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EUROPEAN EMBRYO TRANSFER ASSOCIATION

22ème COLLOQUE SCIENTIFIQUE

22nd SCIENTIFIC MEETING

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Dr Ray Newcomb

Special Celebration

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Zug, Switzerland, 8th and 9th September 2006

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C O N T E N T S

Dr Ray Newcomb

A.E.T.E. Medalist 2006

Ray Newcomb BVSc, MSc, FRCVS
A.E.T.E. Medalist 2006

At this year's meeting, the Society honours Ray Newcomb with the prestigious award of 2006 AETE Medalist and pays tribute to his contribution to the knowledge and practice of farm animal embryo transfer. This distinction, granted only to a select and eminently worthy few, has in the past been known as the "Pioneer Award." Ray, along with other celebrated contemporaries of an exciting scientific era, has indeed been one of the pioneers of our industry.

Born the son of a policeman in Hertfordshire, Ray would have been brought up in an atmosphere of principle, decency and endeavour; attributes which, along with a sublime sense of humour and modest self-deprecation, he retains to this day. He was educated at St Albans Grammar school and Liverpool University from where he graduated as Bachelor of Veterinary Science in 1962. He then spent six years in large animal practice in Cumbria and in Nottinghamshire intervened by two years teaching and research at the University College of Wales in Aberystwyth and at Liverpool University. His research at this time examined the seasonal patterns of sperm production in rams and sperm transport in female rabbits so from an early stage in his career, Ray was to become well-versed in the detailed mechanics of the mammalian reproductive tract.

After declining the offer of a partnership at the practice in Retford, Nottinghamshire, Ray chose to specialise in cattle reproduction and he was selected by the Milk Marketing Board of England and Wales to be Veterinary Officer in charge of an export bull stud at Writtle in Essex. There, he not only supervised semen collection and processing, fertility management and the welfare of resident bulls but he also carried out extensive literature investigation into subjects relevant to the MMB insemination service and the national cattle breeding programme. As a result of studies into methods of early pregnancy diagnosis, Ray was responsible for instigating the development of one of the first milk progesterone PD assays.

At the same time, Ray performed a nation-wide ophthalmologic survey on imported Charolais cattle and their progeny to identify the prevalence of colobamata. This condition, an inherited polygenic trait, had been previously undetected but two Charolais that were totally blind and others with varying degrees of visual defects and resultant hyperexcitability were delivered to the UK during the early imports. The revelations of the existence of colobomas resulted in a temporary export ban on British Charolais semen but subsequent screening and culling programmes, since Ray's initial investigations, have reduced the incidence of this condition to being currently negligible and no longer routinely investigated by the Charolais Society.

In 1972, Ray Newcomb was awarded the Milk Marketing Board Veterinary Research Fellowship to assist the internationally acclaimed scientist, Mr L.E.A.("Tim") Rowson with

his research into bovine embryo transfer at the Animal Research Station in Cambridge. It was here, for the next eight years, that Ray, in association with W.B.Christie, A.O.Trounson, S.M.Willadsen, I.Wilmut, H.R.Tervit, A.Brand and others, produced the work and numerous publications on superovulation and fertilisation, technique of egg recovery and transfer, donor and recipient synchronisation, the use of prostaglandin F2alpha, embryo manipulation, culture and storage, foetal survival and pregnancy; all of which have since become the stuff of day-to-day routine of practitioners throughout the world who make their living out of embryo transfer. The Cambridge team was by no means alone at this time in pursuing the progression of farm animal embryo transfer towards a practical and commercially adaptable reality. There was cooperation and some degree of rivalry amongst researchers internationally, all who were working with inspired optimism after several years of relative disinterest in this field of animal reproduction. Many of these colleagues from different countries visited Ray in Cambridge on more than one occasion during these years to check on the latest progress in his work.

