the transfer pipette, that non-surgical, transcervical transfer rates could be raised to equal those obtained by surgical methods (Pashen et al, 1993; Losinno et al, 2001; Jasko, 2002, McCue and Troedsson, 2003, Lisa and Meadows, 2008).

Thus, the past 15 years has seen a universal change to transcervical transfer methods accompanied by a steady rise in pregnancy rates to around 70-85% in well managed

commercial operations around the world, particularly in North and South America, Australasia and, more recently, in Europe. A number of experimental findings were put forward to try to explain the previous failures of the non-surgical approach, including i) the release of PGF2α (Kask et al, 1997; Koblischka et al, 2008) and/or oxytocin (Handler et al, 2002) by manipulation of the cervix during the transfer procedure leading to premature luteolysis (Hurtgen and Ganjam, 1979; Lagneaux and Palmer, 1989); ii) localised inflammatory responses in the endometrium and/or reflux of the embryo back through the cervix by its surface tension adherence to the tip of the transfer pipette (Squires et al, 1989; Jasko, 2002); iii) infective endometritis established by the passage of commensal vaginal bacteria into the progesterone-dominated uterus at the time of transfer (Allen, 1982a; Lagneaux and Palmer, 1989; Koblischka et al, 2008). On the other hand, Handler et al (2002) showed that pregnancy loss did not necessarily follow cervical dilation in the mare and PGF2α released during sham transcervical transfer did not induce luteolysis (Betteridge et al, 1985; Kask et al, 1997). Thus, operator experience and skill, good ovulation synchrony between donor and recipient mares, ruthless culling of poor quality recipients and strict adherence to sterile practise during the embryo recovery, handling and transfer procedures, were the factors most immediately associated with the rise in non-surgical transfer successes. The requirement for operator skill, acquired from the experience of transferring some hundreds of embryos per breeding season, still proves problematic for equine veterinary practices wishing to utilise embryo transfer for some of their clients. How could such expertise be acquired without the need for these hundreds of transfers per year?

Figure 5



The equipment employed in the 'closed' system of non-surgical embryo recovery from the mare. Note the plastic bag of sterile medium and the in-line embryo filter.

The conundrum stimulated the author's colleague, Sandra Wilsher, to re-evaluate the whole non-surgical procedure to try to simplify and make it more widely applicable and user-friendly. By adapting the commonly used German method of employing Vellcellum forceps to grasp and retract the oestrous mare's cervix for routine artificial insemination (Hans Merkt, personal communication) Sandra designed a set of elongated forceps with a rigid upper jaw for insertion into the external os of the dioestrous cervix and a hinged lower jaw with sharp teeth to close them and grasp the ventral quadrant of the cervix with minimal damage to the

tissue and no release of PGF2α (Figure 7). Pulling back on the forceps straightens the cervical canal (Figure 8a) and allows simple, smooth passage of the transfer pipette through the cervical canal and well up into one uterine horn in a simple, smooth, forward movement (Figure 8b). The plunger of the syringe is depressed rapidly to literally "squirt" a larger-than-normal volume of medium (2.5 - 3.0 ml) containing the embryo further forward up a uterine horn and therefore well away from the pipette tip. The addition of an old-fashioned Polansky's vaginal speculum with a hand-held light allows the cervix to be grasped visually (Figure 8c) and enables the second operator to insert and push forward the transfer pipette (Figure 8d), deposit the embryo and withdraw back out of the uterus, all very simply and in <10 seconds. The whole procedure means that only autoclaved sterile instruments, not a human arm, penetrate the vagina, cervix and uterus, thereby minimising the chance of forward passage into the uterus of bacteria from the vulval lips or vaginal wall (Figure 6).

Figure 6

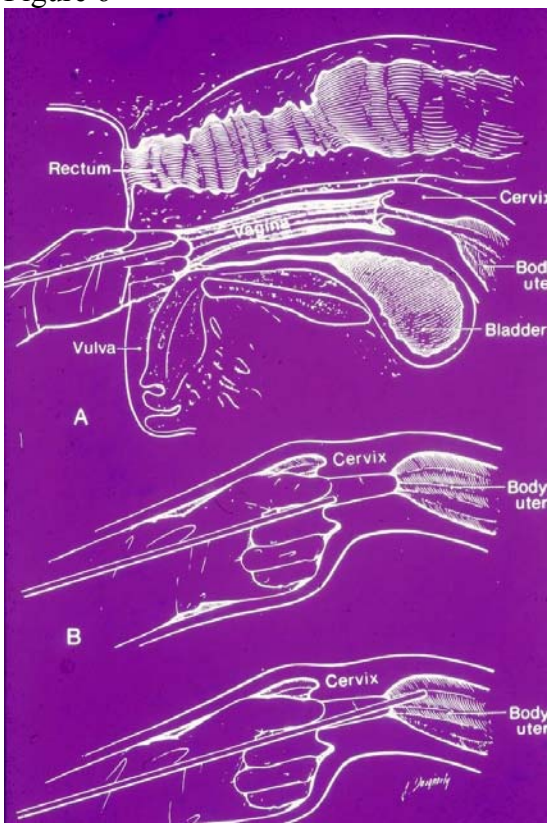


Diagram illustrating the conventional method of non-surgical embryo transfer in the mare. The operator's gloved hand in the vagina guides the tip of the transfer gun through the external os of the cervix before manipulating it transrectally through the cervix and further into the uterus (From Squires 1982).

The other important change in the new method is the use of a much larger sterile insemination pipette, so dispensing with the 0.5 ml straw and the much larger volume of transfer medium which provides cold-shock protection for the embryo and allows it to be squirted well up into the uterine horn and away from the pipette tip (Figure 9). Best of all, the method requires no great experience or manipulative skill to perform and it is well within the grasp of any equine-oriented veterinary clinician. An early trial to evaluate the method gave a pregnancy rate of 85% from the transfer of 20 Grade A embryos to well synchronised recipient mares which compared to a rate of only 40-50% which had been achievable in the

author's laboratory previously using the conventional embryo transfer method (Wilsher and Allen, 2004). In the 4 succeeding years, an overall transfer rate of >80% was achieved using the Wilsher Embryo Transfer Forceps for both experimental and commercial transfers.

Figure 7

The anterior tip of the Wilsher Equine Embryo Transfer Forceps showing the rigid upper jaw for insertion into the external cervical os and the hinged and toothed lower jaw for grasping the ventral quadrant of the cervix.

Embryo splitting

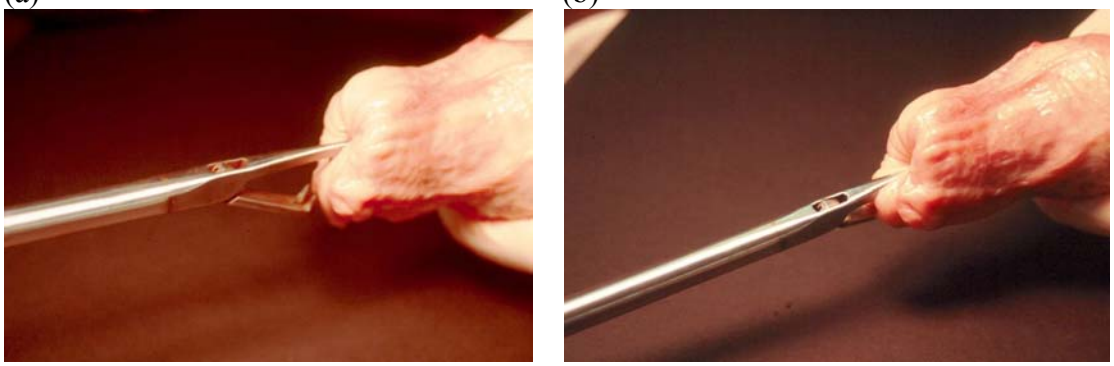
The diffuse epitheliochorial architecture of the equine placenta (Amoroso, 1952; Samuel et al, 1975) renders the mare unable to carry twins, dizygotic or monozygotic, safely to term. When twins are conceived, as occurs spontaneously in some 8% of Thoroughbred pregnancies (Allen et al, 2007), one conceptus usually outgrows the other in early gestation with the result that the disadvantaged fetus literally starves to death from placental inadequacy in mid- to late-gestation, causing abortion of both conceptuses (Jeffcott and Whitwell, 1973). Thus, the ability to bisect a horse embryo and transfer the resulting demi-embryos to separate recipient mares has scientific and practical appeal. First, to produce valuable research animals with genetic identity and, second, as a potential means of doubling embryo recovery rate.

In collaboration with Dr Steen Willadsen, the author and his then research student, Robert Pashen, produced the first horse identical twins in the late 1970s using the Willadsen technique (Willedsen, 1979) to dissect out the individual blastomeres from 4- or 8-cell embryos recovered surgically by flushing the donor mare's oviduct on the second or third day after ovulation. Pairs of blastomeres were then injected into empty zonae pellucida derived from abattoir pig ovaries and the newly constructed 2-cell demi-embryos were embedded in a small plug of agar gel, then inserted into a second agar gel plug and transferred to the oviduct of a dioestrous ewe ("mobile temporary incubator") for 3 or 4 days to allow the 2-cell embryos to develop to the morula or blastocyst stage. The agar plugs were then removed from

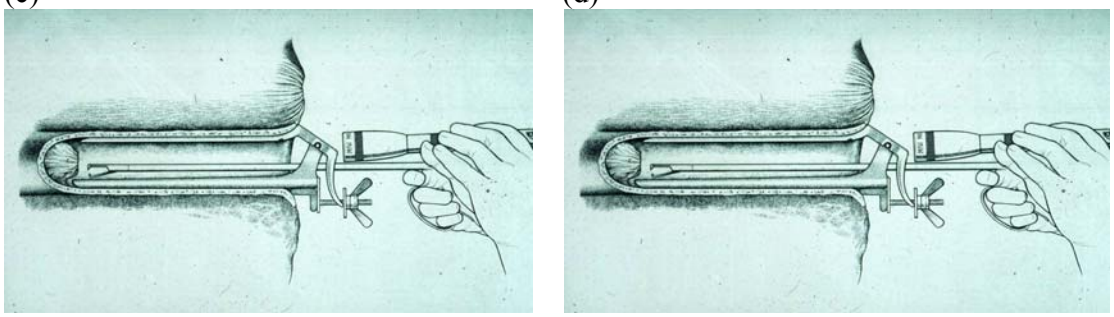
the sheep and the embryos dissected free from the agar before being transferred to the uteri of two recipient mares on the 4th or 5th day after ovulation (Figure 10a). Although cumbersome and time-consuming, and suffering the disadvantage of the requirement for the surgical flushing of the oviduct-stage embryos from the donor mare, the method was remarkably efficient and two sets of identical twins were produced from 5 attempts (Allen and Pashen, 1984). Furthermore, these two sets of twins, a pair of colts and a pair of fillies, displayed some interesting and important features of equine reproductive physiology. In the first pair of colts, one conceptus developed a normal set of active endometrial cups (Allen and Moor, 1972) which secreted high concentrations of eCG into the maternal circulation between 40 and 100 days of gestation (Allen, 1969a) and which, in turn, stimulated a number of accessory ovulations in the maternal ovaries to maintain high progesterone concentrations until mid-gestation (Figure 11a).

Figure 8

(a) (b)

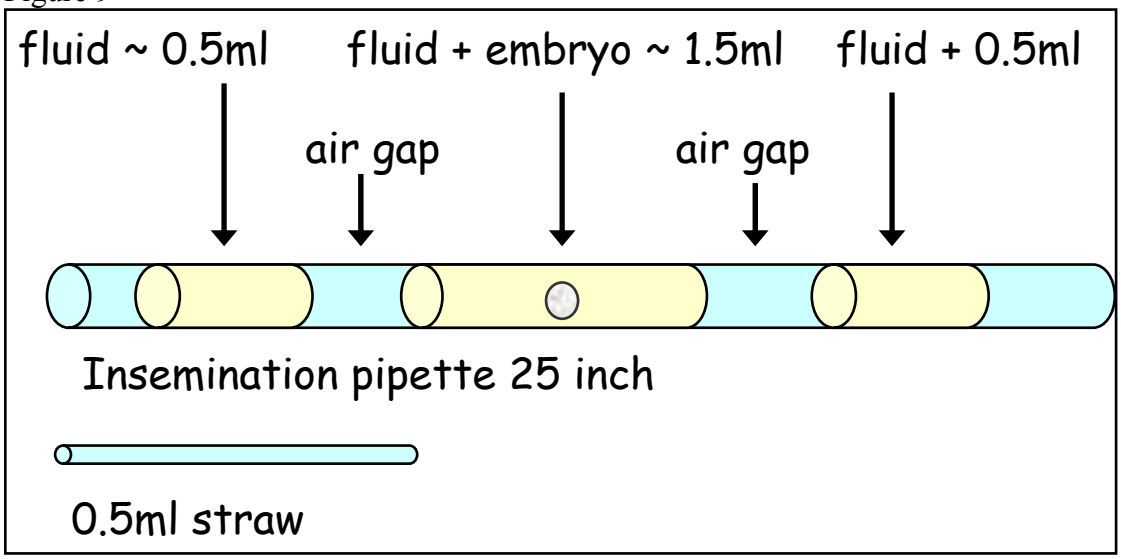


(c) (d)



a & b) Showing grasping and retraction of a post mortem cervix with the Wilsher Forceps; c & d) diagrams showing distension of the vagina with the Polansky's speculum, grasping and retraction of the cervix with the Wilsher Forceps, followed by torch-light directed insertion and forward progression of the embryo transfer pipette (From Wilsher and Allen 2004)

Figure 9

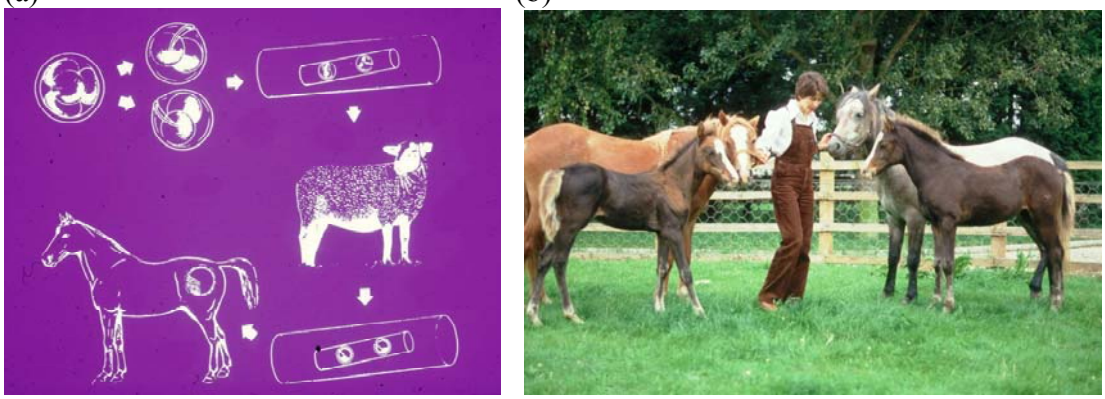


Diagrammatic representation of the larger fluid volumes used in the semi-rigid embryo transfer pipette compared to the conventional 0.5ml straw.

Conversely, the other co-twin in the second recipient showed only the faintest "blip" of eCG in maternal blood (1-2 i.u./ml) between 44 and 53 days of gestation and developed no secondary corpora lutea at all during the first half of gestation, yet the pregnancy was maintained to term and the fetus developed normally, albeit that the uterus exhibited poor tone when palpated per rectum between days 20 and 80 of gestation (Figure 11b).

Figure 10

(a) (b)



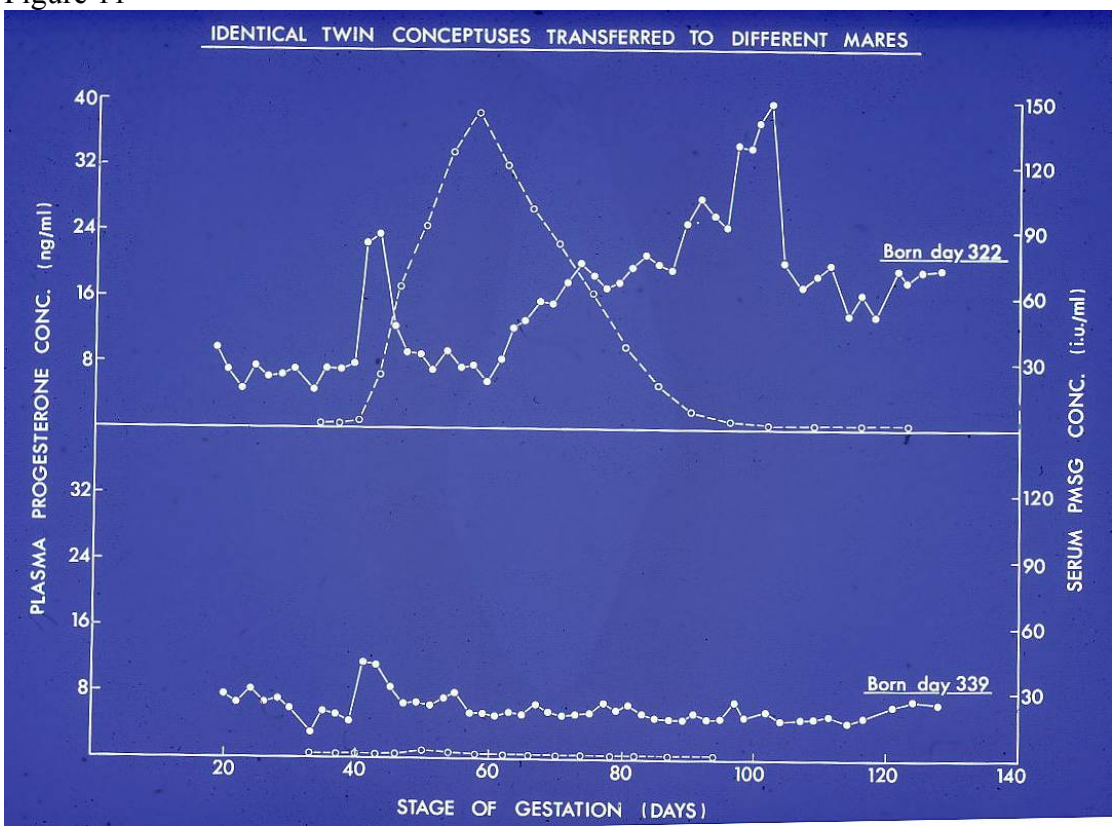
a) Diagrammatic representation of the Willedsen technique for the production of monozygotic twins; b) a pair of identical twins created by the Willedsen technique with their surrogate mothers. Note the smaller size of the twin on the left due to the smaller size of its surrogate mother.

The second important finding in the identical twin fillies was the marked effect of maternal size, and hence uterine capacity, on the size of the offspring, thereby mirroring the original demonstration by Walton and Hammond (1938) in their classical experiments of crossing large Shire horses with much smaller Shetland ponies using artificial insemination. One demi-embryo was transferred to a 13.2hh recipient mare that was similar in size to the genetic sire and dam of the original embryo; this foal was born healthy and robust after 327 days of gestation. The other demi-embryo was transferred to a 12 hh Welsh Pony recipient

mare that was appreciably smaller and less "roomy" than its genetic parents and this foal, born 23 days after its co-twin at 350 days of gestation, was noticeably smaller, showed mild signs of dysmaturity (Rossdale and Silver, 1982) and required assistance to stand and suck during the first 12 hours of life (Figure 10b). Subsequently, it showed a normal growth curve but, at 2 years of age, it remained 3 inches shorter at the withers and 64 kg lighter than its identical co-twin born from the bigger recipient mare.

Thus, this first identical twin experiment showed clearly the necessity, in commercial equine embryo transfer, to place embryos in surrogate mares that are at least as big and "roomy", or more so, than the genetic parents of the embryo to prevent the occurrence of intrauterine growth restriction (IGR) and postnatal "runting" from the development of a smaller than normal placenta. This important influence of recipient size was confirmed in an elegant experiment carried out by Professor Marian Tischner and his colleagues at the Animal Reproduction Laboratory in Krakow, Poland when they produced no fewer than 3 pairs of sex-matched, full sibling foals where one embryo from a Konik Pony donor mare was transferred to a considerably bigger draught-type recipient mare while its next cycle, full sibling brother or sister developed to term in the uterus of the genetic Konik Pony mother. In all 3 pairs of foals, that born from the surrogate draught mare was heavier and taller at birth than its full sibling born from the genetic mother and the size differences persisted to adulthood at 3 years of age (Tischner and Klymczak, 1989).

Figure 11

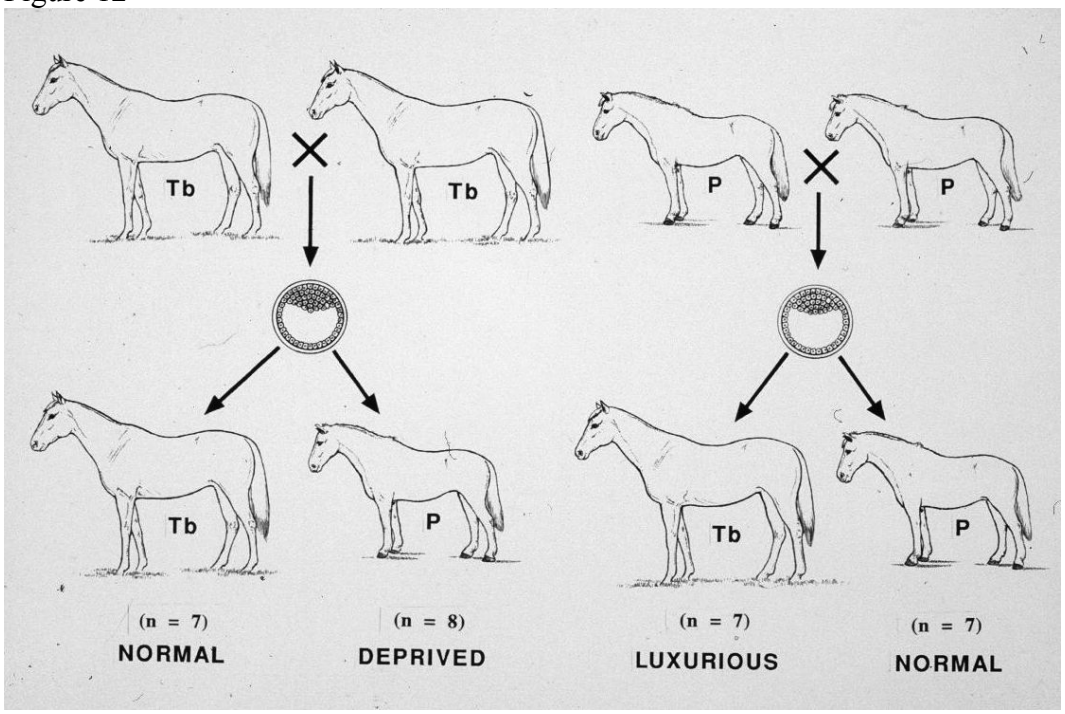


eCG and progesterone profiles measured in the serum of two Pony mares, each carrying an identical twin conceptus created by the Willedsen (1979) technique. Note the lack of eCG in the second mare and the complete absence of secondary ovulations from around 40 days.

The same marked influence of maternal uterine size upon the birthweight and adult size of the foals was similarly highlighted by the author and his colleagues when they transferred

Thoroughbred (Tb) embryos to small Welsh Pony mares (P) to create a model of intrauterine deprivation and cramping, versus P embryos transferred to Tb mares to create the opposite situation of intrauterine excess mediated by a much larger-than-normal placenta (Figure 12). As expected, the Tb-in-P foals were born appreciably smaller and weaker than their Tb-in-Tb controls while, vice versa, the "luxurious" P-in-Tb foals were born much bigger and stronger than their P-in-P counterparts (Allen et al, 2002) and, as shown previously by Walton and Hammond (1938) and Tischner and Klymczak (1989), the size disparities persisted to adulthood at 3 years of age (Figure 13), but without any evidence of organ maldevelopment or malfunction in the deprived Tb-in-P foals (Giussani et al, 2003; Forhead et al, 2004).

Figure 12



Schematic diagram summarising the reciprocal transfer of Thoroughbred and Pony embryos between these two different sized breeds.

Figure 13

(a) (b)

(c) (d)

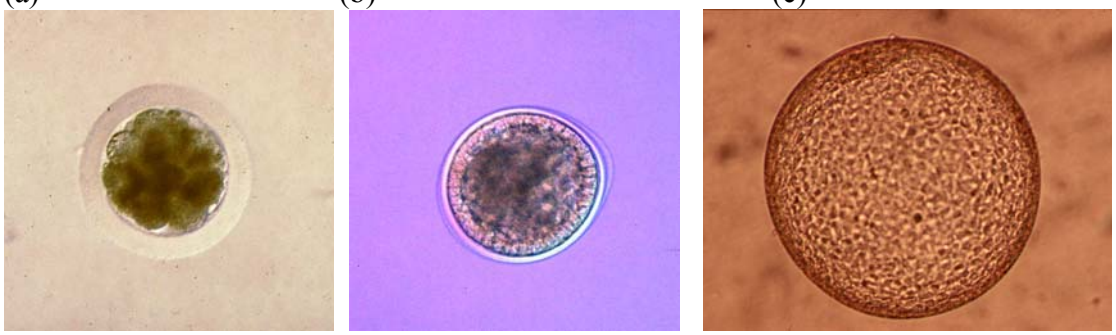
a & b) Thoroughbred-in-Pony (left) and Thoroughbred-in-Thoroughbred controls (right) at a) 16 months and b) 3 years of age. c & d) Larger Pony-in-Thoroughbred foals compared to their smaller Pony-in-Pony controls at c) 16 months and d) 3 years of age. Note the gross fatness of the P-in-Tb offspring at 3 years.

Conversely, however, the "luxurious" P-in-Tb foals all showed marked obesity in adulthood which appeared to be caused by compulsive eating, perhaps entrained during fetal life in utero (Allen et al, 2004).

The modern method of making identical twins by bisecting a morula using the micromanipulator works well in the horse provided this splitting is carried out on true, compact morulae which have not yet undergone any differentiation into inner cell mass (ICM) and trophectoderm (Figures 14a & 15; Skidmore et al, 1989; McKinnon et al, 1989). The big problem is to recover these morulae by non-surgical flushing of the uterus. The demonstration by Battut et al (1997) that the equine embryo enters the uterus 6 days after ovulation when it is already at the late morula/early blastocyst stage of development means that, even if the time of ovulation in the donor mare is determined very accurately, flushing her uterus early on day 6 greatly diminishes the chance of recovering any sort of embryo while flushing later on day 6 greatly increases the chances of recovering an embryo in which ICM differentiation has already commenced but is not yet able to be recognised microscopically (Figure 14b). Thus, bisection of this embryo recovered at the later stage is very unlikely to include accurate splitting of the differentiated cells. The lottery-like nature of the problem was well illustrated in one experiment carried out in the author's laboratory in which 12 Thoroughbred embryos recovered on day 6 after ovulation from 11 mares, with no discernible ICM differentiation, were bisected well. After 3 h in culture to allow the blastomeres to re-compact within the zona pellucida, 19 of the demi-embryos were transferred surgically to the uteri of synchronised recipient mares to give 10 on-going pregnancies. However, only one pair of these embryos developed as monozygotic twins while in 8 other pregnancies only one of the demi-embryos continued to develop normally. This indicated that, by chance alone, only half of the embryo had contained the cells which had already begun, unseen, to specialise into ICM cells (WR Allen and JA Skidmore, unpublished findings).

Figure 14

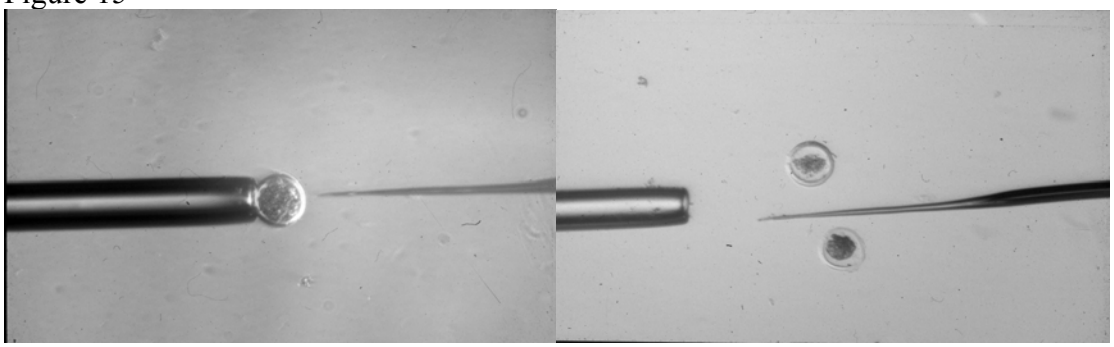
(a) (b) (c)



Equine embryos a) a day 5 compact morula with a thick zona pellucida; b) a day 6 early blastocyst with a palisade layer inside the thinning zona pellucida; c) a late day 7 expanded blastocyst. Note the very small size of the ICM, situated between 11.00am and 12 noon in the blastocyst.

In a parallel experiment, a method was developed to hasten the entry of the embryo into the uterus on day 5, rather than day 6, after ovulation when still at the compact morula stage of development and therefore ideal for both splitting and deep-freezing. In their classical experiments, Webber et al (1991a & b) had demonstrated that the equine embryo secures its own transport through the oviduct by releasing significant quantities of PGE2 from day 4 after ovulation which peristaltically relaxes the smooth, and contracts the longitudinal, smooth muscle of the oviduct wall, thereby driving the embryo on into the uterus. This same group also showed that embryonic entry into the uterus could be hastened by 24 hours by surgically implanting a mini-pump releasing PGE2 onto the mesovarium on day 4 after ovulation (Webber et al, 1991b). Extending this important finding, the author and clinical colleagues in Newmarket achieved the same rapid passage by applying a pharmacological dose of PGE2 in triacetic gel to the ipsilateral oviduct by the means of a rigid laparoscopy inserted through the flank of the mare (Robinson et al, 1999). However, the need to both starve the donor mare for 24 hours and shave a large area of hair off its flank in order to carry out the laparoscopy procedure rather negates the method's commercial applicability to valuable mares, although it has proved valuable to unblock the oviducts of aged, infertile mares (Allen et al, 2006).

Figure 15



Above) Bisection of a horse morula using the micromanipulator.
Below) Three pairs of identical twin horses produced by this technique at The Equine Fertility Unit, Newmarket.

Embryo transfer and freezing

Mimicking the original, successful transport of sheep embryos to South Africa in the oviducts of rabbits in 1961, the author and colleagues commemorated the 40th anniversary of the original flight of freshly collected ram semen from Dr John Hammond's Animal Research Station in Cambridge to Professor Roman Prowahenski's Animal Reproduction Laboratory in Krakow, Poland in 1936 where a single lamb, named AIRMAN, was born five months later, by driving non-stop from Cambridge to Krakow with two female rabbits carrying six 8-16 cell horse embryos in their oviducts; these had been recovered surgically from oviducts of donor mares early in the morning of departure (Figure 16a). Overcoming a number of obstacles en route, including a shortage of petrol and the desire of the guards on the East German-Polish borders to confiscate and eat the rabbits, the team arrived safely in Krakow some 42 hours after leaving Cambridge and immediately recovered 5 of the embryos from the rabbits and transferred these surgically to synchronised recipient mares at the Animal Reproduction Laboratory with the kind help and co-operation of Professor Wadslaw Bielanski and his able second-in-command, Dr Marian Tischner. The three Welsh Pony foals born safely the following year (Figure 15b) became mascots for the 7th International Congress of Animal Reproduction and Artificial Insemination (ICAR) held in Krakow in July 1976. They were the first equine embryo embryos to be transported internationally (Allen et al, 1976).

Figure 16

(a) (b)

a) Transport of horse embryos to Krakow, Poland in the oviducts of two rabbits in July, 1975.
b) One of the resulting Welsh Pony foals with its surrogate mother in Poland 12 months later.

Transport of equine embryos has grown exponentially in the New Millennium, particularly in North America and Europe, and mostly by shipping day 7 or 8 blastocysts in a nutrient-rich medium at 4ºC in an insulated container (Figure 17). These embryos are collected from valuable Quarterhorse or Sporthorse mares at one location and shipped for transfer to suitable recipient mares maintained and monitored in a recipient herd at another location. The systems works well as the equine blastocyst seems well able to survive for 24-48 h at 4ºC (Squires et al, 1999). Two major reasons for this rapid increase in national and international transport of cooled horse embryos are the scarcity of well-maintained recipient herds with experienced operators to carry out the non-surgical transfers, and the relatively low pregnancy rates achieved from deep freezing horse embryos in liquid nitrogen. From the very mediocre pregnancy rate of 48% achieved in the author's laboratory in the 1980s with the help of the eminent Polish embryologist, the late Maria Czlonkowska (Figures 18 & 19; Czlonkowska et al, 1985; Skidmore et al, 1991), little improvement in freezing rates of equine embryo has been achieved worldwide by conventional freezing methods in liquid nitrogen, despite many attempts to do so (Slade et al, 1985; Seidel et al, 1989; Bruyas et al, 1993, 1995, 1997; Meira et al, 1993; Hochi et al, 1994, 1996; Ferriera et al, 1997; and others). Two major equine-specific hurdles seem to impede useful progress. First, the ICM of the equine blastocyst is particularly small compared to that of the embryos of the other large domestic species (Figure 14c) so that damage to only a few ICM cells during the freezing process can easily be fatal to the embryo (Bruyas et al, 1993, 1995); this is exemplified by a high rate of development of anembryonic trophoblast vesicles following transfer of frozen-thawed equine blastocysts to recipient mares (Skidmore et al, 1991; S. Wilsher and W.R. Allen, unpublished observations). Clearly, it would be adventitious to freeze morulae or early blastocysts, rather than expanded blastocysts, to overcome this particular problem and, indeed, all the studies undertaken to date have confirmed improved success rates when freezing smaller embryos recovered on day 6 after ovulation compared to expanding and fully expanded blastocysts recovered on day 7 or 8 (see Squires et al, 1999 for review). However, the problem is exactly as described previously for the recovery of morulae for splitting to produce identical twins. Namely, the recovery rate when flushing on day 6 is significantly lower than that achieved when flushing on day 7 or 8 (Boyle et al, 1989).

Figure 17

A typical, insulated container (Equitainer; Hamilton-Thorn Industries, Maryland, USA) used for successful trans-national and international transport of equine embryos over 24 hours.

The second, and closely related, problem with the attempted freezing of blastocysts is the existence of the equine-unique blastocyst capsule which completely envelops the equine embryo from day 6.5 until day 21-23 after ovulation (Betteridge, 1989). This very tough and elastic, negatively charged, glycoprotein membrane (Oriol et al, 1993a & b) is secreted initially by the trophectoderm cells and is "moulded" into its all enveloping membrane-like configuration by the persistence of the zona pellucida at this early day 6-6.5 day stage of development. Following hatching from the zona, the capsule maintains the spherical shape of the embryo and it provides the elastic strength to enable the latter to withstand the considerable compressive forces of the myometrial contractions which drive it continuously throughout the uterine lumen during days 6 to 17 after ovulation, presumably so it can release its maternal recognition of pregnancy (MRP) signal onto sufficient of the total surface area of the endometrium to suppress the normal, cyclical luteolytic cascade and achieve luteostasis for maintenance of the pregnancy state (Ginther, 1983; Stout and Allen, 2001). The capsule's negative electrostatic charge makes it "sticky" so that it accumulates histotroph, the nutritive endometrial gland secretions essential for embryonic growth, on its external surface (Oriol et al, 1993b; Stewart et al, 1995) as the embryo's pulsatile releases of PGF2α and PGE2 propel it around the uterine lumen (Ginther, 1983; Stout and Allen, 2001). Thus, the blastocyst capsule is an integral and vital component of the young equine embryo which must be present between days 7 and 20 of gestation for embryonic survival (Stout et al, 2005). However, there is a good deal of evidence that its presence on the blastocyst interferes with diffusion of any cryoprotectant into or out of the embryo during the freezing and thawing processes, thereby drastically lowering the success rate of freezing these capsule-enclosed expanded blastocysts (Pfaff et al, 1993; Seidel, 1996; Legrand et al, 2000; Gillard Kigma et al, 2008).

Figure 18

(a) (b)

a) The first born frozen-thawed embryo as a newborn foal with its surrogate Pony mother at the Animal Research Station, Cambridge in 1982. b) A Polish Konik Pony foal with its surrogate mother at the Animal Research Station in 1984 following shipment to the UK as an embryo in liquid nitrogen. Both embryos were frozen by the late Maria Czonklowska.

A ray of hope in the saga of equine embryo freezing occurred when Elaine Carnevale and her colleagues at the Animal Reproduction Laboratory in Fort Collins, Colorado reported encouraging pregnancy rates of 60-75% when applying the technique of rapid vitrification with very high concentrations of the cryoprotectant, ethylene glycol, to horse embryos of <300 um diameter recovered from donor mares 6.5 days after ovulation (Carnevale et al, 2004). A commercial vitrification kit for horse embryos was marketed on the back of that work (Equine Vitrification Kit; AB Technology, Pullman, WA, USA) but, disappointingly, other laboratories around the world were unable to produce equivalent results when using it (Stout, 2006; Sandra Wilsher and WR Allen, unpublished findings) and freezing equine embryos appears to have sunk back into the doldrums. This situation was reflected in the scientific programme of the 7th International Symposium of Equine Embryo Transfer held in Cambridge, UK in July, 2008 when only 3 papers were presented on embryo cryopreservation, one of which interestingly examined the rate of passage of cryoprotectants through isolated capsular material (Gillard Kigma et al, 2008). The transport of cooled embryos is so simple and successful, while the freezing of horse embryos remains relatively unsuccessful and disappointing by comparison, it will take major advances in novel cryoprotectants and/or freezing techniques to reinstate embryo freezing as a viable option in commercial equine embryo transfer operations.

Extraspecies embryo transfer

The rare ability of the many phenotypically and karyotypically diverse member species of the genus Equus to interbreed to produce live, although usually sterile, offspring (Gray, 1972; Allen and Short, 1997) is superseded by their even more surprising capacity to carry a wide range of truly extraspecific, xenogeneic pregnancies created by embryo transfer (Allen, 1982b; Kydd et al, 1985; Allen and Short, 1997). Thus, female horses (Equus caballus, 2n=64) have been shown to be able to carry Przewalski's horse (E. przewalski, 2n=66), donkey (E. asinus, 2n=62) and Grant's zebra (E. burchelli, 2n=46) embryos to term donkeys have carried horse and zebra foals to term (Figure 20; Allen, 1982b, Bennet and Foster, 1985; Kydd et al, 1985, Summers et al, 1987) and the hybrid mule (E. mulus mulus, 2n=63) can carry and give birth to both horse and donkey foals (Antczak et al, 1985; Davies et al, 1985).

Figure 19

Steen Willedsen (standing left), Marion Tischner (standing right) and Twink Allen (squatting) with a newly awakened surrogate mare at The Animal Reproduction Laboratory in Krakow, Poland following surgical transfer of a frozen-thawed embryo transported from Cambridge. The 'operating table' was the pile of straw in the shed behind.

These extraspecific equine pregnancies created wonderful experimental models to highlight immunological effects of equine pregnancy and the dominant role of uterine environment on the differentiation and development of the component membranes of the conceptus, especially the invasive chorionic girdle progenitor tissue of the eCG-secreting equine endometrial cups (Allen, 1969b, 1975, 1982b). For example, transfer of donkey embryos into the uteri of horse mares suppresses chorionic girdle formation to the point that the girdle completely fails to invade the surrogate horse endometrium around day 36 of gestation, with the result that no endometrial cups develop and no eCG is secreted into maternal blood (Allen, 1982b). Conversely, transfer of a hybrid mule embryo from the horse mare in which it is conceived to the uterus of a donkey results in the development of a much bigger and broader chorionic girdle, and hence larger and more eCG-productive endometrial cups, than would have developed had the mule embryo been left in-situ to develop normally in the uterus of its genetic horse mother (Figure 21; Allen, 1969b; Allen et al, 1993) In this way, the technique of embryo transfer has helped to demonstrate the over-riding influence of uterine environment on the early development of the equine .

Figure 20

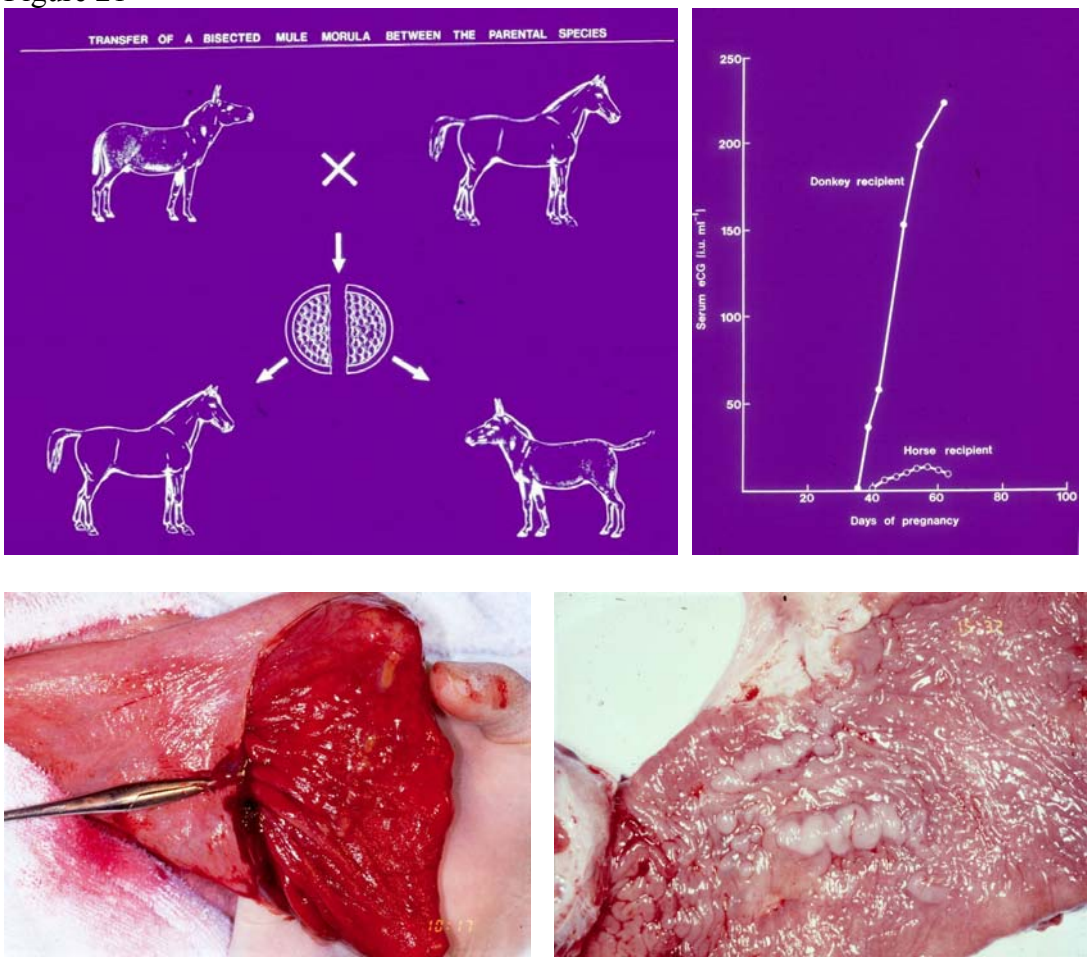
Genetic drift in the nursery! Pony surrogate mares (2n = 64) with their transferred extraspecies Przewalski's horse (2n = 66), donkey (2n = 62) and Grant's zebra (2n = 46) foals.