After the first report of a calf born from a transplanted bovine embryo in 1951 by Willet, Black et al., subsequent achievements in ET were slow to happen. The inevitable limitations of early methods of superovulation, the impracticalities of surgical procedures and persistently poor results from transfers led to the feeling throughout the sixties and early seventies that embryo transfer would stay in the doldrums and probably not advance beyond the level of experimentation. The work that followed by Newcomb, Rowson, Christie and others in Cambridge gave way to renewed enthusiasm in the quest for improved embryo transfer techniques. It was not insignificant that this time of fresh optimism was concurrent with a boom in the popularity of European (Exotic) breeds of cattle, particularly in the USA and Britain, and the potential commercial windfall.

Ray's early work at Cambridge with cattle and sheep embryos was predominately surgical with both donor collection and recipient transfer performed by laparotomy but he was soon to be at the front-line of the later transition from surgical to non-surgical methods. One of his earlier publications (1) describes, with explicit illustrations, his method for the simultaneous flushing of ova from the bovine oviduct and uterus and it is this technique that is still used in sheep, goats and deer where surgery remains necessary for donor embryo collection. It is by way of this surgical approach and a series of exquisite experiments involving utero-tubal and uterine ligation, that Newcomb and colleagues (2) were able to ascertain the timing, developmental stage, position and survival of superovulated eggs entering the uterus. Without this knowledge and a further understanding of the synchrony of egg and uterus (3), the evolution of procedures towards successful non-surgical recovery and transfer would inevitably have been protracted. In the region of 450 surgical donor flushes and their resultant surgical recipient transfers were performed at Cambridge each year, consequently Ray's expertise and hands-on familiarity with the internal and external features of the bovine genital tract would have been unequalled at that time.

The two major theses that Ray produced while at the AnimalResearch Station were "Investigation of Factors Affecting Superovulation, Egg Recovery and Transfer in Cattle", a dissertation submitted to the University of Cambridge for the degree of Master of Science in 1976 and "Non-Surgical Recovery and Transfer of Bovine Ova" submitted for Fellowship of the Royal College of Veterinary Surgeons in 1979. These two works, enhanced by other

associated contemporary publications, in particular those co-authored by Christie and Rowson, covered the breadth of early procedures and techniques that were the basis of the subsequent development of practical embryo transfer. Much of what we, as practitioners, now take for granted was painstakingly fathomed from the uncharted territory that was being explored at Cambridge in the seventies. Today we unquestioningly start superovulation treatments at the mid-cycle stage. We probably rarely consider that the finding-out of this now universally accepted schedule involved a vast amount of work and collaboration with other authors over a considerable period of time. Prior to the involvement of prostaglandin, superovulation, then with PMSG, was started on day 16 of the cycle which resulted in unpredictable timing of oestrus and consequent difficulties with recipient synchronisation. Ray's following work incorporating PGF2alpha into the superovulation regime fine-tuned the optimal response to treatment at the best time of administration for the maximum output of embryos. Will Christie and Ray also investigated donors' ovulation and egg recovery rates after several repeated superovulation programmes (4). Methods of inducing twin pregnancy for what was intended as a potential increase in national beef production formed an integral part of Ray's MSc thesis and an in-depth presentation at the University of Nottingham in 1975 (5). In 1978, the first calves from oocytes matured to metaphase II in-vitro and fertilised in-vivo were produced as a result of work by Newcomb and Christie (6).

Ray's dissertation for Fellowship covered every conceivable aspect of the progression of non-surgical embryo recovery and transfer from the development of the three-lumen catheter, still the most sophisticated of any non-surgical embryo flushing catheter (7), to the ipsilateral uterine horn transfer with the modified Cassou insemination gun that we use today. Trials were done to compare surgical vs. non-surgical recovery and transfer techniques (8), catheter position for maximum recovery, optimal uterine placement of embryos transferred, the survival of embryos after ipsi- or contra-lateral horn transfer, different days for transfer after oestrus and different stages of embryo development for best pregnancy rates. Those of us who have since been involved in commercial bovine embryo transfer benefit every day of our working lives from the very considerable amount of work that went into reaching the conclusions of these investigations.

With the retirement of Mr Tim Rowson in 1980 and the successful progression of embryo transfer methods to practical on-farm adaptation, the ET research team at Cambridge was disbanded. Ray then launched the Milk Marketing Board Embryo Transfer Service, the major ET organisation in the UK at the time, which he ran single-handed for the next six years. At the same time, he was involved in the development of a successful method for the direct transfer of frozen bovine embryos, several years before such methods became commercially available. He also started an embryo transfer club for Eastern Counties Farmers, a large farmers' cooperative where clients from all over the south east of England would transport their donors and recipients to a central market place. In addition, Ray started the first commercial sheep embryo transfer project in the south of England with his colleague, John Hooton. On top of all his work throughout the UK, he has cattle handling facilities and an ET unit at his home in the village of Leavenheath where he has catered for resident and transient donors and recipients for the past 25 years.

During his time at Cambridge and in the following years in commercial ET, Ray has helped and trained people from all over the world in the technique and practice of embryo transfer. He has travelled extensively to China, USA, South Africa, Zimbabwe, Canada, Chile, Peru, New Zealand, Lithuania, Japan and many European countries to lecture, instruct and solve problems for overseas embryo transfer organisations. He has also supervised several export and import projects.

The farm where Ray and his wife, Norma, live in Suffolk is an area of beautiful rolling grassland and woods in an otherwise rather flat and agricultural part of England. As busy as Ray has been away from home, it is hard to imagine how he has been able to find time to look after his ever-expanding herd of beef cattle. To his obvious delight, with fewer commitments away, he has been able to do so much more recently and he is at his most contented when at home on his farm.

Ray Newcomb has been a long-standing member of the A.E.T.E. and was the UK Board Member and former Vice-President. It is a mark of the respect in which he is held and of his international reputation in the field of farm animal reproduction and embryo transfer that Ray's selection, from a list of other deserving and admired candidates, as recipient of this year's A.E.T.E. medal, was uncontested by the Board members from each represented country at the meeting in Hungary last September. We congratulate him and wish him every good fortune.

Robert Brittain

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THE EVOLUTION OF PRACTICAL EMBRYO TRANSFER AT CAMBRIDGE

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Walter Heape was born in Lancashire, England, in 1855. He became a student of histology and botany and then embryology at Cambridge. His first embryo transfer experiment was performed in 1890 with the help of Samuel Buckley, a well known surgeon of Manchester. Two ova were obtained from an Angora doe rabbit, which had been fertilised by an Angora buck 32 hours previously. The ova were recovered on the point of a spear-headed needle and immediately transferred into the fallopian tube of a Belgian Hare doe rabbit, which had been fertilised 3 hours before by a buck of the same breed as herself. In due course, this Belgian Hare doe gave birth to 6 progeny. Four of these resembled herself and her mate, while two were undoubted Angoras – they had long silky hair, peculiar to the breed and were true Albinos. Heape concluded from this experiment that a uterine foster mother has no power of modifying the breed of her foster progeny. The relationship between foster mother and offspring fascinated him and while at Cambridge he had a formative influence on the new science of reproductive biology. He urged that reproductive physiology be recognised as a scientific speciality and that research into animal breeding should be organised and funded by government.

F.H.A. Marshall was a reader in the School of Agriculture of the University of Cambridge in the early 1900's. He was a distinguished reproductive physiologist and wrote the first edition of his book "The Physiology of Reproduction", which later became the "bible" for reproductive physiologists. Sir John Hammond was first a student of Marshall's and then worked with him on subjects such as the oestrous cycle. Later he would become one of the greatest agricultural scientists of the 20th century and did more than anyone to apply science to the improvement of animal production. The Animal Research Station was established in 1932 at the Huntingdon Road, Cambridge, as a small 'field station' for F.H.A. Marshall, John Hammond and Arthur Walton. Hammond and Walton successfully worked on artificial insemination but were unable to obtain support for this from the Ministry of Agriculture simply because of objections raised by pedigree bull breeders and moralists. It was not until the U-boat onslaught on shipping during the Second World War which caused major food shortages in Britain, that support was given for the development of artificial insemination to increase national milk production. This led to the establishment in Cambridge, in 1942, of the first artificial insemination centre in Britain, The Cattle Breeding Centre, of which L.E.A. (Tim) Rowson was Director. The technology was highly successful and developed rapidly. In the early 1950's, A.I. was revolutionised by the development of methods of semen freezing at very low temperatures by Chris Polge, Audrey Smith and Alan Parkes at Mill Hill in London. The technique was then applied at the Cattle Breeding Centre in Cambridge. The first calf to be born from frozen semen was named "Frosty".