Modern breeding technologies

As with conventional embryo transfer, development of the so-called modern breeding technologies of in-vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI), gamete intra-fallopian transfer (GIFT) and nuclear transfer or cloning (NT) initially lagged behind their development and application in cattle and the other farm species. But there has been a significant catch-up in recent years.

Conventional IVF has remained very much a cul-de-sac in the horse, however, despite major research input into this area in the 1980s and 1990s (McKinnon et al, 1986; Bezard et al, 1989; Del Campo et al, 1990; Palmer et al, 1991; Choi et al, 1993; Alm and Torner, 1994; Brinsko et al, 1995; Grondahl et al, 1995a & b; Hinrichs et al, 1995; Li et al, 1995; Meintjes et al, 1995; Bruck et al, 1996; Del'Aquila et al, 1997 and others. The results have been very disappointing indeed with the birth of only two live foals following IVF carried out on in vivo matured oocytes (Palmer et al, 1991). The problems with the technique are multi-faceted and include a relatively low recovery rate of mature oocytes from ovum pickup (OPU) attempts due to the large size of the mature equine follicle and an unusually firm attachment of the cumulus oophorus to the follicle wall (Bruck et al, 1999; Bruck-Bogh, 2003), a relatively low rate of in vitro maturation of oocytes recovered from slaughterhouse ovaries to the MII stage of development in readiness for fertilisation (Li et al, 2000) and, most important, amazing resistance of equine spermatozoa to undergo capacitation in vitro and therefore be able to achieve penetration and fertilisation of the oocyte (Zhang et al, 1989, 1990; Palmer et al, 1991; Alm et al, 2001).

Figure 21



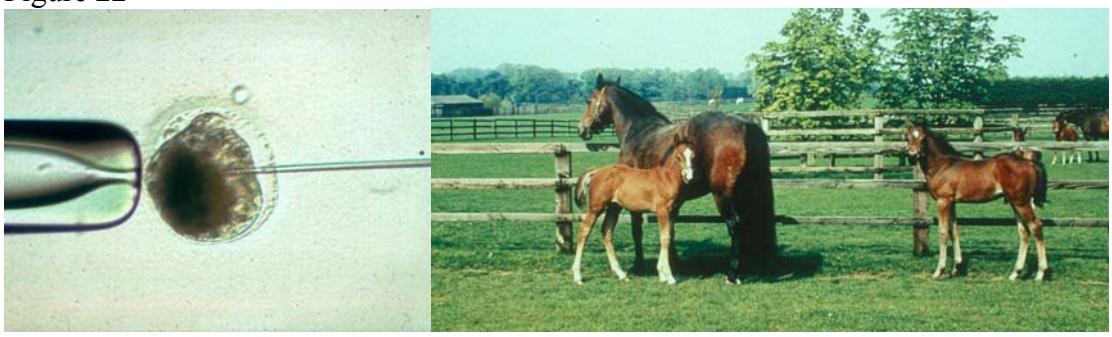
Diagrammatic summary of the transfer of demi-mule embryos, one back to a mare and the other to a Jenny donkey. Note the huge difference in serum eCG concentrations between these two surrogate mothers which is occasioned by the big difference in the size and vitality of the endometrial cups in their uterus, seen here at 60 days of gestation.

Happily, the technique of ICSI has proved much more applicable to the horse and several groups have now reported live foals produced by ICSI (Figure 22; Squires et al, 1996; Grondahl et al, 1995a; Cochran et al, 1998, McKinnon et al, 2000, Li et al, 2000 and Galli et al, 2002). The method has the great advantage that the spermatozoon injected into the oocyte to achieve fertilisation can have been frozen and thawed, gender-selected by fluorescence-activated cell sorting (FACS; Lindsay et al, 2002a & b), and may be totally immotile or even tail-less.

Dr Elaine Carnevale and colleagues in Fort Collins, Colorado have pioneered the technique of GIFT in the horse whereby oocytes recovered by OPU from a genetically valuable donor mare with uterine or oviducal pathology , or is still in active competition, are matured in vitro to the MII stage before being injected into the oviduct of a recipient mare, mated or inseminated previously with genetically superior stallion semen and after the recipient mare's own oocyte has similarly been removed by OPU. Or, alternatively, a low number of washed spermatozoa (0.5-5.0 x106) are injected into the recipient mare's oviduct simultaneously with the in vivo or in vitro matured genetically valuable oocyte. Carnevale's group and others have reported the birth of several live foals produced by GIFT (Carnevale and Ginther, 1993; Carnevale et al, 2000; Hinrichs et al, 2000, 2002; Scott et al, 2001) and the

technique is nowadays offered as a commercial service by the team in Colorado for genetically valuable, but otherwise sterile, donor mares.

Figure 22



Intracytoplasmic injection of a single spermatozoan to achieve fertilisation of an MII stage oocyte. Also, two foals generated by ICSI, all at Mertoun Paddocks in Newmarket.

Following the momentous announcement of the birth of the world's first cloned mammal, DOLLY the sheep, produced by the injection of a diploid mammary gland karyoplast into an enucleated oocyte (Wilmut et al, 1997), equids also joined the cloning fray in 2003 with the birth of three cloned mule foals produced by injecting fetal mule skin cells into enucleated horse oocytes (Woods et al, 2003), and one cloned horse in Italy created by using a cultured adult skin fibroblast as the karyoplast, with the added twist that the resulting clone was carried to term by the mare from which the karyoplast had originated, thus creating a unique pregnancy of complete genetic, and therefore immunological, identity (Galli et al, 2003).

Subsequent studies have revealed that cloning in horses is every bit as inefficient as it is in cattle and other farm species, with low initial pregnancy rates combined with high early and mid-gestation pregnancy losses (Chavatte-Palmer et al, 2004; Hinrichs et al, 2007; Palmer et al, 2008; Skizyszowska et al, 2008). Happily, however, the late gestation abortions caused by hydrops amnion and the early post natal developmental abnormalities and deaths that have occurred commonly in cloned cattle, usually as a consequence of very abnormal development of placental cotyledons (Chavatte-Palmer et al, 2004; Heyman et al, 2004; Wells et al, 2004 and Davies et al, 2004), do not appear to afflict horse clones, perhaps due to the diffuse epitheliochorial nature of the equine placenta (Amoroso, 1952; Samuel et al, 1975). Although accurate published information is not currently available on this subject it nonetheless appears that if a cloned horse embryo does make it safely to mid-gestation it has a high chance of proceeding normally to term with the birth of a healthy and robust foal. Certainly, the first four cloned equids produced by Galli et al (2003) and Woods et al (2003) fell into this category of physical normality and good health. Furthermore, the 9 or more cloned horses produced to date by Professor Katrin Hinrichs and her team at Texas A&M University including an amazing 5 cloned foals all produced from the same karyoplast line (Figure 23; Hinrichs et al, 2007; Choi et al, 2008) have all been live and normal.

Unfortunately, the ill-conceived legislation that forced closure of all horse abattoirs throughout North America in 2007 has severely curtailed further experimental work on equine cloning in Texas and elsewhere due to the resulting shortage of horse oocytes. The pity of such a setback is the fact that Professor Hinrich's laboratory in, Texas and other commercial horse cloning enterprises in Texas and California, are more experienced and better equipped to take cloning forward into the commercial arena of the horse breeding

world where there is an urgent and practical need for the technique to overcome the present severe limitation to genetic advancement in the breeding of most types of Sporthorses by the common practices of castrating young males to improve their competitive performance and retaining the few superior females in competition until a ripe old age, thereby greatly reducing the potential fecundity of both sexes. (Palmer et al, 2008).

Figure 23

The equine reproduction photograph of the New Millennium! Five cloned foals created from a single karyoblast line by Professor Katrin Hinrichs and her team at Texas A&M University, USA (Photograph courtesy of Katrin Hinrichs).

Conclusions

All the so-called modern breeding technologies, from AI through ET and on through ICSI, GIFT and up to NT, have been applied successfully to the horse over the past three decades. But some significant practical problems still exist before it can reasonably be said that all the available methods can be applied efficiently and successfully to the commercial production of elite horses. Most notably, perhaps, the persisting inability to truly superovulate donor mares with exogenous gonadotrophins for embryo transfer or embryo freezing, the difficulty in obtaining sufficient morula-stage embryos for efficient freezing or splitting, the rather low rate of recovery of useable oocytes from valuable donor mares by transvaginal OPU, and the blanket resistance of equine oocytes and spermatozoa to conventional IVF. But despite these hurdles, and very much led by polo pony production in Argentina, straightforward embryo transfer, involving the recovery of one or two viable embryos per cycle from selected donor mares and their simple, and effective transfer to easily synchronised recipient mares, is on the upsurge throughout the non-Thoroughbred equine world. Such visible simplicity and efficiency, together with the clear evidence of the athletic superiority of embryo transfer-produced foals compared to their conventionally bred counterparts in both polo and the other divisions of the Sporthorse industry (Lascombes and Pashen, 2008), may be enough to tip the balance in the Thoroughbred breeding world to accept embryo transfer in order to raise the lifetime fecundity of the very best champion fillies

and so begin to level the huge imbalance in the exploitation of superior male, but not female, genes which has existed for the past 300 years in that sector of the horse breeding industry.

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National Statistical Data of Bovine Embryo Transfer Activity in Europe (2008).

TABLE : 1 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: AUSTRIA ***A.E.T.E 2009***

Data collected by
Dr. Lukas Kalcher

Total number of approved E.T. teams in the country	7
Number of teams providing data	6

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	138	B/A=	11.5
	Embryos collected	**B**	1588	C/A=	7.9
	Embryos transferable	**C**	1096	C/B=	69.0%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	1096	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	314		
In vivo	Frozen	**I**	401		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	715	H+I+J+K=	
Number of frozen stored embryos		**M**	849		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	56.1%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	153
Number of calves born from *in vitro* embryos	
Total	153

TABLE : 2 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: BELGIUM ***A.E.T.E 2009***

Data collected by
Dr. Peter Vercauteren

Total number of approved E.T. teams in the country	
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	142	B/A=	8.6
	Embryos collected	**B**	1225	C/A=	4.8
	Embryos transferable	**C**	681	C/B=	55.6%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**		=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	135		
In vivo	Frozen	**I**	1145		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1280	H+I+J+K=	
Number of frozen stored embryos		**M**	546		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	89.5%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 3 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: CZECH REPUBLIC ***A.E.T.E 2009***

Data collected by
Dr. Jirina Peteliková

Total number of approved E.T. teams in the country	7
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	469	B/A=	10.8
	Embryos collected	**B**	5072	C/A=	5.7
	Embryos transferable	**C**	2654	C/B=	52.3%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	12		
Total i*n vitro* embryos		**F**	12	=(D+E)	
Total number of transferable embryos		**G**	2666	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1131		
In vivo	Frozen	**I**	1063	48.5% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	51		
Total embryos transferred		**L**	2245	H+I+J+K=	
Number of frozen stored embryos		**M**	1522		
% of *in vitro* embryos transferred		**N**	2.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	49.6%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 4 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: DENMARK ***A.E.T.E 2009***

Data collected by
Dr. Henrik Callesen

Total number of approved E.T. teams in the country	14
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	489	B/A=	10.6
	Embryos collected	**B**	5176	C/A=	7.2
	Embryos transferable	**C**	3503	C/B=	67.7%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	3503	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1619		
In vivo	Frozen	**I**	1138		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2757	H+I+J+K=	
Number of frozen stored embryos		**M**	1820		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	41.3%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	2067
Number of calves born from *in vitro* embryos	
Total	2067

TABLE : 5 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: ENGLAND ***A.E.T.E 2009***

Data collected by
Dr. Ian Murphy

Total number of approved E.T. teams in the country	37
Number of teams providing data	

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**	9109	C/B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**	9109	=(C+F)
Number of sexed embryos				
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	6110	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L=

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 6 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: ESTONIA ***A.E.T.E 2009***

Data collected by
Dr. Ulle Jaakma

Total number of approved E.T. teams in the country	1
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2	B/A=	14
	Embryos collected	**B**	28	C/A=	9
	Embryos transferable	**C**	18	C/B=	64.3%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	18	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**			
In vivo	Frozen	**I**	28		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	28	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	100%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 7 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: FINLAND ***A.E.T.E 2009***

Data collected by
Dr. Marja Mikkola

Total number of approved E.T. teams in the country	5
Number of teams providing data	4

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	336	B/A=	12.5
	Embryos collected	**B**	4192	C/A=	8.2
	Embryos transferable	**C**	2761	C/B=	65.9%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	2761	=(C+F)	
Number of sexed embryos			36		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	817		
In vivo	Frozen	**I**	2388		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3205	H+I+J+K=	
Number of frozen stored embryos		**M**	1812		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	74.5%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	1230
Number of calves born from *in vitro* embryos	
Total	1230

TABLE : 8 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: FRANCE ***A.E.T.E 2009***

Data collected by
Dr. Claire Ponsart

Total number of approved E.T. teams in the country	24
Number of teams providing data	22

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5856	B/A=	9.5
	Embryos collected	**B**	55356	C/A=	5.4
	Embryos transferable	**C**	31841	C/B=	57.5%
In vitro	Nb of oocyte donors		28		
(OPU)	Nb of OPU sessions		30		
	Nb of transferable embryos	**D**	99		
In Vitro	Nb of transferable embryos	**E**	18		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	117	=(D+E)	
Total number of transferable embryos		**G**	31958	=(C+F)	
Number of sexed embryos			1310		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	13539		
In vivo	Frozen	**I**	13765	50.4% frozen	
In vitro	Fresh	**J**	90		
In vitro	Frozen	**K**	9	9.1% frozen	
Total embryos transferred		**L**	27403	H+I+J+K=	
Number of frozen stored embryos		**M**	10329		
% of *in vitro* embryos transferred		**N**	0.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	50.3%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	3877
Number of calves born from *in vitro* embryos	47
Total	3924

TABLE : 9 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: GERMANY ***A.E.T.E 2009***

Data collected by
Dr. Hubert Cramer

Total number of approved E.T. teams in the country	45
Number of teams providing data	21

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**	30755	C/A=
	Embryos transferable	**C**	17377	C/B= 56.5%
In vitro	Nb of oocyte donors			
(OPU)	Nb of OPU sessions		1569	
	Nb of transferable embryos	**D**	3124	
In Vitro	Nb of transferable embryos	**E**		
(Slaughtered donors)				
Total i*n vitro* embryos		**F**	3124	=(D+E)
Total number of transferable embryos		**G**	20501	=(C+F)
Number of sexed embryos			376	
EMBRYO TRANSFER				
In vivo	Fresh	**H**	5136	
In vivo	Frozen	**I**	8941	63.5% frozen
In vitro	Fresh	**J**	1072	
In vitro	Frozen	**K**	?	
Total embryos transferred		**L**	15149	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**	7.1%	(J+K)/L=
% of frozen embryos transferred		**O**	59.0%	(I+K)/L=

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 10 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: GREECE ***A.E.T.E 2009***

Data collected by
Dr. Samartzi Fonteini

Total number of approved E.T. teams in the country	2
Number of teams providing data	2

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B= %
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**		=(C+F)
Number of sexed embryos				
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	45	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**		H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	20
Number of calves born from *in vitro* embryos	
Total	20

TABLE : 11 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: HUNGARY ***A.E.T.E 2009***

Data collected by
Dr. Ferenc Flink

Total number of approved E.T. teams in the country	3
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	106	B/A=	11.2
	Embryos collected	**B**	1183	C/A=	8.0
	Embryos transferable	**C**	843	C/B=	71.3%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	843	=(C+F)	
Number of sexed embryos			139		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	274		
In vivo	Frozen	**I**	588		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	862	H+I+J+K=	
Number of frozen stored embryos		**M**	211		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	68.2%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	116
Number of calves born from *in vitro* embryos	
Total	116

TABLE : 12 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: IRELAND ***A.E.T.E 2009***

Data collected by
Dr. Pat Lonergan

Total number of approved E.T. teams in the country	
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	410	B/A=	9.7
	Embryos collected	**B**	3975	C/A=	4.9
	Embryos transferable	**C**	2021	C/B=	50.8%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	2021	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	891		
In vivo	Frozen	**I**	1328		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2219	H+I+J+K=	
Number of frozen stored embryos		**M**	1130		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	59.8%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 13 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: ITALY ***A.E.T.E 2009***

Data collected by
Dr. Giovanna Lazzari

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2089	B/A=	9.5
	Embryos collected	**B**	19803	C/A=	5.9
	Embryos transferable	**C**	12224	C/B=	61.7%
In vitro *(OPU)*	Nb of oocyte donors		84		
	Nb of OPU sessions		295		
	Nb of transferable embryos	**D**	713		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	820		
Total i*n vitro* embryos		**F**	1533	=(D+E)	
Total number of transferable embryos		**G**	13757	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	9129		
In vivo	Frozen	**I**	3224	26.1% frozen	
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	2244	95.7% frozen	
Total embryos transferred		**L**	14697	H+I+J+K=	
Number of frozen stored embryos		**M**	6095		
% of *in vitro* embryos transferred		**N**	15.9%	(J+K)/L=	
% of frozen embryos transferred		**O**	37.2%	(I+K)/L=	%

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	7022
Number of calves born from *in vitro* embryos	
Total	7022

TABLE : 14 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: THE NETHERLANDS ***A.E.T.E 2009***

Data collected by
Dr. Sybrand Merton

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	3498	B/A=	11.9
	Embryos collected	**B**	41496	C/A=	6.8
	Embryos transferable	**C**	23923	C/B=	57.7%
In vitro *(OPU)*	Nb of oocyte donors		235		
	Nb of OPU sessions		1859		
	Nb of transferable embryos	**D**	2815		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	213		
Total i*n vitro* embryos		**F**	3028	=(D+E)	
Total number of transferable embryos		**G**	26951	=(C+F)	
Number of sexed embryos			319		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	4691		
In vivo	Frozen	**I**	12223	72.3% frozen	
In vitro	Fresh	**J**	1040		
In vitro	Frozen	**K**	983	48.6% frozen	
Total embryos transferred		**L**	18937	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	10.7%	(J+K)/L=	
% of frozen embryos transferred		**O**	69.7%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: POLAND ***A.E.T.E 2009***

Data collected by
Dr. Jedrzej Jaskowski

Total number of approved E.T. teams in the country	8
Number of teams providing data	6

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	27	B/A=	6.6
	Embryos collected	**B**	178	C/A=	3.4
	Embryos transferable	**C**	91	C/B=	51.1%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	91	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	77		
In vivo	Frozen	**I**	88		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	165	H+I+J+K=	
Number of frozen stored embryos		**M**	9		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	53.3%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 16 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: ROMANIA ***A.E.T.E 2009***

Data collected by
Dr. Stela Zamfirescu

Total number of approved E.T. teams in the country	3
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	15	B/A=	7.2
	Embryos collected	**B**	108	C/A=	5.1
	Embryos transferable	**C**	77	C/B=	71.3%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	18		
Total i*n vitro* embryos		**F**	18	=(D+E)	
Total number of transferable embryos		**G**	95	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	36		
In vivo	Frozen	**I**	19	34.5% frozen	
In vitro	Fresh	**J**	7		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	62	H+I+J+K=	
Number of frozen stored embryos		**M**	34		
% of *in vitro* embryos transferred		**N**	11.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	29.2%	(I+K)/L=	%

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	28
Number of calves born from *in vitro* embryos	
Total	28

TABLE : 17 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: SPAIN ***A.E.T.E 2009***

Data collected by
Dr. Julio De la Fuente

Total number of approved E.T. teams in the country	
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	633	B/A=	10.1
	Embryos collected	**B**	6374	C/A=	4.5
	Embryos transferable	**C**	2854	C/B=	44.8%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	2854	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	614		
In vivo	Frozen	**I**	1897		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2511	H+I+J+K=	
Number of frozen stored embryos		**M**	1867		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	75.5%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	1038
Number of calves born from *in vitro* embryos	3
Total	1041

TABLE : 18 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: SWEDEN ***A.E.T.E 2009***

Data collected by
Dr. Johanna Geust

Total number of approved E.T. teams in the country	3
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	199	B/A=	6.9
	Embryos collected	**B**	1382	C/A=	3.6
	Embryos transferable	**C**	711	C/B=	51.4%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	711	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3		
In vivo	Frozen	**I**	51		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	54	H+I+J+K=	
Number of frozen stored embryos		**M**	711		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	94.4%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	14
Number of calves born from *in vitro* embryos	
Total	14

TABLE : 19 EMBRYO TRANSFER ACTIVITY IN 2008

COUNTRY: SWITZERLAND ***A.E.T.E 2009***

Data collected by
Dr. Rainer Saner

Total number of approved E.T. teams in the country	5
Number of teams providing data	4

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	485	B/A=	11.4
	Embryos collected	**B**	5541	C/A=	7.3
	Embryos transferable	**C**	3560	C/B=	64.2%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	3560	=(C+F)	
Number of sexed embryos			22		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	696		
In vivo	Frozen	**I**	2004	74.2% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	5	100% frozen	
Total embryos transferred		**L**	2705	H+I+J+K=	
Number of frozen stored embryos		**M**	2752		
% of *in vitro* embryos transferred		**N**	0.2%	(J+K)/L=	
% of frozen embryos transferred		**O**	74.3%	(I+K)/L=	

Number of E.T. calves born (2008)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	1600

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2008

I. EMBRYO PRODUCTION

(Data collected from 19 countries)

In vivo* produced embryos (superovulation)	
- number of flushed donors	14,894
- number of transferable embryos	88,858
- mean number per flushed donor	5.97
***In vitro* produced embryos:**	
From OPU	
- number of OPU sessions	3,753
- number of transferable embryos	6,751
- mean number per session	1.80
From slaughterhouse collected ovaries	
- number of transferable embryos	1081
Total *in vitro*	7,832
Total number of transferable embryos	123,176
Embryos sexed:	2202

* Specified data from two countries not available and not included.

(S. Merton, AETE, Poznan, Poland 2009)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2008

II. EMBRYO TRANSFERS

(Data collected from 19 countries)

***In* vivo produced embryos ***	**Number of embryos transferred** 89,438 (39,102 fresh / 50,336 frozen)
***In vitro* produced embryos**	5,601 (2,314 fresh / 3,287 frozen)
Total number of embryos transferred	101,149
Proportion of IVF embryos transferred	5.5%
Proportion of frozen embryos transferred	56.4%

* Specified data from one country not available and not included.

(S. Merton, AETE, Poznan, Poland 2009)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2008

Species	Embryo Production	Embryo Transfers	Countries
Sheep	419	375	Romania Turkey
Swine	736	28	Czech Republic
Goat	83	75	Romania
Horse	1043 (incl. 47 IVP)	1014 (incl. 48 IVP)	Austria Czech Republic Finland France Hungary Italy Netherlands

(S. Merton, AETE, Poznan, Poland 2009)

INVITED LECTURES

MATERNAL DIET, NUTRIENT UPTAKE AND METABOLISM IN THE BOVINE EMBRYO

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Introduction

Although the nutrient demands of the pre-attachment bovine embryo are quantitatively small they are, nevertheless, qualitatively specific, and reflect the changing needs of the developing embryo during its migration from the oviduct to the uterine lumen. These needs are initially satisfied almost entirely from endogenous reserves accumulated during oocyte growth and, to a lesser extent, from exogenous nutrients available from the female reproductive tract. Subsequently, the elongating embryo is almost totally reliant on uterine sources of nutrients. With respect to endogenous reserves in the oocyte, it is noteworthy that the process of ovarian folliculogenesis, from when primordial follicles leave their resting state to when they reach the preovulatory stage, typically takes between 5 to 7 months in large animal species (including humans), although the active period of growth is estimated to be around 12 weeks (Gougeon, 1996). During this period follicular volume increases around 400-fold (Griffin et al., 2006). Whilst the corresponding increase in oocyte volume (around 40-fold) may appear modest, it nevertheless represents a significant increase in biomass and highlights the extent of cellular biosynthesis that takes place in the oocyte which is by far the largest single cell in the mammalian body. It further emphasises the protracted period of time during which environmental determinants of egg quality, such as maternal diet, can exert their effects. With respect to nutrient provision from the female reproductive tract, one might expect that the precise nature and quantity of nutrient provision, and how these is affected by maternal diet, are well characterised in the ruminant. However, this is not the case. What is known has been comprehensively reviewed elsewhere (e.g. Sinclair et al., 2003) and is briefly summarised in Table 1.

With the foregoing discussion in mind, the current article provides a contemporary overview of how maternal diet influences nutrient provision to the growing, follicle-enclosed oocyte and pre-implantation embryo in the cow, in a manner that determines pregnancy outcome. The nutrient composition of reproductive fluids and how this influences embryo metabolism is considered initially, followed by a discussion of how measurements of embryo metabolism can be used to predict embryo quality and pregnancy outcome. Finally, the effects of maternal diet on egg and embryo quality are considered in nulliparous cattle and maiden heifers. Data are drawn from other species where necessary but, in the interests of brevity, the reader is referred to other articles (e.g. Garnsworthy et al., 2008) for a detailed description of how nutrition can influence fertility in the lactating cow.

Nutrient composition of reproductive fluids and embryo metabolism

Follicular and oviductal fluid-composition is each hormonally regulated. Oviductal secretions, for example, increase during pro-oestrus and decrease during the luteal phase of the cycle. Each fluid comprises a serum transudate and secretory products from surrounding

granulosa cells, within the follicle, and from the oviductal epithelium (e.g. Andersen et al., 1976; Brantmeier et al., 1987; Wise, 1987; Killian et al., 1989; Staros and Killian, 1998; Elhassan et al., 2001; Leese et al., 2001; Leroy et al., 2004; Orsi et al., 2005; Alavi-Shoushtari et al., 2006; Hugentobler et al., 2007 and 2008; see Table 1). Although the relative contribution of serum and locally produced metabolites in each of these two biological fluids has not been fully quantified, the metabolite composition of each fluid is known to vary greatly between individual animals and, significantly, between studies probably reflecting differences in methods of sample collection and in analytical procedures.

Oxygen tension

Oxygen consumption by the oocyte increases during follicle development, although metabolic rate is greater in oocytes from primary follicles when cellular volume is accounted for (Harris et al., 2009). Poor follicular vascularity (determined by Doppler ultrasongraphy) and low intra-follicular oxygen content have been found to correlate with increased chromosomal abnormalities and decreased pregnancy rates in humans (Chui et al., 1997; Van Blerkom et al., 1997). Increasing oxygen concentration from 2, through 5, 10 to 20% during in vitro maturation (IVM) reduced the number of trophectoderm cells but had no consistent, biologically significant effect on either embryo or fetal development in the mouse (Banwell et al., 2007). Controversy surrounds the optimal oxygen tension for the maturation of bovine oocytes and for zygote culture, with recent studies (e.g. Mingoti et al., 2009; Goovaerts et al., 2009) advocating contrasting (20 vs 5%) oxygen levels. Such inconsistencies may merely reflect the method of culture (i.e. single vs group), basal media formulation, macromolecule supplementation, and the presence or absence of somatic support cells, reflecting the non-physiological nature of current systems for gamete/embryo culture. Indeed oxygen tension in the uterus is lower than that of the oviduct, although the magnitude of these differences varies considerably between species (Fischer and Bavister, 1993). In the cow, oxygen tension is greatest in growing antral follicles, with similar values for large and pre-ovulatory follicles, and uterine fluids (Table 1), possibly reflecting species differences in the nature and timing of implantation.

Carbohydrates and fatty acids

The discussion above is central to the 'quiet embryo hypothesis' first proposed by Leese (2002), which states that pre-implantation embryo survival, and indeed long-term development, is enhanced when metabolism during this very early period is minimal. Much of this metabolic effect is thought to be related to the production of reactive oxygen species. In contrast to the growing oocyte, energy metabolism in the pre-implantation embryo is characterised by a significantly greater reliance on carboxylic acids such as pyruvate and citrate at the expense of glucose (Table 1). The low metabolic activity of the early cleavage-stage embryo, nevertheless, belies its dependence on oxidative phosphorylation for the generation of > 90% of ATP (Sinclair et al., 2003). Around 40% of glucose uptake by the embryo during this period is metabolised to lactate. The principle function of glucose during this period is thought to be the generation of reducing equivalents (in the form of NADPH) and ribose sugars, both via the pentose phosphate pathway, and as a source of 3-carbon precursors, all for biosynthetic purposes. ATP production on a per embryo basis increases during compaction and blastulation although, on a cellular basis, oxidative and glycolytic metabolism alters little during this period. The embryo, nevertheless, becomes increasingly reliant on glycolysis following compaction, accounting for between 15 and 20% ATP production, possibly reflecting the lower oxygen tension of uterine as opposed to oviductal fluids.

In contrast to the fate of carbohydrates, the contribution of lipids as a metabolic fuel in the early pre-implantation embryo is less well understood. Fergusson and Leese (1999) reported a 42% reduction in triglyceride content by the 2-cell stage in bovine embryos, but there was no subsequent net change in triglyceride content to the hatched blastocyst stage. The close apposition of mitochondria to lipid droplets in the mature oocyte suggests a role of triglyceride oxidation in ATP production during fertilisation and early development. The β-oxidation of fatty acids is also thought to generate much of the water and at least some of the energy necessary for blastocoel formation (Wiley, 1987).

Proteins and amino acids

Amino acid content of bovine oviductal and uterine fluids have been comprehensively studied (Elhassan et al., 2001; Hugentobler et al., 2007). So called non-essential amino acids, including glycine, glutamte and alanine, account for around 80% of total amino acids in the oviduct, a value that drops to around 60% in uterine fluids. Glycine concentrations are particularly high where, in addition to protein synthesis, glycine is thought to play an important role in osmoregulation. However, amino acids also function as important energy sources for the early embryo and threonine and glutamine are readily metabolised in this way. One of the products of such metabolism is alanine, significant amounts of which are secreted as the pre-implantation embryo attempts to sequester harmful ammonium ions.

The relative contribution of various amino acids for biosynthetic purposes is very likely tempered by the uptake of proteins such as albumin by endocytosis and the subsequent release of amino acids following hydolysis (Thompson et al., 1998), and possibly through the recycling of ammonia nitrogen by the embryo, although the extent of the latter has not been fully quantified (Leese, 1991). Albumin certainly improves embryo development in vitro (Orsi and Leese, 2004a), although the variability in embryotrophic effects between studies may have as much to do with batch variation in albumin purity, as even highly purified albumin is known to retain small and poorly defined molecules (Bavister, 1995). The metabolism of amino acids invariably leads to the production of ammonium, particularly during in vitro culture, which is detrimental to subsequent development (to be discussed later). Bovine embryos can release free ammonium, but also fix amino nitrogen/ammonium by the pyruvate dependent synthesis of alanine, and also possibly via the synthesis of glutamine from glutamate (Orsi and Leese, 2004b).

Embryo metabolism as a predictor of early development

The quest for non-invasive, objective and quantitative methodologies to identify the most viable embryos for transfer following culture has rekindled interest in measurements of embryo metabolism. Although there has been a trend to move away from metabolic target analysis (i.e. analysis of single or selectively defined metabolites) for diagnostic purpose towards metabolite profiling (Singh and Sinclair, 2007), significant advances have been made by profiling one particular class of metabolite (i.e. amino acids) by HPLC analysis of culture media that warrants its consideration in this article.

This method of embryo selection is based on the depletion/appearance of amino acids in media during culture, where there appears to be great similarity in the metabolism of amino acids between pre-implantation embryos of contrasting mammalian species (cow, Partridge and Leese, 1996; pig, Booth et al., 2005; human, Brison et al., 2004). This latter study involved 53 cycles of IVF in human subjects using ICSI. HPLC analysis of spent culture media identified three amino acids (i.e. asparagine, glycine and leucine) that were

significantly correlated to pregnancy outcome in a manner that was independent of other known determinants of pregnancy establishment such as maternal age, ovarian reserve, embryo cell number and morphological grading. It follows that a combination of assessments (e.g. morphological grade and amino acid metabolism) could further enhance the chances of selecting developmentally competent embryos. Recently, this lead to the derivation of a logistic regression model that predicts the probability of porcine blastocyst formation in vitro from measurements of cell number, cell division, cellular fragmentation (all in cleavage-stage embryos), and amino acid turnover, determined around 24 hours post-insemination (Booth et al., 2007). In this study 85% of those embryos predicted to become blastocysts did indeed go onto form blastocysts, which was significantly greater than the number expected by chance.

Given the importance of oocyte quality in determining post-fertilisation development (Merton et al., 2003), we recently evaluated the potential of metabolite concentration of bovine follicular fluid at the point of egg recovery as a predictor of post-fertilisation development to the blastocyst stage (Sinclair et al., 2008). A total of 445 cumulus-oocyte complexes (COCs) were aspirated from visually healthy follicles and underwent IVM/IVF/IVC singly using the well-of-the-well system (Vajta et al., 2000). Of those (78%) that cleaved following insemination, 32% went onto form blastocysts. Follicular fluid was analysed for amino acids using gas chromatography-mass spectrometry and fatty acids by gas chromatography. In contrast to fatty acids, which had little predictive value, the amino acid composition of follicular fluid was associated with morphological assessments of COC quality and with post-fertilisation development to the blastocyst stage. Principal component analysis further identified two amino acids (i.e. alanine and glycine) that had the highest value in predicting post-fertilisation development. Furthermore, the predictive value of these two amino acids, in terms of the percentage oocytes that cleaved following IVF, was greatest for COCs with the poorest morphological grades but, with respect to blastocyst yields, was independent of morphological grade, where glutamate and valine also featured, and so may serve as a useful additional non-invasive marker of COC quality.

Alanine, glycine and glutamate are three of the most abundant amino acids found in follicular fluid, as well as in oviductal and uterine fluids (Table 1). In addition to protein synthesis, they are believed to have a number of cellular functions including nucleotide biosynthesis, osmoregulation (glycine), and ammonia detoxification (alanine, glutamate) (Steeves et al., 2003; Orsi and Leese, 2004b). Furthermore, in chemically defined bovine embryo culture media both alanine and glycine have been shown to improve blastocyst yields (Lee and Fukui, 1996). However, the relative importance of specific amino acids, and indeed other metabolites, as predictors of embryo development will very likely vary between species, biological fluids, predicted outcomes and be influenced by environmental factors such as maternal diet and ambient temperature (e.g. Shehab-El-Deen et al., 2009). Furthermore, it remains to be determined if these metabolic markers can predict pregnancy outcome following embryo transfer in cattle.

Table 1. Typical micro-environmental conditions and nutrient supply in the ovary, oviduct and uterus of cyclic but non-lactating. Data derived from a variety of sources (see text for details). Follicular fluid levels pertain to the largest follicles for the periods in question.

Day of oestrous cycle	1-3		4-7		Pre-ovulation
Location	**Follicular fluid**	**Isthmus**	**Follicular fluid**	**Uterus**	**Follicular fluid**
Oxygen tension (mm Hg)†	80	55	55	52	52
Protein content (mg/ml)	65	71	60	72	68
Albumin (mg/ml)	42	8.8	40	9.1	46
Principal essential amino acids (mM)					
Threonine	0.4	0.8	0.3	1.7	0.2
Leucine	0.3	0.5	0.2	1.8	0.2
Arginine	0.2	0.3	0.2	1.4	0.1
Principal nonessential amino acids (mM)					
Glycine	0.9	14.0	0.6	12.0	0.5
Glutamate	0.2	5.5	0.1	4.2	0.1
Alanine	0.7	3.7	0.5	3.1	0.5
Glucose and carboxylic acids (mM)					
Glucose	4.7	1.6	5.1	2.2	4.8
Pyruvate	0.01	1.1	0.04	1.7	0.03
Lactate	8.2	8.3	5.0	11.7	5.1
Lipids					
Cholesterol (μg/ml)	23	143	65	175	-
Phospholipid (nM)	426	324	633	90	-
Phospholipid:cholesterol ratio	18.5	2.3	9.7	0.5	-

† mmHg = 7.5 ($\%O_2$/100 x 101.3)

Maternal diet, egg and embryo quality

Given the technical difficulties of studying in vivo embryo development in cattle there have been few studies in this area and so our understanding of how maternal diet can affect pre-implantation embryo development has been directed largely to effects on the follicle-enclosed growing oocyte.

Level of feeding, body composition and metabolic hormones

In one of the few studies that were able to assess the effects of nutrition on in vivo embryo development in cattle, beef heifers were superovulated and, following artificial insemination, Day 7 embryos flushed from the reproductive tracts and cultured for 24 hours in vitro (Nolan et al., 1998). Blastocyst yields and blastocyst cell numbers were significantly greater (73 vs 42%) for heifers on the Low (40 MJ metabolisable energy (ME) per day) diet compared with heifers on the High (120 MJ ME per day) diet. A subsequent study from the same group (Yaakub et al., 1999), in collaboration with researchers from Germany and North America (Wrenzycki et al., 2000), showed that ad libitum feeding of high starch diets significantly reduced blastocyst yields following superovulation in beef heifers. This occurred as a consequence of deranged embryo metabolism, with increased transcripts for the antioxidant enzyme Cu/Zn-superoxide dismutase detected in blastocysts. Clearly, therefore, high planes of nutrition are detrimental for early embryo development.

In contrast to the paucity of data on the direct effects of diet and body-composition on the pre-implantation embryo there is a reasonably comprehensive body of literature regarding dietary effects on the follicle-enclosed oocyte in cattle, where the focus at the author's laboratory has largely been on effects mediated via insulin and components of the insulin-like growth factor system. Circulating levels of both insulin and IGF-I fluctuate during the oestrous cycle, with peak concentration coinciding with oestrus and ovulation (Armstrong et al., 2001). Indeed, circulating concentrations of insulin in particular were found to fluctuate with each follicle wave, as oestradiol is known to stimulate the expression of mRNAs encoding both insulin and IGF-I in pancreatic and liver cells respectively (Johnson et al., 1998; Morimoto et al., 2001). Both insulin and IGF-I interact synergistically with gonadotrophins to promote follicular growth and steriodogenesis within the follicle. In the study of Armstrong et al. (2001) the high energy (1.6 x maintenance) diet significantly increased plasma insulin and IGF-I concentrations, relative to the low energy diet, and increased growth rate and maximum size of the pre-ovulatory follicle. However, increased global nutrient supply reduced oocyte quality, defined as the proportion of oocytes which, following IVF, developed to the blastocyst stage in vitro. In addition to increases in peripheral insulin and IGF-I, the high energy diet reduced steady-state concentrations of mRNA encoding IGFBP2, IGFBP4, insulin receptor and type 1 IGF receptor in granulosa and thecal cells, changes expected to increase the bioavailability of intra-follicular IGF. Consequently, diets formulated to optimise follicle development may compromise oocyte quality.

We have since shown that antral follicle development and egg quality are both impaired in obese and hyperinsulinaemic (> 37 μIU/ml plasma insulin) young female cattle (Adamiak et al., 2005). In that study, oocytes were retrieved from donors using ultrasound guided follicular aspiration, and matured, fertilised and cultured *in vitro*. Detailed analysis revealed that the negative relationship between insulin and egg quality (defined as the proportion of inseminated oocytes that developed to the blastocyst stage) increased over time (Figure 1); i.e. as the animals became fatter. This effect could have been due to the duration of exposure of oocytes to elevated levels of insulin, but the data also suggest that oocytes

exposed to high levels of insulin during the pre-antral stages of follicular development may be most sensitive to the negative effects of this hormone. Studies in France, also working with overfed heifers, broadly concur with our data, where the benefits of a short-term period of dietary restriction on the post-fertilisation developmental of oocytes have been demonstrated (Freret et al., 2006).

Due to the dramatic increase in obesity levels in human subjects, a considerable amount of research effort has been devoted to understanding the effects of obesity on fertility. Overweight women are more likely to encounter menstrual dysfunction and anovulation (Clark et al., 1995). Furthermore, women with a BMI $\geq$ 25 kg m^{-2} have a lower chance of pregnancy following IVF and have an increased miscarriage rate (Maheshwari et al., 2007). A contributing factor in these cases is impaired egg quality associated with insulin resistance. However, much of the human data in this area is derived from patients with PCOS (Jungheim and Moley, 2008). Recently, the ability of insulin sensitising agents 5-aminoimidazole 4-carboxamide-riboside (AICAR), sodium salicylate and rosiglitazone to enhance the post-fertilisation developmental potential of oocytes was determined in obese C57BL/6 mice offered a high-fat diet (Minge et al., 2008). Rosiglitazone, a potent agonist for the nuclear receptor peroxisome proliferator activated receptor-γ (PPAR-γ), was most effective in lowering blood insulin and triglyceride concentrations, and restoring post-fertilisation development of *in vivo* derived zygotes cultured *in vitro*. Within the mouse ovary PPAR-γ is most highly expressed in granulosa cells (Minge et al., 2006), where it can interact with target genes such as *Cd36* and *Scarb1* involved in lipid uptake and metabolism (Minge et al., 2008).