One of the chief objectives of Hammond's Animal Research Station (ARS) was to develop techniques for superovulation and embryo transfer in farm animals, to achieve for female animals what A.I. had achieved for males, in the hope that this would lead to much greater progress in animal breeding. Rowson joined the staff of the ARS on a part-time basis to develop the technology in farm animals. Hammond retired in 1955 and Thadeus Mann (with considerable knowledge of the biochemistry of semen) was made Director. This caused some concern within the farming community, but their fears were allayed by the appointment of two practical deputy directors, Rowson and Walton. In many ways Rowson was way ahead of his time. He had developed a non-surgical collection device in 1949 with his colleague Dr. Des Dowling, which was commercially available from Arnolds and with which he obtained a 50% embryo recovery rate. It was in fact a very cumbersome device made of red rubber with a metal stylet insert to stiffen it. It was at least 1.5m long, difficult to sterilise and waved around, impeding one's sensitivity and ability to pass it through the cervix dextrously. In the same year (1949), the National Egg Transfer Breeding Conference was held at San Antonio in Texas. Some popular articles were suggesting that 30 calves per year might be obtained from the best cows (Hervey 1949).

The first report of a calf having developed to term from a transplanted bovine embryo appeared in 1951. These early workers used homologous serum to transfer embryos. It wasn't until 1969 that the ground-breaking success of Rowson and colleagues was published. They compared the pregnancy rate when using Medium 199 or bovine serum in the surgical collection and transfer of embryos. The comparison was incontrovertible and absolute. Using serum, there were no pregnancies; using Medium 199, the success rate was 91%. It is extremely surprising that the toxicity of bovine serum to embryos had not been recognised because it had been observed by Chang 20 years earlier in 1949; a lesson to us all, to "read the literature." Chang started his PhD at Cambridge in 1939 under the direction of Hammond and Walton and was a regular visitor to the Animal Research Station.

Rowson's early success set interest in embryo transfer ablaze. This coincided with great interest in the English speaking countries in European (exotic) breeds of cattle. The first training course on Embryo Transfer was run by Rowson, Brand & Tervit in the late spring of 1972 which I was privileged to attend along with other veterinarians from Britain, Canada, USA, Ireland and New Zealand. In October that year, I was delighted to receive a Milk Marketing Board (MMB) Senior Veterinary Research Fellowship at the Animal Research Station, initially for a period of three years, which ultimately extended to eight years. Tim Rowson's health was not robust at that time and my brief was to support him by doing much of the routine preparation of cattle and surgical recovery of embryos and to assist another MMB scholar, Ian Wilmut, by supplying him with embryos for his freezing experiments. The first calf from frozen semen at Cambridge, "Frosty," stimulated Chris Polge, together with veterinary colleagues from the MMB and French colleague Robert Cassou, to move to a completely frozen insemination service in England and Wales in 1972. In 1973, "Frosty 2nd" was born from a blastocyst frozen and thawed by Ian Wilmut at the Animal Research Station. I supervised the birth of this calf which was to generate huge national interest. At the Royal Show that year, Ian attended the important first days of the show and I covered the rest. Even towards the end of the show, the press were clamouring for information and schoolchildren continued to file in to see the miracle calf, "Frosty 2nd". In many ways, it was a miracle calf

because it resulted from the transfer of a Day 10 frozen embryo, which is far from being the most suitable developmental stage to freeze.