Insulin resistance can also manifest in the pre-implantation embryo. Chronic exposure of mouse embryos to either insulin or IGF-I leads to decreased IGF-I receptor expression and impaired insulin-stimulated glucose uptake (Pantaleon and Kaye, 1996). Insulin-resistant embryos, as a consequence, undergo apoptotic cellular death, manifesting in lower implantation rates when transferred to pseudo-pregnant females (Pinto et al., 2002).

Dietary carbohydrates and lipids

Follow-up studies to those described above in heifers at the author's laboratory have investigated the effects of carbohydrate and lipid source, and frequency of feeding on the post-fertilisation developmental potential of oocytes. A consistent feature of these studies was that oocytes were recovered from heifers by trans-vaginal, ultrasound-guided follicular aspiration, and matured, fertilised and cultured to the blastcosyst stage in vitro. In summary, whereas the effects of dietary carbohydrate source (i.e starch vs fibre) and supplemental fatty acids (in the form of calcium soaps of palm oil) were effective in altering post-fertilisation development of oocytes recovered from moderately thin heifers (high starch diets and saturated fatty acids were both detrimental), they had little effect on oocyte quality from moderately fat heifers (Adamiak et al., 2006). Interestingly, the incorporation of the branched-chain amino acid leucine into the high starch based diet offered to moderately thin heifers reversed the negative effects of this diet on oocyte quality (Rooke et al., 2009). This was associated with a significant reduction in the insulin:glucagon ratio. However, the feeding of high fibre or high starch diets as two, as opposed to four, meals per day, whilst effective in altering plasma concentrations of metabolic hormones such as insulin, glucagon, IGF-I and leptin, had no effect on post-fertilisation developmental potential of oocytes (Rooke et al., 2008).

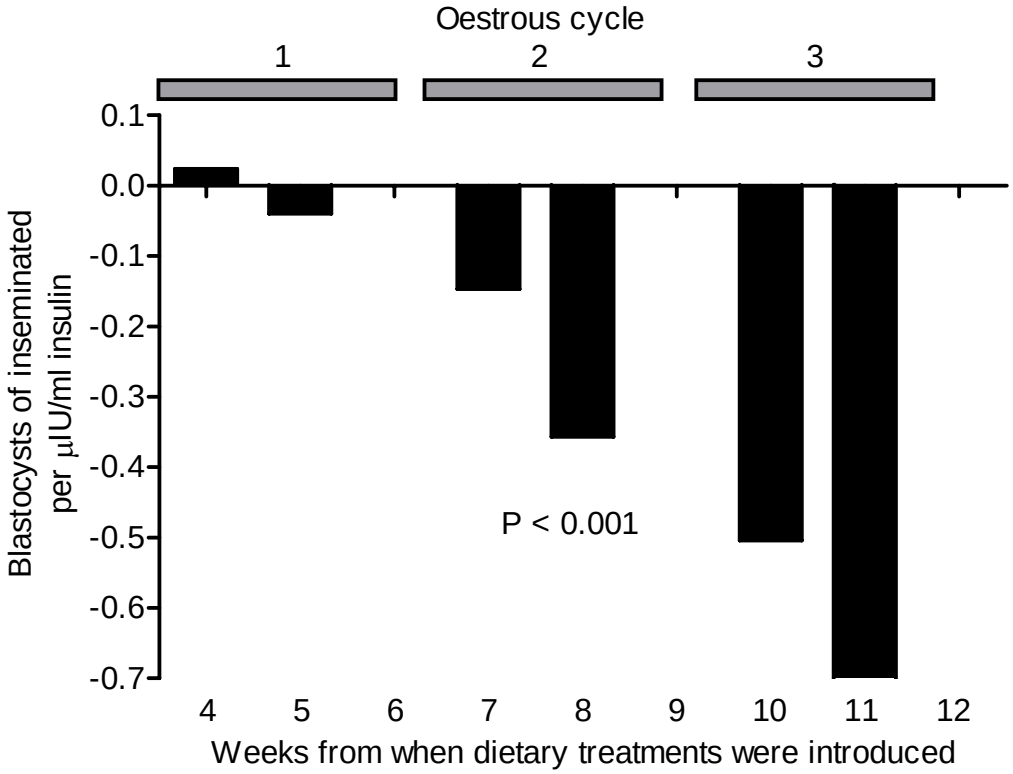

Figure 1
Regression coefficients for blastocysts of cleaved against plasma insulin concentrations determined at each of two oocyte recovery sessions within each of three successive oestrous cycles from the study of Adamiak et al. (2005). Heifers were moderately fat at the beginning of the experimental period and were offered a high calorie diet at a level equivalent to twice their metabolisable energy requirements for maintenance. Oocytes were matured, fertilised and cultured to the blastocyst stage *in vitro*. Mean plasma insulin concentration for these animals was 48 µIU/ml.

An important additional observation from the study of Adamiak et al. (2006) was the apparent existence of selective uptake mechanisms and/or *de novo* synthesis within the ovarian follicle and oocyte that favoured saturated fatty acids (FA) over polyunsaturated FA (PUFA). Saturated FA as a proportion of total FA in that study were greater in oocytes than in either granulosa cells or plasma, an observation we have since confirmed when offering PUFA enriched diets to sheep. This latter sheep study which, at the time of writing, is currently under peer review, looked at the effects of offering omega-3 and -6 PUFA on ovarian follicular development in vivo, and granulosa cell and embryo development in vitro. The study was initiated on the premise that the feeding of diets with PUFA from these two contrasting families would alter inflammatory mediators within the ovarian follicle associated with the terminal stages of oocyte maturation and ovulation. We failed, however, to observe a differential effect of PUFA source on ovarian response to FSH, although there was an effect on ovarian steroidogenesis (omega-3 > omega-6). High density lipoproteins (HDL) were fractionated from sera of ewes offered these diets and incorporated into embryo culture media. Although there was no differential effect of omega-3 and-6 PUFA enriched HDL on the FA composition of embryos, fewer zygotes cultured in the presence of omega-6 PUFA developed to the blastocyst stage. Our failure to detect a differential effect of omega-3 and-6 PUFA on ovarian response to stimulation is consistent with the observations of Childs et al

(2008) in beef heifers. These authors compared the effects of substituting a saturated form of dietary FA (palmitic acid) with a partially rumen protected form of omega-3 PUFA. Transcript expression of a number of genes involved in cell adhesion, viability and metabolism in Day 7 embryos flushed form the reproductive tract were also unaltered by diet. Consequently, whilst omega-3 and-6 PUFA can differentially influence a number of reproductive processes through a variety of mechanism (Wathes et al., 2007), direct effects on the oocyte and pre-implantation remain to be firmly established.

Dietary nitrogen

Feeding of high nitrogen diets, particularly those high in ruminally degradable nitrogen, has been shown to reduce uterine pH around Day 7 after oestrus in dairy heifers (Elrod and Butler, 1993). First-service conception rates in this study were also significantly reduced for heifers on the high compared with the low nitrogen diet (61 vs 82 %). In heifers, high plasma concentrations of ammonium and urea during both the pre-antral and antral stages of follicular development were associated with reduced cleavage rates following *in vitro* maturation and fertilisation, and reduced blastocyst production rates following *in vitro* culture (Sinclair et al., 2000a and b). Both glucose and protein (uptake of ^{35}S-methionine) metabolism were increased in surviving embryos, each indicative of metabolic stress. Mean plasma ammonium concentrations in these latter studies peaked at around 300 to 350 μM (for some individuals values peaked at around 500 μM) within two hours of feeding, and this suppressed the normal postprandial rise in insulin release (Sinclair et al., 2000c) consistent with earlier observations in cows receiving intra-ruminal infusions of urea (e.g. Choung and Chamberlain, 1995). Jugular vein infusion of either ammonium chloride or urea for several hours in beef heifers led to peak plasma ammonium and urea concentrations of around 800 μM and 14 mM respectively, with similar levels recorded in oviducal fluid (Kenny et al., 2002). However, with the exception of calcium, these treatments had no effect on oviductal glucose, lactate and electrolyte concentrations. These observations, together with those that have shown direct effects of ammonium exposure on mouse embryo metabolism and development during culture (Lane and Gardner, 2003; Zander et al., 2006), point to direct actions of this metabolite on the follicle-enclosed oocyte and pre-implantation embryo although, in the case of the former, effects may also be mediated through actions on granulosa and cumulus cells which, when pre-cultured in the presence of ammonium chloride, were subsequently less able to support oocyte and early embryo development (Rooke et al., 2004). Direct actions of ammonium can also lead to impaired fetal development as has been shown in both the mouse and sheep (Lane and Gardner, 2003; Powell et al., 2006).

Conclusions

The foregoing discussion illustrates how maternal diet and body composition can each affect oocyte quality and early embryo development. The responses described in the current article pertain to the non-lactating animal and it is recognised that many of these nutrient and metabolic-hormone mediated responses would differ in the lactating animal. Future studies should focus more on the effects of specific nutrients to provide a greater mechanistic insight into how maternal diet can affect egg and embryo quality. More emphasis should also be placed of measures of pregnancy outcome and on metabolic markers of egg and embryo quality that may of value in assisted reproduction.

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REPRODUCTION TECHNIQUES AND INBREEDING

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1. Abstract

The world is faced with the challenge to meet the increasing demand for livestock products while conserving animal genetic resource diversity and maintaining environmental integrity. Reproductive techniques have a major impact on the structure of breeding programs, the rate of genetic gain and dissemination of genetic gain in populations. This manuscript reviews the impact of reproductive technologies on the underlying components of genetic gain and inbreeding. It is argues that evaluation of alternative breeding schemes should be based on genetic gain while constraining inbreeding. Optimum breeding schemes can be characterised by: decreased importance of sib information; increased accuracy at the expense of intensity; and a factorial mating strategy.

2. Introduction

The general aim of the livestock sector is to meet the aspirations of the world's population for increased availability of animal products in a sustainable manner while ensuring food safety, animal welfare and the maintenance of rare and specialist breeds. The expected worldwide increase in demand for animal products for the next decade is 7% annually. Human population growth, increasing urbanization and rising incomes in the developing world are the main reasons for this increase (Speedy, 2003). The availability of marginal land suitable for producing this increase is limited, and hence there is a need to produce more from the same resources. For genetics to contribute half the required acceleration would mean a doubling in genetic gain (from the current figure of 1.5%). This illustrates both the need and the opportunity for farm animal genetic improvement, and placed alongside the requirements for sustainability, the need for precision animal breeding. Sustainability in livestock production implies meeting production targets while ensuring targets are also met for environmentally significant outputs, human feed efficiency, animal health and welfare and maintenance of biodiversity, in both farmed livestock and wild species affected by animal husbandry.

In recent decades, genetic improvement of farm animals has contributed to meeting the needs of the growing world population. Genetic improvement programmes exploit genetic variation among animals. By knowing breeding values of animals, improvements have been made in traits without knowing the genes that are responsible, and this is what has been exploited throughout domestication: the heritability of traits and the similarity of relatives. Genetic improvement implies change. For the genetic change to be an improvement, the overall effects must bring benefits to the owners of the animals. The desired direction of

change of a particular breed depends on the social, economic and environmental context in which livestock production takes place.

FAO's State of the World's Animal Genetic Resources (FAO, 2007) highlights issues confronting the use and conservation of diverse animal genetic resources. Two important driving forces for the livestock sector are the unprecedented growth in demand for livestock products (Delgado et al., 1999) and global environmental issues (De Haan et al., 1997; Steinfeld et al., 2006). Especially countries in the developing world are faced with the challenge to meet the increasing demand for livestock products while conserving animal genetic resource diversity and maintaining environmental integrity.
Reproductive technologies play an important role in genetic improvement programs (Nicholas, 1997; Van Arendonk and Bijma, 2003). In essence, the most basic effect of using reproductive technologies in males or females is an increase in fecundity. This means that fewer parents are needed to produce a given number of offspring. The application of reproductive techniques has had a major impact on the structure of breeding programs, the rate of genetic gain and the dissemination of genetic gain in livestock production and aquaculture. Research on application of reproductive technologies has initially focussed on genetic gain while little attention was paid to inbreeding (i.e. longer-term consequences of selection for genetic diversity). However, it is now well accepted that the evaluation of alternative breeding schemes should be based on genetic gain while constraining the rate of inbreeding.
This manuscript reviews the impact of reproductive technologies on the underlying components of genetic gain and inbreeding.

3. Creation and dissemination of genetic gain

In within-breed genetic improvement programs, two activities need to be distinguished. The first is the creation of genetic improvement by selecting animals based on their estimated breeding value for the relevant traits. Secondly, there is the dissemination of superior genetic material from the genetically elite animals to the commercial population. In most breeding schemes, only a small fraction of the population (referred to as nucleus animals) is involved in creating genetic improvement. This can be illustrated most clearly for poultry where genetic populations kept on a small number of nucleus farms are used for creating genetic improvement. Due to the high fecundity of hens, the superior genetic material of a hen in the nucleus can be disseminated to a million laying hens through a number of steps of multiplication.

The impact of genetic improvement made in the nucleus depends on the dissemination of genetic material from the nucleus to the target population, i.e. the animals kept by the local farmers which are responsible for the production of animal products (milk, meat, eggs, wool). The value of superior animals in the nucleus is limited if they do not efficiently contribute to improvement of the genetic level of the target population. Reproductive technologies, especially AI, are very important for effective dissemination. However, their impact varies between species. In sheep and goat breeding, the dissemination of genetic material largely depends on the trade in live animals. In the case of cattle, an exceptional bull can have over 100,000 progeny spread over a number of countries.

3. 1. Steps involved in genetic improvement scheme

The activities that constitute a program for genetic improvement can be summarized in 6 major steps:

1. Analysis of production system: what kind of animal is “desired”
2. Choice of breeding system: pure breeding or use of crossbreeding.
3. Definition of breeding goal (desired direction of change)
4. Evaluation of selection candidates (estimating the breeding value)
5. Select of best animals as parents for next generation
6. Use superior animals to produce (crossbred) animals in the target population

The steps 4 and 5 relate to within-breed selection to create of genetic improvement in the breeding goal and involve only the nucleus animals and relatives on which phenotypic information is collected for estimating breeding values. In case of pure-breeding, within-breed selection can be practiced in one nucleus population. However, when crossbreeding is used, within-breed selection needs to be practiced in each breed and the breeding goal could differ between breeds depending on the role of the breed in the crossbreeding scheme. When crossbreeding is used in pigs, for example, more emphasis is placed on litter size in the dam line(s) and more emphasis is placed on growth characteristics in the sire line(s).

3.2. Prediction of rate of Genetic Gain

Prediction of the rate of genetic gain in a population is based on Fisher's infinitesimal genetic model, in which the trait is assumed to be determined by an infinite number of genes, each with an infinitesimally small effect. Under that model the phenotypic trait value of an individual, P, is the sum of a heritable component, A, referred to as ‘‘breeding value’’ and a residual component, E, referred to as ‘‘environment’’: $P = A + E$.
Under this model, the rate of genetic gain (ΔG) in a population under selection can be predicted from the intensity of selection (i), accuracy of selection (ρ), additive genetic standard deviation (σA) and generation interval (L) in the different paths of selection (j) as (Rendel and Robertson, 1950):

$$\Delta G = \frac{\sum i_j \rho_j \sigma_{Aj}}{\sum L_j}$$

The intensity of selection basically depends on the proportion of selection candidates that are needed as parents to produce the next generation and the way in which they are selection, i.e. truncation or random selection. Accuracy of selection depends on the heritability and the amount of information available on the individual and its relatives to estimate A. From the above equation, it follows that finding the breeding schemes that maximizes ΔG involves finding the optimum combination of number of parents to produce the next generation and number (and type) of individuals on which phenotypic information should be collected.

The prediction of an animal's breeding value is based on phenotypes of the animal itself and/or those of its relatives where the contribution of observations on relatives depends on the additive genetic relationship, i.e. the proportion of genes identical by descent, and the heritability of the trait. Since 1970, the statistical technique best linear unbiased prediction (BLUP) is used to estimate the breeding value of animal from phenotypic information collected in a pedigreed population. The BLUP method utilizes phenotypic observations on individuals and genetic relationships between individuals to obtain estimates of breeding values for the entire population while simultaneously adjusting observations for systematic environmental influences. BLUP procedures are widely used in genetic evaluation systems for livestock in developed countries. The current BLUP procedures requires knowledge on pedigree relationships in a population which posses a serious limitations on its application in developing countries (Van Arendonk, 2009).

3.3. Rate of Inbreeding

The magnitude of inbreeding at the population level is measured by the rate of inbreeding (ΔF). Only in the absence of selection ΔF is related directly to the number of sires and dams. In selected populations, this equation is no longer valid because parents contribute unequally to the next generation. Wray and Thompson (1990) introduced methods to predict rates of inbreeding in selected populations, based on the concept of long-term genetic contributions. Recently, Woolliams et al. (1999) and Woolliams and Bijma (2000) developed a general theory to predict rates of inbreeding in populations undergoing selection. These methods facilitate a deterministic optimisation of short and long-term response of breeding schemes. Bijma and Woolliams (2000) demonstrated that with BLUP selection, the number of candidates per parent (selection intensity) may be as or more important than the number of parents. Doubling the number of parents failed to halve the rate of inbreeding.

Reduction of inbreeding in a selection schemes requires methods uses the information on selection candidates at the time selection decisions are made to arrive at the optimum decisions. This has lead to an algorithm for selecting animals based on their estimated breeding values (EBV) and their relationship to each other. The relatedness and inbreeding coefficients of animals in a pedigreed population can be estimated from pedigree information. The aim of the algorithm is to maximize the mean EBV of the selected animals while minimizing the mean relationship among them, because it is this mean relationship that determines the long-term inbreeding in the population (Wray and Goddard 1994, Meuwissen 1997). Simulation studies have shown that these methods can substantially reduce the rate of inbreeding while losing little selection response. Software to do the calculations is now used by several breeding companies (Brotherstone and Goddard, 2005). Application in a population requires reliable information on genetic relationships between animals.

3.4. Optimisation of Breeding Schemes

Under the infinitesimal model, inbreeding reduces genetic variation, which in turn reduces genetic gain. Furthermore, when inbreeding depression is present, fitness of the population may reduce to an extent where it affects the selection differentials, i.e. indirectly inbreeding may also reduce genetic gain.In the short term, inbreeding and genetic gain have an unfavourable relationship. For example, maximising short-term response by selecting fewer parents increase inbreeding which reduces long-term response (e.g. Verrier et al. 1993). To balance the short and long-term response a restriction on the rate of inbreeding is required (e.g. Quinton and Smith, 1995). The objective in optimisation of breeding strategies should be to obtain maximise genetic gain while restricting the rate of inbreeding. Acceptable levels of inbreeding are difficult to determine and are discussed by Bijma, (2000), who indicated that inbreeding depression is probably the most important issue. Though detailed knowledge of the relevant parameters to determine the level of the constraint is lacking, different approaches point towards values around 0.5% and 1% per generation. **.** Maximising genetic gain while constraining the rate of inbreeding will change the layout of breeding schemes compared to simply maximising genetic gain. Following the pioneering work of Nicholas and Smith (1983) and subsequently many others, breeding schemes for dairy cattle have moved towards selection based on sib information which enables higher short-term genetic gain. However, for species such as dairy cattle where the trait of interest cannot be measured on the male selection candidate, maximising genetic gain while restricting inbreeding is likely to move optimum selection schemes back to progeny testing, in particular when population size is small and the constraint on ΔF is stringent (Meuwissen and Sonesson, 1998). This situation

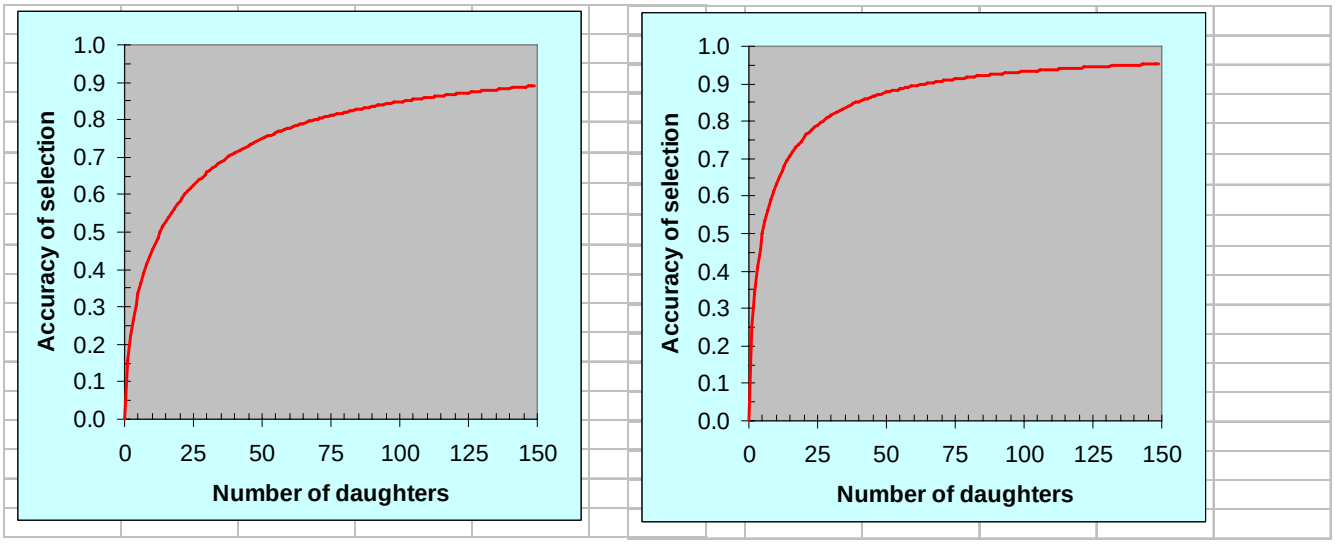
changes when traits of interest can be measured early in life on both sexes, as is the case in beef cattle and pigs. The situation would also change when molecular information was available to predict the Mendelian-sampling component of selection candidates at an early age (e.g. Spelman et al., 1999). The combination of increased female reproductive rate and DNA markers offers good opportunities to increase gain while restricting inbreeding.

4. Role of Artificial Insemination

The first reproductive technique that had a major impact on animal breeding schemes was Artificial Insemination (AI). In combination with pedigree registration and milk recording, AI offers the opportunity to obtain accurate estimates of breeding values of young bulls (Figure 1). Rendel and Robertson (2) proposed the progeny-testing scheme to make efficient use of the possibilities offered by AI combined with pedigree registration and performance recording. Since then, progeny-testing schemes have been widely adopted for creating genetic improvement in dairy cattle in the developed world, and many studies have been directed at optimising the design of those schemes from a genetic and economic point of view.

The generally accepted method of improving dairy cow populations relies on the two-stage gains achieved by first selecting among all possible young bulls (pedigree selection) to create the annual group of young sires, and then, several years later, selecting within that group the bulls with the best daughter proofs. The best proven bulls are used as sires of the next generation of young bulls and thereby contribute to the creation of genetic improvement. Furthermore, the proven bulls contribute to the dissemination of genetic improvement through the production of large numbers of second-crop daughters facilitated by the use of AI.

Figure 1: Accuracy of estimated breeding value of bulls dependent on progeny group size for two different heritabilities (h2=0.1, left and h2=0.25 right).



Implementing a progeny testing requires the commitment of a large group of farmers that are willing to participate in routine milk recording and pedigree registration. From figure 1 it follows that we need records on 100 to 250 daughters in order to get a reliable estimated breeding value of a bull. For a breeding program in which 100 young bulls are tested annually this corresponds to 10,000 to 25,000 first lactation cows or 40,000 to 100,000 cows. These

numbers show that a progeny testing scheme requires pedigree registration and performance recording on a large number of farms. The progeny testing schemes in place in developed countries capitalize on the large-scale use of milk recording by individual farmers for management purposes. This means that information on milk recording on daughters of young bulls is available for genetic evaluation at no or very low costs for the breeding organization. When the costs of milk recording have to be carried by the breeding programme it is very questionable whether progeny testing is still the economically most attractive option for generating genetic progress. Routine milk recording is not in place in most developing countries which hinders the implementation of progeny testing.

Correct pedigree information is paramount in a successful breeding program, and its importance has increased with the introduction the animal model for national genetic evaluation of dairy cattle. Usually, the correct assignment of the sire and dam to a calf is not questioned. However, the proportion of errors in sire identification may vary from a few percent to as much as 22% (Banos et al., 2001; Visscher et al., 2002). Errors, particularly in sire identification, will slow down genetic progress by reducing the accuracy of the estimated breeding values of cows and bulls (Van Vleck, 1970, Visscher et al., 2002). Banos et al. (2001) showed that pedigree errors also lead to a downward bias of genetic correlations between countries. This bias leads towards selection of domestic bulls and the inability to identify truly superior animals that are available internationally reduces the rate of genetic gain. DNA tests can be used to reduce the number of pedigree errors in a population.

5. Female reproductive capacity

An increase in reproductive rate of females offers the opportunity to reduce the number of dams that need to be selected for the next generation. At the same time, it leads to an increase in the amount of information available on sibs for estimating the EBV of male as well as female selection candidates. Among the early studies of the genetic implications of multiple ovulation and embryo transfer (MOET) were those by Land and Hill (1975) for beef cattle and Nicholas and Smith (1983) for dairy cattle. The general conclusion from these studies was that MOET could produce substantial increases in genetic improvement. However, it was noted that the rate of inbreeding would also be substantially increased. Since the early studies, there has been a great deal of activity amongst quantitative geneticists to perform more sophisticated studies taking into account several important genetic phenomenon that were ignored in the initial calculations (Nicholas, 1996). Today, it is clear that it is important to account for the Bulmer effect - the effect of correlated EBV on selection intensity and rate of inbreeding. The initial studies in particular underestimated the extent to which inbreeding would be increased and overestimated the value of information on sibs as compared to own and progeny information.

Following the pioneering work of Nicholas and Smith (1983) and subsequently many others, breeding schemes for dairy cattle have moved towards selection based on sib information which enables higher short-term genetic gain. However, for species such as dairy cattle where the trait of interest cannot be measured on the male selection candidate, maximising genetic gain while restricting inbreeding is likely to move optimum selection schemes back to progeny testing, in particular when population size is small and the constraint on ΔF is stringent (Meuwissen and Sonesson, 1998). This situation changes when traits of interest can be measured early in life on both sexes, as is the case in beef cattle and pigs. The situation would also change when molecular information was available to predict the Mendelian-sampling component of selection candidates at an early age (e.g. Spelman et al., 1999). The combination of increased female reproductive rate and DNA markers offers good opportunities to increase gain while restricting inbreeding.

6. Semen sexing

The availability of sexed semen in dairy cattle has been eagerly anticipated for many years, and recent developments in fluorescence activated cell sorting have brought this technology to commercial application. For a long time, the large-scale application has been hindered by the low number of straws of X-bearing semen that can be produced and the lower conception rates (Weigel, 2003). In recent years, a number of AI-companies have started to offer sexed semen to their farmers.

Semen sexing provides the potential to increase the numbers of offspring of one sex in a closed population, thereby increasing the intensity of selection for that sex. A number of studies have shown that the effect of semen sexing on rate of genetic gain is limited (Nicholas, 1997). Semen sexing, however, enhances the farmers' ability to obtain a larger number of replacement heifers from its own herd. This enables farmers to expand their herd size without the need for buying replacement heifers from other farmers. In a herd with a stable herd size, semen sexing could be used to breed replacement heifers from the cows with the highest genetic merit. This will create a one-time lift of the genetic level of the herd and thereby a reduction of the genetic lag between nucleus and commercial herds. The largest economic benefit of using sexed semen in pure-bred herds would come from the ability to use the remaining dairy cows for the production of cross-bred animals for meat production (De Boer and Van Arendonk, 1994).

Semen sexing can be used for to increase the efficiency of producing F1 dairy hybrids. For F1 scheme to be sustainable, part of the purebred population needs to be mated to bulls of that breed to produce replacements. The number of cows that need to be mated for breeding replacements can be nearly halved by the use of sexed semen. In addition, the number of F1-females that are produced can be nearly doubled by using sexed semen. In other words, the number of purebred cows that need to be kept for the production of F1 hybrids can be reduced by 60 to 75% depending on the sex ratio resulting from the use of sexed semen. The economic benefit of this reduction is largest when purebred cows and crossbred cows are competing for the same resources. Benefits are smaller in a stratified crossbreeding system as used in Brazil where dairy farms buy replacement F1 females, as in the poultry or swine industry (Madalena et al., 1990). Those females are produced in areas of less expensive land, using Holstein semen on Brazilian dairy zebu breeds.

7 Creating genetic gain with limited pedigree recording

The costs of implementing pedigree and performance recording are high. It is, therefore, important to look at alternative schemes for creating genetic improvement which do not involve progeny testing. Nucleus breeding schemes in which phenotypes are recorded on sibs have been suggested as an alternative. The number of animals for which pedigree and performance recording needs to be implemented is a lot lower in such a scheme compared to a progeny testing scheme. A number of simulation studies have shown that these schemes can yield acceptable levels of genetic improvement. Examples of a number of actual breeding schemes in developing countries are summarized in the publication on the state of the world's animal genetic resources (FAO, 2007).

8. The use of molecular information

Over the past few years, we have seen spectacular advancements in molecular genetics. Major advancements have taken place in the high-density single-nucleotide polymorphism (SNP) technology, which enables genotyping of an individual at many thousands of SNPs at low cost. It is anticipated that in a couple of years, an entire individual

genome will be sequenced for less than 1000 US$. SNP gene chips with over 50,000 SNPs are being used in cattle and poultry and will soon also be available for other farm animals. These recent advancements in the field of molecular genetics enable revolutionary changes in genetic analysis of populations and genetic improvement programmes. We are at the threshold of an era in which these developments in molecular genetics will enable us to completely revise our assumptions about which traits can be addressed by breeding, how breeding values are estimated and the impact breeding may have. In the following, two applications of molecular information are presented which circumvent the need for pedigree registration, namely pedigree reconstruction and genomic selection.

8.1. Using molecular information for pedigree reconstruction

The breeding schemes described so far all rely on pedigree information for exploiting phenotypic information collected on animals in the population in estimating of breeding values of selection candidates. In situations where the quality of the pedigree registration is poor, genotyping of animals enables the identification and correction of pedigree errors which results in a higher genetic gain (Van Vleck, 1970; Visscher et al., 2002).

Many methods have been developed in the past three decades to estimate the current, past and ancient effective population sizes using different information extracted from some genetic markers in a sample of individuals (Wang, 2005). Marker information can also be used for reconstruction of pedigrees within a population and the estimation of marker-based relationships between animals. Over the last decade, there has been growing interest in the field of population genetics in using marker-based relationship information to enable estimation of the components of variance for quantitative traits. Statistical methodologies allowing molecular marker data to be combined with phenotypic information allow estimation of heritabilities in certain natural populations (e.g., Ritland, 1996; Thomas & Hill, 2000). Comparisons of real data and simulated studies, however, clearly indicate that not all populations are suitable for marker-based analysis. According to Ritland (1996), the most important single feature that allows for marker-based analysis is having adequate numbers of relationships within the sample. With expanding amounts of marker data becoming readily available (e.g., with SNPs) and the reduction in costs of genotyping, there is a growing opportunity to use pedigree reconstruction in a breeding program to overcome the need of pedigree registration (Dodds et al, 2007). Pedigree reconstruction can be greatly improved when DNA samples are available of the sires that have been frequently used in the population. It, for example, enables the use of information collected on offspring that resulted from the natural matings in a group of females that kept with a number of sires. The marker information can also be used to assign individuals to a specific breed or breed combination which is highly relevant in situation where crossbreeding occurs.

Pedigree reconstruction resulting in marker-based relations could, for example be used in a situation where a number of sires have contributed to the generation of offspring. Based on genetic markers, the paternal relationships can be reconstructed especially when genotypes of the putative sires are known. This would circumvent the need to collect pedigree information through the registration of matings and resulting offspring and to track animals from birth until the age at which performance is recorded. The genotypes of an offspring could be taken at any stage from birth, during herd life, and at slaughter, thus superseding the costly and error-prone tracking of animals by ear tagging. The genotypes could be collected at the time of recording the first phenotype. From that point onwards, a system is needed to trace the identity of an animal, e.g. by ear tagging, to ensure that traits that are subsequently recorded can be traced to the animal and to monitor movement of animals between farms. Collection and analysis of molecular information requires an adequate infrastructure and accurate tracking of samples to ensure the reliability of information. Application of molecular

information for pedigree reconstruction requires a low-cost and accurate sampling and analysis systems.

Adaptation is an important trait in the breeding goal especially for populations that are kept and produce under harsh environments. Potential selection candidates might be kept under relative good management which hampers the opportunity to record the adaptive capacity on these animals. Estimation of breeding values of selection candidates for adaptive capacity can be improved by using information from animals in the general population. Breeding values for adaptive capacity can be based on information on animals that suffered over even died from lack of adaptive capacity. By collecting DNA on these animals and matched controls, breeding values can be estimated without the needed of pedigree registration.

Pedigree reconstruction based on DNA information offers a unique opportunity to kick-start a genetic improvement program in a population which has not been under selection. In traditional breeding schemes, a considerable amount of time is needed for pedigree relationships to build up which needed for including information on relatives in estimating breeding values. For example, in a progeny testing scheme one needs to wait 3 to 6 years depending on the trait before information on progeny becomes available. By genotyping a large number of selection candidates and animals with performance records one can start selection and thereby creating genetic chain soon after the start of the program. The selection of animals can be based on breeding values estimated from the performance records collected on related animals (e.g. half sibs) in that population and the marker-based relationships. The genotypes of the selection candidates also provide information on relationships between these founder animals. These relationships should be taken into account in making selection decisions to avoid that selection results in a too large loss of genetic diversity.

In conclusion, marker-based relationships offer an unique opportunity for creating genetic improvement programs in un-pedigreed populations. Further research is needed to determine the optimal design of a breeding scheme based on marker-based relationships from a genetic and economic point of view.

8.2. Genomic selection

With the availability of high-density marker maps and cost-effective genotyping, genomic selection (GS) methods may provide faster genetic gain than can be achieved by traditional selection methods (Meuwissen et al., 2001). GS may be defined as the simultaneous selection for many (tens or hundreds of thousands of) markers, which cover the entire genome in a dense manner so that all genes are expected to be in linkage disequilibrium (LD) with at least some of the markers. Simulation results and limited experimental results suggest that breeding values can be predicted with high accuracy using genetic markers alone, but more validation is required, especially in samples of the population different from that in which the effects of the markers was estimated (Goddard & Hayes, 2007). Implementation of genomic selection is likely to have major implications for genetic evaluation systems and for genetic improvement programmes.

There are two issues regarding GS that are relevant here. In the first place, the approach requires linkage disequilibrium (LD) between marker loci and quantitative trait loci. Most simulation studies on GS have considered a single population of relatively small effective size. In developing countries, populations may consist of subpopulations and the number of markers in LD with quantitative trait loci (QTL) might be relatively small compared to purebred populations. This is supported by the relative small fraction of the genetic variance explained by the recent genome-wide association study in humans

(Wellcome Trust Case Control Consortium, 2007). Habier et al. (2008) demonstrated that markers can capture genetic relationships among genotyped individuals which partly explains the accuracies of genome-assisted breeding values even when markers are not in LD with QTL. They showed that in some cases, accuracy of genomic-assisted breeding values (GEBV) can result entirely from genetic relationships captured by markers. There result also demonstrates that GS can be applied in unpedigreed populations which offers great opportunities for breeding schemes in developing countries. To validate the potential advantage of using GEBV, it is necessary to increase our understanding of (1) capturing relationships and (2) capturing LD to the accuracy of GEBVs. In order to exploit the full potential of GS in developing countries, we need to develop more sophisticated genomic selection models that can use information from purebred as well as crossbred individuals. For a more detailed discussion on GS, reference is made to paper by Van der Werf et al. (2008) which was presented at the same conference and included in this same issue.

9. Discussion

Reproductive technologies can play an important role in the implementation of genetic improvement programmes in developing countries. In the paper, I have mainly focused on the optimal structure of such programmes from a genetic point of view while taking into account the difficulties encountered with implementing pedigree and performance recording at a large scale.

Breed development is a dynamic process of genetic change driven by environmental and market conditions and selection practised by humans. Evolution of breeds results from changes in the ecosystems and human preferences. However, in the past 100 years there has been a net loss of diversity resulting from an increase in the rate of extinction of breeds. Genetic variation can be conserved in two different ways. It can be maintained within the commercial livestock population or it can be maintained in a population that is not used for commercial livestock production. Among the second group of methods for genetic conservation, semen from a wide range of bulls can be stored in liquid nitrogen so cheaply that there is no excuse for not using this method. However, this semen is unlikely ever to contribute substantially to the genetic composition of future populations of dairy cows in developed countries (Hill, 2000; Brotherstone and Goddard, 2005). This implies that genetic variation, if it is to be useful, must be conserved in commercial cattle populations. However, it is undesirable and unrealistic to expect cattle owners to practice policies for genetic conservation, unless these policies maximize their current and future profit. This certainly holds for farmers in developing countries. Characterization of animal genetic resources including description of natural habitats and production systems is important for setting conservation priorities. Characterization of local genetic resources enables policy decisions on management of animal genetic resources. Policy decision should aim to promote further breed development in order to meet the needs of present and future generations.

In this paper a number of options are discussed to use reproductive and other technologies in genetic improvement programmes for indigenous breeds in developing countries. Within-breed improvement programmes can contribute to improved livelihood of people that depend on low-input systems. These breeding programmes must have outputs which are consistent with the producer's objectives and need to be driven by incentives from the market to justify the producer's investment. The bottom line is that successful adoption of a technology depends on its compatibility with the needs of the farmer and the production system. It has to be relatively simple, relatively cheap, and above all, involve relatively low risks. It is necessary to look at the production system holistically, and involve the producer at

every stage in the planning and operation of the breeding program, integrating traditional behaviour and values.

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EMBRYONIC LOSS IN CATTLE: A COW OR EMBRYO-INDUCED PHENOMENON?

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Approaches to understanding embryo mortality

Poor reproductive efficiency is a worldwide problem affecting the dairy industry. Embryo mortality in cattle, reflected in reduced conception rate/calving rate per service, is a major cause of economic loss. The well reported decline in dairy cow fertility over the past several decades has occurred in association with a dramatic increase in milk yield per cow as a result of intensive selection (see (Lamming & Royal 1999, Diskin & Morris 2008). Infertility in dairy cattle is a multifactorial problem which may be linked to various checkpoints along the developmental axis including suboptimal follicle development associated with poor oestrus exhibition, suboptimal oocyte quality, altered sperm transport and fertilization and/or suboptimal reproductive tract environment. One of the obstacles to achieving a better understanding of the causes of poor fertility is the difficulty in separating these various issues from each other.

One approach to identifying at which stage along the developmental axis problems arise has been to inseminate animals and either flush embryos at given stages after insemination to determine fertilization failure and timing of embryonic mortality or to continually monitor pregnancy to pinpoint the period of loss. Published data indicate a fertilisation rate of 90% and average calving rates of about 55% are normal for heifers and moderate yielding dairy cows, indicating an embryonic and foetal mortality rate of about 35% (Sreenan & Diskin 1986). Very few embryos are lost between fertilization and Day 8 of gestation (corresponding to the blastocyst stage); 70-80% of the total embryonic loss is estimated to occur between days 8 and 16 after insemination (corresponding to the day of maternal recognition of pregnancy in cattle); a further 10% between days 16 and 42, by which time implantation is complete, and further 5-8% between day 42 and term (reviewed by; (Diskin *et al.* 2006, Diskin & Morris 2008).

Dunne *et al*. (2000) reported that embryo survival rates on Days 14, 30 and at full-term were similar (68%, 76%, 72%, respectively), indicating that most embryo loss, at least in beef heifers, occurs before Day 14. Silke *et al*. (2002) reported embryonic loss of 6-7% between Day 28 and 84 of gestation. Starbuck *et al*. (2004) reported embryonic loss of about 11% between Day 30-60 and related this loss to concentration of progesterone at week 5 of gestation, twin ovulation, body condition, age and sire.

The sparse data from flushed early embryos from normally ovulating (i.e., non-superovulated), high-yielding lactating dairy cows (Wiebold 1988, Ryan *et al.* 1993, Sartori *et al.* 2002) indicate that fertilization rate is also high, but few studies have directly compared lactating and nonlactating dairy cows. In the study of Sartori *et al.* (2002), comparing lactating and nonlactating (either nulliparous heifers or dry cows) Holstein cattle, fertilization was only reduced during summer in lactating dairy cows; however, lactating dairy cows had

poorer embryo development than nonlactating females. This last observation is interesting as it suggests that the ability of the reproductive tract in supporting normal embryo development may be impaired in lactating cows. However, oocyte quality cannot be ruled out as a contributing factor, as it is clear from IVF studies where typically 80% of inseminated oocytes cleave and 30-40% develop to blastocysts, that fertilization success is no guarantee of future development (Lonergan 2007).

One way of experimentally separating potential issues surrounding the follicle and/or oocyte from issues relating to the reproductive tract environment is to use ovum-pick up coupled with IVF. Differences in development seen in this scenario would by definition not be related to post-ovulation issues i.e., the reproductive tract. While several authors have reported development of OPU IVF embryos in dairy cows, e.g., (Fouladi-Nashta *et al.* 2007), few have compared development from dairy cows and heifers. In one study from our group (Rizos *et al.* 2005) there was no difference in the proportion of good quality oocytes undergoing fertilization and development to the blastocyst stage between lactating cows and heifers. Snjiders *et al*. (2000) found that a lower proportion of oocytes recovered from dairy cows with a higher genetic merit for milk production underwent cleavage or developed to the blastocyst stage in vitro than those from cows of average genetic merit.

The use of embryo transfer allows one to remove any confounding effects of the endogenous oocyte from the picture. Several studies from Florida compared embryo transfer (ET) with artificial insemination (AI) in order to overcome poor conception rate of lactating dairy cows due to heat stress (Putney *et al.* 1989, Ambrose *et al.* 1999, Drost *et al.* 1999, Al-Katanani *et al.* 2002). In all of these studies, conception rate was higher for ET than AI when fresh or frozen in vivo produced embryos were used; transfer of frozen (Ambrose *et al.* 1999, Drost *et al.* 1999) or vitrified (Al-Katanani *et al.* 2002) IVF embryos had no advantage over AI. Increased pregnancy rate of recipient lactating cows indicates that the bovine embryo is sensitive to maternal heat stress during the first 7 d after estrus. Embryo transfer may bypass this period of embryonic sensitivity and provide an alternative to AI to partially circumvent heat stress-induced infertility in cattle. In contrast, Sartori *et al.* (2006) compared ET with AI in dairy cows in Wisconsin at cooler times of the year and found no difference in CR.

Type of embryo

The origin of the embryo (e.g., in vivo derived following superovulation *vs* in vitro produced (IVP) *vs* nuclear transfer) can have a significant impact on the dynamics of embryo mortality. Heyman *et al.* (2002) monitored the evolution of pregnancy following the transfer of embryos derived from somatic cell cloning, embryonic cloning and IVP in order to detect the occurrence of late gestation losses and their frequency. On the basis of progesterone concentrations on Day 21, there were no significant differences in the percentages of initiated pregnancies between the groups (55.6-62.7%). Confirmed pregnancy rate by Day 35 using ultrasound scanning was significantly lower in the two somatic cloned groups (27.5-33.8%) compared with the embryonic clones (49.2%) and IVF embryos (52.9%). This pattern was maintained at Days 50, 70 and 90. The incidence of loss between Day 90 of gestation and calving was 43.7% for adult somatic clones and 33.3% for foetal somatic clones compared with 4.3% after embryonic cloning and 0% after IVP. As pointed out below, some indications of the subsequent developmental fate of the embryo can be seen by looking at the endometrial transcriptome. We have observed a similar fall off in pregnancy rates up to Day 75 in SCNT pregnancies compared to in vivo derived embryos (Lonergan *et al.* 2007).

Interaction of embryo with endometrium

The ability to transfer an in vitro derived embryo to a synchronized recipient and obtain acceptable pregnancy rates would suggest that the embryo is somewhat autonomous for at least the first week of life and that direct contact with the maternal reproductive tract is, to a certain extent, unnecessary. Indeed, pregnancies have resulted from the transfer of embryos at Day 12 (Lazzari *et al.* 2002) and even as late as Day 16 (Betteridge *et al.* 1980).

In support of this, when we compared the transcriptome of the endometrium in pregnant and cyclic heifers on various days from oestrus (Day 5, 7, 13 and 16) we could only detect differentially expressed genes in the endometrium on Day 16, coincident with a filamentous embryo secreting large amount of interferon-tau (Forde *et al.* 2009). This would suggest that the cow, or more specifically her uterus, is always an optimist regarding likelihood of pregnancy i.e., that the temporal changes occurring in the endometrium are similar in pregnant and cyclic cows up the point when luteolysis normally occurs.

Preparation of the endometrium for embryo attachment and implantation in all studied mammals, including ruminants, involves carefully orchestrated spatiotemporal alterations in transcriptome profiles. Two recent key papers provide strong evidence that the endometrium reacts differently to different embryo types (Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009); in other words, embryos of different quality (i.e., with divergent developmental fates) signal differently to the endometrium and in turn elicit a different response in terms of the transcriptome of the endometrium. In this way, the endometrium can be considered as a biological sensor able to fine-tune its physiology in response to the presence of embryos whose development will become altered much later after the implantation process (Mansouri-Attia *et al.* 2009) i.e., the endometrium can be viewed as a mirror reflecting the quality of the conceptus. These data suggest that placental abnormalities in bovine clone pregnancies may originate from disturbed embryo–maternal communication during the peri-implantation period.

Role of progesterone in establishing pregnancy

The steroid hormone progesterone plays a key role in the reproductive events associated with pregnancy establishment and maintenance. High concentrations of circulating progesterone in the immediate post-conception period have been associated with an advancement of conceptus elongation, an associated increase in interferon-tau production and higher pregnancy rates in cattle (Lamming & Royal 1999, Mann & Lamming 2001, Inskeep 2004, Stronge *et al.* 2005, McNeill *et al.* 2006) and sheep (Ashworth *et al.* 1989, Satterfield *et al.* 2006).

The effects of elevated progesterone shortly after conception on the advancement of conceptus elongation have been convincingly demonstrated in cattle and sheep. Garrett *et al.* (1988) administered 100 mg progesterone on Days 1, 2, 3 and 4 of pregnancy which resulted in an increased peripheral plasma progesterone concentration on Days 2 to 5 and significantly larger conceptuses on Day 14. Using a progesterone implant on Day 3 of pregnancy, Carter et al. (Carter *et al.* 2008) significantly elevated progesterone concentrations until Day 8 and this was associated with a larger conceptus recovered at slaughter on Day 16. Similarly, when ewes received daily injections of 25 mg progesterone from 36 h postmating, blastocyst diameter increased by 220% on Day 9 and the time of elongation of blastocysts to a filamentous conceptus on Day 12 was advanced (Satterfield *et al.* 2006); these effects of

progesterone treatment on blastocyst development were blocked by administration of RU486, a progesterone receptor antagonist.

From the above, it is clear that the concentration of circulating progesterone has an effect on the developing embryo. This effect is likely as a result of downstream effects of progesterone-induced changes in gene expression in the tissues of the uterus (Bauersachs *et al.* 2006, Satterfield *et al.* 2006, Forde *et al.* 2009) resulting in changes in the composition of histroph to which the developing embryo is exposed. The importance of histotroph for conceptus development was demonstrated in the uterine gland knockout (UGKO) model in sheep in which embryos fail to develop beyond the blastocyst stage in adult UGKO ewes (Spencer & Gray 2006). Whether any of the effect of progesterone is directly on the embryo or whether the observed advancement of development is mediated solely through changes induced in the endometrium is, as yet, unclear. In an attempt to answer this question, several authors have added progesterone to embryo culture medium in vitro and examined development to the blastocyst stage. Results, however, have been varied and contradictory with some authors reporting positive effects of progesterone (Ferguson *et al.* 2005, Merlo *et al.* 2007) while others have reported no effect (Reggio *et al.* 1997, Goff & Smith 1998). These contradictory observations may be partially explained by the culture system used as steroids are known to be lipophilic and culture under oil, the system employed in many laboratories, may result in depletion from the medium (Shimada *et al.* 2002).

In a series of recent experiments using in vitro and in vivo models we addressed the issue of whether the effects of progesterone on conceptus elongation could be due, at least in part, to a direct effect of progesterone on the embryo (Clemente *et al.* 2009). mRNA for progesterone receptors was present at all stages of embryo development raising the possibility of a direct effect of progesterone on the embryo. Exposure to progesterone in vitro in the absence or presence of oviduct epithelial cells did not affect the proportion of embryos developing to the blastocyst stage, blastocyst cell number or the relative abundance of selected transcripts in the blastocyst. Furthermore, exposure to progesterone in vitro did not affect post-hatching elongation of the embryo following transfer to synchronised recipients and recovery on Day 14. In contrast, transfer of in vitro derived blastocysts to a uterine environment previously primed by elevated progesterone resulted in a 4-fold increase in conceptus length on Day 14. These data provide clear evidence to support the hypothesis that progesterone-induced changes in the uterine environment are responsible for the advancement in conceptus elongation reported previously in cattle and that, interestingly, the embryo does not need to be present during the period of high progesterone in order to exhibit advanced elongation.

Thus, it is clear that the reproductive tract environment undoubted plays a significant role in determining developmental outcome as evidenced by the well described role of progesterone in modifying endometrial gene expression (Satterfield *et al.* 2006, Forde *et al.* 2009) and the association of progesterone in the days immediately following conception and subsequent conceptus elongation (Mann & Lamming 2001, Satterfield *et al.* 2006, Carter *et al.* 2008) and pregnancy rate (Stronge *et al.* 2005). We hypothesized that that part of the difference in fertility between heifers and post partum dairy cows could be explained by differences in the ability of the reproductive tract (oviduct and uterus) to support early embryo development. To test this hypothesis in single-ovulating animals would be challenging due to the numbers of animals required. Therefore, using endoscopy, we transferred 1800 in vitro fertilized embryos to the oviducts of nulliparous Holstein-Friesian heifers and post partum Holstein-Friesian cows (100 per recipient) and assessed their

development to the blastocyst stage following nonsurgical recovery on Day 7 (unpublished). Interestingly, the recovery rate was significantly lower from cows (57%) compared to heifers (79%) and of the structures recovered only 18% had developed to the blastocyst stage in cows compared to 34% in heifers.

Conclusion

Many factors are likely to impact on the success or otherwise of pregnancy. It is clear that both maternal factors (e.g., oocyte quality, reproductive tract environment) can have a strong influence on the likelihood of embryo survival. However, the inherent quality of the embryo (e.g., in vivo derived, IVP or cloned) can also affect the chances of it surviving, for example, by failing to elicit the correct response from the endometrium to ensure an optimal environment.

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SHORT COMMUNICATIONS

ASSOCIATION BETWEEN BULL FIELD FERTILITY AND IN VITRO MEASURES OF VIABILITY AND FERTILITY

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The aim of this study was to identify a reliable *in vitro* assay to predict bull field fertility. Two approaches were taken: Firstly, we examined the metabolic activity of sperm from high and low fertility bulls in terms of their ability to penetrate artificial mucus, their mitochondrial activity as well as their survivability after the freeze thaw process. Secondly, we examined the ability of sperm from high and low fertility bulls to fertilise oocytes *in vitro* and support embryo development to the blastocyst stage.

Six bulls used in commercial AI in Ireland were classified as having 'high fertility' (n=3) or 'low fertility' (n=3) according to 90 day non return rates following insemination with frozen semen (mean: 75.6% vs 65.6%, respectively). The ability of sperm from each bull to penetrate mucus composed of 70% MAP-5 (Labstock MicroServices, Ireland) and either 30% PBS (n=7 replicates) or 30% of a semen extender (Androhep; n=3 replicates) was assessed. Glass capillary tubes (n=2/bull/replicate) were filled with artificial mucus and incubated with sperm stained in 1% Hoechst 33342 for 30 min at 37^{o}C. The number of sperm was subsequently counted at 10 mm intervals along the tube between 40 and 80 mm. Sperm mitochondrial activity of each bull was assessed by the MTT assay. MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) is reduced to purple formazan crystals in the mitochondria of living cells and the colour change in the medium is representative of the level of mitochondrial activity. Sperm were incubated with MTT for 1 h at 37^{o}C following which the absorbance was read using a spectrophotometer (n=4 replicates). Sperm viability following the freeze thaw process of each bull was assessed using the live/dead sperm viability kit (SYBR 14/ Propidium Iodide; Molecular Probes). In each of 3 replicates a minimum of 250 cells was assessed and classified as either live or dead. Finally, the ability of sperm from the high and low fertility bulls to fertilise oocytes and their ability to produce competent embryos was assessed (n=5 replicates with 279-306 oocytes/bull).

The mean number of sperm counted at each 10 mm mark was significantly higher in the high fertility bulls irrespective of the composition of the mucus (mucus containing (a) PBS - 46.8 ± 5.24 and 21.45 ± 5.24 respectively ($P < 0.05$) and (b) androhep 87.8 ± 5.49 and 46.7 ± 5.6, respectively; $P < 0.01$). There was no difference in the ability of sperm from high and low fertility bulls to reduce MTT to formazan (mean absorbance 0.19 ± 0.020 and 0.18 ± 0.020, respectively). In addition there was no effect of bull fertility on the proportion of live sperm per straw (mean 43.1 ± 8.73% and 36.5 ± 8.7%, respectively). Oocyte cleavage following insemination with sperm from high fertility bulls tended to be higher than with low fertility bulls (72.4 ± 3.52% and 60.7 ± 3.49% respectively, $P = 0.07$). There was no effect of bull fertility on blastocyst rate (mean 30.4 ± 4.69% and 26.8 ± 4.63% for the high and low fertility bulls respectively). In conclusion, under the conditions of the current study, the assessment of the ability of sperm to penetrate artificial mucus was the most reliable in vitro assay to discriminate between bulls varying in their field fertility.

Notes

REPEATABILITY AND PERFORMANCE TREND IN SUCCESSIVE LAPAROSCOPIC OVUM PICK-UP SESSIONS IN SHEEP

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The ovum pick-up performance in repeated aspiration of follicles has been studied in sheep. Twelve cyclic Rasa Aragonesa ewes in the breeding season (BS) and 13 non-cyclic ones in the non-breeding season (NBS) were subjected to five laparoscopic ovum pick-up (LOPU) sessions one week apart. Before the first LOPU session, ewes were treated with 40 mg FGA sponges for 13 days. In the BS, 125 μg cloprostenol was applied at sponge insertion. In both seasons, ewes were randomly allocated to two groups: continuous progestagen treatment (sponges replaced every session; BS: n=6; NBS: n=6) and control (sponges removed at the first LOPU session; BS: n=6; NBS: n=7). Continuous progestagen treatment was intended to avoid ovulations and preovulatory follicles (Roche et al., 2004; Reprod Fertil Develop 16(4): 515). All visible follicles were aspirated (Alberio et al., 2002; Small Rumin. Res. 46: 81–87) and recovered cumulus-oocyte complexes (COCs) were morphologically evaluated (Stangl et al., 1999; Theriogenology 52: 709-716).

The number of aspirated follicles per ewe and session (AF), recovered COCs (RC), COCs scored 1 to 3 (suitable for IVP; COCs1+2+3), COCs scored 1-2 (COCs1+2) and recovery rate (RR) were tested for repeatability throughout the five LOPU sessions by the Kendall´s concordance coefficient (W). Repeatability was not significantly affected by either progestagen treatment or season. All the studied variables showed a significant repeatability (AF: W=0.81, RO: W=0.6, COCs1+2+3: W=0.53, COCs1+2: W=0.49; each, P<0.01, RR: W=0.34; P<0.05). Hence, these variables differed significantly between donors, displaying similar rankings in the five LOPU sessions.

The trend of the studied variables with the number of sessions was also studied. In the first session, there were no significant differences between seasons or treatments. Pooled means of the studied variables per ewe at the first session were 24.8, 11.2, 9.0, 5.1 and 45.8%, respectively. Irrespective of treatment and season, the numbers of AF and RC per ewe and session significantly decreased in the successive sessions (-0.89 and -0.44, respectively; each, P<0.05). The number of COCs1+2+3 per ewe and session also decreased significantly in the NBS in both, progestagen (-1.80, P<0.05) and control (-0.57, P<0.01) groups. This decrease was significantly higher in progestagen than in control group (-1.80 vs. -0.57; P<0.05).

In conclusion, ewes could be ranked according to their performance in LOPU and selected or rejected for subsequent sessions based on the results of the first session. The number of COCs suitable for IVP of embryos significantly decreased with the number of sessions in the NBS, when using the treatments and protocols described here.

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Notes

ARE WE ABLE TO SUCCESSFULLY OBTAIN IVP OVINE EMBRYOS BY USING SEMI-DEFINED SEMEN DILUENTS? PRELIMINARY RESULTS

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Semen diluents for most animal species usually contain fructose or glucose, egg yolk (EY), and glycerol. However, preparation of uniform semen diluent containing EY is difficult, because individual EY quality may vary depending on the number of days after laying and the storage conditions. Removal of chicken egg yolk from semen diluents would be advantageous in producing a more uniform, reliable product. A number of researchers have reported the positive and direct effect of albumin on frozen-thawed spermatozoa, and BSA is one of the proteins available for replacement of EY (Blank, *et al*., 1979; Davis *et al*., 1979; 1980; Singleton *et al*., 1983). The objective of this work was to examine frozen-thawed spermatozoa from semi-defined diluents (different concentrations of BSA) *vs.* undefined diluent (EY) in the in vitro embryo production (IVP) process.

Ovaries of Sarda breed sheep were collected from a slaughterhouse. Recovered oocytes were matured in TCM199 supplemented with 10% (v/v) heat oestrus sheep serum, 10 μg/ml FSH, 10 μg/ml LH, and 100 μM cysteamine. Matured oocytes were divided into four different groups and fertilized from a single ram ejaculate in SOF with 2% estrous sheep serum by using four frozen-thawed ram semen diluents (10, 15, 20% BSA, and 20% EY as control group). Presumptive zygotes were cultured in SOF medium supplemented with 0.4% BSA (w/v), supplemented with essential and non-essential amino acids. The embryos that reached expanded blastcyst stage (Day 6 - 8) of each group were recorded and vitrified.

There were no significant differences among groups in the rate of development to the blastcyst stage. There were significant differences ($P < 0.001$) in the cleavage rates of the 10% and 15% *vs.* 20% BSA groups (Table 1). However, the cleavage rates in the 10 or 15% BSA groups did not differ significantly from the EY control.

In conclusion, groups with the addition of all concentrations of BSA attained the blastocyst stage at rates comparable to those of the control group. The use of 10 or 15% BSA (but not 20%) produced cleavage rates comparable to the egg yolk control. Subsequent results will clarify if diluents using different concentrations of BSA are superior to egg yolk diluent in numbers of embryos produced.

Table 1: Percentage of cleavage and blastocyst development rates using different frozen - thawed semen diluents.

Diluents Treat.	No. Oocytes	Cleavage Rate	Blastocyct s 6d	Blastocyct s 7d	Blastocyct s 8d	Blastocyct Rate
10 % BSA	132	111/132 (84.09)[a]	5	27	14	53/132 (40.15)
15 % BSA	86	66/86 (76.74)[a]	7	22	-	29/86 (33.72)
20 % BSA	167	113/167 (67.66)[b]	9	41	9	59/167 (35.32)
20 % EY	84	69/84 (82.14)[ac]	-	26	-	26/84 (30.95)

a-b Values in a row of column with different letters differ ($P < 0.001$).
b -c Values in a row of column with different letters differ ($P < 0.01$).

Notes

THE EFFECT OF ANTIOXIDANT ADDITIVES ON THE CYTOLOGICAL AND ULTRASTRUCTURAL PARAMETERS OF FROZEN-THAWED BUCK SEMEN

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In mammals, the semen membrane contains a large number of unsaturated fatty acids, facilitating thus the lipid peroxidation in the presence of species reactive to oxygen (ROS). The consequence of this process is the reduction of the quality of the seminal material or even the death of the sperm cell. The antioxidant capacity of the sperm cell is insufficient to prevent the lipid peroxidation during the freezing-thawing process. The purpose of this study was to determine the influence of antioxidant additives (BSA, Cystein, vitamin E) added in different concentrations to the freezing media on the semen cytological parameters post-thawing (motility, viability, membrane integrity, anomalies). The experiments were done on 62 ejaculates collected by artificial vagina from 3 bucks (Alpine) during the normal reproduction season. After collecting, the samples were washed of the seminal plasma and diluted in medium based on Tris in which antioxidants were added (6 experimental versions) or in medium without added antioxidants (witness). The diluted semen was cooled at 4º C, placed in vials and frozen in fine 0.25 ml French vials and then stored in liquid nitrogen. The 6 experimental versions were tested for each buck taking into account how suited each animal is for the freezing of the seminal material. The results show that adding BSA in a concentration of 5 mg/ml, Cystein (10mM) and vitamin E (1mM) have positive protection effects on the semen characteristics consequently to the freezing-thawing process for 2 of the 3 bucks, leading to the improvement of the technology to freeze buck semen. Since the international research regarding the involvement of the oxidative stress associated to freezing-thawing in the reduction of the fertility of buck semen post-thawing is at its beginnings, other studies are necessary to confirm the obtained results. The studies on electron-microscopy have demonstrated that after Cystein and vitamin E supplementation, they protect very well the membrane systems of the sperm cell.

Key words: ram, semen, cryopreservation, antioxidants

Notes

IS THE MORPHOLOGY OF PORCINE OOCYTES ASSOCIATED WITH *ZONA PELLUCIDA* GLYCOPROTEIN 3 (PZP3) AND INTEGRIN BETA 2 PROTEIN LEVELS?

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The morphology and quality of oocytes is set during oo- and folliculogenesis, and completed during final maturation. Early embryonic development is associated with the morphology of the cumulus-oocyte-complex (COC). However, the knowledge of the possible relationships between oocyte morphology and the level of proteins within the oocyte, which may reflect fertilization ability, is insufficient.

Using western-blot analysis and confocal microscopic observation, we determined the levels of integrin beta-2 (integrin β2) and porcine *zona pellucida* and glycoprotein 3 (pZP3) protein levels in four morphologically different types of oocytes, graded according to their cytoplasm composition and cumulus structure. The level of integrin β2 protein was increased in grade-I and -II oocytes as compared to other grades ($P<0.05$). Moreover, the level of pZP3 protein was 3-4 fold higher in grade-I oocytes ($P<0.01$).

We suggest that COC morphology may be associated with oocyte fertilization ability with respect to its sperm-oocyte interaction gene expression, which is the result of increased accumulation of specific proteins prior to fertilization in higher quality oocytes. Higher quality oocytes may also reflect better fertilization ability.

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Notes

INFLUENCE OF eCG *vs.* TRANSIENT WEANING METHODS ON OVARIAN FOLLICULAR ATRESIA AND OOCYTE MATURATION IN PRIMIPAROUS LACTATING DOES AT EARLY *POSTPARTUM* PERIOD

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High-yield lactating does need effective estrus synchronization methods to improve their reproductive outcome by enhancing ovarian function. The aim of the present work was to analyse ovarian follicular atresia and oocyte maturation after hormonal and non-hormonal estrus synchronization regimes in primiparous lactating does in the early *postpartum* period. A total of 46 females were randomly treated with: 1) 25 IU equine chorionic gonadotropin (eCG) 48h before artificial insemination (AI) (eCG group, n=23), or 2) by doe-litter separation 24h before AI (Bio group, n=23). At day 11 *postpartum* (AI time) animals were euthanasized according to the bioethics committee of the University. One ovary was fixed in paraformaldehyde solution for the detection of cell apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling (TUNEL; In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH). Follicular atresia was studied in a total of 1926 follicles (eCG group: n=830; Bio group: n=1096) and follicles were clasified as: late-atetric (with > 50% of the granulose cells labelled including the theca cells) or mid-atretic (with < 50% of the granulose cells labelled). Cumulus-oocyte complexes (COC) of the counterpart ovaries were *in vitro* matured in TCM199 with 100ng/ml EGF, 10ng/ml IGF-I and 10% FCS. Afterwards, a total of 339 COC (eCG group: n=168; Bio group: n=171) were treated progressively with 2mM hyaluronidase, 0.5% pronase, 4% paraformaldehyde, 0.02% Triton X-100 and 7.5% BSA for confocal study. Oocytes were incubated with 100μg/ml FITC-LCA for cortical granule (CG) staining and with 10μg/ml Propidium Iodide for nuclear staining. Chi-square test was performed. Rates of late atretic follicles were not different between treatments (11.7 *vs.* 11.7%), but the eCG group showed a significantly higher number of mid-atretic follicles compared to the Bio group (7.1 *vs.* 4.7%, respectively; $P<0.05$). Nuclear *in vitro* oocyte maturation (measured as metaphase II rate), did not show significant differences between treatments (60.4 *vs.* 67.1%, eCG and Bio groups respectively). Rate of oocytes with peripheral distribution of CG (considered as cytoplasmically matured) was significantly higher in the Bio group compared to the eCG treatment (29.1 *vs.* 13.9%, respectively; $P<0.05$). The number of oocytes with cortical (partially matured), homogeneous (cytoplasmically immatured) or anomalous distribution of CG (poor quality or degenerated oocytes) were similar between eCG and Bio treatments (16.7, 54.2, 15.3 *vs.* 9.3, 39.5, 22.1%, respectively). In conclusion, transient doe-litter separation seems to reduce ovarian follicular atresia and improve oocyte competence compared to the eCG treatment. Therefore, a 24h-long transient weaning could be a reliable and alternative non-hormonal method for synchronizing estrus in primiparous lactating rabbit does. This work was supported by MEC project AGL07-60168, AGL08-022283 AGL. MAA is granted by CM and FSE, and RMGG was supported by "Juan de la Cierva" MEC Program.

Notes

BOVINE SEX RATIO IS NOT ALTERED FOLLOWING THREE DIFFERENT MODIFICATIONS IN IVF PROTOCOL

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Y- and X-bearing spermatozoa are known to differ in their DNA content, which allows their separation following flow-cytometry sorting. The different DNA content implicates a difference in spermatozoa weight which has been suggested to be responsible for differences in motility patterns, which raises the possibility to alter the sex ratio by modifications in IVF conditions. Swim-up separation selects highly motile spermatozoa, and thus it has been suggested to skew sex ratio in favor of males due to a putative higher linear motility of Y-bearing spermatozoa. Similarly, shortening coincubation time could skew the sex ratio if Y-bearing spermatozoa are able to penetrate faster than their counterparts. Lastly, sperm incubation prior to fertilization could skew the sex ratio in favor of those spermatozoa that aged later.

The aim of this study was to determine the effect of different modifications in IVF conditions on the sex ratio. In vitro matured cumulus-oocyte complexes were fertilized with frozen-thawed sperm from the same bull under our routine conditions (Percoll separation and 20 hours of coincubation) or under three different modifications: 1) Swim-up sperm separation (1 hour in 1 ml); 2) Short coincubation times (2.5 hours); and 3) Preincubation (sperm incubation for 6 hours before coincubation). Sexing was performed in zona-free in vitro produced blastocysts with a single PCR using the male specific primer BRY4a and a satellite (Sat1).

A preliminary study testing different coincubation times was conducted to determine the minimum coincubation time necessary to maintain normal blastocyst yields. A sharp drop (from 39% to 5 % blastocysts) in developmental kinetics occurs for this bull between 2:45 hours and 2:30.

The percentage of male embryos was 52.8% (67 out of 127) in our routine conditions, 56.5% (48 out of 85) for Swim-up, 72.7% (8 out of 11) following short coincubation, and 52.5% (52 out of 99) after preincubation. No significant differences from the expected 1:1 sex ratio were obtained by Chi-square test.

In conclusion, following IVF with sperm separated by Swim-up or preincubated during 6 hours, no sex ratio skew was observed. The same situation was found when coincubation was restricted to 2.5 hours, although the number of embryos sexed in this group was not enough to obtain proper conclusions.

Notes

TRANSPERITONEAL SPERM MIGRATION IN THE PIG – MYTH OR REALITY?

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Deep intrauterine insemination with special designed catheters (Vazquez et al., Theriogenology 2005) allows the deposition of sperm only into one uterine horn. Nevertheless, bilateral fertilization of oocytes occurs. How the spermatozoa reach the contralateral oviduct remains unclear although there is supposition for both transperitoneal and transuterine migration. The aim of the present experiment was to study these migration ways.

In altogether 24 peripuberal German Landrace gilts (age 185 days) follicle growth and ovulation were induced by 1,000 IU eCG and 500 IU hCG 72 h after eCG. Gilts were endoscopically inseminated 31-32 h after hCG injection with 20 ml of extended fresh boar semen (60 x 10^6 spermatozoa, > 70 % motility) as followed. Group CONTROL: intrauterine insemination (IUI) into the right uterine horn (approximately 10–15 cm caudal from the utero-tubal junction); the left uterine horn served as a non-treated control. Group LIGATURE: IUI into the right uterine horn; the left horn was closed by endoscopic double ligature 3–5 cm lateral of the bifurcation. Group INTRAPERITONEAL (IP): IUI into the right uterine horn; the left horn was closed by double ligature and semen was deposited intraperitoneally at the surface of the left ovary. The genital tracts were surgically removed 65-66 h after hCG, the oviducts flushed and the oocytes were analyzed after staining for fertilization (one pronucleus + one deconsed sperm head and all stages up to ≥ 4 cells) and cleavage. Furthermore, accessory spermatozoa count/oocyte was graded as 0: without, 1: < 5, 2: 5-50, 3: 50-100 and 4: > 100 spermatozoa. Results of cleavage and fertilization of oocytes depending of insemination and ligature treatments are presented in Table 1.

Table 1. Grade of accessory spermatozoa and fertilization results (n = 23 gilts)

Group	Uterine horn	Acc. sperm (mean grade)	No. oocytes	Fertilized n (%)	Cleaved %	≥ IV-cell %
CONTROL	right – IUI[1)]	2.75	49	39 (79.6)	47.0	16.6
	left – untreated	1.57	55	35 (63.7)	40.0	7.3
LIGATURE	right - IUI	2.50[a]	62	44 (71.0)[a]	56.6[a]	8.1
	left - ligature	0.12[b]	63	9 (14.3)[b]	1.6[b]	1.6
IP	right - IUI	3.17[c]	41	31 (75.6)[c]	63.4[c]	14.6
	left – ligature+IPI[2)]	0.29[d]	50	7 (14.0)[d]	4.0[d]	0

[a,b]; [c,d] $p < 0.05$; [1)] – Intra-Uterine Insemination, [2)] – Intra-Peritoneal Insemination

Results indicate that low dose IUI into one uterine horn provides a high degree of accessory spermatozoa and a lower one in the contra-lateral control side. Here in tendency, cleavage to stages ≥ 4-cell-embryo was delayed. Ligation of the contralateral uterine horn and intraperitoneal sperm deposition significantly limits the presence of spermatozoa and fertilization. The limited number of fertilized oocytes recovered after ligation of the uterine horn originates from only 3/15 gilts of the LIGATURE and IPI groups, and could be due to insufficient endoscopic closure procedure. Transuterine but not transperitoneal sperm migration and fertilization seems to be real after (deep intrauterine) deposition of low dose semen in the pig.

Notes

EFFECT OF DIFFERENT IN VITRO BOVINE EMBRYO CULTURE SYSTEMS WITH REDUCED NUMBER OF EMBRYOS

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Recent interest for the in vitro production (IVP) of reduced number of bovine embryos is growing among researchers, associated mainly to the development of the ovum pick-up technique (OPU) in which normally a small number of oocytes is available (4-10). It has been widely reported that development of bovine embryos is suppressed in cultures with single or reduced number of embryos. The aim of this work is to study the effect of different in vitro embryo culture systems for reduced number of embryos on development. COCs from ovaries collected from a slaughterhouse were in vitro matured and fertilized. After fertilization, presumptive zygotes were in vitro cultured in different culture systems. Systems compared were: r5IVC, reduced number (n=5) of embryos put in culture in 50 µl drops; r5IVC+EGF+ITS, reduced number of embryos incubation with supplementation of 10 ng/ml Epidermal Growth Factor (Sigma, E-4127) and ITS (9 µg/ml insulin, 5 µg/ml transferring, 6 ng/ml selenium) in the culture medium; WOW (Well of Well system with a handmade steel rod, 5 embryos per well, Vajta et al., 2000); gIVC, control grouped incubation (50 embryos in wells of 500 µl). The basic culture medium was Synthetic Oviduct Fluid (SOFaaci, Holm et al., 1990. Theriogenology, 52; 683-700) supplemented with 3mg/ml bovine serum albumin (BSA).

Culture System	COCs (n)	% Cleavage (n)	% Blastocyst (n)	Nº cells/blastocyst (±SEM)
gIVC	196	89.3 (175)[ab]	42.8 (75)[a]	92.4 (±4.3)
r5IVC	120	84.2 (101)[a]	19.8 (20)[b]	80.5 (±7.8)
r5IVC+EGF+ITS	190	85.3 (162)[a]	24.1 (39)[b]	97.6 (±6.1)
WOW	164	93.9 (154)[b]	26.6 (41)[b]	88.4 (±7.8)

[a,b]: Different superscripts in the same column indicate a statistical difference ($P < 0.05$)

Embryos cultured in gIVC and WOW showed higher cleavage rate than those cultured in r5IVC and r5IVC+EGF+ITS conditions (89.3%; 84.2%; 85.3% and 93.9%, respectively). In our conditions, embryos in gIVC showed the higher blastocyst rate, whereas no statistically significant difference was observed among r5IVC, r5IVC+ EGF+ITS and WOW (42.9%; 19.8%; 24.1% and 26.6%, respectively, $P<0.05$). Regarding quality of blastocyst (number of cells/blastocyst) no difference was observed among the culture systems tested. In conclusion, we observed that culture of reduced number of embryos impaired embryo development. Although no statistical significance was reached it seems that WOW system might improve embryo development of reduced number embryo culture.

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Notes

GENOME ACTIVATION IN PREIMPLANTATION CANINE EMBRYO

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Genome activation is essential for the development of the preimplantation embryo. However, in the canine species, the stage at which activation takes place is still unclear. Because in vitro fertilization and embryo development are inefficient in the dog, collection of embryos in vivo is thus required to study embryonic features. This embryo collection is complicated by the fact that only natural cycles may be used every 6-8 months (induction of heat is difficult in the bitch) and superovulation does not work. In this work, timing of embryonic development was studied and transcription activity, as assessed by BrUTP incorporation, was measured in canine embryos from the 2-pronuclei (2-PN) to the blastocyst stage.

Material and Methods

Bitches follow-up

During estrus, the time of ovulation was determined by transabdominal ultrasonography. Ovariectomies were performed from 102h (4.5 days) to 274h (11.5 days) after ovulation. Embryos were collected by tubal flushing.

Transcriptional activity

Transcription activity was evaluated using BrUTP incorporation in nascent RNAs, without microinjection. In each experiment, anoestrus oocytes were processed similarly as positive controls.

Immuno-cytochemistry

Embryos were then fixed and submitted to immuno-cytochemistry (monoclonal primary antibody directed against BrdU) and DNA was stained by ethidium homodimer. The embryos were observed and transcriptional activity was quantified using confocal microscopy.

Results

Embryos (n=160, from 30 bitches) were collected and the timing of development is described in the table below.

		2-PN	2-cells	4-cells	8-cells	Morula	Blastocyst
	n embryos (n bitches)	37 (7)	33 (8)	12 (5)	34 (7)	22 (4)	22 (5)
Time after ovulation	hours	102h-132h	120h-161h	133h-154h	153h-225h	230h-266h	230h-274h
	days	4.5-5.5	5-7	5.5-6.5	6.5-9.5	9.5-11	9.5-11.5

No transcription activity was detectable in 2-PN, 2-cell and 4-cell embryos. In early 8-cell embryos (in cohorts of 4-cell and 8-cell embryos), transcription of the nuclei was heterogeneous within the same embryo. In late 8-cell embryos, in morulae and in blastocysts, all nuclei were transcriptionally active.

Discussion

In this work, the timing of development of the canine preimplantation embryo was recorded precisely, based on a close follow-up of the timing of ovulation using ultrasonography. Based on BrUTP incorporation, the gene expression appears to take place primarily during the 8-cell stage.

Notes

EFFECT OF ELEVATED PROGESTERONE IN VIVO ON SURVIVAL AND ELONGATION OF BOVINE BLASTOCYSTS PRODUCED IN VITRO FOLLOWING EMBRYO TRANSFER

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Progesterone (P_4) is the key hormone responsible for maintenance of pregnancy in mammals and high concentrations of circulating progesterone in the immediate post-conception period have been associated with an advancement of conceptus elongation, an associated increase in interferon-tau production and higher pregnancy rates in cattle and sheep. In order to test the hypothesis that a modified uterine environment is capable of advancing the post-hatching elongation of Day 7 blastocyst, the objective of this study was to examine the effect of artificially elevated circulating progesterone on Day 3 and subsequent embryo survival and development of blastocysts produced *in vitro*. Blastocysts were produced by IVM/IVF and IVC in SOF and were transferred in groups of 20 to the ipsilateral uterine horn of synchronized recipients (n=9 recipients/group) with normal or elevated progesterone concentration. To obtain divergent progesterone concentrations in heifers, a validated model was used where approximately half of the animals received a progesterone-releasing intravaginal device (PRID) on Day 3 of the estrous cycle. A daily blood sample was taken from all animals by jugular venipuncture from Day 0 (day of oestrus) to Day 14 to establish the recipient progesterone profile. On Day 14 (the initiation of elongation in cattle, a critical checkpoint in development) animals were slaughtered and the embryos were recovered by flushing the uterus. The recovery rate and the dimensions of all embryos were recorded and embryos individually were snap-frozen in LN_2 for further studies. Data were analysed by mixed models ANOVA and Student's/t-test where appropiate. Insertion of a PRID on Day 3 increased progesterone concentration 4- to 5-fold within 24 h. The concentration remained significantly higher than control animals until Day 7 after which time concentrations were no longer different between control and treated heifers. The recovery rate of embryos on Day 14 was not significantly different for both groups (control: 61.7±5.9% vs P_4-treated: 43.9±7.5%). Elevation of progesterone prior to embryo transfer on Day 7 resulted in a significant increase in mean conceptus length and area on Day 14 compared to those recovered from control animals (Length: control: 4.27±0.42 mm vs P_4-treated: 15.46±1.61 mm; Width: 1.41±0.06 mm vs P_4-treated: 1.88±0.07 mm; Area: Control: 7.02±0.77 mm vs P_4-treated: 27.22±2.6 mm; $P<0.001$). These results provide clear evidence to support the hypothesis that progesterone-induced changes in the uterine environment are responsible for the advancement in conceptus elongation and interestingly, the embryo does not need to be present during the period of high progesterone in order to exhibit advanced elongation.

Notes

EFFICIENCY OF OPU-ICSI-IVP TECHNOLOGY APPLIED TO MARES WITH REPRODUCTIVE PATHOLOGIES

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In the last few years, the introduction of equine Ovum Pick Up (OPU) combined with intracytoplasmic sperm injection (ICSI), in vitro embryo culture (IVC) and embryo transfer has allowed to obtain offspring from subfertile or infertile donors that could not reproduce by natural mating, artificial insemination or conventional embryo transfer (ET). In addition, not only a broad spectrum of male and female reproductive problems can be overcome, but also it is possible to extend the reproductive season throughout all the year since OPU can be performed at any stage of the reproductive cycle and equine in vitro produced embryos can be successfully cryopreserved.

In this study we present data from 162 commercial OPU-ICSI-IVP sessions and we evaluate the outcome in terms of embryo production and pregnancy rate comparing donor mares with reproductive problems versus healthy mares. Reproductive problems included cervix laceration, uterus aplasia, endometritis, embryo resorption, no embryo production with conventional ET, abnormal oocytes, history of no pregnancy. Mares older than 20 years were also included in this group.

Data are shown in table 1. We found no statistically significant difference between absence or presence of reproductive problems in the rate of oocyte maturation (66.28% vs. 64.29%), cleavage (61.98% vs. 65.76%) embryo development (19.06% vs. 15.49%) and pregnancy rate (52.17% vs. 69.44%) following transfer of frozen-thawed embryos.

Therefore, we conclude that OPU-ICSI-IVP is an efficient and reliable technique for producing embryos/pregnancies from mares affected by a variety of reproductive problems.

Table 1. Efficiency of UPU-ICSI-IVP technology and pregnancy rate from healthy mares (Healthy) versus mares affected by various reproductive pathologies (Pathol).

Type of donor mare	N° of OPU	N° of donors	N° of follicles	N°of oocytes (rec.rate)	N° of Injected (%MII)	N° of Cleav.	N° of Blastocyts (%Bl/Inj) (Bl/Cl)	N° of Transf.	N° of Pregn. (%)
Healthy	80	50	1340	881 (65.74)	584 (66.28)	362 (61.98)	69(11.81) (19.06)	46	24 (52.17)
Pathol.	83	42	1578	967 (61.28)	628 (64.94)	413 (65.76)	64 (10.19) (15.49)	36	25 (69.44)

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Notes

EFFECT OF INSULIN, TRANSFERRING AND SELENIUM AND/OR ASCORBIC ACID ON DEVELOPMENT OF PREPUBERTAL CALF OOCYTES TO THE BLASTOCYST STAGE

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In cattle, the media used for maturation and culture of embryos is usually supplemented with sera obtained from fetal calves or other sources, which are known to contain essential hormones, growth factors, amino acids, trace elements and other unidentified factors. However, the biological activity of these sera differ unpredictably from lot to lot and it would be useful to develop improved maturation media which do not contain serum to reduce the variations within and between laboratories and to ensure biosafety. Although prepubertal calf oocytes are capable of undergoing nuclear maturation and fertilization at similar rates to cow oocytes, blastocyst yields from such oocytes are significantly reduced compared with that of adult oocytes. This deficiency in developmental capacity of oocytes from calves may be due to abnormal cytoplasmic maturation of these oocytes. Thus, calf oocytes represent a good model for elucidating the mechanisms which govern the quality of cytoplasmic maturation.

The aim of this study was to determine the effect of the addition of insulin, transferrin selenium (ITS) and/or ascorbic acid to the maturation medium on the first phase of the bovine oocyte in vitro maturation (IVM) and subsequently, on in vitro fertilization (IVF) and embryo development. Cumulus-oocyte complexes (COCs) were matured in TCM 199 supplemented with PVA and EGF (control), and with the addition of ITS (ITS) or ITS and ascorbic acid (ITS+ASC) for 12 hours at 38.5ºC and 5% CO_2. Then, oocytes from the 3 experimental groups were transferred to TCM199 supplemented with PVA and EGF for additional 12 hours. After IVM, oocytes were in vitro fertilized and at 18 to 20 hpi were transferred to SOF microdrops. Embryo cleavage was evaluated at 48 hpi and blastocyst yield at day 8 post-insemination. No statistical difference in cleavage rate (cleavage/total oocytes) between the tree groups was observed (68.7%, 65.9% and 69.0% for control, ITS and ITS + ASC, respectively). However, the percentages of embryos reaching the blastocyst stages were significantly higher in the ITS+ASC group (14.2%) compared with the control (8.5%) or the ITS group (8.5%). These data suggest that the exposure to ITS and ascorbic acid for the first 12 hours of in vitro maturation resulted in a better embryo developmental competence of prepubertal calf oocytes.