Tim Rowson and Ian were later both awarded the highest British scientific award of FRS, Fellowship of the Royal Society, along with Thadeus Mann, Robert Moor and Christopher Polge. This was undoubtedly a most commendable achievement in such a small research station. That same year, in the summer of 1973, Ian Wilmut left the Animal Research Station for a permanent post in Edinburgh and was succeeded by a Dane, Steen Willadsen, a brilliant, hyperactive workaholic MMB scholar. On many a Monday morning, when other scientists had booked one of the three operating theatres to continue their experiments, they would arrive, only to find that not a single sterile surgical instrument was available. Steen had been working on his own all weekend. It was then a case of 'all hands on deck' to try to retrieve some order back into the working system of the unit by sterilising sufficient material to continue the days work. After a year 'feeling his way', Steen was able to improve the technique of freezing embryos. He was extremely dextrous and produced, with his micromanipulators, sheep/goat chimeras and the first farm animal clones. He would walk around with an embryo in a glass pipette attached to a rubber bulb and never alter his pressure on the bulb or lose an embryo until one occasion when I was flushing a cow at the Cambridge Veterinary School with Ramon Castro (Fidel's brother), the Minister of Agriculture for Cuba, in attendance. I only recovered one egg which was duly dispatched to Steen to split, who then proceeded to squirt the embryo accidentally onto the laboratory floor.

After taking up my fellowship at the Unit, I was 'thrown in at the deep end' and asked to deputise for Tim Rowson by visiting China in 1973 with a British Industrial Technology Mission along with 1000 British businessmen. I was required to give a three hour lecture on recent advances in artificial insemination followed by three hours of questions and a practical demonstration. I was also asked to establish an ET unit in Georgia USA in that same year. My visit to China was most interesting for we were some of the first Westerners to visit the country. US President Nixon had visited previously followed by Britain's Prime Minister, Sir Alec Douglas-Hume. At the fantastic concert and circus performances that were put on for the British contingent, it must have been thought that we all came from the aristocracy because the Eton Boating Song was always played as a musical introduction. During a trip to the zoo, we were of far more interest than the hairy inmates in cages. From China, I flew to Hong Kong, then on to Japan to visit Sugie Soma and Fukumitsu. They had already achieved success with minor surgical transfer and had a recovery device which was beautifully made but had some inherent problems with its design.

On my return to Cambridge, I combined some features of the Sugie apparatus with those of the Rowson/Dowling non-surgical recovery device. Initially, we made embryo recovery catheters within the ARS workshop with the assistance of the multi-skilled Paul Miles, workshop manager. Ultimately, with all the charm I could muster, I managed to persuade a British manufacturer to make catheters for me. Unlike the Americans as a race, the Brits are extremely cautious and only embark on new projects somewhat tentatively. The device worked extremely well with many advantages over other devices particularly with regards to collection as a sterile procedure. When demonstrated in Onderstepoort, South Africa, this 3-way catheter became the method of choice for collecting sterile uterine samples. However

supplies of the catheter were erratic and publication on its use only ensued years after its initial development (Newcomb, Christie & Rowson 1978).

At that time, the standard procedure for the induction of superovulation at Cambridge was to use PMSG as gonadotrophin. In the past, all PMSG treatment started in the follicular phase on Day 16 of the oestrous cycle and then one had to wait for the cow to come into heat which caused considerable problems in obtaining sufficient numbers of recipients for any reasonable trial work. Fortunately, the studies of Rowson & Moor on luteolysis in sheep made the ARS the ideal location for manufacturers to investigate the value of their prostaglandin. Initially, their products were so lacking in potency that they could only induce luteolysis when injected non-surgically into the uterine horn ipsilateral to the corpus luteum. Imperial Chemical Industries (ICI) were the most progressive organisation in this field despite the first prostaglandin being developed by the Americans. Ultimately, we had plenty of products from ICI, Upjohn & May & Baker and they were sufficiently potent to induce luteolysis systemically by intra-muscular injection. Some products caused ischaemia and necrotic areas in muscle which we were able to examine after the animals had been slaughtered, giving useful feedback to the manufacturers. The availability of prostaglandins (PgF2alpha) analogues and epimers enabled me to determine the interval required between the injection of gonadotrophin and oestrus for optimal results (4 days) and to determine the optimum stage of the oestrous cycle on which to commence gonadotrophin treatments to obtain maximum superovulation (mid-cycle). Also, the availability of prostaglandins enabled a more controlled ovulation to be achieved in attempts to produce twins, at which Gordon, William & Edward (1962), were unsuccessful in their very large MMB trial in Wales. For all their efforts, they simply demonstrated the great variability of superovulation. My approach to controlled ovulation to induce twin pregnancy was, to a large extent, achieved by giving sufficient PMSG to induce follicle stimulation but to allow insufficient time for all but the largest follicles to mature, by administering prostaglandin some 18 hours before treating with PMSG.