Notes

HISTONE DEACETYLASE INHIBITOR, VALPROIC ACID, INCREASES *IN VITRO* DEVELOPMENT OF MOUSE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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Somatic cell nuclear transfer (SCNT) is a promising technology, but still a rather inefficient process. In part, this is caused by an incomplete or incorrect reprogramming of the differentiated somatic nucleus into a totipotent embryonic state. Previous studies in the mouse revealed that increasing histone acetylation in SCNT embryos by applying a histone deacetylase inhibitor (HDACi), trychostatin A (TSA), significantly enhances *in vitro* embryonic development and cloning efficiency (*Kishigami S et al., 2006 BBRC 340, 183-189*; *Rybouchkin A et al., 2006, Biol Reprod. 74:1083-1089*). On the other hand, the HDACi valproic acid (VPA) was able to induce reprogramming of mouse fibroblasts with only three transcription factors with M.J. *Huangfu D et al., 2008 Nat Biotechnol. 26, 795-797*). The purpose of our study was to compare the effects of VPA and TSA treatments on the *in vitro* development of mouse SCNT embryos.

B6CBAF1 (C57BL/6JxCBA/J) female mice were used as oocyte and somatic-cell donors. Enucleated oocytes were individually injected with a cumulus cell nucleus and then cultured in KSOM medium (37ºC, 5% CO_2). In a first set of experiments, SCNT embryos were activated at 1-2 h post-NT in Ca^{2+}-free-CZB medium (with 10 mM $SrCl_2$ and 5 µg/ml cytochalasin B) supplemented with either 0.5mM VPA or 5nM TSA. At 6 h post-activation, the embryos were moved to KSOM for another 2 h of HDACi treatment. In a second set of experiments, SCNT embryos were treated with 2mM VPA or 100nM TSA in KSOM for 2-3 h immediately after NT. Then, they were activated for 6 h in the presence of the same concentration of HDACi. At the end of each treatment the embryos were washed and cultured in KSOM medium until the blastocyst stage. SCNT embryos that were not exposed to HDACi were used as controls. Results are shown in table1. In the first set of experiments, the rates of embryonic development were very similar between all groups until the morula stage. In terms of blastocyst formation, 5nM TSA treatment was statistically similar to 0.5mM VPA but significantly higher than the control group (p=0.028). In the second set of experiments, the blastocyst rate for 2mM VPA treatment was increased when compared to 0.5mM VPA (P=0.005) and both 2mM VPA and 100nM TSA treatments resulted in a significantly higher blastocyst development rate than the control group (p=0.001 and p=0.035, respectively).

In conclusion, VPA, as TSA, is beneficial for *in vitro* development of mouse SCNT embryos. Studies are currently being performed to determine whether this improvement in blastocyst rates correlates with an increased development to term. So far, one live cloned pup has been obtained from VPA-treated embryos.

Table 1. In vitro development of mouse SCNT embryos treated with VPA or TSA.

HDACi Treatment	No. reconstructed	No. (%) Activated	SCNT-embryos with pronuclei developed to (%)				No replicates
			Two cell	Four cell	Morula	Blastocyst	
Control	274	240 (87.6)[a]	192 (80)[a]	149 (62.1)[a]	128 (53.3)[a]	55 (22.9)[a]	12
VPA 0.5mM	289	253 (87.5)[a]	197 (77.9)[a]	163 (64.4)[a]	128 (50.6)[a]	69 (27.3)[a,b]	11
TSA 5nM	328	292 (89)[a]	233 (79.8)[a]	189 (64.7)[a]	162 (55.5)[a]	93 (31.8)[b]	12
Control	251	231 (92)[a]	182 (78.8)[a]	149 (64.5)[a]	127 (55)[a]	58 (25.1)[a]	12
VPA 2mM	420	376 (89.5)[a]	297 (79)[a]	254 (67.9)[a]	239 (63.6)[b]	144 (38.3)[b]	12
TSA 100nM	273	238 (87.2)[a]	177 (74.4)[a]	149 (62.6)[a]	133 (55.9)[a,b]	82 (34.5)[b]	10

[a–b] Values with different superscripts within the same column and category differ significantly between treatments ($p < 0.05$).

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Notes

DOES FLUNIXIN MEGLUMIN CHANGE THE RELATION BETWEEN PREGNANCY RATE AND A TIME OF PASSAGE THE TRANSSFER GUN THROUGH UTERUS CERVIX INTO THE PLACE OF EMBRYO DEPOSITION IN RECIPIENT HEIFERS?

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The purpose of our study was to investigate the relation between pregnancy rate and the time passing the transfer gun through uterus cervix into the place of embryo deposition in recipient cows. The experiment was carried out in 86 recipient heifers at the age of 14 to 18 months and BCS of 2.52. Heat was synchronized by two *i.m.* injections of 0.5 μg of cloprostenol given in 14 days apart. Seven days after the heat all heifers were ultrasonographically selected before transfer to examine the absence or presence of *corpus luteum* (CL) and its diameter. Only good fresh or frozen embryos were used in this experiment. In all heifers the time needed to pass the transfer gun through uterine cervix to the place of embryo transfer and the depth of catheter insertion ("shallow" or "deep") were recorded. Directly after the transfer all heifers were divided into two groups. The first group (FM group; n=46) received an *i.m*. injection of 830 mg Flunixin-Meglumin (10 ml of Finadyne®RP, Schering-Plough) and the second group (CNTR; n=40) served as control. Ultrasonographic pregnancy examination was performed 6 weeks after the embryo transfer. The mean size of the corpora lutea on Day 7 was 19.6± 0.36 mm. The time of passing the transfer gun through the cervix to the place of embryo deposition was 95.1±62s (94.4±62s and 95.6±63s resp. in group FM and CNTR). Regarding the "shallow" and "deep" insertion of the catheter we needed an average time of 102.1±51.3s and 82.1±36s. The time spent for embryo transfer was less in recipients having a greater corpus luteum compared to heifers with a smaller corpus luteum (CL > 20 mm - 84.9±53.8s; CL < 16 mm - 96.6±65.7s). The pregnancy rate in the FM group and CNTR group was 63% and 57.5%. However, if the time of manipulation was longer than 60s the pregnancy rate was 46.6% in the CNTR group and 63.6% in the FM group, whereas if the transfer time was below 60s the pregnancy rate was 61.5% and 64.2% in the FM group and CNTR group. We have not found any relation between the time needed for embryo transfer, the size of CL and/or the depth of insert of the transfer gun to the uterus. More investigations are needed to clarify a possible relation between the passage time of the transfer gun through uterus cervix to the place of embryo deposition and pregnancy results in recipient heifers in respect to the preventive use of FM. However, it seems that Flunixin Meglumin may be suitable to improve the pregnancy rate after prolonged time of manipulation on the uterus.

Notes

EMBRYO COLLECTION IN HOLSTEIN COWS AFTER ADMINISTRATION OF UTEROTONIC DRUGS - EFFECTS ON EMBRYO QUALITY AND PREGNANCY RATES AFTER EMBRYO TRANSFER

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In several studies negative effects of prostaglandin F2α (PGF2α) on embryo quality and pregnancy rates were reported. Since PGF2α and Oxytocin (OT) were used in experiments to improve embryo recovery rates in superovulated Holstein cows (Wolgast et al., AETE 2008), the question arose whether embryo transfer (ET) results obtained with those embryos are lower in comparison to results achieved with control embryos.

Donors cows (1-12 lactations) were superovulated with Folltropin-V® (630 IU FSH i.m.), and were inseminated after estrus induction with 500 µg Cloprostenol i.m. (Estrumate®) and 25 mg Dinoprost i.m. (Dinolytic®). The animals were divided at random into six experimental groups: Group 1 (n = 34) got a luteolytic dose of Dinoprost (25 mg in 5 ml Dinolytic®, i.m.) 12 h - 16 h before embryo collection and furthermore an injection of 10 IU OT (1 ml Oxytocin® Albrecht, i.v.) at the beginning of the flush. Group 2 (n = 32) was treated with Dinoprost and placebo (1 ml 0.9 % NaCl-solution, i.v.). Group 3 (n=30) received placebo (5 ml 0.9 % NaCl-solution, i.m.) and OT. Group 4 (n = 25) got a reduced dose of Dinoprost (8.5 mg i.m.) and OT (4 IU i.v.). Group 5 (n = 24) got the placebo twice, while Group 6 (n = 29) served as standard control. Timing of treatments in Groups 2 - 5 was equivalent to the timing in Group 1. All embryos were evaluated according the IETS criteria and the transferable embryos (class 1 - 3) were transferred fresh or after freezing and thawing into breeder owned recipients. The statistic analysis was performed with SAS® (Chi-square-test, CATMOD procedure and maximum-likelihood variance analysis).

A total of 2159 embryos were recovered on D 7 of pregnancy (174 flushings). No differences in embryo quality were noted between Groups 1 - 6. Pregnancy rates came up to 55 % (68/123), 60 % (97/162), 53 % (67/126), 62 % (76/123), 50 % (62/124) and 54 % (74/136) after ET of embryos (794 transfers) collected in Groups 1 - 6, respectively. The differences were not significant ($P = 0.41$).

Neither prostaglandin nor oxytocin treatment used in order to improve embryo recovery rates negatively influenced embryo quality or pregnancy rates.

Notes

ARE HOLSTEIN COWS WITH TWIN CORPORA LUTEA AT THE START OF SUPEROVULATION INFERIOR EMBRYO DONORS?

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Double ovulations are an increasing problem in dairy cattle which is probably associated with high milk production. Prior to this study we recorded that 18 % (5/28) of donor cows with twin corpora lutea (TCL) at the start of superovulation did not show estrus and 21 % (6/28) of them were poor responders with delayed estrus.

Therefore, the suitability of Holstein embryo transfer donors maintained under commercial conditions and having TCL present on the ovaries at the start of superovulation (23/205 animals, 11.2 %) was analyzed in comparison to the suitability of donors with single corpora lutes (SCL; 182/205, 88.8 %). All cows were stimulated with 630 IU FSH, and estrus was induced with 500 µg Cloprostenol and 25 mg Dinoprost. Ovarian control was done ultrasonographically. The animals were presented for embryo collection on D7 of pregnancy.

Statistic analyses were performed with SAS® (Chi-square-test, CATMOD Procedure and maximum-likelihood variance analysis).

Cows with TCL at the start of superovulation treatment were predominantly in their 4th or 5th lactation (52 %) and were flushed later during lactation (284 ± 152 d p.p.) than the cows with SCL (250 ± 150 days p.p.).

Superovulation reaction (12.4 ± 7.9 CL vs. 13.6 ± 7.7 CL) and the total recovery rate after uterus flushing (73.1 % vs. 77.4 %), as well as the numbers of oocytes/embryos collected (9.1 ± 7.8 vs. 10.5 ± 7.7) and the numbers of transferable embryos (4.4 ± 5.5 vs. 5.9 ± 5.4) were lower in animals with TCL than in cows with SCL at the start of superovulation, respectively. More unfertilized oocytes (101/209: 48.3 %, vs. 745/1916: 38.9 %) were found in the flushings of animals with TCL in comparison to the cows with SCL, and also a significantly lower embryo quality was observed (18/83, 21.7 % vs. 162/1072, 15.1 % fair and poor embryos; $P < 0.05$).

Transfer of embryos from donors with TCL resulted in a significantly reduced pregnancy rate (42/93, 45% vs. 396/693, 57%; $P < 0.03$).

These results indicate that cows with TCL at the beginning of superovulation might be less suitable embryo donors.

Notes

EFFECT OF ACTIVINE IN CULTURE ON DEVELOPMENT AND CELLULAR POPULATION OF IN VITRO PRODUCED BOVINE EMBRYOS

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Granulosa cells, cumulus cells and epithelial oviductal cells are a major site of activin expression. Furthermore, activin receptors are expressed in bovine granulosa cells, oocytes and preimplantatory embryos, suggesting that activin may exert autocrine / paracrine roles in bovine oocyte maturation and embryonic development (Yoshioka et al., 1988 Biol Reprod 59:1017-1022). The aim of this study was to investigate the effects of activin-A on bovine embryonic development. In vitro produced presumptive zygotes (n=1119) were cultured up to Day 8 (192h) in SOF with aminoacids, inositol and 3 g/l of BSA. Culture medium was supplemented with 10 ng/mL of activin from 24-72h (group 24-72), from 72-192 h (group 72-192), from 24-192 h (group 24-192), and without additives (control group). Development was assessed on days 3, 6, 7 and 8. At this time, hatched and expanded Day 8 blastocysts were fixed and stained (n=99; 4 replicates) for differential cell counts (Thouas et al., Reprod Biomed Online 2001;3:25-29). Data were analyzed by GLM and results presented as LSM±SE and shown in the table

		Day-3		Day 7		Day 8		
Activin	R	Cleaved	≥5 cells	Early	Blastocysts	Blastocysts	Expanded	Hatched
24h-72h	6	86.7 ±1.1^{d}	68.1 ±1.9^{d}	27.24±4.1^{x}	14.30±3.4^{a}	30.79±3.4	19.91±2.8	5.10±1.06^{a}
24h-192h	6			34.68±4.1	26.22±3.4^{bc}	34.06±3.4	22.01±2.8	5.46±1.06^{a}
72h-192h	11	92.2 ±1.0^{e}	79.1 ±1.7^{e}	43.01±3.0^{ya}	30.12±2.4^{c}	39.46±2.4	27.97±2.0^{a}	2.48±0.83
No activin	11			30.31±3.0^{b}	21.10±2.4^{ab}	32.38±2.4	20.23±2.0^{b}	1.51±0.83^{b}

R: replicates; N: cultured oocytes; data are proportions of cultured presumptive zygotes: a,b: $p<0.05$; x,y: $p<0.01$; d,e: $p<0.001$.

The absence of activin during the first 72h of culture significantly improved Day 3 cleavage and >5-cell rates. When activin was added from 72h to 192h significantly more early blastocyst were obtained on Day 7. This trend was maintained for the Day 7 blastocysts rates and also for the Day 8 expanded blastocyst rates. Interestingly, hatching rates were higher in those groups into which activin was present from the first 24h of the culture. Culture conditions did not affect blastocyst differential cell counts, (i.e., no differences were observed between cells in the inner cell mass and the trophectoderm). Effects of activin during in vitro bovine embryo development, depends on timing of its addition to the culture medium.

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Notes

SEX RATIO OF LAMBS BORN FROM FROZEN EMBRYO TRANSFER

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It is desirable to control the sex of embryo transfer offspring. Embryo sex ratio after superovulation has not been widely investigated in sheep. The objective of the present study was to investigate sex ratio of embryos resulting from superstimulated donors from two different breeds. Romanov (n=5) and Charollais (n=6) (2-7 years of age) donors were superovulated using FSH-p (total of 20 mg) (Folltropin-V; Vetrepharm, Canada) applied in eight decreasing doses of 2.4, 2.4, 1.8, 1.8, 1.6, 1.2, 1,1 mg i.m. at 12 h intervals, starting 60h before sponge withdrawal. Donors were undergone intrauterine insemination with fresh diluted semen 40h after sponge removal. Six-day embryos were recovered surgically and Grade 1 embryos (IETS classification) were frozen by direct transfer method. Embryos (n=104) were transferred into recipient ewes in pairs. Proportional data for the sex-ratio of offspring lambing rate were analyzed by Chi-square analysis. Percentages for female sex ratio of offspring born from frozen thawed embryos was 64% ($P < 0.05$) and lambing rates for recipient carried Romanov embryos (79%) was significantly higher ($P < 0.05$) than those carried Charollais embryos (57%). Mean number of Grade 1 embryos did not differ for Romanov ewes (11.2 ± 1.0) and Charollais ewes (8.8 ± 0.9; $P > 0.05$). Age of the donors did not significantly effected sex ratio of offspring but there was a skew of sex ratio from male to female in embryos from youngest (2 year) or oldest (7 years) donors. In summary, there is an advantage in the sex ratio obtained when using superstimulated ewes.

Notes

DIFFERENCES IN GLOBAL TRANSCRIPTOME PROFILE OF BOVINE BLASTOCYSTS DERIVED FROM SUPEROVULATED OR SYNCHRONISED CYCLIC HEIFERS

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Superovulation coupled with embryo transfer is widely used to generate embryos from high genetic merit cows. In addition, it has become a useful tool for research to produce in vivo cultured embryos to be used as golden standards. However, the yield and quality of the embryos while they are still in the oviduct or once they reach the uterus might be affected due to this procedure. So, this study was conducted to investigate the gene expression profile of bovine blastocysts derived from superovulated or synchronised cyclic heifers.

For this, bovine Affymetrix array analysis was performed using two groups of blastocysts, namely: bovine blastocysts produced after superovulation of Simmental heifers (n=9) and flushed at day 7 by non-surgical endoscopic method (n=32) and bovine blastocysts (n=49) derived from synchronized Simmental heifers (n=4) after transfer of 2-cells stage embryos from superovulated donor Simmental heifers (n=9). Total RNA was extracted from three pools of embryos from each experimental group (6 embryos per pool) using Pico-pure RNA isolation kit (Arcutrus, Sunnywale, CA). A total of 6 Biotin labelled cRNA samples were hybridized on 6 bovine affymetrix arrays consisting of 24128 probe sets. The microarray data normalization and background correction was performed using Guanine Cytosine Robust Multi-Array Analysis (GCRMA) and the data analysis was performed using LIMMA written on R package which maintained the Bioconductor.

Array data analysis revealed a total of 454 transcripts to be differentially expressed ($P \leq 0.05$, fold change ≥ 2) between the two groups. Of these 429 and 25 were up and down regulated, respectively in blastocysts derived from superovulated heifers compared to those derived from synchronized animals. Genes which involved in response to stress (HSPA14 and HSPE1), cellular and metabolic processes (CPSF3, ATPIF1, POMP, CDC2 and MDH2), ribosome pathway (RPL10, FAU, RPS3 and RPLP1), oxidative phosphorylation pathway (COX4, ATP5HA, NDUFA12 and UQCR), proteasome pathway (PSMD4, PSMA7 and PSMB4) and cell communication (FN1, KRT8, KRT18 and DSG2) were enriched in blastocysts derived from superovulated animals. On the other hand, protein metabolic processes related genes (CLGN) were found to be enriched in blastocyst derived from synchronized group. Real-time PCR will be done to validate the transcript abundance of 15 selected genes involved in different biological processes.

In conclusion, blastocysts cultured in synchronized animals post 2-cells stage showed significant differences in transcriptome profile compared to their counterparts remained in superovulated heifers until day 7. Further functional analysis of some selected candidate genes could give new insights into mechanisms regulating embryos ability to survive after transfer.

Notes

RELATIONSHIP BETWEEN FOLLICLE SIZE, INTRAFOLLICULAR TESTOSTERONE CONCENTRATION AND SEX RATIO IN INDIVIDUALLY CULTURED BOVINE EMBRYOS

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Recent studies have suggested a relationship between bovine follicular fluid testosterone concentration and the likelihood of the oocyte being fertilized by an X- or Y-bearing spermatozoa (Grant et al., Biol Reprod 2008; 78:812-5). However, this theory has been challenged (Bermejo-Alvarez et al., Biol Reprod 2008; 79:594-7).

To further test this hypothesis, follicles were dissected from the ovaries of slaughtered heifers. Each follicle was measured and carefully ruptured. Once the cumulus-oocyte complex (COC) was located and removed the follicular fluid was collected and stored at -80°C for testosterone analysis by radioimmunoassay. COCs were matured, fertilized and cultured in an individually identifiable manner using microwells formed on the bottom of a culture dish (so-called Well-of-the-Well system). All cleaved embryos (2- to 4-cell stage, n=140) were frozen and stored at –80ºC until sex determination by PCR.

Testosterone concentrations were positively skewed. In order to determine if testosterone concentrations were different between subsequently males and female embryos data were analyzed with SAS using the Wilcoxon signed rank test in Proc NPAR1WAY. Linear, quadratic, and cubic logistical regression analysis using PROC LOGISTIC was done to determine if testosterone concentrations could be used to predict that the sex of an embryo. The odds ratio and the coefficient of determination (R^2) were used to determine the adequacy of the logistical regression models.

There was no significant difference between follicular fluid testosterone concentrations in male and female embryos (mean±s.e. 51.5±5.59 and 49.5±7.42 ng/ml, respectively). Linear, quadratic, and cubic logistical regression showed that follicular testosterone concentration could not reliably predict the sex of the embryo with odds ratios of 1.001, 1.013, and 1.066 respectively and R^2 values of 0.0003, 0.0126, and 0.0567, respectively. Follicular size and testosterone concentration were not related (R^2=0.087). Finally, follicular size had no influence on embryo sex determination (p=0.7).

In conclusion, under the conditions of this study, the likelihood of an oocyte being fertilized by an X- or Y-bearing sperm was not affected by the size of the follicle from which it was derived or the testosterone concentration in the follicular fluid.

Notes

SIGNIFICANCE AND APPLICATION OF BIOTECHNOLOGY OPTIONS IN THE SMALL RUMINANTS IN TURKEY

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Turkey ranks eight and tenth in the world with the sheep and goat populations, respectively. The numbers do not reflect the same order in terms of productivity per animal. Almost 98% of the small ruminant population consists of local-native breeds known with their adaptability to harsh environment, restricted feed supplies feeding regimes and extensively managed pasture lands. Range lands in Turkey have been declined from 40 to 12 millions hectares and this situation enforced the breeding system to be switched to intensive system. Sheep and goat breeds raised in Turkey are characterized by low in fertility, growing rate and survivability. Lamb and mutton are main resources to overcome meat shortages in the country. Thus, subject to these constraints, technological improvements are highly desirable to increase overall productivity in small ruminants. Breeding strategies such as crossbreeding and breed substitution have been conducted in al large scale for five years in the country. Prolific and Terminal breeds have been established via imported frozen embryos and MOET was started for speeding up to establishing nuclei flocks. Laparoscopic artificial inseminations (LAI) widely spread out all over the country for producing F1 fertile ewes. Up until the 2009, countrywide 100 000 LAIs and approximately 500 ETs were recorded in sheep. The five years intensive application of LAI and ET technology indicated that breed substitution and crossbreeding programmes are successful thanks to compatibility of the genotypes with farmers' breeding objectives and production system. Litter size and lambing frequency were increased per enterprise to stay straight in the current meat markets. The LAI and MOET technologies applied in breeding strategies under the Turkey condition were able to increase reproductive efficiency and rates of animal genetic improvement, thereby contributing to an increased output from the livestock sector. They also offer potential for greatly extending the multiplication and transport of genetic material and for conserving unique genetic resources in reasonably available forms for possible future use.

Notes

DEEP INTRAUTERINE SEMEN DEPOSITION AND INSEMINATION ORGANIZATION IN RESPECT TO PERCENTAGE OF EMBRYOS SUITABLE FOR TRANSFER, DEGENERATED EMBRYOS AND UNFERTILIZED OVA IN SUPEROVULATED DONOR COWS

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The superovulatory results under farm conditions were compared in donor cows after insemination conducted on the basis of heat signs or after the time of oriented insemination with regards of the site of semen deposition. Total 58 Polish Holstein breed cows were used for this experiment. The donor cows were divided into three groups. The superovulation started on 11th day of oestrus cycle. Eight intra muscular (i.m) injections of FSH (Pluset, Bionic) were given every 12 hr in decreasing doses. Cloprostenol (0.5 μg, 2 ml of Estrumate, Intervet-Schering) was simultaneously injected together with the fifth and sixth FSH-application. In group I (CONV, n=26) and II (DEEP, n=22) the animals were inseminated 48hr and 64hr after the initial injection of PG. In group III (CNTR) donor animals (n=10) were inseminated 12hr after heat signs were observed and again 16hr later. In group I donors were inseminated into the uterine body – the first insemination was done using two semen straws containing 10x10^6 sperms each and the second AI was performed using one semen straw. The AI in group III was done according to group I, however, the first AI was accomplished by deep intrauterine insemination using one straw per uterine horn. We used a quick lock 2000 catheter for semen deposition in the uterus body and a flexible catheter (IMV) for deep intrauterine insemination. On Day 7 donors were flushed. The total numbers of ova and embryos (TOE) in group I-III were 7.13, 5.3 and 10.1 and the numbers of embryos suitable for transfer were 5.2, 5.1 and 3.8, respectively. Simultaneously the rates of degenerated embryos and unfertilized ova among TOE in group III (CNTR: 16% and 48.1%) were higher ($p<0.05$) compared to group I (CONV: 18.5% and 5.5%) and group II (DEEP: 7.1% and 0%).

In conclusion, deep intrauterine insemination doesn't improve the number of embryos suitable for transfer, percentage of degenerated embryos and unfertilized ova compared to the insemination into the uterus body in respect to the time of oriented insemination of donors. A high proportion of unfertilized ova in donors inseminated on the basis of visible heat signs can be connected with false observations of donor cows in farm conditions.

Notes

ESTIMATE OF THE OVULATORY RESPONSE THROUGH THE TRANSRECTAL ULTRASONOGRAPHY IN RECIPIENT GOATS SUBMITTED TO PROTOCOLS OF SHORT DURATION

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The evaluation of the ovulatory response is essential for determination of the efficiency of the protocols of synchronization of the estrous and of the ovulation. In this context, ultrasound was applied to scan the reproduction of small ruminants to contemplate the use of non invasive methods. Therefore, the objective of this work was to evaluate the efficiency of the transrectal ultrasound scan to estimate the ovulatory response, as well as, of hormonal protocols of short duration to synchronize the estrous and the ovulation in goats. 40 goats distributed randomly were used in two experimental groups (GI and GII). GI was constituted by pluriparous females (n=10) and nuliparous animals (n=10) that were treated with a reusable CIDR, for 5 days and in the moment of its withdrawal they received the administration 200 U.I. of eCG and 0.05 mg of Cloprostenol, for via IM. In GII also composed by pluriparous (n=10) and nuliparous (n=10) females, the animals received an intravaginal sponge containing 50 mg of MAP for 5 days and in the moment of its withdrawal 200 U.I. of eCG and 0.05 mg of Cloprostenol, via i.m. The ultrasonographic screening was accomplished in all the females of both groups (GI and GII) 24 hours after the withdrawal of the devices, quantifying the visualized follicles. As ultrasound equipment a Honda HS-1101 AutoScan was used equipped with a linear transducer of 5.0 MHz. Simultaneously, the observation of the estrous occurrence was accomplished with the aid of vasectomized males. At the 7th day after initiating the estrous, in all females laparoscopy was performed to evaluate the ovulatory rate (OR) and the embryo transfer (ET). The pregnancy diagnosis was accomplished through transrectal ultrasound in the 25th day after the ET. The statistical analyses were made through the SAS (1999), being the averages compared to 5% of probability by the test F. As for the estrous occurrence percentages of 100% were observed in GI and 80% in GII. The ultrasonographical evaluation showed that the mean follicular response was of 2.3 ± 0.68 in GI and of 1.3 ± 1.16 in GII. Being, observed in the laparoscopical evaluation, a mean OR of 1.6 ± 0.84 in GI and of 0.9 ± 0.74 in GII. There was no significant difference (P>0.05) among the methods. However, in the evaluation for category mean OR of 1.90 ± 0.28 were registered (GI) and of 2.0 ± 0.26 (GII) for nuliparous and of 1.5 ± 0.22 (GI) and 0.7 ± 0.33 (GII) for pluriparous animals, inferior results were registered (P <0.05) in the pluriparous of GII. Though, there was no difference in the pregnancy rate that was of 60.0% (12/20) in GI and of 55.0% (11/20) in GII. The presence of non-ovulated follicles was observed in most of the recipients of both groups (GI and GII). Therefore, it may be concluded that the transrectal ultrasonography is efficient in estimating the ovulatory response, as well as, the appraised protocols were satisfactory to synchronize the estrous and the ovulation in recipient-goats.

Notes

BIOLOGICAL FACTORS AFFECTING OOCYTE COLLECTION AND EMBRYO PRODUCTION IN A COMMERCIAL OPU-IVP SYSTEM IN HOSLTEIN AND MONTBELIARD BREEDS

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The aim of this work was to identify biological factors affecting oocyte collection, embryo production and subsequent pregnancy rates through a retrospective study conducted from 381 commercial OPU-IVP sessions performed on high genetic Holstein and Montbeliard donors.

Oocytes were retrievied using an ultrasound scanner SC 200 (Pie medical) equipped with a 7.5 MHz annular-array transducer. Donors were superovulated at day 12 of pre-synchronized cycle with FSH (Stimufol®) divided in 5 decreasing doses over 2.5 days. Cumulus-oocyte complexes (COCs) were collected 12h after last FSH injection and matured for 22h in M199 Hepes plus FCS, FSH, oestradiol and EGF. They were then fertilized in fert-TALP with frozen-thawed semen. Zygotes were cultured for 6 days on a monolayer of Vero cells in B2 medium. Day 7 embryos were transferred as fresh into recipients. The effects of donor breed, dominant follicle puncture (DFP) and physiological state of females on oocyte and embryo production were analysed by ANOVA (proc GLM, SAS).

From all collected animals, most (80.6%) of the donors were cows (55% collected as early pregnant and 31.9% previously infertile) and 19.4% were heifers (from those 86.5% pregnant). The mean number of collected COCs per session was 13.3±8.3 (mean±SD), 57.8% of which had ≥ 3 layers of cumulus cells (grade 1 and 2). A mean of 10.8±6.9 COCs were subjected to IVM-IVF-IVC which resulted in 3.5±3.5 blastocysts per session (77% were graded as 1 and 2) whereas 2.8±2.5 embryos were transferred into recipients. An overall pregnancy rate of 50.4% (544/1080) was obtained at day 90: 59.1%, 50.4% and 27.9% for grade1, G2 and G3 blastocysts respectively. OPU sessions resulted at least one day 90 pregnancy in 61.2% of the cases. The breed had no effect on the number of collected COCs but a higher percentage of grade 1 and 2 COCs were observed for Montbeliard than for Holstein donors (60 vs 53%). Dominant follicle puncture 48 h prior to superovulation treatment significantly increased the number of COCs (15.7±9.9 vs 10.9±5.6; $p<0.05$), the number of developed and transferred embryos and the number of pregnancies per session (4.3±3.9 vs 2.7±2.8; 3.3±2.6 vs 2.4±2.4; 1.7±1.6 vs 1.2±1.4, respectively). This effect was particularly impressive for Montbeliard heifers. Higher numbers of COCs were collected from infertile cows, non pregnant cows as for early pregnant heifers (16.9±10.4; 13.8±7.6 and 13.8±8.6, respectively) when compared to pregnant cows and non pregnant heifers (11.0±6.1; 10.5±6.6, $p<0.05$). The mean number of developed embryos was also influenced by parity of the donor and were: 4.5±4.5; 3.4±3.7; 3.1±2.8 for infertile, non pregnant and pregnant cows and 3.0±3.4; 2.5±2.3 for pregnant and non pregnant heifers, respectively.

In conclusion, oocyte collection and embryo production were mainly influenced by parity and physiological status of Holstein and Montbeliard donors. Puncture of dominant follicle at the start of superovulation treatment increased the overall quantity of COCs and improved the efficiency of the IVP procedure.

Notes

DIRECT TRANSFER OF FROZEN IN VITRO PRODUCED BOVINE EMBRYOS: AN EFFECTIVE METHOD TO DEVELOP A TARENTAISE PURE BREED NUCLEUS IN TUNISIA

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In cattle, in vitro embryo production (IVP) has become important for the production of embryos from live donors as an alternative to or integrated with multiple ovulation and embryo transfer (MOET). Large numbers of embryos of average genetic merit can be also produced from ovaries of slaughtered donors and can be commercialized at very competitive prices to set up a pure breed nucleus in developing countries. In Tunisia, a program has been implemented to develop the French dairy breed Tarentaise which is well adapted to difficult climatic and nutritional conditions. The first part of this project was to validate the UNCEIA IVP system from oocytes collected from slaughtered Tarentaise cows to the direct transfer of frozen/thawed embryos in one Tunisian herd to estimate the efficiency of the whole procedure.

Ovaries from 12 Tarentaise cows were collected at slaughterhouse and treated separately as the female parental origin was required and for sanitary precautions. Cumulus-oocyte complexes (COCs) were aspirated from 2 to 6 antral follicles and grade 1 to 3 COCs were matured for 22h in M199 + FCS, FSH/LH, estradiol and EGF. Matured oocytes were fertilized in fert-TALP. Frozen semen from 2 different bulls were used for insemination according to the genetic origin of the female donor. Zygotes were cultured for 6 days on Vero cells monolayers in B2 medium. At day 7, grade 1 embryos were selected for slowly freezing (UNCEIA patent FR2809744). Frozen/thawed embryos were directly transferred to synchronized Holstein heifers in Tunisia. Estrus synchronization was performed by a progesterone-releasing intravaginal device (Ceva Interchem) for 10 days. 24 hours before the removal of the PRID, a single injection of PG (Enzaprost®, Ceva Interchem) was administered. Pregnancy was diagnosed by ultrasonography 35 days after estrus.

Table 1. Overall efficiency of IVP obtained from separated ovaries of Tarentaise cows and pregnancy rates after single direct transfer of frozen/thawed embryos in Tunisia

	Inseminated oocytes	Cleaved embryos	Developed embryos	Frozen G1 embryos	Pregnancy Day 35
Total (12 cows)	290	247	60	32	12/32 (37.5%)
mean±SD (per cow)	24.2 ± 14.7	20.6 ± 11.9	5.0 ± 5.8	2.7± 4.6	

Great variations in the number of oocytes and embryos produced from individual cows were observed (none G1 embryos were obtained for 6 cows). At day 35, the pregnancy rate was 37.5%. These results demonstrate the feasibility and interest of using direct transfer of IVP frozen/thawed embryos to set up a pure breed nucleus in a foreign country with adapted logistics.

Notes

EFFECT OF PERIFOLLICULAR BLOOD FLOW ON DEVELOPMENTAL COMPETENCE OF BOVINE COC AND QUALITY OF THE CORRESPONDING OOCYTE

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Approximately 20% of the cumulus-oocyte complexes (COC) collected from living animals during repeated ovum pick-up (OPU) sessions develop to the blastocyst stage. An increase in the blood supply to individual follicles appears to be associated with follicular growth rates, while a reduction seems to be closely related to follicular atresia. The purpose of this study was to determine whether qualitative perifollicular blood flow changes can be used to predict the developmental competence of COC collected during repeated OPU sessions once weekly and to analyze the quality of the corresponding oocyte at the molecular level.

Lactating Holstein cows (n=10) were used as oocyte donors. After dominant follicle removal, OPU was performed once weekly for six weeks employing a 7.5 MHz transducer (GE 8C-RS) of an ultrasound scanner (GE Logiq Book). Follicle size and Doppler characteristics were recorded by transvaginal ultrasonography just before COC collection using the colour flow imaging. Owing for technical limitations for measurement of blood flow in small individual follicles, only the presence or absence of blood flow was assessed for each follicle. Follicles with or without detectable blood flow from each individual cow were aspirated separately. After morphological classification of COC, standard protocols for IVP were used for embryo production. Cleavage and blastocyst rates were recorded at day 3 and day 8, respectively. In addition, after denudation of COC, immature oocytes were also frozen at -80°C to analyze the relative transcript abundance using RT-qPCR. The transcripts studied play important roles during oocyte development [DNA methyltransferase 1a, 1b, 3a (DNMT1a, DNMT1b, DNMT3a); histone deacetylase 2 (HDAC2); growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15); maternal effect gene zygotic arrest (ZAR) and heat shock protein 70.1 (HSP)].

In total, 243 (125 with and 118 without detectable blood flow) follicles ≥ 3 mm were aspirated. Morphology of the COC was similar in both groups. Cleavage rates were similar for COC originating from follicles with and without detectable blood flow, 54.3% (25/46) and 51.5% (34/66), respectively. However, developmental rates up to the blastocyst stage did show a significant difference, 23.9% (11/46) and 15.2% (10/66) for COC aspirated from follicles with or without detectable blood flow ($p \leq 0.05$). Relative abundances of BMP15 and HDAC2 transcripts were increased in oocytes stemming from follicles with visible blood flow compared to those from follicles without visible blood flow. All other mRNAs did show similar expression patterns.

These results show that using COC originating from follicles with detectable perifollicular blood flow collected once weekly may have a higher developmental competence and quality compared to those from follicles without detectable blood flow. Within the detection limits of this study, differences in perifollicular blood flow during repeated OPU sessions once weekly were predictive of oocyte competence.

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Notes

EFFECT OF SLOW FREEZING AND VITRIFICATION ON THE QUALITY OF EQUINE EMBRYOS

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Embryo transfer is an increasingly widely used technique for producing multiple offspring from genetically valuable and/or competing mares. Presently, the vast majority of equine embryo transfers are performed soon after recovery or after chilled transport for up to 24 h. Cryopreservation is not widely used, partly because recovering embryos at the appropriate stage is labour intensive, but primarily because pregnancy rates after thawing are disappointing. Embryos > 300 μm in diameter are especially susceptible to the damaging effects of freezing and thawing, irrespective of whether they are frozen by a slow-cooling technique or by vitrification. While the reasons for the poor post-thaw viability of equine embryos are not fully understood, freezing and thawing has been reported to damage mitochondria and irreversibly disrupt the actin cytoskeleton. The present study investigated the causes of reduced embryo viability by comparing the effects of slow freezing and vitrification on cytoskeletal integrity, mitochondrial activity, and the percentages of dead or apoptotic cells in small (≤ 300 μm) versus large (> 300 μm) equine embryos.

Grade 1-2 embryos recovered from Dutch Warmblood mares on days 6-7 after ovulation were classified on the basis of size (≤ or > 300 μm) before being slow-frozen or vitrified (n= 8 for each of the groups ≤ 300 μm and > 300 μm), or used as non-treated or cryoprotectant (CPA) exposed controls (n = 5 for both ≤ 300 and > 300 μm). After thawing or warming, the embryos were stained with the following fluorescent markers: 1) 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) to determine the number of dead cells and evaluate nuclear morphology; 2) Mitotracker Red CMHX-Ros to identify active mitochondria; 3) Mitotracker Deep Red to label all mitochondria; 4) Alexa Fluor 488 to visualize the actin cytoskeleton. The percentage of dead cells and the number of grossly apoptotic nuclei per embryo were evaluated using a conventional fluorescence microscope. The distribution of active and total mitochondria and the integrity of the cytoskeleton were evaluated using a multiphoton microscope.

Multivariate analysis revealed that the percentage of dead cells was increased ($p<0.05$) by both exposure to CPA and cryopreservation; large embryos were particularly susceptible to cell death after both slow cooling and vitrification and also suffered significant damage after exposure to the vitrification media. While small embryos suffered less cell death, the percentage of cells showing evidence of apoptosis (nuclear fragmentation or condensation) was significantly increased by vitrification or exposure to the vitrification media. Freezing by either method negatively affected cytoskeleton quality and also tended to reduce mitochondrial activity ($p = 0.061$) in large embryos. For equine embryos > 300 μm in diameter, there was no significant advantage of vitrification or slow-cooling in terms of preserving embryo quality. On the other hand, vitrification resulted in a higher incidence of apoptotic nuclei in small embryos than slow-cooling and may not, therefore, be the best way to cryopreserve small equine embryos.

Notes

ENDANGERED "GOCHU ASTUR-CELTA" PIG BREED GENETIC RESOURCE BANK: PRELIMINARY RESULTS

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The need to conserve farm animal biodiversity is accepted by many countries through the ratification of the convention of biological diversity, and sperm quality is known to be an important criterion in breeding soundness evaluation. The Gochu Astur-Celta pig is an endangered autochthonous breed from the north of Spain and its semen has never been described before. For the conservation of its genetic biodiversity and long-term survival, sperm parameters must be studied to establish a germplasm bank.

Sperm-rich ejaculate fractions were collected using the gloved-hand technique from six boars (aged 13-24 months), twice a week (N= 109), were extended (1:1, vol/vol) in Beltsville Thawing Solution (BTS) and evaluated for conventional semen characteristics. Volume (V) was assessed from a graduated collection test tube; sperm concentration (C) was evaluated by a spectrophotometer (Accucell, IMV Technologies, France) and the percentage of motile sperm (TM) was evaluated after collection, at 4ºC and after thawing from samples of the extended semen placed in a heated microscope stage set at 38ºC. Morphological abnormalities of sperm head (HA), midpiece (MA), tail (TA) and cytoplasmic droplets (CD) were determined by direct count of 200 cells from random fields of an immobilized smear, using 1000x magnification in a phase contrast microscope and the same for acrosome integrity (percentage of normal acrosome, NAR). Membrane functionality was evaluated by the Hypoosmotic Swelling test (HOST) to detect the presence of swollen tails in a 100 mM citrate–fructose solution at 37 ºC during 30 minutes (percentage of HOST-positive spermatozoa, HOST-PS). For freezing, semen was centrifuged at 17ºC for 3 minutes at 2,400 x *g* and the supernatant was removed by aspiration. Sperm pellets were re-extended with lactose-egg yolk (LEY, 20%, vol:vol egg yolk) extender. Ejaculates were cooled to 4ºC in 90 minutes, and resuspended with LEY-Glycerol-Orvus ES Paste (9% glycerol, 1.5% Equex STM) extender to a final concentration of 1000 x 10^6 cells/mL, before packaging into 0.5 ml straws and freezing for storage in liquid nitrogen.

Data are expressed as means ± standard error. Fresh semen characteristics were: V = 82.5 ± 4.0 mL; C = 560.7 x 10^6 ± 22.4 spz mL^{-1} and TM = 85.0% ± 1.0. Percentage of HA was 1.2% ± 0.1; MA 0.6% ± 0.1; TA 2.8% ± 0.3; CD 2.8% ± 0.4; NAR 98.9 ± 0.2 and membrane integrity 89.1% ± 0.6. After refrigeration, the % of TM was 71.4 ± 1.2, and the post-thawing survival rate was 32.8 ± 1.4.

However complementary studies are needed to ensure that banks are correctly created, our results indicate the possibility of collecting sperm that survive freezing/thawing procedures with satisfactory quality to use it as fresh and for its cryopreservation.

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Notes

USING OF SEX-SORTED INSEMINATION DOSES IN SUPEROVULATION

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The aim of this study was to evaluate the effect of insemination with sex-sorted semen on the production of transferable embryos in superovulated cows.

In total, 26 Holstein cows were used in a MOET programme. All donors were synchronized by a single intramuscular injection of PGF2alfa (Oestrophan, Leciva Prague). The superovulation treatments were started between day 9 and day 11 of the estrous cycle (heat detection: Day 0). The animals received pFSH (Pluset, Calier, Spain) administered over 8 injections in decreasing dosages at 12 hour intervals. The luteolysis was induced by intramuscular injection of PGF2alfa simultaneously with the 5th and 6th pFSH injection. For insemination frozen sex sorted semen of different bulls was used. Donor animals of the control group (n=22) were inseminated with one straw of sexed semen each at 48 hrs, 60 hrs and 72 hrs after the injection of the first PGF2alfa. Donor animals of the experimental group (n=4) were examined by ultrasound beginning from the last application of FSH to the expected time of ovulation. Animals of this group were inseminated two times, just before ovulation and 12 hrs later with one straw of sexed semen each. Embryos were non-surgically recovered on Day 7 of the oestrus cycle in the control and the experimental group. Embryos were assessed following the IETS guideline.

In the control group 22 flushing sessions were performed and in total 213 embryos were recovered. Only 30 embryos (14 %) were classified as “transferable”. In the experimental group 4 flushings were done resulting in 19 embryos. Fourteen (74 %) of them were assessed as “transferable”.

Our first data show that heat detection following insemination close to ovulation promise higher numbers of transferable embryos in superovulation programmes using frozen sex sorted semen.

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Notes

FOLLICLE SIZE EFFECT ON OOCYTE RECOVERY AND DEVELOPMENTAL COMPETENCE OF OOCYTES RETRIEVED FROM SLAUGHTERHOUSE OVARIES OR BY OVUM PICK UP

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The aim of this research was to investigate follicle size effect on oocyte recovery and developmental competence of oocytes recovered from slaughterhouse ovaries (SO) and those recovered in vivo by Ovum Pick Up (OPU). Immature cumulus-oocyte-complexes (COCs) were obtained by aspirating follicles ≤ 5 mm or > 5 mm from the ovaries, either after slaughter or using OPU. OPU was performed in 17 donor cows synchronized with $PGF_2\alpha$ and stimulated with pFSH, twice a day in consecutive two days (Folltropin®, Bioniche). Total of 4 OPU sessions were performed on each cow. The number of aspirated follicles, retrieved oocytes and oocyte recovery rate were recorded. COCs of grade 1 and 2 were matured in TCM199 with FCS, FSH/LH and cysteamine, fertilized with frozen/thawed semen prepared on gradient of BoviPure™ and subsequently cultured for 9 days in SOFaaBSA. The number of cleaved embryos was recorded at 24, 27, 30, 33, 36 and 48 hpi. Embryo yield was recorded on day 5, 6, 7, 8 and 9. Data were analyzed by Chi-square test. Significantly more oocytes were retrieved from donor cow follicles >5 mm, with higher recovery rate comparing to follicles ≤5 mm. On contrary, more oocytes were obtained from follicles ≤5 mm of slaughterhouse ovaries (Table 1). There were no significant differences between oocyte quality, with highest proportion of grade 1 and 2 oocytes in both SO and OPU, regardless to follicle size. Kinetics of early cleavage shown that significantly more oocytes from follicles >5 mm, either from SO or OPU, cleaved in significantly higher proportion 30 hpi (74.03% SO; 83.80% OPU) compared to follicles ≤ 5 mm (61.22% SO; 74.26% OPU). Overall cleavage rate 48 hpi and embryo yield on day 5, 6, 7, 8 and 9 was also significantly higher when oocytes originate from follicles > 5 mm (Table 1).

Table 1. Effect of follicle size and oocyte origin on oocyte recovery and embryo production

Oocyte origin	Follicle Size (mm)	Aspirated follicles	Retrieved oocytes	Recovery rate (%)	Cleaved (%)	Day 5 (M)	Day 6 (M/BL)	Day7/8 (M/BL)	Day 9 (H/BL)
OPU (n=17)	≤5 (n=216)	4.21 ± 0.53[a]	2.42 ± 0.31[a]	54.0[a]	82.2[a]	15.8 [a]	30.7[a]	41.6[a]	12.9[a]
	>5 (n=319)	6.27 ± 0.60[b]	3.59 ± 0.48[b]	58.3[b]	89.4[b]	28.9[b]	42.9[b]	47.9[b]	26.1[b]
SO (n=173)	≤5 (n=1692)	19.37 ± 1.59[c]	12.87 ± 1.06[c]	69.8[c]	78.3[c]	11.2[c]	16.1[c]	20.2[c]	7.5[c]
	>5 (n=501)	5.74 ± 0.71[d]	3.87 ± 0.47[d]	69.8[c]	86.1[d]	19.9[d]	31.8[d]	36.1[d]	15.9[d]

Values with different superscript within columns are significantly different, P <0.05

The results demonstrate that the oocytes recovered after OPU are more competent to develop to the blastocyst stage than those derived from SO. The majority of oocytes collected from SO originated from small follicle, which had impaired developmental competence. This was confirmed by lower cleavage and embryo production rate compared to large follicles.

Notes

MITOCHONDRIAL AGGREGATION PATTERNS AND ACTIVITY IN IN VITRO CULTURED BOVINE OOCYTES FROM EARLY ANTRAL FOLLICLES

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Mitochondria play a vital role in the oocyte to provide ATP for fertilization and preimplantation embryo development. Data in human, bovine and pig oocytes suggest that the efficiency of mitochondrial respiration in oocytes is closely related to the rate of embryo developmental competence (Wilding et al, 2001; Stojkovic et al., 2001; Torner et al., 2004). It is expected that evaluation of mitochondrial activity would allow more precise determination of in vitro culture conditions for bovine oocytes originating from early antral ovarian follicles.

The study was aimed to determine the distribution and oxidative activity of mitochondria in non-cultured and in vitro cultured bovine oocytes originating from early antral ovarian follicles using the fluorescence probe MitoTracker CMTM Ros Orange. The meiotic configuration of the oocytes was also evaluated.

Cumulus-oocyte complexes surrounded with mural granulosa cells (COCGs) were recovered from slaughtered cattle ovaries by isolation from small fragments of ovarian cortex of early antral follicles with diameter 0.4 to 0.8 mm. Collected follicles were evaluated on the basis of their morphological appearance and size and than were opened under stereomicroscope to free COCGs. Selected COCGs were placed in growth culture (Alm et al., 2006) for 7 days (Day 7) and 14 days (Day 14). For the control (Day 0) oocytes recovered from freshly collected COCGs were fixed and stained. Following growth culture COCs with normal appearance were freed from culture, placed in maturation medium for 24 h and then fixed and stained.

The total number of evaluated oocytes was 257, i.e. 61 Day 0; 69 Day 7; 32 Day 7+IVM; 65 Day 14 and 30 Day 14+IVM. The percentage of oocytes with immature meiotic configuration after growth culture decreased in time and resulted in 96.7; 72.5 and 35.4% respectively for Day 0, Day 7 and Day 14 cultures. The type of mitochondrial distribution in the oocytes (n=257) changed from homogeneous to heterogeneous as the culture time increased. Fine homogeneous mitochondrial aggregation was seen in 78.7; 42.0; 34.4; 10.8 and 0% oocytes respectively after Day 0; Day 7; Day 7+IVM; Day 14 and Day 14+IVM cultures. Analysis of relation among nuclear configuration and mitochondrial distributions showed that 56.8% diplotene oocytes had fine homogeneous mitochondrial aggregation while 61.4% of oocytes with degenerated chromatin showed heterogeneous, granulated aggregation of mitochondria. This result led to the conclusion that in vitro culture of COCGs prolonged up to 14 days affected both mitochondrial distribution and activity as well as chromatin configuration – the main negative effects manifested by degeneration.

References:

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Notes

QUANTITATIVE ASSESSMENT OF TRANSCRIPTS AND PROTEINS CONTRIBUTING TO CELL CYCLE CONTROL AND GAP JUNCTION CONNECTIONS IN MORPHOLOGICALLY VARIABLE GROUPS OF PORCINE CUMULUS OOCYTE COMPLEXES

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Oocytes and somatic cumulus cells are connected by an extensive network of gap junctions. These connections considerably contribute to oocyte maturation and developmental competence.

Cumulus-oocyte complexes (COCs), recovered after slaughter of puberal gilts, were cultured in standard porcine IVM culture medium (TCM 199) for 44 h. COCs were graded regarding to the morphology as grade-I with a homogeneous cytoplasm and a complete cumulus oophorus; grade-II with homogeneous cytoplasm and an incomplete but compact cumulus oophorus with more than two layers; COCs of grade-III are characterized by a heterogeneous cytoplasm and a greater-than-one-cell-layer cumulus oophorus; and grade-IV oocytes have a strongly heterogenous cytoplasm and either a partial or completely absent cumulus oophorus.

By assessing the activity of glucose-6-phosphate dehydrogenase (G6PDH) using brilliant cresyl blue (BCB) test, and using real-time quantitative PCR (RQ-PCR) reaction methods and confocal microscopic observations, we determined the transcript levels of connexins 43 and 45, cyclin dependent kinases (cdk5 and cdk5r), and cdk inhibitors 1 and 3 (p27kip1 and cdkn3), as well as cdk4 protein, in morphologically different groups of porcine oocytes isolated from puberal gilts.

We found statistically increased cdkn3, cdk5 and connexin 45 mRNA levels in oocytes graded as I as compared to grades II, III, and IV. The cdkn1, cdk5r and connexin 43 transcript contents were higher only when comparing between oocytes graded as I, III and IV.

Our results demonstrate that the levels of cdk4 protein and all of the investigated transcripts are associated with COCs morphology and may be related to further maturation ability.

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Notes

NON-INVASIVE METHOD OF OOCYTE QUALITY ASSESSMENT USING MICRO-FLUIDIC CHIP SYSTEMS-NEW INSIGHT INTO THE USE OF MICROTECHNOLOGY IN REPRODUCTIVE BIOLOGY

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The lab-on-chip (LOC) used in our experiments is a silicon/glass device with two integrated optical fibers. The silicon chip is micromachined in a monocrystalline silicon substrate by DRIE etching (120 μm deep), forming 120 μm wide inlet/outlet microchannels and a gamete trap. Additionally by the same process, 110 μm wide channels for optical fibers are formed. After etching, the silicon chip is oxidized to passivate the chip surface. The LOC allows optical chracterization of single oocyte by transmission/absoption VIS/NIR spectra measurements. After characterization, the porcine oocyte can be drawn back to the tip without damage to its structure.

Porcine cumulus oocyte complexes (COCs) were recovered by puncturing individual follicles. The COCs were washed three times in modified PBS supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamycin, and 0.5 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). After measurements, the collected COCs were cultured in Nunclon™Δ 4-well dishes in 500 μl standard porcine IVM culture medium; TCM-199 (tissue culture medium). Wells were covered with a mineral oil overlay and cultured for 48 h at 38 ^{o} C under 5% CO_2 in air. COCs were incubated with hyaluronidase Sigma-Aldrich Co. (St. Louis, MO, USA) for 2 min at 38^{o} C to separate cumulus cells and granulosa cells. The cells were removed by vortexing the oocytes in 1% sodium citrate buffer and by mechanical displacement using a small-diameter glass micropipette.

The results of the micro-fluidic chip measurements of porcine oocytes demonstrate for the first time the new parametric non-invasive methods for quality assessment of mammal gametes. Therefore, there is no other literature data to be compared with obtained results. Some changes of transmission spectra in the range of 500 nm to 600 nm were observed and can be correlated with the quality of measured oocytes. For example, shift of the transmission peaks obtained for small size porcine at 600 nm to 613 nm for large porcine has been observed. ÷ Results of these tests revealed various differences in these parameters in specific relation to oocyte cytoplasmic ultrastructure. In conclusion, the porcine oocytes demonstrated high heterogeneity in the color and ultrastructure of cytoplasm, which may result in differences of oocyte growth, development, successful fertilization, reaching of blastocyst stage and rate of successful implantations.

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CAN PORCINE OOCYTES ISOLATED FROM VARIOUS SIZES OF FOLLICLES BE ASSESSED BY MICRO-FLUIDIC CHIP SYSTEMS?

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In a spectrophotometric measurement, which is decribed here, a silicon/glass microfluidical chip with two fibers is used. One optical fiber is connected to a halogen lamp and the second fiber is connected to a miniaturized VIS/NIR spectrophotometer working with a computer system to collect spectrograms. In the case of fluorometric investigations, one of the fibers is connected to an excitating 490 nm fluorescence LED with 500 nm short-pass interference filter. The fluorescence light is collected by a VIS/NIR CCD minicamera equipped with 500 nm long-pass interference filter to eliminate undesired light. The CCD-based detection units work with a computer and specialized software for real-time processing of fluorescence images.

Ovaries and reproductive tracts were recovered from gilts immediately after slaughter and transported to the laboratory within 20 min at 38^{o} C in 0.9% NaCl. The ovaries were then placed in 5% fetal bovine serum solution (FBS; Sigma-Aldrich Co. St. Louis, MO, USA) in phosphate buffered saline (PBS). Follicles were classified into three size categories: small (<3mm), medium (3-5 mm), and large (>5 mm). The selected grade I COCs (with a homogeneous cytoplasm and a complete cumulus oophorus) were cultured in Nunclon™Δ 4-well dishes (Nunc, GmbH, Co. KG, Germany) in 500 µl standard porcine *in vitro* maturation (IVM) medium (TCM-199). Wells were covered with a mineral oil overlay and cultured for 44 h at 38^{o} C under 5% CO_2 in air.

We compared the quality of oocytes isolated from various sizes of follicles by using the gamete's cytoplasmic ultrastructure according to the obtained measurements of light spectrum absoption and light transmission. We determined the presence of several differences in all of the investigated parameters between the gametes isolated from various sizes of follicles. We also found high heterogeneity within each of the investigated groups. Our results show morphological differences in relation to cytoplasmic ultrastructure based on spectrophotometric measurement between porcine oocytes collected from large, medium and small follicles. The presented results may describe a new parametric method for evaluation of the developmental potential of mammalian gametes.

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EFFECT OF TYPE OF HATCHING ON PREGNANCY RATE AFTER TRANSFER OF FRESH AND FROZEN BOVINE IN VITRO PRODUCED HATCHING BLASTOCYSTS

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In vitro production of embryos has made it possible to study the different steps of embryo development like compaction of the morula, blastulation and expansion of the blastocyst extensively. The next step of development is the hatching process. Only few studies reported however on this process. Hatching is a prerequisite for placentation and occurs in vivo between day 8 and day 10. The fact that hatching can occur in vitro indicates that forces involved originate within the embryo itself. Massip et al. (1982, J. Anat. 134:399-405) described two types of hatching in bovine blastocysts, hatching through a large slit and herniation through a small slit.

In our lab, in vitro production of embryos was performed after fertilization of oocytes collected by ovum pick-up, in SOF without serum as described earlier (van Wagtendonk et al. 2000, Theriogenology 53: 575-597). At day 7 and 8 morula and blastocysts were collected and transferred either fresh or frozen-thawed in recipient cows. In our in vitro system hatching before transfer is observed in 8% of the transferable embryos. The type of hatching was assessed as hatching through a wide slit or a small slit (when hatching occurred through a small opening in the zona). Such hatching embryos were transferred (n=312) between 2001 and 2008 and pregnancy results were noted.

Of the hatching blastocysts 69% had a small opening in the zona. A significant increase of pregnancy rate occurred after transfer of a fresh blastocyst hatching through a small slit. No effect was observed after transfer of frozen blastocysts (see table 1)

Table 1: Effect of type of hatching on pregnancy rate of fresh and frozen hatching blastocysts.

Type of hatching	Pregnancy rate		
	fresh (n)	frozen (n)	total (n)
Wide slit	34% (n=32)	33% (n=66)	34% (n=98)
Small slit	61% [a](n=62)	31%[b] (n=152)	40% (n=214)

[a,b]: values in rows with different superscript are significantly different using chi^2 test, $P<0.05$.

We hypothesize that the high percentage of hatching via a small opening could be due either to zona damage during in vitro oocyte/embryo handling or zona pellucida hardening during IVC. The increased pregnancy rate after transfer of fresh embryos that were hatching through a small slit is spectacular. The mechanism causing this increase needs to be studied but we can hypothesize that by this type of hatching the embryo could divide in two parts by this herniation and this could lead to a partitioning of the embryo and thereby, when the ICM is split, to the formation of monozygotic twins or when the ICM is not split, in a proper embryo and an empty vesicle. In both cases enhancing signals involved in maternal-fetal recognition could increase the change of the animal to become pregnant. After freezing the pregnancy rate was much lower, probably these hatching blastocysts, especially the part out of the zona, were vulnerable for freezing and therefore more prone to degeneration.

Notes

IN VITRO DEVELOPMENT OF NUCLEAR-TRANSFERRED RABBIT EMBRYOS FOLLOWING CHEMICAL POST-ACTIVATION

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The aim of the present study was to determine the effect of chemical post-activation on developmental potential of rabbit cloned embryos. In the somatic cell cloning procedure the *in vivo* -matured oocytes were used as source of recipient cells.

To induce superovulation, mature New Zealand female rabbits were injected intramuscularly with 100 IU of PMSG (Serogonadotropin, Biowet) and intravenously with 100 IU of hCG (Biogonadyl, Biomed) 72 h later. Ovulated oocytes were recovered from the oviducts 17-18 h after hCG injection by flushing with Dulbecco's PBS (solution). Enucleation of Metaphase II-staged oocytes was accomplished by demecolcine-induced microsurgical method. The sources of nuclear donor cells were foetal and adult skin fibroblasts. A single somatic cell was introduced into the perivitelline space of the enucleated oocyte and membrane fusion of the donor cell and recipient oocyte was induced by three DC pulses of 3.2 kV/cm, for 20 μs each. The reconstructed oocytes were incubated in B2 medium for one hour and subsequently treated with 5 μM calcium ionomycin for five minutes to be artificially activated. Then nuclear-transferred oocytes were incubated in 2 mM 6-dimethylaminopurine (6-DMAP) for one hour. The cloned embryos were *in vitro* cultured in 50 μl droplets of B2 medium for five to six days.

Developmental abilities of nuclear-transferred rabbit embryos were assessed by cleavage rate and morula/blastocyst formation yields.

Donor cells	No. of oocytes fused (%)	No. of embryos cleaved (%)	No. of morulae (%)	No. of blastocyst (%)
Foetal fibroblasts	77/100 (77.0)	57 (74.0)	37 (77.0)	33 (42.9)
Adult skin fibroblasts	101/139 (72.7)	50 (49.5)	31 (30.7)	28 (27.7)

In conclusion, nuclear-transferred rabbit embryos, which were derived from oocytes activated using ionomycin and 6-DMAP, were able to develop *in vitro* to morula and blastocyst stages. The rate of NT blastocysts reconstructed with fetal fibroblasts was significantly higher ($P<0.05$) than reconstructed with adult skin fibroblasts.

Notes

PARTHENOGENETIC DEVELOPMENT OF BOVINE EMBRYOS PRODUCED FROM IN VITRO MATURED OOCYTES DEPENDING ON CULTURE SYSTEM AND AGE OF ANIMALS

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In previous studies we demonstrated a beneficial effect of rbST (recombinant bovine somatotropin "Monsanto") and follicle somatic cells (granulose cells) during IVM of bovine oocytes from prepubertal animals on subsequent embryo development to the blastocyst stage after IVF (Kuzmina et al., 2003). It was shown that in bovine oocytes from adult animals matured in the presence of rbST and somatic cells increased levels of respiratory activity were associated with decreased levels of calcium from intracellular stores (Kuzmina et al., 2007, J Reprod Develop 53:309-316). The aim of this study was to compare the developmental competence of parthenogenetic embryos produced from oocyte matured in vitro depending on culture system and age of animals-donors.

Cumulus-oocyte complexes (COCs) were collected from calves (3 to 5 months), heifers (1.5 to 2 years), and cows (3 to 4 lactation). Only those oocytes having a compact cumulus were used in the experiments. COCs were incubated in TCM 199 containing 10% (v/v) heat-treated FCS (Sigma) and 10^6/ml granulosa cells (control group). The culture medium of the experimental groups was modified by supplementation of the control medium with 10 ng/ml rbST. Oocytes were activated to parthenogenetic development by cold shock according to Ernst et al. (Ernst et al., 1983). Embryos were cultured using standard protocols. Oocyte maturation, cumulus morphology and quality of early embryos including status of chromatin (cytogenetic analysis by Tarkowsky, 1966) were evaluated. Data were analyzed by Chi^2 – test.

In total, 610 oocytes were activated by cold shock after 24 hours of cultivation. There were no differences in percentages of oocytes reaching the metaphase II stage in all groups of experiments. Only 14% (16/112) of embryos from prepubertal animals reached the morulae stage after 5 days of cultivation. When oocytes from this group were matured in the presence of rbST the percentage of embryos increased to 31% (31/99, $P<0.01$).

We could not find differences between percentage of parthenotes produced from cow oocytes matured with or without rbST (35 %, 34/98 vs. 37%, 38/103). 17% (16/97) oocytes from heifers developed to early preimplantation embryos after activation. rbST stimulated the potentiality of bovine oocyte from prepubertal animals and heifers to parthenogenetic development after activation. We found that supplementation of rbST during IVM significantly increased the percentage of parthenotes after activation oocyte from heifers (34%, 34/101, $P<0.01$). Two embryos were produced from 201 activated cow oocytes developed to blastocyst, 1 embryo at the blastocyst stage was produced from heifer oocytes (n=198) after activation. The proportions of intact 2 to 4 cell embryos on day 2 and of intact morulae on day 5 were higher in the group of embryos originating from oocytes cultured with rbST ($P<0.05$).

Based on the results of the present study we concluded that competence of bovine oocytes to induce parthenogenesis depends on the age of donors and culture system of oocytes.

Notes

STUDYING EFFECTS OF DIFFERENT CULTURE CONDITIONS ON CELL STRUCTURES OF BOVINE EMBRYOS USING A COLOUR ANALYZER SYSTEM

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Stages of the early life, like the very first days of embryo development, have an enormous impact on the future existence. Early embryo development is a very sensitive indicator to show how environmental impacts can lead to short and long term (memory-) effects.

The objective of this study was to evaluate differences in (I) the amount of cytoskeleton and nuclei, (II) the accumulation of lipid in droplets, and (III) the diameter of Day 7 bovine blastocysts cultured either in vitro or under a combination of in vitro and in vivo conditions.

In vitro matured oocytes (using mPM+10%OCS) were fertilized with $2x10^6$/ml swim-up separated spermatozoa and cultured in CR1+5%OCS until Day 2. Then presumptive embryos were divided into two groups:

Embryos of the "IVP-Group" were continuously cultured under in vitro conditions until Day 7. In the so called "In vivo-Group" cleaved stages were endoscopically transferred to bovine oviducts of synchronized heifers at Day 2 and in vivo cultured for 5 days. At Day 7 embryos were recovered by flushing the oviducts and uterine horns by using the means of endoscopy (Day 0 = day of fertilization).

Day 7 blastocysts (grade I) were stored in Formol overnight, stained with Phalloidin and DAPI or Nile-Red and DAPI to dye the cytoskeleton plus nuclei or lipid accumulations plus nuclei.

After the blastocysts were stained with fluorescent dye they were evaluated using a confocal laser microscope that scanned pictures of the embryos' cross sections every 2 µm. A new computer software (Color Analyzer®) was created that counts the number of pixels according to the colours of the cell structures for detecting differences in embryo development.

In total 336 oocytes were constantly cultured in vitro. Within the transfers 379 presumptive embryos were transferred to the oviducts of synchronized heifers, 267 (70.4%) complexes were recovered by flushing the oviducts and horns of the uteri. The blastocyst rate at Day 7 was 31.8% in the In-Vitro-Group and 20.6% in the D2-7-Group.

The Color Analyzer® showed to be suitable to quantify cell structures according to the used blue or green dye. However, the preliminary results show that the different culture conditions had no effect on the content of lipid-droplets, the amount of nuclei and quantity of cytoskeleton of the bovine embryos.

Although the preliminary data could not reveal an effect of different culture conditions on early embryo development, main attention will be paid on the performance of more detailed comparative studies including additional groups, higher numbers of embryos, resistance to cryopreservation and the expression of genes.

Notes

FOLLICULAR FLUID ANTI-MÜLLERIAN HORMONE AND CUMULUS CELL TRANSCRIPT EXPRESSION AS PREDICTORS OF BOVINE *IN VITRO* EMBRYO DEVELOPMENT

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Ovarian follicular fluid (FF) is discarded following aspiration and cumulus cells removed following *in vitro* maturation. Therefore, as an initial step to develop non-invasive markers of early post-fertilisation development FF concentrations of anti-müllerian hormone (AMH), together with transcript levels of two cumulus-cell expressed genes (progesterone receptor (*PGR*) and hyaluronic acid synthase 2 (*HAS2*)), were related to the proportion cleaved oocytes that followed insemination and zygotes that developed to the blastocyst stage *in vitro*. FF and cumulus-oocyte complexes (COC) were aspirated from individual follicles (visual diameter of 6 to 10 mm), and COC morphologically graded, matured, fertilised and cultured individually to Day 8 using the well-of-the-well (WOW) system (n = 67) or as groups of 25 to 30 (n = 248), both in SOFaaCiBSA as previously described (Sinclair et al., 2008; RBM-Online 16: 859-868). Of these COC, 272 cleaved and 93 went on to form blastocysts. Data were analysed using GLM assuming binomial errors and with logit transformations. Individually cultured oocytes that cleaved following insemination were classified as followed: 2-3 cells (27%), 4-7 cells (66%), 8-12 cells (7%). Method of culture (WOW vs group) did not affect percentage cleaved of inseminated oocytes (83.6 ± 5.3 vs 87.5 ± 2.4) or blastocysts of cleaved oocytes following insemination (35.7± 6.9 vs 33.6 ± 3.5).

FF volume (an objective proxy of follicle size) was determined at the time of aspiration and FF AMH, progesterone (P4) and oestradiol (E2) concentrations determined by ELISA. E2 concentrations increased ($P < 0.001$) with follicular size, and were greater ($P = 0.002$) in follicles with ≥ 200 μl FF than follicles with < 200 μl FF (61.4 ± 12.3 vs 10.2 ± 8.5 ng/ml). FF AMH concentrations decreased ($P < 0.001$) with follicle size, and were negatively ($P = 0.01$) associated with FF P4 concentrations in the larger (≥ 200 μl FF) but not smaller (< 200 μl FF) ovarian follicles, consistent with previous reports in cattle. However, FF concentrations of these three hormones were not related to post-fertilisation development.

Cumulus-cell transcript expression was determined by quantitative real-time PCR. *HAS2* transcript expression (relative to the housekeeping genes *ACTB* or *GAPDH*) was positively ($P = 0.027$) related to the proportion of inseminated oocytes that cleaved but did not predict blastocyst yields. In contrast, *PGR* mRNA expression (relative to either *ACTB* or *GAPDH*) was positively ($P = 0.003$) related to the proportion blastocysts of cleaved for oocytes from large ((≥ 200 μl FF) but not small (< 200 μl FF) antral follicles. Furthermore, relative *PGR* transcript expression was positively ($P = 0.017$) related to FF E2 in large but not small follicles. These novel findings indicate that *PGR* mRNA expression, when considered in relation to follicular size, can predict post-fertilisation development to the blastocyst stage. It remains to be determined if these developmental markers can predict pregnancy outcome following embryo transfer.

Notes

INFLUENCE OF EMBRYO STAGE ON PREGNANCY RATES FOLLOWING TRANSFER OF BOVINE BIOPSIED EMBRYOS UNDER ON-FARM CONDITIONS

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Embryo sexing is used in France since the 90's by using a PCR method based on a specific Y DNA sequence (INRA patent 1987). The sexing procedure was progressively simplified within the last 5 years by the R&D department of UNCEIA to facilitate its on-farm use. The present protocol (centrifugation of tubes containing biopsy cells, denaturation of DNA, addition of the reaction mix, amplification of sequences of interest by PCR, sample deposit on gel and electrophoresis) allows identifying the sex of embryos within 140 min. This study aimed to describe retrospectively the association between characteristics of sexed embryos and pregnancy rates following embryo transfer (ET) under field conditions. Data were analysed from 677 sexed embryos, in vivo produced by MIDATEST ET team between 2000 and 2009. Embryos were collected by conventional techniques (donor cows inseminated twice on observed oestrus following a standard superovulatory treatment and collected on day 7). Quality of embryos was assessed on the IETS morphological criteria: excellent, good (with 1 defect as death cell or dark colour), fair (with 2 defects) or poor (discarded for sexing). Biopsy was realized with a steel blade by a single operator (from 3 to 10 cells) and put in a microtube for sex determination. Embryos were transferred as fresh (n=284) or frozen-thawed (n=393; straws in air for 5-10 s and in a water bath for 30 s). Freezing method used ethylene glycol (1.5 M) plus fetal calf serum (40%) from 2000 to 2004 (n=53), or plus sucrose (0.1%) since 2004 (n=340). Biopsies were realized preferentially at the morula stage (64.8 %), compared to young blastocysts (22.9%) or later stages (12.3%). Excellent embryos represented 77.8 % of the sexed embryos (Table 1), whereas fair embryos were rarely used (18/677). Pregnancy rates averaged 59 and 50 % in fresh and frozen-thawed embryos respectively. There was no effect of year, breed of donor cows, and protocol of freezing on pregnancy rates. A multivariate logistic regression model tested the combined effects of embryo stage and quality, together with parity of recipients and season of ET. When embryos were transferred as fresh, pregnancy rates tended to be related to embryo stage and quality (p=0.07), whereas no effect was observed in frozen-thawed embryos (Table 1). Parity of recipients was significantly related to ET results when embryos were transferred as frozen (heifers (n=379): 51.2 % vs cows (n=14): 14.3 %; p=0.01). A similar trend was observed for fresh embryos, but this was not significant (61.3 %, n=238 vs 47.8 %, n=46). Season was influencing pregnancy rates when embryos were used as fresh (P=0.05). For frozen embryos, a tendency was observed (P=0.07).

Table 1: Characteristics of sexed embryos and pregnancy rates (PR) following ET (*stage: M=Morula, YB=Young blastocyst, B=Blastocyst and expanded ; quality : 0=excellent, 1=good, fair)

PR (Freq)	Stage and quality of sexed embryos *					
	M0	M1	YB0	YB1	B0	B1
Fresh	55.8 (104)	45.6 (57)	70.4 (71)	61.5 (13)	66.7 (39)	- (0)
Frozen	49.3 (209)	50.7 (69)	54.8 (62)	44.4 (9)	45.2 (42)	50 (2)

To conclude, there was no effect of embryo stage and quality on ET results, when embryos were transferred as frozen. When used as fresh, embryos at morula stage seem to lead to decreased pregnancy rates compared to blastocysts. These results may modify the choice of embryos to be micromanipulated before ET: more advanced stages when embryos are transferred as fresh and more flexibility when they have to be frozen.

Notes

ATTEMPTS TO CULTURE BIOPSIED CELLS FROM IN VITRO BOVINE BLASTOCYSTS FOR GENOTYPING

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Genomic tools now become available for most livestock species and are being used routinely for marker assisted selection in cattle. One major challenge in bovine selection is the possibility to detect multiple markers from biopsies of pre-implantation stage embryos which allows to transfer only selected embryos following genotyping. Preliminary studies have shown that DNA collected from few biopsied cells may not be sufficient for genotyping based on many markers. The aim of this work was to test different in vitro culture conditions of biopsied cells from bovine blastocysts to produce a large number of cells for genotyping.

Bovine embryos were produced *in vitro* according to our standard protocol. Only grade 1 embryos were biopsied using a microblade under a stereomicroscope. Biopsies had from 5 to 10 cells. Biopsied embryos were *in vitro* cultured in B2+2.5%FCS seeded with VERO cells for 48 h to assess the survival rate. Individual biopsies were cultured in vitro in 4-wells culture dishes (Nunc) coated with collagen type 1 at 39°C in a humidified air atmosphere and 5% CO2 under 3 medium conditions. Intact hatched D8 to D10 blastocysts were culture under the same conditions as controls. In condition 1, 43 biopsies and 30 control blastocysts were cultured in DMEM/F12+10%FCS and 0.25% ITS (Insulin, Transferrin, Selenium. In condition 2, 30 biopsies and 35 control blastocysts were cultured in DMEM/F12+20%FCS supplemented with 1mM sodium pyruvate, 1µg/ml heparin and 1µg/ml FGF4. In condition 3, 30 biopsies and 43 control blastocysts were cultured in 30% of DMEM/F12+20%FCS and 70% DMEM/F12+20%FCS conditioned medium using mitomycined VERO cells supplemented with 1mM sodium pyruvate, 1.5µg/ml heparin and 1.5µg/ml FGF4 (adapted from Oda et al., 2006, Meth in Enzy.Vol 419). Medium was replaced every 3 days. Outgrowths were physically detached and isolated cells were cultured using condition 3. For further passages, monolayers were trypsinised (0.025%) and cells were analysed by immunofluorescence using anti- cytokeratin 1-8 antibodies.

After biopsy and 48h of in vitro culture, 97.1% (100/103) of embryos survived. For all culture conditions, none of the biopsied cells attached to the coated dishes and no colony was observed after culture. Control intact blastocysts adhered and formed outgrowths in 33.3%, 85.7% and 93% for condition 1, 2 and 3 respectively. After several passages, 3 cell lines were produced and we observed a network of cytokeratin filaments by immunofluorescence suggesting an epithelial cell type for this network.

These results demonstrate that the three tested culture conditions are favourable for the production and multiplication of cells from intact bovine blastocysts and that condition 3 seems to be suitable condition for embryonic cell culture. However, the production of a large number of cells from biopsies was not efficient enough for genotyping.

Notes

EFFECT OF ROUTE OF SUPEROVULATORY GONADOTROPHIN ADMINISTRATION ON THE EMBRYO RECOVERY RATE OF BOER GOAT DOES

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Seventeen Boer goat does were used in this study to compare the effect of an intramuscular or subcutaneous route of administrating a superovulation treatment during the natural breeding season (autumn). The oestrous cycles of all does were synchronised for 17 days using CIDR dispensers and superovulated with pFSH administered in 7 dosages, at 12 h intervals initiated 48 h prior to CIDR removal. Does were teased for the onset of oestrus at 8 h intervals, starting at CIDR removal and cervically inseminated with fresh undiluted Boer goat semen at a fixed time (36 h and 48 h) following CIDR withdrawal. Embryos were surgically flushed on day 6 following the second AI, evaluated and classified according to their morphological appearance. The route of gonadotrophin (pFSH) administration had no effect on response to oestrous synchronisation. The number of ovulations, structures and embryos recovered and fertilisation rate were also similar via both routes of gonadotrophin administration. The number of unfertilised ova per donor on the other hand, was significantly higher in does administered pFSH using the intramuscular (3.3±4.8), than the subcutaneous route (0.3±0.8). However, the number of degenerated embryos was higher when pFSH was administered subcutaneously (5.9±4.5), compared to intramuscularly (2.6±2.3). The overall number of transferable embryos was not affected by the route of gonadotrophin administration. It could therefore be concluded that, in Boer goats pFSH superovulatory treatment can be administered utilising either of the routes. Although subcutaneous route of gonadotrophin administration led to a lower number of unfertilised ova, this beneficial effect was negated by increase in the number of degenerated embryos – resulting in both routes of administration leading to a similar number of transferable embryos.

Notes

EFFECTS OF ULTRASOUND IN POST-THAWING EQUINE SPERM PARAMETERS

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Therapeutic ultrasound (TUS) consists of inaudible high mechanical vibration at a frequency range of 0.7-3 MHz. When applied on tissues TUS promotes the metabolic function. Few experiments refer to its effect on cells; furthermore, we are unaware of previous reports on the effect of TUS application on semen.

The aim of this study was to investigate the effect of different therapeutic ultrasound parameters on frozen/thawed equine spermatozoa.

Frozen semen in 0.5 ml straws of eight stallions was used. One straw of each stallion was thawed, diluted, stored in glass specimen collection tubes and divided in five groups (n=40): one untreated spermatozoa (control) and four treated groups. In a thermostat-regulated water bath (37°C) experimental groups were ultrasonically treated for 3 min respectively at intensity of 0.5, 2.5, 4.5, 6.5 W using a transducer 2.5 cm^2, frequency 1 MHz (Vetrison-portable Physiomed, Elektromedizine AG, Germany). The control group was left untreated. In all groups semen parameters (motility, progressive motility, RAPID-cells, VAP-Velocity Average Path, VSL-Straight Line Velocity, VCL- Curvilinear Velocity, ALH-Amplitude of Lateral Head Displacement, BCF-Beat Cross Frequency, STR-Straightness, LIN-Linearity) were evaluated one hour after TUS applications by computer assisted sperm analyzer (CASA, Ivos 12.2, Hamilton Thorne Biosciences Inc., USA). In the meanwhile semen was placed in 37°C incubator. The data were analyzer using one-factor analysis of variance (ANOVA), at level of significance $P<0.05$.

In all samples semen parameters (control *vs* treated) were 13% lower ($p<0.05$) for spermatozoa treaded with TUS at 0.5 and 2.5W; 18% lower at 4.5 and 6.5W. These results indicate that TUS semen applications at chosen intensity have a negative impact on its quality.

Future studies will aim to understand TUS effect on spermatozoa inner structure and cell functionality. It is well-known that ultrasound treatment can enhance cell membrane permeability (sonoporation). Our future goals will be to use TUS on thawed semen with the intent to improve its quality; sonoporation might allow the spermatozoa uptake of some chemical agents which are known to positively affect sperm quality.

Notes

LANOSTEROL INDUCED Δ7-STEROL REDUCTASE GENE EXPRESSION IN CUMULUS CELLS DURING MEIOTIC RESUMPTION OF PORCINE OOCYTES

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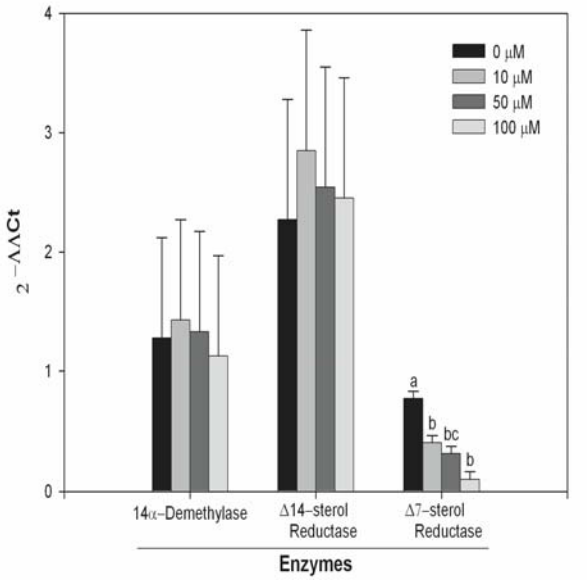
The mechanism whereby meiosis activating sterols mediate in the resumption of meiosis in the oocyte is largely unknown (Grøndahl et al., 1998). The aim of this study was to determine the effect of lanosterol on the relative expression of several enzymes involved in the conversion of acetate to cholesterol in the sterol biosynthetic pathway. COC´s, obtained from prepubertal gilt, were in vitro matured in NSCU37 medium, supplemented with eCG, hCG, FCS and EGF. The maturation medium was supplemented with 10, 50 and 100 µM of lanosterol. The COCs were cultured covered with mineral oil at 39.0°C in a humidified atmosphere of 5% CO_2. After 22 h cumulus cells from 35 COCs were removed using hyaluronidase. Total RNA was isolated from cumulus cells using the Trizol® method. Reverse transcription was performed using Quantitec Kit (Qiagen). The samples were treated with DNAse and the reaction was inactivated by incubation at 95ºC for 3 min. Cumulus cells cDNA were stored at -20ºC. Quantitative real-time PCR analysis was performed in 25 µl which consisted of SYBR Green Master Mix, 25 µM each of sequence-specific primers (Table 1) and 1 ng of cDNA. The PCR protocol is show in Table 1. The relative expression ratio was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). That is, to determine a normalised arbitrary value for each gene, every data point was normalised to the reference gene GADPH (housekeeping), as well as to their respective control. We found only an increase in the level of Δ7 sterol-Reductase transcript in cumulus cells when lanosterol was added to the maturation medium ($P<0.05$, Figure 1). Our observations suggest that exogenous lanosterol could be used by the cumulus cells to incorporate in the cholesterol biosynthetic pathway and must enhance the production of T-MAS.

Table 1. Oligonucleotide primers used for gene expression analysis

Gene	Primer	Sequence (5'-3')	FS (bp)	AT (ºC)	Gba
GADPH	F	CAAGGTCATCCATGACAACT	483	60	AF069649
	R	CTGTTGCTGTAGCCAAATTC			
Δ7-sterol Reductase	F	TTGACTTCAAGCTGTTCTTCAATG	511	56	AF034544
	R	CAGTAGGCCAGGCTGCCCATCAGG			
14α-Demethylase	F	CAATCCAGAAACGCAGACAA	171	60	NM_214432
	R	CCATCCAGGCACTGGTAGTT			
Δ14-sterol Reductase	F	CAGTTGCTCTACGTGGGTGA	233	60	AF096304
	R	GCCCCACGGAAGATGTAGTA			

FS: Fragment size. AT: annealing Temperature. Gba: Gene bank accession number

Figure 1. Quantification of relative mRNA expression of 14α-Demethylase, 14Δ-Reductase and 7Δ-Reductase gene in cumulus cells during 22 h of *in vitro*. Data represent the least square mean ± standar error mean. [abcd] Groups with different superscripts are significantly different ($P < 0.05$).