In my early years at Cambridge, I would glean experimental data from my routine embryo collection procedures for other scientists. At this time, all procedures were surgical under general anaesthesia and embryos were consistently flushed by exteriorising the uterus and securing it to a stainless steel bar. The oviduct was canulated via the fimbria, occluding the uterus with thumb and forefinger and forcing fluid through the oviduct via the canula into a round-bottomed egg collection cup. Looking at our collection results, it soon became apparent that our maximum recovery of embryos by the method described was achieved when eggs were recovered at Day 3 after oestrus. (Day 3: 73.6 +/- 3.1% cf. 58.2 +/- 6.5% on Day 4) I suggested to Mr Rowson that it might be worth recovering embryos only at Day 3 after oestrus if there were no disadvantages. Mr Rowson's reply was that there had been no problems in sheep at this stage and of the few cow transfers done at this time, the results were normal. On receiving this advice, we changed to what we believed would be a more efficient system. During my trip to Georgia USA, I set up operations on the same basis. On my return to Cambridge, we had had a period of few pregnancies and two months after the Georgia project, we were informed that my 7 weeks of work there had resulted in none of the recipients being in calf. As usual, the cause of such a failure was not immediately apparent because other changes had also been made such as the antibiotic levels in the media. In consequence, it took 10 months trial work to identify the problem. It became apparent that

there was an effect of age of egg on the ability to withstand the uterine environment. No pregnancies resulted when Day 2 embryos were transferred into a synchronous recipient uterus. Rather surprisingly, the transfer of Day 4 embryos into asynchronous recipients was more successful ($p<0.01$) than were Day 3 eggs transferred into synchronous uteri. (47% cf. 11.8%) The low conception rate resulting from the uterine transfer of Day 3 cow eggs may partly be due to their expulsion from the uterus but it does appear that Day 3 eggs are too immature to withstand the uterine environment. In a small experiment, we ligated the utero-tubal junction on one side and flushed from oviduct to uterus and then ligated the utero-tubal junction on the other. On recovery at Day 7 after oestrus, eggs secured within the oviduct were normally developed whereas many of those remaining in the uterus were degenerate. In sheep, most eggs are present in the uterus 66 hours after ovulation (Holst 1974) whereas in the cow, which ovulates about 12 hours after the end of standing oestrus, Hamilton and Laing (1946) could find no eggs in the uterus before 96 hours after the end of oestrus. Thus, although cleavage rates of eggs are approximately similar in relation to oestrus in both the sheep and the cow, sheep eggs are entering or are already present in the uterus at Day 3 but cow eggs of a comparable age remain in the oviduct for a further 24 hours. In order to examine where eggs are situated within the tract on different days after oestrus, a method of simultaneous flushing of different sites was devised. 123 heifers were used as donors in the trial. The proportion of eggs recovered from the oviduct rapidly declines between Days 3 and 5 after oestrus but thereafter only gradually decreases so that even at Day 7 and 8 after oestrus a small proportion of ova were still recovered from the oviduct. (Day 3: 96.5%, Day 5:17.4%, Day 6: 14.2%, Day 7: 7.9%, Day 8: 6.7%).

We were aware that there were significant hormone changes in superovulated animals and together with Derek Booth (Booth et al. 1975) we examined total unconjugated oestrogen and progesterone in superovulated crossbred Hereford heifers during and after superovulation. The results were both illuminating and dramatic, making me wonder how we ever recovered normal embryos. At Day 6 after oestrus, oestrogen levels were increased many fold compared with unsuperovulated heifers and the same applied for mid-cycle (post superovulation) levels of progesterone. When we examined intervals from prostaglandin-induced oestrus in heifers to subsequent oestrus, the mean interval was 21 days but after superovulation, the interval was 27 days. The level of oestrogen at Day 6 in superovulated heifers was 7-fold higher than at normal oestrus and it would appear that these high oestrogen levels might be the trigger for the next 21 day cycle. However, it must be realised that there is enormous variability in the interval to return-to-oestrus in superovulated heifers. We had some indication of the formation of corpora albicans-like structures after superovulation. Superovulated donors only rarely maintain a pregnancy in that cycle as the uterine environment is so abnormal hormonally. Most people, after flushing, will inject the donor with a normal luteolytic dose of prostaglandin. I prefer to follow the findings of Reuben Mapletoft that if one injects a small dose of prostaglandin after flushing, the hormone levels then become normal. If the donor does not subsequently return to oestrus at the appropriate time, it should be given a normal dose of prostaglandin. In my experience as a practitioner, when a donor has only one or two ovulations after superovulation treatment, the embryos are seldom recovered. I believe that this is because the abnormal hormone levels have interfered with normal tubal transport and that the embryos are locked in the oviduct.

At the Animal Research Station, surgical transfers were also made under general anaesthesia. The uterus was exposed and, after noting the position of the corpus luteum, eggs were transferred using a fire polished Pasteur pipette attached with a rubber tube to a 1ml or 2ml plastic syringe. The egg was drawn into the Pasteur pipette in a small volume of medium (0.05ml). The uterus was punctured with a blunt 18 guage needle 5cms from the utero-tubal junction and the tip of the Pasteur pipette introduced through this puncture and the contents expelled in the direction of the body of the uterus. Normally transfers were made at the tip of the uterine horn ipsilateral to the corpus luteum but no comparison had been made between transferring to the tip of the contralateral horn vs. the ipsilateral horn. In our studies, no pregnancies were established (Newcomb and Rowson 1976) after transfer to the contralateral horn only ($p<0.02$) but this experiment was conducted during the winter of 1974/75 at a time of fodder shortage in Britain. Similar results were obtained by Sreenan et al. (1976). After bilateral surgical egg transfer and where only one foetus survived, more ($p<0.005$) unilateral pregnancies established in the uterine horn ipsilateral to the corpus luteum than in the contralateral horn (10 ipsilsteral vs. 3 contralateral pregnancies). We had assumed that failures occurred soon after transfer to the contralateral uterine horn but our views were challenged by the publication by Del Campo (1977) which indicated that when eggs are transferred to the contralateral uterine horn, the embryonic signal maintaining the corpus luteum was transmitted via the uterine lumen to the ipsilateral horn. The signal is therefore very potent and is produced at a time when the embryo is quite small and located entirely within the contralateral uterine horn. In Del Campo's study, the recipients were slaughtered at D24. Repetition of this study by Christie et al. (1979) indicated that at slaughter on D24 or D26, the embryos which had been transferred at D7 still survived and had extended the life of the corpus luteum, yet in other animals which were examined at D42, only 20% remained pregnant. This was a surprising finding for the losses occurred when the embryo was comparatively large and the trophoblast extended throughout the length of both uterine horns. Examination of the survival of eggs transferred non-surgically to the ipsilateral uterine horn on day 7 after oestrus showed a similar pattern to that observed after surgical transfer to the tip of the contralateral uterine horn. When recipients were slaughtered at D16, before the normal time of luteolysis, a high proportion (80%) of these embryos still survived. At days 24-26 after the normal time of luteolysis, a smaller but still high proportion (67%) survived. Yet by D42, survival was reduced to 50%, some 15% lower than survival after surgical transfer. (Christie et al., 1980)

Human chorionic gonadotrophin (hCG) or progesterone has been given to recipients of surgical transfers to the contralateral horn (Christie et al.1979) and to non-surgical recipients (Christie et al. 1980). No effect on foetal survival was observed when progesterone was administered from D13 to D35 but daily treatments over the same period with hCG at a level known to extend the life of the corpus luteum (1000 iu daily) increased the foetal survival rate to that achieved after surgical transfer to the tip of the ipsilateral uterine horn. The mechanisms concerned are not understood but results suggest that the administration of some artificial signal in a slow release or sporadic release form, may in future ensure a high survival rate after non-surgical transfer to any site.