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Notes

THE EFFECTS OF LEPTIN SUPPLEMENTATION IN *IN VITRO* MATURATION MEDIUM ON MEIOTIC MATURATION AND APOPTOSIS OF BOVINE OOCYTES EXPOSED TO HEAT STRESS

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Numerous studies suggest that leptin enhances developmental capacity of bovine oocytes matured *in vitro*. The purpose of this research was to determine whether leptin supplementation can protect oocytes from negative impact of heat stress during the process of maturation. In this study COCs (n = 310) were aspirated from the slaughterhouse ovaries. The COCs were morphologically assessed, stained with BCB and selected accordingly - BCB staining classified them as blue (or grown, BCB^+) or unstained (growing, BCB^-). Only BCB^+ oocytes (n=260) were used in the experiment. In general, 260 COCs were divided into four groups matured in *in vitro* maturation medium (OMM: TCM 199, FBS 10%, sodium pyruvate, penicillin, streptomycine, FSH, LH, β – estradiol). In both control 1 group and experimental 1 group (CONT 1 and EXP 1) oocytes were exposed to physiological temperature of 38.5˚C, whereas COCs of the control 2 group and of the experimental 2 group (CONT 2 and EXP 2) were exposed to moderate heat stress (41˚C) for 12 hours. Culture medium in EXP 1 and EXP 2 was supplemented with 1 ng/ml of leptin. Small groups of 25 – 30 COCs were placed in four – well plates containing 500 µl of *in vitro* maturation medium per well. After 24 hours of maturation two types of staining were used. Half of the total number of cells was examined with Annexin V (An-V) used in conjunction with Propidium Iodide (PI) to distinguish viable (An-V negative), apoptotic (An-V positive) and necrotic oocytes (permeable to PI). These binding effects were analyzed by flow cytometry. In the second staining the COCs were treated with 4' – 6' – diamidino - 2 - phenylindole (DAPI). It was used to analyze meiotic maturation of oocytes (presence of the first polar body observed under UV light in fluorescence microscopy). Among the groups exposed to moderate heat stress, a higher rate of matured oocytes ($p<0.05$) was observed in the group with leptin addition to OMM (68%) compared to the group cultured without leptin (60%). There was the same level of apoptosis in the groups cultured in high temperature (CONT 2 and EXP 2 16% resp.). However, leptin significantly ($p<0.001$) reduced the percentage of necrotic cells among oocytes exposed to moderate heat stress (CONT 2 – 16%, EXP 2 - 8%). There were also more intact cells (CONT 2– 68%, EXP 2 – 76%). In conclusion, leptin administration during oocyte culture at 41˚C had a little effect on their development, but it protected COCs from necrosis.

Notes

MEIOTIC COMPETENCE OF PORCINE OOCYTES INFLUENCES THE EFFICIENCY OF IN VITRO FERTILIZATION

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In recent years a great effort has been made to improve the efficiency of in vitro embryo production in pigs, but low normospermy in inseminated porcine oocytes still remains a major problem (Gil et al., 2008; Suzuki et al., 2005). In our previous study we showed that, in cyclic donors, the late luteal phase was more suitable for oocyte recovery because more oocytes with greater meiotic competence were collected compared with the other phases of follicular development (Machatkova et al., 2008). The present study was designed to compare the efficiency of fertilization in porcine oocytes with lesser or greater meiotic competence collected from different-size follicles during the luteal phase.

Ovaries from adult cyclic sows were examined for morphology and classified as those at early (Days 1–5), middle (6–10) or late (11–15) stages of the luteal phase. Oocytes were isolated from small (2–4 mm) or medium (5–9 mm) follicles separately by aspiration or cutting. Only healthy cumulus-oocyte complexes with a dark, evenly granulated cytoplasm and at least two compact layers of cumulus cells were selected for experiments. They were used for both the detection of cortical granules by confocal microscopy after FITC-PNA staining and the assessment of fertilization efficiency. Oocytes were matured in TCM-199 medium supplemented by additives, fetal calf serum and P.G. 600 for 43 h. The frozen-thawed semen of a boar with proven in vivo and in vitro fertility was used for fertilization. The oocytes were co-incubated with spermatozoa for 3 h by a standard protocol. The presumptive zygotes were cultured in PZM3 medium for 16 h, fixed in glutaraldehyde, stained with 33258-Hoechst and examined by epifluorescence (× 400). Only oocytes with one male and one female pronucleus and two polar bodies were determined as monospermic. The data were expressed as mean percentage ± S.D. values and were analyzed by the Chi-square test.

In oocytes collected from small follicles, the mean % of monospermic oocytes increased and the mean % of polyspermic oocytes decreased during the luteal phase. Besides the asynchrony rate (8.2, 0 and 5.3), the monospermy and polyspermy rates were 24.5%, 48.7% and 58.7%, and 67.3%, 51.3% and 36.0%, respectively, for the early, middle and late luteal phases. In oocytes collected from small follicles at the late luteal phase, the mean % of monospermic oocytes was lower (58.7%) and the mean % of polyspermic oocytes was higher (36.0%) than in oocytes collected from medium follicles in the same period in which the monospermy rate was 77.1% and the polyspermy rate was 22.9%. The differences between the two oocyte categories were significant ($p < 0.05$). The differences in localization of cortical granules between oocytes from small and those from medium follicles were also observed. The diffuse distribution of cortical granules in the whole cytoplasm of oocytes with lesser meiotic competence as opposed to the peripheral localization of cortical granules in the cytoplasmic cortex of oocytes with greater meiotic competence was found.

It can be concluded that porcine oocytes with greater meiotic competence had a greater ability to be normally fertilized beside other in consideration of the peripheral localization of cortical granules in their cytoplasm. Collection of oocytes from individual donors in the late luteal phase can improve the efficiency of fertilization in terms of normospermy.

References:

Gil et al. (2008), Theriogenology 70, 1260-1268

Machatkova et al. (2008), Vet Med Czech 53, 70-76

Suzuki et al. (2005), Theriogenology 64, 1287-1296

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Notes

IN VITRO CULTURE SYSTEM AFFECTS LAMBING AND LAMB BIRTH WEIGHT OF VITRIFIED OVINE EMBRYOS

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It has been reported an increased birth weight of lambs following transfer of cultured embryos in synthetic oviductal fluid (SOF) with serum.

The aim of this study was to test lambing rate and body weight of lambs born from three different in vitro culture systems.

Ovaries of Sarda sheep were collected from slaughterhouse. The oocytes were matured in TCM-199 supplemented with 4 mg/ml BSA (BSA4 and BSA8 groups) or with 10% FBS (Serum group), 100 mM cysteamine, 0.3 mM Na Pyruvate, 0.1 UI/ml r-FSH, 0.1 UI/ml r-LH, 1 □g/ml estradiol-17β. Matured oocytes were fertilized with fresh semen in SOF with 20% of heat inactivated estrous sheep serum. The presumptive zygotes were cultured for 6-7 days until blastocyst stage in medium consisting of SOF supplemented with 1% BME, 1% MEM, 1mM glutamine and 8 mg/ml fatty acid-free BSA (BSA8 and Serum groups) or 4 mg/ml fatty acid-free BSA (BSA4 group). In the third and fifth day of culture (day 0: day of fertilization) 10% of charcoal stripped FBS was added to the medium into the Serum group, while 8 mg/ml fatty acid-free BSA and 4 mg/ml fatty acid-free BSA was added in BSA8 and BSA4, respectively. The embryos produced were vitrified and a total of 133 blastocysts (50 from BSA4 group, 49 from BSA8 group and 34 from Serum group) were transferred in pairs into synchronized ewes during the reproductive period.

The lambing rate was not significant different between BSA groups while Serum group showed significant differences when compared with BSA groups ($P<0.05$ and $P< 0.001$ for BSA4 and BSA8 respectively).

The percentages of heavy lambs (≥ 4.5 kg) were: 0%, 17.3% and 0% for BSA4, BSA8 and Serum respectively with significant differences between BSA groups ($P<0.05$).

In conclusion, our data demonstrated that different culture systems can lead to differences in lambing rate and in body birth weight of lambs.

Culture medium	N. Vitrified blastocysts transferred	Lambs born/transferred embryos (%)	Heavy lambs (%)
BSA 4 mg (vitrified)	50	21/50 (42) a	0/21 (0) a
BSA 8 mg (vitrified)	49	23/49 (46.9) a	4/23 (17.3) a,b
Serum	34	7/34 (20.6) b, c	0/7 (0) a

Values in the same column with different letters differ: (a,b) $P<0.001$, (a,c) $P<0.05$

Notes

LECTIN-BINDING PATTERNS OF RABBIT UTERUS DURING PREIMPLANTATION

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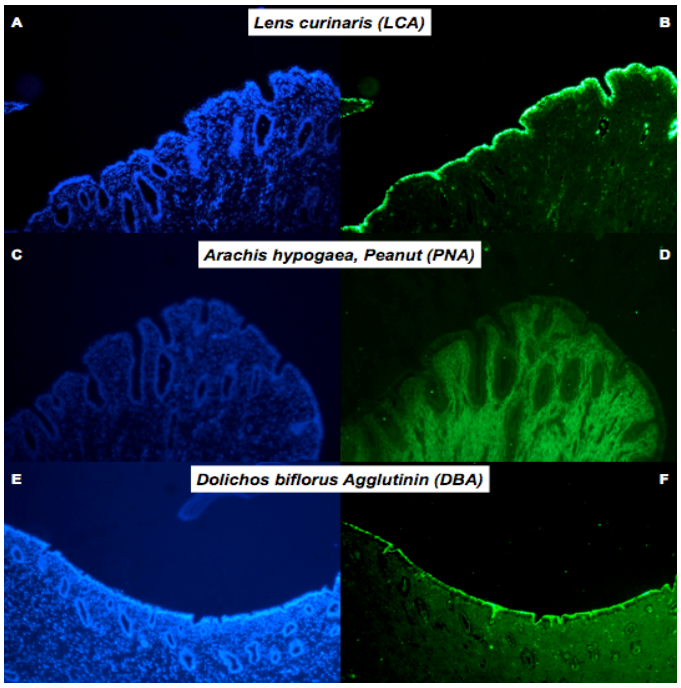
Ten sexually mature New Zealand strain female rabbits were used. Receptivity was stimulated using eCG before insemination. Females, with red colour of vulvar lips, were divided in two groups: (i) females without hormonal induction of ovulation and (ii) females with ovulation induced with an i.m. injection of 1 µg buserelin acetate at AI time. All females were slaughtered at 72 h after AI. The reproductive tract was removed, ovulation rate was recorded and embryos were recovered by flushing each oviduct (and a small part of the uterus) with 5 ml PBS. The uterus was dissected, fixed in 4% (w/v) neutral formalin and embedded in paraffin. Paraffin sections were cut at 3 µm thick sections and mounted on poly-L-lysine-coated slides. After rehydration, sections were rinsed several times in PBS, pH 7.2. Thereafter, the sections were incubated in 1% BSA for 20 min at room temperature to minimise nonspecific staining. They were then incubated for 30 min at 37ºC with FITC-conjugated lectins at a final concentration of 10 µg⁄ml (*Arachis hypogaea, Peanut (PNA):* D-galactose*, Dolichos biflorus Agglutinin (DBA):* N-acetyl-galactosamine*, Lens curinaris (LCA):* D-mannose, D-glucose). All sections were counterstained with DAPI mounting medium (Vector Laboratories Inc.). The preparations were examined with an inverted fluorescence microscope using a 100x lens (Figure 1). The lectin binding profile at endometrium was analysed and scored as follows: (–) none, (+) positive. Neither ovulations nor embryos were detected at Group 1 females. The average value for ovulation and embryo recovery rates were 12±2.8 ovulations and 85±3.2 %, respectively, for Group 2 females.

Figure 1. Lectin staining in the rabbit endometrium. B: positive reaction for LCA. D: Negative reaction for PNA. F: positive reaction for DBA. A, C and E: DAPI used to visualize nuclei.



Extensive and uniform binding of LCA and DBA to the glycocalyx and apical cell surface of the endometrium for ovulated and non-ovulated females were evident, with no apparent difference in intensity between the two female groups. However, only the apical cell surface of females with ovulations and embryos bound PNA. These results suggest that D-galactose expression may be controlled by sex hormones and probably has a specific function in the embryo adhesion processes. The observation of variability in glycoprotein distribution in endometrium indicates their specific physiological functions in reproductive processes.

Notes

MECHANISM OF ACTION OF LINOLENIC ACID IN IMPROVING BOVINE OOCYTE MATURATION

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Dietary polyunsaturated fatty acids have been shown to influence reproductive performance in various ways including a direct effect on oocyte development. Our previous studies have shown that supplementation of α-Linolenic acid (ALA; 18:3 *n*-3) to oocyte maturation medium improves oocyte nuclear maturation and results in an increased blastocyst yield in vitro. This was associated with higher levels of phosphorylated Erk1 and Erk2 in the oocytes prior to GVBD and an increased prostaglandin E_2 (PGE_2) concentration in the spent media.

The current study investigated the involvement of the MAPK pathway and PGE_2 in mediating the effects of ALA on oocyte maturation. U-0126 (a selective MAP kinase kinase (MEK) inhibitor, 10 μM) and/or NS-398 (a selective inhibitor of cyclooxygenase-2; 10 μM) were added to the bovine COCs during maturation in the presence or absence of ALA (50 μM) for 24 h ($n \geq 20$ oocytes per treatment group, 6 repeats). Oocytes nuclear maturation was assessed and PGE_2 concentration in spent media were analysed. Treatment of COCs with each of the inhibitors significantly inhibited ($P<0.001$) oocyte nuclear maturation (U-0126, 38±8%; NS-398, 63±1%). This was associated with a significant decrease in the PGE_2 concentration in the maturation media (48±2% and 36±5%, respectively, as a percent of control; $P<0.05$). Supplementation of ALA increased oocyte nuclear maturation when added in the presence of either NS-398 or U-0126 (59±6% and 87±4%, respectively). This was also associated with higher PGE_2 concentration compared to NS-398- or U-0126-treated groups (205±47% and 107±2%, respectively, as a percent of control), but not significantly different from controls. Treatment of COCs with both NS-398 and U-0126 resulted in significant inhibition of nuclear maturation (11±1%) compared to the control and other treatment groups ($P<0.05$). Addition of ALA in the presence of both inhibitors did not show any significant difference (12.1±2%; $P>0.05$) compared to NS398 + U0126 treated group. These data shows that ALA can improve oocyte maturation both directly through MAP kinase pathway, and indirectly through PGE_2 synthesis.

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Notes

INDIVIDUAL CULTURE OF BOVINE EMBRYOS IN A POLYESTER MESH

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The ability to culture oocytes/embryos in an individually identifiable manner has important implications for the identification of markers of oocyte developmental competence. The objective of this study was to compare the effect of culture in different sizes of woven polyester mesh (SEFAR Petex, UK) on the development of IVP bovine embryos. Cumulus oocytes complexes (COCs, n = 381, 6 replicates) or IVP presumptive zygotes (n = 256, 4 replicates) were cultured in groups of 20 in a 100 µl droplet of medium under oil by placing each structure between the filaments of the mesh (Figure 1). In Experiment 1, the effect of embryo culture in two sizes of mesh on development was investigated. Presumptive zygotes were cultured in synthetic oviduct fluid (SOF) in a mesh size of (i) 170 µm opening and 96 µm wire thickness (S mesh) (Booth *et al*. 2007 Biol. Reprod. 77, 765-779), (ii) 240 µm opening and 73 µm wire thickness (M mesh) or (iii) control, without mesh. In Experiment 2, oocytes/embryos were cultured individually for the entire IVP process. Due to the presence of cumulus a larger mesh size was necessary for IVM and IVF. Thus, COCs were allocated to 1 of 3 groups: IVM/IVF in a mesh size of 500 µm opening and 220 µm wire thickness (L mesh), and IVC in S mesh (L-L-S mesh), (ii) IVM/IVF in L mesh and IVC in M mesh (L-L-M mesh) or (iii) control. Data (means±SE) for rates of cleavage (Day 2) and blastocyst development (Day 7-9) in each replicate were compared by Tukey's test after ANOVA. In Experiment 1, development in the mesh was similar to the control and was not affected by mesh size (cleavage 88.7, 81.0, 78.4% and blastocysts 33.1, 25.0, 23.3% in S mesh, M mesh and control, respectively). In Experiment 2, IVC in S mesh resulted in a lower cleavage rate than the control (70.7 vs 80.8%; $P<0.05$) while culture in the M mesh was intermediate (75.6%). There was no difference ($P>0.1$) in blastocyst development between the groups (21.2, 21.1 and 23.7%, respectively). In conclusion, it is possible to culture oocytes to the blastocyst stage in this simple system while maintaining the individual identity of the oocyte

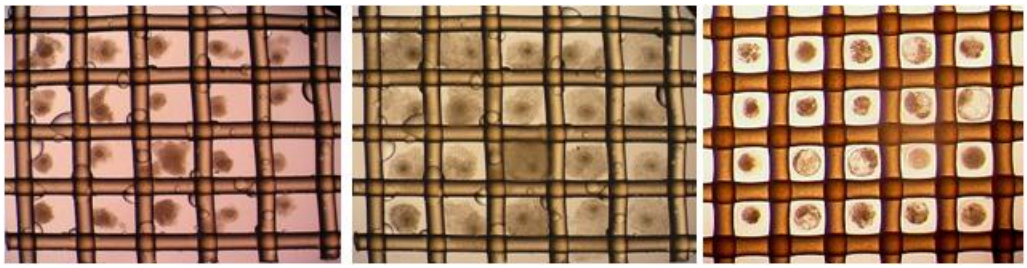

Figure 1. Immature COCs, mature COCs and Day 7 embryos cultured in polyester mesh.

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Notes

POST THAW SURVIVAL OF BOVINE IVP EMBRYOS WITH A NEW SLOW FREEZING PROGRAMME BASED ON MEMBRANE PERMEABILITY FOR WATER AND GLYCEROL

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Although conventional slow freezing programmes are successfully applied on bovine IVP blastocysts, post thaw survival and development of IVP morulae is still much too low. In a previous study (Mullaart et al. AETE 2007) we measured membrane permeability coefficients for water and cryoprotectant, the respective activation energies, the aqueous volume of the embryos, and membrane surface area. We applied the theoretical model of Woelders and Chaveiro (Cryobiology 49 (2004), 258-271) to predict the optimal freezing programme for a glycerol concentration of 10% (v/v), in which at every subzero temperature the freezing rate is maximised (to prevent 'slow-cooling damage'), while intracellular ice formation should be precluded.

In the present study, we compared the conventional linear slow freezing programme (straws seeded at -7°C, held 10', cooled −0.5°C/min to −30 °C, plunged) with the new non linear freezing curve (seeded at −6°C, held 7', non-linear cooling (−1.7 to −5.0 °C/min) to −40 °C, held 5', plunged). In order to achieve the high cooling rates of the new freezing curve, a controlled rate nitrogen vapour freezer (Planer, IBF100) was used for the new and the conventional freezing curves. In addition, we used the conventional method, i.e. the conventional programme in a programmable alcohol bath. IETS class 1 & 2 Day 6 morulae, and mid- and expanded Day 7 blastocysts were produced in vitro (SOFaaBSA culture system) from oocytes from slaughterhouse ovaries. Post thaw survival was measured by (re-) expansion rate at 24h (embryos of all qualities or only IETS class 1 & 2 embryos) and hatching rate at 72h (all qualities). Results were analysed by Chi-square.

Using the conventional freezing programme, fewer ($P<0.05$) blastocysts survived and hatched after freezing in the nitrogen freezer, compared to the alcohol freezer. In the nitrogen freezer, the new freezing programme resulted in a lower ($P<0.05$) blastocyst survival and hatching rate than the conventional programme. Also, fewer ($P<0.05$) morulae survived and hatched using the new programme in the nitrogen freezer, compared to the conventional programme in the alcohol freezer.

Table 1: Effect of freezing program on post thaw survival and hatching of IVP embryos.

System	Freezing programme	n Thawed Embryos	Re-expanded 24h		Hatched 72h
			all qualities	class 1&2	all qualities
		Blastocysts			
Alcohol freezer	Conventional	71	66 (93%) [a]	59 (83%) [a]	57 (80%) [a]
Nitrogen freezer	Conventional	75	58 (77%) [b]	49 (65%) [b]	46 (61%) [b]
Nitrogen freezer	New	51	26 (51%) [c]	15 (29%) [c]	5 (10%) [c]
		Morulae			
Alcohol freezer	Conventional	56	51 (91%) [a]	31 (55%) [a]	20 (36%) [a]
Nitrogen freezer	New	42	24 (57%) [b]	12 (29%) [b]	3 (7%) [b]

[a,b,c]: Within blastocysts or within morulae, different superscripts in the same column indicate $P<0.05$.

Application of the theoretical model had not rendered an improved freezing method for morulae (or blastocysts). Possibly, the theoretically determined freezing rates were too high, but negative effects of the nitrogen freezer also seem to play a role.

Notes

CELL DEATH IN VITRIFIED WARMED BOVINE BLASTOCYSTS AT DIFFERENT STAGES OF DEVELOPMENT.

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This experiment was conducted to determine whether the stage of blastocyst development could influence the cell death rates of vitrified/warmed bovine blastocysts. Blastocysts were produced *in vitro* and cultured in synthetic oviductal fluid containing 5% of foetal calf serum. On Day 8 after fertilization, blastocysts were classified into three categories according to the degree of expansion of the blastocoele: *early blastocysts* with a blastocoele less or equal to half of the volume of the embryo, *expanded blastocysts* with a blastocoele larger than the volume of the embryo and with a thin zona pellucida and *hatched or hatching blastocysts*. Blastocysts were vitrified using the cryotop system (Kuwayama et al. 2005). Survival rates as well as cell damage in vitrified/warmed bovine blastocysts were assessed 3 h after warming.

When the results according to the developmental stage were analyzed, 48.1% of expanded blastocysts and 53.6% of hatched blastocysts were fully re-expanded within 3 h of warming ($P<0.05$). In contrast, only 29.7% of early blastocysts survived after warming. Cell death was monitored in blastocysts 3 h after warming by TUNEL labelling of cells with damaged DNA. TUNEL staining revealed a higher proportion of DNA fragmentation (TUNEL positive blastomeres/total number of blastomeres) in vitrified early blastocysts (12.03% ± 13.75) and expanded blastocysts (10.38% ± 5.90). In contrast, cell damage was minimal in those blastocysts vitrified at hatched stage (8.64%,± 4.31 respectively). For control fresh blastocysts, the proportion of DNA fragmentation was 7.87% ± 6.75; 4.53% ± 4.25 and 5.36% ± 5.31 for early, expanded and hatched blastocyst stage respectively).

Blastocyst maturity at the time of vitrification was a key factor influencing outcome parameters. Significant differences in post-warming re-expansion and DNA damage were noted between non-expanded blastocysts and expanded and hatched blastocysts.

Notes

DIFFERENT METHODS OF NUCLEAR TRANSFER ALTERS THE GENE EXPRESSION DURING BOVINE PREIMPLANTATION DEVELOPMENT

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The majority of embryos prepared in vitro differ in gene expression in comparison to their in vivo counterparts. As a consequence of the altered transcription, especially of the developmentally important genes, many of these embryos fail to reach transferable blastocyst stage. Somatic cell nuclear transfer is often associated with poor results due to incomplete nuclear reprogramming of somatic donor cells that is a prerequisite for the correct development of mammalian embryos.

In our previous study using SSH, we found a set of genes differentially expressed between MII oocyte and 4-cell stage bovine embryos. We assessed the expression of several genes with known or predicted function in somatic cells or during the development of in vitro preimplantation embryos. Among them, four genes with known role during cell cycle, transcription regulation, mRNA processing and cytoskeletal structure were selected for the present study. We compared the gene expression among early 8-cell, late 8-cell stage embryos and blastocysts prepared under different conditions. Hand made cloning (HMC^{TM}), micromanipulator-based somatic cell nuclear transfer (SCNT), in vitro fertilized (IVF) embryos and their in vivo counterparts were analyzed using real-time RT-PCR (RotorGene 3000).

During the in vitro embryo cultivation, CENPF (Centromere protein F, 350/400ka), HMGN2 (High mobility group nucleosomal binding domain 2), TMSB4 (thymosin beta 4) and SRFS3 (splicing factor, arginine/serine-rich 3) showed the increase of their mRNAs content during major genome activation at the 8-cell stage. The transcription of splicing factor SRFS3 showed a transient increase also during minor genome activation at the 4-cell stage. Our results obtained from an analysis in single embryos demonstrate that individual HMC^{TM} and SCNT embryos in the same batch of cloning showed disparities in gene expression. The levels of studied genes in IVF embryos were similar to those with in vivo. SRFS3, CENPF and TMSB4 expression in SCNT embryos resembled those in naturally fertilized ones. HMC^{TM} embryos showed elevated levels of mRNA content of all the studied genes at late 8-cell stage, which correspond to the transition from maternal to zygotic control of gene expression. Moreover, the expression of SRFS3 mRNA was upregulated already at the early 8-cell stage. The transcript level of selected genes was significantly altered by the methods of nuclear transfer.

The alteration of gene expression in SCNT and HMC^{TM} embryos can lead to the compromised efficiency of these methods. The genes studied are known to have important functions during preimplantation and/or early postimplantation development in mammals and thus have the potential to be used as genetic markers of embryo viability.

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Notes

CHANGES IN TRANSCRIPT EXPRESSION OF POLG, TFAM, NRF1 AND COX1 CORRELATED WITH BOVINE OOCYTE SELECTION BY BCB TEST[a]

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This study was conducted in order to determine whether the level of G6PDH activity in immature bovine oocytes is correlated with the transcript relative abundance (RA) of the mtDNA replication related genes, POLG,TFAM, NRF1and mtDNA encoded COX1 in immature and mature oocytes. G6PDH activity was assessed by the BCB test. Transcript RA was assessed by real-time PCR.

Bovine immature cumulus-oocyte complexes (COC) recovered from slaughtered ovaries were incubated in 26 μM BCB in PBS for 1 h at 37°C. Then COCs were separated according to the cytoplasm coloration (BCB+ and BCB-). COCs not subjected to BCB staining served as a control (C). The oocytes of all groups immature and after in vitro maturation were stripped off cumulus cells, collected in minimal volumes of PBS, snap frozen in liquid nitrogen and stored at –80°C for qPCR analysis.

Seventeen μl of SideStep Lysis & Stabilization Buffer (Stratagene, La Jolla, CA, USA) was added to 10 μl of oocyte sample (27 oocytes) in PBS. The SideStep Buffer was used to lyse the oocytes and ensure RNA stabilisation. The one-step Brilliant II SYBR Green QRT-PCR Master Mix Kit (Stratagene, La Jolla, CA, USA) was used to perform relative quantification of gene expression. Each PCR reaction (total volume of 25 μl) consisted of 1 μl of cell lysate and 24 μl of reaction mixture, which consisted of 12.5 μl 2x SYBR Green QRT-PCR master mix, 0.4 μl of each 200 nM forward and reverse primer, 1 μl of RT/RNase block enzyme mixture and 9.7 μl RNase-free water. Thermal cycling conditions were as follows: 30 min at 50°C (for the first-strand synthesis); 10 min at 95°C; 40 cycles of 30 sec at 95°C for denaturing, 60 sec at 57°C for annealing and 30 sec at 72°C for extension.

In immature oocytes, significant differences were noted in RA of three out of four of the genes analyzed: TFAM mRNA expression differed ($P<0.01$) between BCB-, BCB+, and the control group; COX1 expression differed ($P<0.05$) between all analyzed groups, and NRF1 transcript levels differed ($P<0.01$) between BCB- and BCB+, and between BCB- and the control group ($P<0.05$). No significant difference in RA of POLG gene between the analyzed groups was noted. In mature oocytes, a significant difference ($P<0.05$) was noted only for RA of TFAM gene between BCB- and BCB+, and between BCB- and the control group. The results suggest that immature BCB- oocytes do have significantly lower transcript level of genes involved in mitochondrial biogenesis, suggesting that this may be one of the reasons for their low developmental competence compared to BCB+ and control oocytes.

[a] *This research was supported by the Scientific Net "Animal Reproduction Biotechnology".*

Notes

EFFICACY OF TRADITIONAL AND MODIFIED (VITMASTER) METHODS OF RABBIT EMBRYO VITRIFICATION

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Methods of rabbit embryo freezing were introduced over 30 years ago. However, the highest known proportion of rabbit embryos able to develop after cryopreservation into viable fetuses *in vivo* reached approximately 65%. Recently developed, minimum volume vitrification systems combined with using of supercooled liquid nitrogen (cooled down to about –206ºC, e.g. due to VitMaster device) resumed a question of a real, final optimization of rabbit embryo cryopreservation methods.

Effects of traditional vitrification method and vitrification using supercooled liquid nitrogen (VitMaster) applied for rabbit morula embryos were compared here. Embryos were equilibrated in Isotonic Equilibration Medium (IEM) containing 1,2 propanediol (2.72 M) and glycerol (1.36 M) for 7 min. and vitrified in 0.25 ml insemination straws after 1 min. exposure to vitrification medium (VM) containing additionally 1.0 M sucrose (Papis et al. 1993, Cryobiology, 30, 98-105). All solutions were modified by addition of 10% FCS. Briefly, 5 to 8 embryos were subjected to equilibration in IEM for 7 min. and subsequently - for 60 sec. in VM. Embryos were inserted into a middle column of VM in preloaded 0.25 mL straw (CryoBio System, France) which was heat sealed and plunged directly (but in stepwise manner) into liquid nitrogen (LN2) (-196ºC) or into supercooled LN2 (- 206ºC, VitMaster system, IMT Ltd, Israel). After a period of storage, straws were warmed in air (10 sec.) and subsequently in 20ºC water bath (7 sec.). Isotonic solutions of sucrose (ISS) in concentrations of 1.0 and 0.25 M were used for stepwise dilution of cryoprotective agents after warming.

Out of 65 traditionally vitrified embryos, all were intact after warming and after *in vitro* culture 61(93.8%) and 54 (83.1%) of them developed to the blastocyst and expanding/ hatching blastocyst stage within 48 h, respectively .

Table 1.
Number (%) of rabbit embryos developed *in vitro* after traditional or VitMaster vitrification*

	Intact embryos after warming	Blastocysts at 24h	Blastocysts at 48 h	Expanding/hatching blastocysts at 48h
Traditional vitr.	65 (100)	42 (64.6)	61 (93.8)	54 (83.1)
VitMaster vitr.	24 (100)	16 (66.7)	23 (95.8)	22 (91.7)
Control	Not vitrified	20 (62.5)	30 (93.8)	29 (90.6)

***** Data presented within columns are not different (P>0.05).

A hundred percent of embryos vitrified using VitMaster (24/24, 100%) were intact after warming and 23 of them (95.8%) developed *in vitro* to the blastocyst stage within 48 h, all but one being expanding and/or hatching blastocysts.

In conclusion, we describe here simple and efficient method of vitrification of rabbit embryos which is easy to perform, free from contamination risk and ready for practical use.

Notes

NUMERICAL CHROMOSOME ABERRATIONS IN COMPETENT (BCB+) OOCYTES OF PERI-PUBERTAL GILTS AFTER MATURATION IN VITRO*

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Oocytes of prepubertal females of many domestic species show reduced developmental competence in relation to their adult counterparts (ie. cattle, sheep, goat, pig). This problem still remains unresolved but recent data suggests insufficient cytoplasmic maturation as a main factor of reduced *in vitro* embryo production efficiency. The main source of porcine oocytes for a routine IVP are ovaries of commercially slaughtered, peri-pubertal gilts. Up to 60% of such females are prepubertal (non-cycling NCL) gilts. It is well documented that their oocytes are more frequently chromosomally unbalanced. Moreover chromosomal imbalance negatively affects quality of resulting embryos. Although the composition of porcine IVM media has been substantially optimized (ie. supplementation with Follicular fluid, EGF, cystein) a significant improvement in embryo quality has not been noticed. It is suggested that embryo developmental potential depends mainly on oocyte intrinsic quality therefore this factor has recently gained a big importance.

The objective of this study was to find out whether donor puberty affects the rate of chromosomal numerical abnormalities among competent porcine oocytes selected by BCB test and matured in vitro.

Ovaries of peri-pubertal gilts (age of 6-7 months, weight about 100kg) were classified based on their morphology (NCL gilts – lack of the corpora lutea, cycling CL gilts – presence of corpora lutea). Oocytes were aspirated with syringe/needle, selected under a stereomicroscope and incubated for 90min in brilliant cresyl blue solution (BCB test). Only competent (BCB+) and control oocytes (morphologically normal, non-BCB treated) were subjected to in vitro maturation (44h, NCSU23). After incubation oocytes were denuded, fixed on slides and frozen. So far, a total of 267 in vitro matured oocytes were analyzed using Fluorescence In Situ Hybridization (FISH) with probes specific for pig chromosome pairs no 1 and 10. More than 85% of fixed oocytes were successfully analyzed. Analysis of control oocytes is in progress.

We assumed, that in order to receive convincing data following cytogenetic analysis, only slides comprised of oocyte chromosome set along with chromatin of the first polar body or a diploid chromosome set were included into investigations. In all cases, an excepted set of clear fluorescent signals was observed. Surprisingly, donor puberty did not influence the rate of aneuploid oocytes (NCL gilts 5.6% vs. CL gilts 8.54%). However a significantly higher incidence of diploidy among NCL oocytes (33/140 – 23.57%) was observed (CL gilts 10/127 – 7.87%, $p<0.001$).

In conclusion porcine oocytes display generally significantly higher rate of genomic mutations when compared to cattle or horse. The findings of this work on aneuploidy do not support the previously published evidence. Since this experiment included only competent BCB+ oocytes, it may be suggested that the phenomenon is related to at least 2 factors: intrinsic features of BCB+ porcine oocytes or applied maturation conditions.

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Notes

RELATIONSHIP BETWEEN BLUETONGUE VIRUS (BTV-8) INFECTION AND FOETAL MORTALITY IN CATTLE

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Infection by Bluetongue virus (BTV) may affect reproductive performances but little is known about its real impact on fertility and occurrence of abortions. The aim of this study was to describe the effects of BTV infection on foetal mortality in 53 herds located in the east of France and infected by the virus between August and November 2007. In 2008, two visits were performed 50 days apart by local veterinary surgeons and pregnancy was checked by ultrasonography. The first visit aimed to record data on pregnancy and infection status and to register clinical signs. A total of 1939 cows were classified within 3 groups: non pregnant (n=262), early pregnant (< 3 months, n=1041), late pregnant (>3 months, n=636). For each cow, a blood sample was taken about 8 days after the first visit and BT status was determined by ELISA.

BT status was found positive in 69.5 % of the cows (80 % of beef vs 67 % of dairy cows). Prevalence was highly variable between herds as 19 % of herds had 100% of cows with positive serological results and 10 herds presented at least 70 % of seronegative results. Clinical signs were reported by farmers in 40 % (457/1141) of seropositive cows compared to 20 % (90/460) of seronegative cows ($p< 0.001$). As a consequence, clinical signs should not be considered as a reliable tool to diagnose BT infection with enough accuracy.

At first visit, ultrasonography was performed in cows which were supposed to be pregnant. At 2d visit, a positive pregnancy status has been confirmed in 78 % of the cows, but this proportion was higher in seronegative cows when compared to seropositive cows (81 % vs 76 %, $p<0.02$). This proportion was associated with the presence of clinical signs. Out of 1311 pregnant cows at first visit, only 37 presented a foetal mortality at 2nd pregnancy check (2.8 %). The rate of foetal mortality was also related to the BT seropositive status of cows ($p<0.05$: Table 1).

Table 1: Rate of foetal mortality observed between 2 pregnancy diagnosis 50 days apart (negative pregnancy check during the 2nd visit in cows which were pregnant at first visit)

2nd pregnancy check	Seronegative females	Seropositive females
Pregnant	412/418 (98.6 %)	862/893 (96.5 %)
Non pregnant	6/418 (1.4 %)	31/893 (3.5 %)

These results show that a seropositive BTV status assessed by serological test (ELISA) is associated with an increased frequency of foetal mortality in beef and dairy cattle. However further studies are needed to quantify effects on other reproductive variables such as calving to calving interval and number of AI's per conception.

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Notes

METHODOLICAL ADVANCES IN QUANTITATIVE ANALYSIS OF LIPID CONTENT IN THE PORCINE OOCYTES AND PREIMPLANTATION EMBRYOS

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Porcine oocytes and early stage embryos contain a high content of lipids in the form of cytoplasmic droplets with a hydrophobic core enclosed by a phospholipid monolayer. Different types of lipids may participate in several cellular processes: they play an important role as building material storage, as an energy source vital for proper embryo development and interact with various other organelles, mainly mitochondria. Moreover, the success rate of the cryopreservation of pig embryos appears to be highly correlated with their cytoplasmic lipid content. Unfortunately, the main technique for quantitative evaluation of lipid composition, i.e. gas chromatography is very hard to use for investigating very small and labile objects like mammalian oocytes and embryos. Therefore we developed several novel techniques based mainly on scanning confocal microscopy with using the specific fluorochromes to quantitatively evaluate the level of different classes of lipids in the porcine oocytes and embryos.

The experiments were carried out on immature oocytes (IO) collected from ovary follicles of non-stimulated gilts and mature oocytes (MO) as well as on zygotes (zyg), 2-4 cell embryos (2-4c), morula (m) and blastocysts (bl) collected from superovulated gilts and sows. All selected oocytes and embryos were fixed with 3% formaldehyde in phosphate buffered saline, washed and stained with 10 μg/ml nile red, 50 μg/ml filipin and with 2 μg/ml Bodipy 493/503 (Molecular Probes, Eugene, OR, USA) and analyzed in confocal microscope LSM 510 Meta (Zeiss, Germany). To evaluate the level of different types of lipids nile red, filipin and Bodipy 493/503 were excited using a 514 nm, 405 nm and 480 nm laser lines, respectively. Emission spectrums (originating from nile red) were recorded and deconvoluted into two bands corresponding to neutral and polar lipids, mainly triglycerides and phospholipids, respectively. Additionally, the intensity of filipin and Bodipy 493/503 fluorescence we used to evaluate the content of triglycerides and free cholesterol.

Analysis of variance has shown that total amount of triglycerides, phospholipids and cholesterol significantly decrease during cleavage, especially after morula compaction, but during oocyte maturation the amount of phospholipids remains unchanged (confidence level $p < 0.01$). The level of triglycerides (TG) and phospholipids (PH) during oocyte maturation and early embryonic development were shown in table.

	IO ±SE	MO ±SE	z ±SE	2-4c ±SE	m ±SE	bl ±SE
TG	1.33 ±0.03	1.19 ±0.02	1.0 ±0.04	0.98 ±0.02	1.03 ±0.03	0.96 ±0.02
PH	1.08 ±0.04	1.07 ±0.02	1.0 ±0.03	0.95 ±0.03	0.86 ±0.02	0.66 ±0.02

SE: standard error of the mean. Data were normalized by the mean value estimated for zygote.

The above, described techniques enable to detect small changes in the levels of different types of lipids during oocyte maturation and early embryonic development in the single porcine oocytes and embryos. In comparison to the previously used qualitative, histochemical methods, confocal microscopy techniques are quantitative and more precise.

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Notes

THE NOVEL METHOD OF PSEUDOPHYSIOLOGICAL ACTIVATION APPLIED TO GENERATION OF PORCINE NUCLEAR-TRANSFERRED EMBRYOS DERIVED FROM ADULT DERMAL FIBROBLAST CELLS

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The physicochemical agents such as electric pulses or calcium ionophore antibiotics, which are commonly used for artificial activation of porcine nuclear-transferred (NT) oocytes, can affect detrimentally or cytotoxically the clonal cybrids (nuclear-cytoplasmic hybrids) and thereby inhibit the development or decrease the quality of cloned embryos. Therefore, we have recently developed the novel method of pseudophysiological transcomplementary (transcytoplasmic) activation to stimulate the developmental program of porcine oocytes reconstructed by the somatic cell cloning. The mechanism underlying this original technique of activation is transcytoplasmic influx of sperm-derived proteins triggering intracellular calcium oscillations, among others, glucosamine-6-phosphate isomerase or deaminase (GNPI or GNPDA) termed as oscillin/oscillogen and isoform ξ of phospholipase C (PLC-ξ). These proteins are accumulated in the cytoplasm and nucleoplasm of fertilized metaphase II-staged oocytes and remain active post resumption of II meiotic cell division not only to meiotic-to-mitotic transition of cell cycle control, but also throughout all the first cleavage mitotic cycle of zygotes. That is why the oscillatory changes in the cytoplasmic concentration of free calcium cations $[Ca^{2+}]_c$ persist up to the interphase of second cleavage mitotic cycle, before they cease in the early 2-blastomere embryos following gradual ubiquitin-dependent proteasome biodegradation, which begins at anaphase of first cell cycle of zygotes. The transmission of oscillogenic proteins is mediated *via* rabbit zygote-descended cytoplasts into cytosolic microenvironment of porcine somatic cell nuclear-transferred oocytes. The aim of our study was to determine the *in vitro* developmental capability of porcine cloned embryos following pseudophysiological transcytoplasmic activation of oocytes receiving sow ear cutaneous fibroblast cell nuclei. In the cloning procedure, *in vitro*-matured oocytes were used as recipient cells for cell nuclei of cultured (confluent) somatic cells. The reconstruction of enucleated oocytes was performed by intracytoplasmic injection of either the somatic cell-derived karyoplast or whole tiny nuclear donor cell. The activation of NT oocytes was achieved by fusion of them with the cytoplasts isolated from *in vivo*-derived rabbit zygotes (i.e., with the so-called zygoplasts), which was induced by application of two successive DC pulses of 1.2 kV cm^{-1} for 60 μsec. Cloned embryos were cultured *in vitro* in NCSU-23/BSA/FBS medium for 6-7 days up to morula and blastocyst stages. A total of 136/165 (82.4%) oocytes reconstructed with fibroblast cell nuclei were successfully fused with zygoplasts. Out of 136 cultured NT embryos, 94 (69.1%) were cleaved. The rates of cloned embryos that reached the morula and blastocyst stages yielded 67/136 (49.3%) and 35/136 (25.7%), respectively. In conclusion, the original method of pseudophysiological transcomplementary activation of porcine NT oocytes turned out to be relatively efficient which has been confirmed by the high percentages of cloned embryos developed *in vitro* to morula and blastocyst stages.

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Notes

RELATIVE mRNA ABUNDANCE IN OVINE BLASTOCYSTS PRODUCED IN VIVO OR IN VITRO IN DIFFERENT CULTURE MEDIA

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Recent studies have demonstrated that the in vitro culture is associated with changes in gene expression profile which may reduce the quality of the in vitro produced embryos and may result in large offspring syndrome (LOS). The objective of the present study was to examine the relative abundance of candidate gene transcripts in ovine blastocysts derived from 5 different culture media.

Oocytes were obtained from ovaries of slaughtered adult ewes, matured in vitro in TCM-199 with different supplements for 24h (see Table) and then fertilized with fresh ram semen (day 0= fertilization day). Zygotes were cultured for 6-7 days in 20 µl droplets of synthetic oviduct fluid (SOF) with different supplements at 39°C under 5% CO_2, 7% O_2 and 88% N_2. Day 6-7 blastocysts in vitro produced and day 7 blastocysts in vivo derived, used as control, were snap frozen in groups of 10. Poly(A)mRNA was extracted and relative mRNA abundance was analyzed by Quantitative RT-PCR and normalized to the housekeeping gene *H2A.z*. Seven genes related with LOS, imprinting, DNA methylation and apoptosis were analyzed: *IGF2R* (Insulin-like growth factor receptor), *DNMT3A* (DNA methyltransferase 3a), *IL6* (Interleukin-6), *UBE2A* (Ubiquitin-conjugating enzyme 2A), *LAMA1* (Laminin alpha 1), *FGF4* (Fibroblast growth factor 4) and *BAX* (BCL2 associated X protein).

IGF2R and *UBE2A* transcript abundance was significantly higher in the 5 groups cultured in vitro than in vivo derived group. Furthermore, *IGF2R* transcript abundance was significantly higher in group D than in group C whereas *UBE2A* was significantly upregulated in D and E groups compared with group B. *LAMA1* was significantly upregulated in group D compared with groups A, B, E and F. No differences were observed in *DNMT3A*, *IL6*, *FGF4* and *BAX*.

In conclusion, embryo transcription is significantly affected by different supplemented culture media.

Table

Groups	IVM	IVC Day 1	IVC Day 3-5-7
A	0.4% BSA	8mg/ml BSA	8mg/ml BSA
B	0.4% BSA	8mg/ml BSA	8mg/ml BSA + 6mg/ml HA
C	0.4% BSA	8mg/ml BSA	8mg/ml BSA + charcoal stripped FBS10%
D	0.4% BSA	4mg/ml BSA	4mg/ml BSA
E	10% FBS	10% FBS	10% FBS
F	In vivo	In vivo	In vivo

BSA: Bovine Serum Albumin; FBS: Fetal Bovine Serum

Notes

INFLUENCE OF FSHP DOSE ON THE NUMBER OF ANOVULATORY FOLLICLES AND CORPORA LUTEA IN SANTA INÊS SHEEP

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The experiment was realized on a private farm that is localized in north area of Brazil. The study was conducted in order to examine the effect of the follicle stimulating hormone (FSHp) dosage on the number of corpora lutea (NLC) and the number of anovulatory follicles (NAF) in Santa Inês sheep at the age of 2 to 6 years old. Twenty four sheep received an intravaginal implant containing 60mg of Medroxiprogesterone Acetate for 14 days. The sheep were assigned to three treatments (eight females/treatment) according to the FSHp dosage (T1:0 IU; T2: 100 IU and T3: 200 IU). After the superovulation treatment all the sheep were monitored. Animals which exhibited oestrus signs were mated. Seven days later the numbers of corpora lutea and anavolatory follicles were determined using laparotomy.

The mean values observed in the NCL-group were 1.25±0.46 CL, 1.25±0.46 CL and 4.50±3.74 CL for the treatments T1, T2 and T3, respectively. The number of CLs of treatment T3 was significant higher compared to the treatments T1 and T2 ($P<0.05$).
However, there was not difference between the treatments with regard to the numbers of follicles that did not ovulate (0.63±0.74, 1.00±1.7, 1.13±1.04).

The higher ovulation rate in animals treated with 200 IU FSHp (T3) compared to the other treatments (T1 and T2) can be considered as normal. Either animals which received no FSHp (T1) or sheep of the treatment T2 which received only half of the full dosage of FSHp responded similarly and showed a low number of CLs. The number of ovulation sites was tripled when doubling the dosage from 100UI FSHp to 200UI FSHp.

Wierzchos et al. (1992) also studied the effect of the FSH dosage on ovarian response. Polish Mountain sheep were treated with either 250, 500 or 750 IU of FSHp which resulted in 7.6, 8.5 and 8.3 corpora lutea. However, it has to be kept in mind that numerous factors are discussed which may exert a crucial effect and/or modify the ovarian response such as animal nutrition, management, breed and climate. We must emphasize that animal individuality is one of the most important factors responsible for the high rate of variability to the superovulatory treatment.

In conclusion, more research has to be done in order to study the properties of reproduction physiology in Santa Inês sheep in the north of the Brazilian area.

Keys words: ovulatory response, ovulation, Medroxiprogesterone Acetate.

Notes

EFFECT OF HEIFER PLUS™ ON BULL SEMEN PARAMETERS ACCESSED BY CASA ANALYZER

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In a bovine farm economy, that lives by its milk and derived products, it is important to have a tool that can increase the population of female animals. Recently, a commercial kit, that can be used for AI, immediately after post-thaw, was purposed. It is called Heifer-Plus™. Its characteristic seems to alter the sex ratio of offspring increasing motility of only the X-chromosome bearing (female) sperm and slows the motility of the Y-chromosome bearing (male) sperm.

When inseminated, the sperm are sorted in the female reproductive tract, and more oocytes are fertilized by the X-chromosome bearing sperm. Several studies have been made about the efficacy of this methodology but still results are controversial. In our study, we tested and compared motility of three bulls (thawed semen) before and after incubation with Heifer-Plus™ (Heifer plus group) following the manufacturer recommendations or without adding any substances (control group), by Computer Assisted Semen Analyzer (CASA, Hamilton Thorne Research, Beverly). Motility analysis was conducted at time 0, post-thawing, and after 20 minutes of incubation at 37°C with Heifer-Plus™ (time recommended by producers to have wanted effect). Control group were tested at same condition but without adding any substances. All parameters were analyzed statistically with *t-student* for data non paired and chi square method ($p<0.05$ as threshold). Except for VAP and VCL recorded after 20 minutes incubation, all others parameters did not show any statistical differences between control group and Heifer-Plus™ group. At time 0 we recorded a VAP of 84.7+/- 8.2 μm/sec and VCL of 142.5 +/- 9.5 μm/sec for both group, while at 20 minutes VAP was 68.5 +/- 1.3 μm/sec and the VCL was 109.5 +/- 11 μm/sec in Heifer-Plus™ group. The values of VAP between time 0 and after incubation were statistically significant ($p<0.01$) for the Heifer-Plus™ group. The values of VCL between time 0 and after incubation were statistically significant ($p<0.001$) for the Heifer-Plus™ group. The control group did not show any differences in VAP and VCL values between time 0 and after 20 minutes of incubation.

Our study demonstrated that the treatment of post-thawed bovine semen with Heifer-Plus™ slow down the VAP and VCL, of all sperm's population. Our results are in contrast with authors that report "there was no difference in progressive sperm motility between control semen or Heifer-Plus™ treated samples"(*E. Curry, et al. 2008* Proceedings IETS). However, based on our results we can not speculate on the reliability of Heifer-Plus kit to treat semen following thawing to alter the sex ratio.

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Notes

DEVELOPMENT OF CAPRINE CLONED EMBRYOS FOLLOWING THE PSEUDOPHYSIOLOGICAL ACTIVATION OF OOCYTES RECONSTRUCTED WITH TRANSGENIC FETAL FIBROBLAST CELL NUCLEI

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The purpose of the study was to examine the *in vitro* developmental potential of caprine cloned embryos following pseudophysiological transcomplementary (transcytoplasmic) activation of oocytes receiving transgenic fetal fibroblast cell nuclei. Somatic cells were genetically modified by the lipofection method with *pAlb-GFPBsd* gene construct. Positively-selected transgenic fibroblast cells were *in vitro* cultured up to a total confluence state and then used for the somatic cell nuclear transfer (SCNT) as a source of nuclear donor cells. *In vitro*-matured caprine oocytes were the source of recipient cells. The reconstruction of the previously enucleated oocytes (i.e., ooplasts) was performed by microinjection of either the somatic cell-derived karyoplasts or intact whole tiny nuclear donor cells directly into the cytoplasm. The reconstructed oocytes were incubated in Upgraded B2 INRA medium for 1 h before their pseudophysiological transcomplementary activation. The activation was achieved by electrofusion of clonal cybrids (nuclear-cytoplasmic hybrids) with the allogeneic cytoplasts isolated from caprine *in vitro*-fertilized ova (zygotes). Single zygote-descended cytoplasts (the so-called zygoplasts) were inserted into the perivitelline space of previously reconstituted oocytes. The resulting zygoplast-clonal cybrid couplets were subsequently subjected to plasmolemma electroporation, which was induced by application of a single DC pulse of 2.4 kV cm^{-1} for 15 μsec. The electropermeabilization of zygoplast and reconstructed oocyte plasma membranes occurred in an isotonic dielectric solution deprived of Ca^{2+} ions. The transcytoplasmically-activated clonal cybrids were *in vitro* cultured in Upgraded B2 INRA medium for 2 days at 38.5°C in a 100% water-saturated atmosphere of 5% CO_2 and 95% air. Afterwards, cleaved embryos were co-cultured with *Vero* epithelial cells in the same medium supplemented with 10% FBS for 5-7 days up to morula/blastocyst stages under the same thermal and atmospheric conditions. A total of 64/83 (77.1%) oocytes reconstructed with fibroblast cell nuclei were successfully fused with zygoplasts. From among 64 cultured cloned embryos, 31 (48.4%) were cleaved. The rates of SCNT embryos that reached the morula and blastocyst stages yielded 23/64 (35.9%) and 14/64 (21.9%), respectively.

In conclusion, the method of pseudophysiological transcytoplasmic activation of nuclear transfer-derived oocytes seems to be feasible for efficient stimulation of preimplantation development of clonal cybrids. Moreover, the relatively high frequencies of transgenic morulae and blastocysts were noticed among *in vitro* cultured caprine cloned embryos produced by this strategy of activation of oocytes reconstructed with transgenic fetal fibroblast cell nuclei.

Notes

SOURCES OF VARIATION OF EQUINE EMBRYO PRODUCTION AND PREGNANCY RATES AFTER TRANSFER: A TEN YEARS RETROSPECTIVE STUDY IN FRANCE

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The use of equine embryo transfer (ET) is particularly interesting in Selle Français mares which can produce offspring via embryo transfer for later running competitions. A French ET team named AMELIS started a commercial equine ET activity in 1999 in Normandy. This ten years retrospective study aims to investigate the sources of variation of embryo transfer results under field conditions. A total of 4920 flushes were realised between 1999 and 2008 by 6 operators. Donor mares were inseminated with fresh semen (immediately used or stored at 4°C) or frozen. Only 70 donor mares were covered by stallions. Embryos were collected on Day 7 or 8 after ovulation and transferred mainly as fresh into recipients by cervical method. Recorded data included flushes characteristics (physiological status of donor mares, rank of cycle, breeding method, season and operator), stage and size of the embryos. Pregnancy was diagnosed on Days 14 and 50 after ovulation by ultrasonography. Khi 2 tests were performed to identify the potential variation factors of transfer results. Significant factors were further analysed by a multivariate model of logistic regression (SAS, Logistic procedure). A total of 2596 embryos were produced out of 4920 flushes (53 %). At least one embryo has been collected in 45.5 % of flushes (1, 2 and 3 embryos in 38.5, 6.7 and 0.3 % of flushes respectively). 42.3 % of flushes were performed in mares running competition. Most frequently, frozen semen has been used for AI (59.7%). The proportion of flushes with at least one embryo was associated with physiological status of donor mares, rank of cycle and breeding method (Table 1), whereas there was no effect of year, season and operator. Pregnancy rates averaged 68.5 % (from 63.3 in 2001 to 74.5 % in 2008) and 57.8 % on D14 (from 2600 ET) and 50 (from 2369 ET) respectively. Pregnancy rates on D 14 were influenced by embryo stage (morulas ; 59.2%, young blastocysts (BL) : 71.6 %, BL : 73.9% ; exp. BL : 66.2%, $p<0,01$), as well as month of flushing (3: 73.5%, 4:72.0%, 5:72.8%, 6:66.8%, 7:64.3%, 8:65.5%, 9:70.5%, $p<0.05$) and stud farms. There was no global effect of year, stage and breed of recipient on pregnancy rates.

To conclude, status of donor mares, AI method and rank of cycle were found as major factors influencing embryo recovery. The morula stage led to decreased ET results in comparison to later stages, independently of the stage of recipients.

Table 1 Significant variation factors related to embryo production after multivariate logistic regression analysis (*P<0.05)

Variable	Reference	Adjusted OR (freq., % flushes with at least 1 embryo)			
Status of donors	Mares running competition	Maiden	Mares with foals	Mares without foals	Mares with problems
	1 (2082, 47)	0.9 (422, 45)	0.9 (320, 46)	0.8* (1076, 42)	0.5* (548, 33)
Breeding method	Fresh semen, immediate use	Stallion	Fresh semen, +4°C storage	Fresh semen, transported	Frozen semen
	1 (990, 53)	1.1 (70, 49)	0.6* (344, 42)	0.7* (365, 45)	0.6* (2939, 42)
Rank of cycle	first	second		third and more	
	1 (1671, 46.4)	0.8* (1074, 40.9)		0.8* (1480, 40.8)	

Notes

SOME STANDARD PARAMETERS OF TRANSGENIC BOAR SEMEN

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The aim of the study was to examine the quality of semen from transgenic boars with a gene construct containing the human α1,2-fucosyltransferase gene.

Semen collected from 5 transgenic boars (F1 and F2) was compare to semen of 3 non-transgenic (2 Landrace, 1 Large White) boars. All boars were between 11 – 27 months old. There were 20 ejaculates obtained from transgenic boars (2-7 ejaculates from each one) and the 24 ejaculates from non-transgenic boars (4-13 ejaculates from each one). Volume, concentration, motility (visual assessment), pH, osmotic pressure and morphology of raw semen were investigated. All ejaculates were diluted in BTS extender and kept at 15° C to examine the motility during storage and determine a survival time (a day period when the motility decreased to 30 %). The data were analyzed statistically with the T test.

The results are shown in table below.

Table 1. Comparison of the assessment of transgenic and non transgenic boar semen quality.

Parameters	Transgenic boars	Non-transgenic boars	p
Volume of ejaculate (ml)	185.5 ± 72.2	199.4 ± 46.5	0.4454
Concentration (x 10^3/mm^3)	368.8 ± 98.0	394.8 ± 82.4	0.3443
Motility of raw semen (%)	69.5 ± 9.0	72.7 ± 15.7	0.4240
Osmotic pressure (mOsm)	316.6 ± 12.0	315.5 ± 11.0	0.7598
pH	7.60 ± 0.18	7.71 ± 0.20	0.0643
Morphology:			
normal spermatozoa (%)	76.5 ± 19.3	66.2 ± 22.3	0.1106
major sperm defects (%)	6.5 ± 2.9	12.2 ± 9.1	**0.0103**
minor sperm defects (%)	15.8 ± 18.2	21.6 ± 20.1	0.3223
Motility of preserved semen (%)			
after 1 day (%)	63.8 ± 17.8	57.1 ± 21.3	0.2726
after 4 days (%)	49.2 ± 22.7	45.2 ± 22.4	0.5631
after 7 days (%)	39.0 ± 25.6	40.2 ± 21.2	0.8569
Survival time (days)	8.0 ± 5.2	7.6 ± 4.2	0.7748

Only for morphological anomalies (major sperm defects) differences were significant.

In conclusion, we can suppose that construct containing the human α1,2-fucosyltransferase gene don't affect on all assessed parameters of boar semen.

Notes

THE SPECIFICS OF CENP-F PROTEIN EXPRESSION DURING MAMMALIAN PREIMPLANTATION DEVELOPMENT

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Centromeric protein F (CENP-F; mitosin) is crucially important for correct chromosome alignment and segregation during mitosis. Its expression and localization is cell cycle dependent. The proper progress through mitosis is essential for oocyte maturation and consequent embryo development.

To uncover the role of CENP-F during bovine preimplantation development, we injected a 3´end terminus-homologous dsRNA into the zygotes. The efficiency of mRNA degradation was confirmed using single-embryo qRT-PCR and an average decrease by 94.9% ($p<0.001$) was achieved relative to controls. There was no disparity between individual groups in the developmental competence until the 8-cell stage but only 28.1% ± 6.19 of *CENPF* dsRNA injected 8-cell embryos were able to develop to the 16-cell stage or beyond. The depletion of the protein was verified by two different antibodies – targeted to N-terminus and C-terminus of CENP-F respectively. When using antibody against C-terminus no or very slight signal was detected in CENP-F dsRNA injected embryos. When using antibody against N-terminus the intensity of the signal did not differ in control embryos and CENP-F dsRNA injected embryos. This suggests existence of at least two splicing variants of CENP-F in bovine preimplantation embryos.

Using western blot analysis, we have found that there are several splicing variants of CENP-F in cattle. According to GenBank bovine CENP-F (xp_612376) has more than 350kDa. We have performed three CENP-F specific antibodies – two of them were specific to C-terminus of CENP-F, the third one was specific to N-terminus of the protein. We have found striking differences in the size of protein detected using C-terminus specific antibodies and N-terminus specific antibody in embryos. The non-truncated CENP-F protein was almost not detectable using N-terminus specific antibody, however there was a strong double band at 180 and 190 kDa respectively. We have found several splicing variants in both embryonic and fibroblast CENP-F in analysis of mRNA as well.

These findings suggest that CENP-F activation at late 8-cell stage is important for subsequent correct development and that we have found new splicing variants of CENP-F that outweigh the only till now known variant of CENP-F in embryos.

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Notes

INFLUENCE OF DIFFERENT FOLLICLE POPULATIONS UPON QUALITY OF EQUINE OOCYTES

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During the last few years horse breeders became more interested in assisted reproduction techniques. However, in vitro embryo production is not very successful in the horse and only few foals have been born by using this technology. While the rate of chromosomal maturation of in vitro cultured horse oocytes is high, fertilization rates in vitro are still not efficient. This could be due to an impaired cytoplasmic maturation in vitro. In contrast to cattle, equine oocytes with expanded cumulus cells reached higher fertilisation rates in vitro and the cytoplasmic maturation also depends on the cumulus morphology. Expanded cumulus-oocyte-complexes show a consistent increase of mitochondrial activity during the in vitro maturation.

The aim of our study was to characterize cytoplasmic and structural changes of horse oocytes during the maturation of the follicle in vivo.

Therefore, 14 Mecklenburger Warmblood mares underwent repeated transvaginal ultrasound guided follicle aspiration. Aspiration sessions were performed 44 times during the heat of the mares to obtain oocytes from preovulatory follicles and subordinant follicle populations and 79 times oocytes were recovered from growing, progressive follicle populations before a dominant follicle had developed. We expected to obtain oocytes of dominant, atretic and viable follicles in these different aspiration sessions. At least steroid analysis of the follicle fluid could confirm the clinical deviation of the follicle populations.

Every single oocyte was analysed for structural and cytoplasmic maturation. Therefore the oocytes were divided in groups depended of its cumulus morphology and incubated with brilliant cresyl blue stain immediately, to evaluate the glucose-6-phosphate dehydrogenase activity (G-6-PDH). For measuring the mitochondrial activity and aggregation as well as to evaluate the chromatin configuration, the oocytes were stained with Mito Tracker Orange and Hoechst 33342 stain parallel and fixed.

Our results could not show an impact of the follicle population upon the G-6-PDH-activity and no link between the G-6-PDH-activity and other parameters could be proofed. But we observed that progressive follicle populations consist of viable follicles, contained significant more oocytes with a compact cumulus, fibrillar chromatin and a higher mitochondrial activity, while subordinante follicle populations consist of atretic follicles, had more oocytes with a expanded cumulus, condensed chromatin and a decreased mitochondrial activity ($p<0.05$). Higher levels of mitochondrial activity were found in oocytes with an expanded cumulus or with a corona radiata than in oocytes with a compact cumulus. As the analysis showed no significant differences between oocytes with expanded cumulus or corona radiata, it could be inferred that they belong to the same oocyte population. Moreover, expanded cumulus-oocyte-complexes had a significant higher mitochondrial activity, if they come from a progressive follicle population ($p<0.05$).

In conclusion, the follicle population originating the oocyte significantly influences parameters of its quality.

Therefore the origin of the oocyte should be more noticed in programmes of in vitro embryo production in the future.

Notes

TRANSCRIPTIONAL REACTIVATION DURING IN VIVO MATURATION OF CANINE OOCYTE

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In vitro maturation rates obtained with canine oocytes remain very low to date compared to those obtained in other species. In parallel, basic data on the process of vivo maturation phenomenon are lacking in this species. In a previous work, we performed an ultrastructural study of in vivo meiosis resumption by TEM. A transient structural modification of oocyte nucleoli occurring after ovulation was evidenced: the nucleoli that presented a dense aspect since the LH peak recover a reticulated structure between 34 and 68 hours post ovulation (Viaris de Lesegno et al., 2008). The present study was designed to determine whether these modifications were associated with a transcriptional activity.

Materials and Methods

Bitches follow-up

During oestrus, blood LH was assayed and the time of ovulation was determined by transabdominal ultrasonography. Ovariectomies were performed in bitches during anoestrus (n=7), during oestrus before the LH peak (n=2), after LH peak but prior to ovulation (n=3) or following ovulation (n=10), from 7 to 48 hours post ovulation. Oocytes were collected by slicing for anoestrus ovaries, by follicular puncture before ovulation and by tubal flushing after ovulation.

Transcriptional activity

Transcriptional activity was evaluated through BrUTP incorporation in nascent RNAs without microinjection. For 10 anoestrus oocytes, RNA polymerase II inhibitor alpha-amanitin was added to check the signal specificity.

Immunocytochemistry

Oocytes were then fixed and submitted to immunocytochemistry (monoclonal primary antibody directed against BrdU) and DNA was stained by ethidium homodimer. Oocytes were observed under confocal microscopy and transcriptional activity was expressed in Arbitrary Unit (AU).

Results

All analysed oocytes (n=157) were at the Germinal Vesicle stage (prophase I). In those that were transcriptionally active, we evidenced a transcriptional activity in both the nucleoplasm and the nucleoli. Transcriptional levels in these two compartments were correlated ($r=0.77$; $p<0.0001$).

During anoestrus, oocytes were found transcriptionally active (n=41; around 200 AU in nucleoplasm and 40 AU in nucleoli). In estrus, the situation varied between periods. Prior to the LH peak, transcriptional activity was low. It increased significantly after the LH peak. After ovulation, transcription remained at the preovulatory level for the first 10 hours. But between 27 and 33 hours post-ovulation, transcriptional activity peaked and then returned to previous levels.

Discussion

The main result of this work is the transcriptional reactivation in the oocyte between 24 and 39 hours after ovulation. Since this reactivation occurs very close in time to meiotic resumption of the oocytes, it may be that it plays an important role in this phenomenon. The determination of the genes transcribed during this post-ovulatory transcriptional window would be of great interest for a better understanding of canine meiotic resumption.

Notes

CAN MICRO-FLUIDIC CHIP SYSTEMS BE USEFUL IN EVALUATION OF BOVINE OOCYTE AND EMBRYO QUALITY?

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Microspectrometry seems to be an ideal method of non-invasive examination of cattle embryos. To date, no methodology of spectrophotometric characterization of cattle embryos has been developed because there was no existing device for spectral measurements of a single embryo. In this paper we present for the first time a lab-on-chip (LOC) for optical characterization of cow embryos. The chip works together with external miniaturized instrumentation enabling spectrophotometric and fluorometric characterization of a single embryo. The aim of this study was to determine the possible influence of micro-fluidic chip measurements on the rate of successful fertilized bovine oocytes.

Immature oocytes of follicles 2 mm or greater were collected from naturally cycling Holstein cows (donors). The animals were slaughtered and the ovaries were transported to the laboratory within 1 h at 38.5°C. Groups of 50-70 good quality cumulus oocyte complexes (COCs) were matured in 500 µl IVM culture medium over 24 h at 39°C in humidified atmosphere containing 5% CO_2. After maturation, oocytes were inseminated with washed, frozen–thawed semen of a bull of known *in vitro* fertility (final sperm concentration of 1.5 mln/ml) and co-incubated for 20 h at 39°C in humidified atmosphere containing 5% CO_2.

Measurements were performed after oocyte collection, following maturation, and after fertilization. After IVF, presumptive zygotes were mounted on glass slides in VECTASHIELD with DAPI (4',6-diaminoino-2-fenylindol) drop and observed under fluorescent microscope OLYMPUS CKX41.

The results of these measurements did not indicate any differences in the rate of successful fertilizations between oocytes which were measured by the micro-fluidic chip system and unmeasured control oocytes. In this case, the micro-fluidic chip measurements were used to determine if this new non-invasive method for successful evaluation of bovine oocytes and embryos before reaching the blastocyst stage following implantation is effective. We demonstrated the micro-fluidic chip system as the first parametric non-invasive method for evaluation of gametes and embryos used in embryo transfer.

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Notes

MICRO-FLUIDIC CHIP SYSTEM: A NEW METHOD OF MEASUREMENT OF APOPTOSIS IN PORCINE OOCYTES

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In order to verify conception of the measurement methodology and LOC (lab-on-chip) construction, being actualy discovered, spectrophotometric and fluorometric measurements of single porcine oocytes was carried out. For the first time, the spectral characteristics of the single gamete before and after treatment in fluoresceine solution from Annexin-V-FLUOS staining kit are presented.

The ovaries were collected from thirty selected crossbreed Landrace puberal gilts. The ovaries and reproductive tract were recovered and transported to the laboratory at 38^{o} C in 0.9% NaCl within 10 minutes of sacrificing the animals. The follicles were then opened by puncturing of individual follicles in a sterile petri dish, and the cumulus oocyte complexes (COCs) were recovered. The COCs were washed three times in modified PBS supplemented with 36 µg/ml pyruvate, 50 µg/ml gentamycin, and 0.5 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). After measurements, the collected COCs were cultured in Nunclon™Δ 4-well dishes in 500 µl standard porcine IVM culture medium; TCM-199 (tissue culture medium). COCs were incubated with hyaluronidase Sigma-Aldrich Co. (St. Louis, MO, USA) for 2 min at 38^{o} C to separate cumulus cells and granulosa cells. The cells were removed by vortexing the oocytes in 1% sodium citrate buffer and by mechanical displacement using a small-diameter glass micropipette.

Significant differences in spectral characteristics were observed in the VIS wavelength region, which can probably be used for the determination of oocyte quality. Fluorencence biosignal was clearly observed, and the fluorencence images were very sharp and enabled us to distinguish individual cells.

Presented here for the first time, LOC working together with optical miniaturized instrumentation enables non-invasive optical-parametric characterization of the single gamete. Spectral characteristics of the single oocyte have been successfully obtained. Sharp images of the fluoresceine-labeled oocyte have been observed. Preliminary methodology and LOC for microspectrometric measurements of the single porcine oocyte, never before reported in literature, have been presented. We believe that the LOC and instrumentation developed by us will lead to the construction of a portable device for single oocyte examination for point-of care application. This will open a new way for true and subjective characterization of porcine oocytes in the selection of high quality porcine oocytes for IVF and IVP programs.

This study was supported by grant No. 1682/B/PO1/2007/33 from the Polish Ministry of Scientific Research and Information Technology. The work was financed by POIG 01.03.01-00-014/08 subproject 2B APOZAR. Bartosz Kempisty acknowledges support from the Fellowship of the Polish Science Foundation.
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Notes

EFFECTS OF LASER PUNCTURE OF THE EMBRYONIC CAPSULE ON VITALITY AND SENSITIVITY TO ETHYLENE GLYCOL OF THE EQUINE CONCEPTUS[A]

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Deep freezing of horse conceptuses exceeding 300 µm in diameter revealed unsatisfactory post-thaw survival rates. Two possible causes for this phenomenon are under discussion: the glycoprotein capsule, which protects the early blastocyst from about day 6.5 to 22, and the endodermic layer of the bilaminar trophoblast. The aim of the present study was to investigate growth patterns and viability of equine embryos in culture after laser puncture of the embryonic capsule and/or short time exposure to ethylene glycol (EG). From March to August, forty embryos were flushed from 7.5 to 9.5 days after ovulation representing a recovery rate of 54.4%. The conceptuses were assigned randomly to four treatment groups: group A (control I) – culture; group B (treatment I) – laser puncture; group C (control II) – exposure to EG for ten minutes; and group D (treatment II) – laser puncture followed by EG treatment. All conceptuses were cultured in TCM 199 for 48 hours at 38.5 °C, 5% CO_2 and maximum air humidity. Development patterns differed significantly among groups after 48 hours of culture ($p = 0.0381$) in contrast to 24 hrs period ($p = 0.0823$). After 48 hrs culture embryos showed continuous growth (A: 3/10; C: 4/10; D: 4/10), rupture (A: 4/10; B: 2/10; C: 4/10), shrinkage (A: 3/10; B: 8/10; C: 1/10; D: 6/10), and stagnation of growth (C: 1/10). According to the data of 24 hrs culture, successive rupture of the conceptuses might be a sign of good rather than poor quality. However, the lack of uterine pressure on the capsule during in vitro culture could also contribute to this particular reaction. All embryos of group A showed growth after 24 hrs of culture. Four of them continued growing and ended up in rupture of the capsule before 48 hrs. Laser puncture followed y EG revealed slightly better results compared with laser treatment alone. This might be due to the fact, that embryos of group B were punctured twice and those of group D only once. The present study demonstrates that laser puncture adversely affected the growth of embryos. Although treatment with EG resulted in a significant loss of size, it did not induce the collapse of the embryo, as expected. Thus it is not very likely that ethylene glycol sufficiently penetrated the embryo via the laser defect.

Additional studies are needed to demonstrate the kinetics of embryonic EG uptake. Furthermore, deep freezing and transfer of the embryos after laser puncture should be undertaken in order to test the survival of the embryos in vivo.

[a] *The study was supported by Minitüb (Tiefenbach, Germany), Ibidi (Munich, Germany), Haupt- und Landgestüt Schwaiganger (Ohlstadt, Germany) and the K. Wilhelm Schaumannstiftung (Hamburg, Germany)*

Notes

REPEATABLE LAPAROSCOPIC OVUM PICK-UP (OPU) IN GOAT – CLINICAL ASPECT

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New techniques for repeated, noninvasive oocyte recovery in living donor goats are necessary as an alternative to the traumatic surgery method. However, the use is restricted to the relatively low efficacy and reproducibility of the results. The aim of the study was to develop a new laparoscopic technique for repeated recovery of goat oocytes useful for in vitro culture and fertilization and cloning and for the evaluation of clinical aspects such as tissue damaging, adhesions in the abdominal cavity, scar formation and disorders in the reproductive tract of the same donors after 2 – 5 ovum pick-up sessions.

Oocytes were aspirated with an originally designed catheter for ovum pick-up. The catheter equipped with a needle /(length: 10mm, 21 – 22G, bevel 30^0) allowed to recover oocytes without damaging the ovary and to obtain oocytes of high quality. Seventeen goats served as oocyte donors. Groups were assigned according the frequency of oocyte collection: one time (Group I, n=17), two times (Group II, n=17), three times (Group III, n=10), four times (Group IV, n=8) and 5 times (Group V, n=2). Estrus was synchronized using intravaginal sponges (Chronogest CR, Intervet) for 14 days. Superovulation was induced by a single injection of eCG (1000 IU im) 16 - 24 hours before the removal of the sponges. Oocytes were collected 24 hours after sponge removal. Animals were anaesthetized by infusion, the duration was about 15 to 20 min. The endoscope was inserted into the abdominal cavity through the umbilicus. Two trockars which were used for the manipulators were inserted 15 cm cranial of the udder. Oocytes were collected by aspiration of the follicular fluid from the ovarian follicles. Depending on the size, the single aspiration of up to 8 follicles was performed. The quality of the oocytes was assessed according to the following classification: class I – homogenous cytoplasm, at least 3 layers of the granulosa cells; class II – homogenous cytoplasm, 1-2 layers of granulosa cells; class III – homogenous cytoplasm, no granular cells; class IV – heterogeneous cytoplasm, independent of the granulosa cells. Oocytes of the class I, II and III were used for the culture.

Group	Number aspirated follicle	Recovered oocytes (%)	Morphology recovered oocytes			
			I class (%)	II class (%)	III class (%)	IV class (not qualified) (%)
I	172	78 (45.0)	39 (50.0)	27 (35.5)	10 (13.2)	2 (2.6)
II	81	45 (55.6)	2 (4.4)	31 (68.9)	9 (21.4)	3 (6.7)
III	64	35 (54.6)	11 (17.2)	13 (37.1)	10 (29.4)	1 (2.9)
IV	84	68 (80.9)	21 (25.0)	39 (57.4)	6 (9.1)	2 (2.9)
V	12	12 (58.3)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)
Total	413	233 (56.4)	73 (32.4)	117 (52.0)	35 (15.6)	8 (3.5)

After the first three endoscopic sessions there was no complication detectable. In Group IV one animal (1/8) showed adhesions between the omentum and the peritoneum closed to the location where the grasping forceps were inserted. In one animal in Group V (1/2) adhesions occurred between the infundibulum and the ovary. The ovaries per se did not show any pathological signs. It is conclude that the proposed technique represents a suitable technique for collecting oocytes of good quality that can be used for IMV/IVF techniques and cloning. Furthermore, the method can be useful for multiple recoveries of oocytes in goats.

Notes

NON-SURGICAL TRANSFER OF DAY 10 HORSE EMBRYOS TO ASYNCHRONOUS RECIPIENT MARES

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A level of synchrony between the embryo and its uterine environment is essential for the establishment and maintenance of pregnancy and maximum pregnancy rates are achievable when transferring horse embryos to recipient mares that ovulated +1 to -3 days with respect to the donor. How far the degree of asynchrony can be stretched when an equine embryo is transferred to a recipient mare has not been fully investigated. This study reports the results of transferring day 10 embryos to recipient mares that had ovulated -9 to + 4 days with respect to the donor.

Grade 1 day 10 embryos were transferred non-surgically to recipient mares that had ovulated 9, 7, 5, 3, or 1 day after the donor, on the same day as the donor, or 2 or 4 days before the donor (n = 6 or 8 per group). Transrectal ultrasound scanning examinations were commenced 2 days after transfer and continued on alternate days until an embryo with a viable heartbeat was present, or embryonic death had occurred.

The number of recipient mares pregnant with a viable embryo was lowest in those recipients that had ovulated 4 days before (0/6; 0%) or 7 and 9 days after (3/8; 38% and 0/6; 0% respectively) the donor mare. Pregnancy rates for the remaining groups, ovulating between 5 days before and 2 days after the donor, were not significantly different and ranged from 63 -100%. The 3 pregnancies established following transfer to a recipient that had ovulated 7 days after the donor showed slow embryonic development and delayed differentiation of the fetal membranes. Furthermore, the survival of all 12 embryos transferred to either a day 10 or a day 12 uterus (0 or +2 asynchrony) illustrated that the maternal recognition of pregnancy signal can prevent luteolysis much later in dioestrus than had been considered previously.

Notes

DISTRIBUTION OF ACTIVE MITOCHONDRIA IN DOG OOCYTES BEFORE AND AFTER IVM

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The nuclear and cytoplasmic maturation of mammalian oocytes is a complex and well-orchestrated process involving redistribution of chromosomes and organelles. In particular, organization and continued metabolic activity of mitochondria are necessary features of cytoplasmic maturation and resumption of meiosis. It appears that there are large differences in the timing of changes in activity and in the distribution of mitochondria during oocyte maturation among species (cattle, human, sheep, goat, pig). The distribution of active mitochondria may be indicative of the energy or ion requirement of various key events during mammalian oocyte maturation, fertilization and early embryo development. In this study, we evaluated mitochondrial distribution and activity in dog oocytes before and after *in vitro* maturation (IVM).

COCs (n=365) were collected from ovaries after ovariohysterectomy, 315 of them were incubated in a maturation medium and assigned to the control group (n=115) and to two experimental groups (n=250). The COCs of the control group were matured in a basic medium Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham(DME/F-12), 15mM HEPES, 25 mM Na-pyruvate, penicillin, streptomycin, FSH (0.5µg/ml), LH (0,5µg/ml) and estradiol (2.0 µg/ml). The oocytes in experimental group I were incubated using 100ng/ml EGF and 3% BSA, whereas in the experimental group II the culture medium was supplemented with 100ng/ml EGF, 3% BSA and 2.0µg/ml progesterone. After 0, 24, 48 and 72 h IVM, COCs were processed to evaluate active mitochondria aggregation and chromatin configuration in oocyte. COCs were incubated for 30 min in phosphate-buffered saline (PBS) containing 3% BSA and 200 nM MitoTracker Orange CMTM Ros (Molecular Probes) under culture conditions. The mitochondrial-specific fluorescent and cell- permeant probe MitoTracker Orange – fluorescent tetramethylrosamine is readily sequestered only by actively respiring organelles, depending upon their oxidative activity. After exposure of the COCs to the probe, cumulus cells were mechanically removed from the oocytes by repeated pipetting and subsequent treatment with 3% sodium citrate. The denuded oocytes were washed three times in pre-warmed PBS without BSA and fixed for 15 min at 37^{o}C using freshly prepared 2% of paraformaldehyde. After fixation, the oocytes were washed three times in PBS and then mounted between slide and cover slip in Vectashield with DAPI. Statistical analysis was performed with PROC FREQ in SAS 9.1 (SAS Institute Inc., Cary, NC 1998) using Chi-square test of goodness of fit.

Immediately after oocytes collection we observed peripheral, perinuclear, homogenous and mixed distributions (72, 14, 10 and 2% resp.; $p<0.001$). The percentage of oocytes with peripheral distribution, after maturation in medium with EGF and BSA was 46.6, 16.7 and 16.7% ($p<0.05$) respectively, after 24, 48 and 72 hours of incubation. We observe also a high percentage of oocytes with aggregation of mitochondria in perinuclear distribution after 48 hours of incubation, respectively 13.3%, 56.7% and 50% ($p<0.05$) in the control group, and both experimental groups.

Notes

THE RETROSPECTIVE SCIENTIFIC RESULTS OF THE RESEARCH CONCERNING REPRODUCTION BIOTECHNOLOGIES IN SHEEP AND GOAT IN ROMANIA

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In Romania, the assisted reproduction technologies in sheep and goats (ART) have been used since 1978 with the purpose of maximizing the productive potential in small ruminants. The first generations of reproduction biotechnologies, the synchronic induction of estrus with artificial insemination in succession, have been successfully applied in Romania since the 1960s-1970s by Paraschivescu Marcel and Otel V (1964). After 1978, the technologies for collection, dilution, preservation and use of semen were doubled by the more intense use of second generation biotechnologies, semen freezing and embryo transfer. In parallel with the technological and scientific development of the reproduction biotechnologies, after 1994 the following began to be used: the biotechnologies associated to the embryo transfer such as *in vitro* fertilization and embryonic bisection (Zamfirescu S., 1979,1987,2000,2001). The ART advantages are unanimously recognized (sanitary and veterinary, zootechnical, scientific and economical), but their application in practice is still limited because of the financial obstacles but also because of the lack of super-qualified labor. The researches done in Romania in the field of artificial insemination in sheep and goats have been extremely numerous such as: the optimization of dilution media, the optimal gycerol level which ensures the protection of the sperm cells, the freezing of the buck semen with or without seminal plasma, the establishment of the best methods and type of freezing, the optimization of the insemination methods for cryopreserved semen and the quality assessment of frozen-thawed semen by different methods (Zamfirescu S., 2000,1998). Our results in the freezing of the ram and buck semen are very good, comparable to those obtained at international level (Leboeuf B, 1998). We founded a cryobank for ram and buck semen (over 10 000 straws/stock), which served to deposit semen from active populations and from population in genetic preservation. Over the past 15 years, the results in the sheep and goat embryo transfer were from a number of 450 sheep, highlighting an ovulation rate of 3-12 after treatments with eCG and 5-16 after treatments of superovulation with FSHo and FSHp. The number of viable embryos was between 3-8, according to treatment and especially according to the individual variability of the donors. The pregnancy rates were between 45-65 % in sheep and 65-70% in goats. The embryo survival was between 35-32 % (Zamfirescu S., 2004)) *In vitro* fertilization is a very complex biotechnology studied on local sheep and goats in Romania. The oocytes are collected from ovaries of slaughtered females, are matured, fertilized *in vitro* and cultured for 6–7 days when the fertilized oocyte develops into preimplantation morula or blastocyst. In Romania, the coefficient of transformation from complex cumulus-oocytes *in vitro* maturation and fertilization is 73% (maturation) 47% (cleavage in 2-4 cells) 26 % (preimplantation morulae) and 9 % (blastocysts). This transformation of complex cummulus-oocytes is very unsatisfactory because it is conditioned by many factors.

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