We (Newcomb, Christie & Rowson 1980) conducted an interesting trial transferring surgically single D7 embryos bilaterally to each uterine horn thus:

1. To the tip of the horn ipsilateral to the corpus luteum and to the tip of the contralateral horn.
2. To the ipsilateral tip and contralateral base.
3. To the ipsilateral base and contralateral tip.
4. To the ipsilateral base and contralateral base.
   (20 recipients in each group)

Maximum foetal survival occurred after transfer to the tip of the ipsilateral horn. All other sites had a lower embryo survival rate but were similar to one another. Twinning rates were virtually the same for all groups. Despite the fact that the tip of the ipsilateral uterine horn is the optimal site for embryo development, no attempt should be made to transfer to it non-surgically, for pregnancies will not ensue. (de la Rey. Personal Communication).

The interpretation of the experiment using hCG when an embryo was transferred to the contralateral uterine horn is somewhat speculative. It is known that in the bovine, an embryo must be present in the uterus by D16 or 17 after oestrus in order to extend the life of the corpus luteum. (Betteridge et al. 1976). In the sheep, the equivalent time is before Day 13 of the cycle. The early signal given by the bovine embryo in the contralateral uterine horn is probably luteotrophic and delivered via the lumen of the uterus to the ipsilateral uterine horn and transmitted via the same counter-current mechanism associated with the uterine luteolysis. How daily hCG treatment will allow for a normal pregnancy rate when the embryo has been transferred to the contralateral uterine horn is not easily explained but may be via some immunological effect of hCG or an antiluteolytic effect i.e., altering the nature of prostaglandin within the uterus thereby preventing luteolysis.

It was long believed that non-surgical transfer gave poor results compared to surgery because eggs were ejected by the oxytocic effect of traversing the cervix. Tervit (1973) at Cambridge injected radioactive microspheres into the uterus resulting in ejection but this only happened in the few days after oestrus. In my own studies, I cannulated the uterine vein and posterior vena cava but only up to Day 3 after oestrus did I get a release of PGF2alpha when I performed sham transfers. (Newcomb 1976) In addition, Prof Arie Brand (Brand et al. 1976), after leaving Cambridge, demonstrated that uterine myometrial electrical activity was quiescent by Day 4 after oestrus.

The Sacrewell Project.

The work of Hammond had demonstrated that in the horse, the size of the foal was determined by the uterine capacity and size of the mare used. The assumption was made that the same applied to the cow. We were asked to operate a joint exercise with the Cambridge Veterinary School and The Royal Agricultural Society of England, converting a Jersey herd at their Sacrewell Farm, in one generation, to a British Friesian Herd. Full Friesian weight calves were born from these Jersey cows with considerable dystocia problems when bull calves were born. The stockmen were extremely concerned but the project was very good for the Veterinary School. At that time they would normally only anaesthetise one or two cows per annum whereas at the ARS, we would anaesthetise in excess of 450 heifers per annum. The social interaction between the parties involved was very good but the Vet School, doing everything to the letter, were painstakingly slow. Whereas, at the ARS, we would

anaesthetise and intubate a cow within 5 minutes, on one occasion, the Vet School team took 45 minutes to do the same. Waiting for them was fully rewarded if only to see the saucy glint in Tim Rowson's eyes as various attractive young lady vet students struggled in their attempt to insert an endotracheal tube. The dystociae during this project were of great concern to the Jersey Society and they requested that I write an article for their journal to warn members of the problem.

Tim Rowson, Bill Christie and I had developed an understanding of much of the physiological influences on embryo transfer, an efficient non-surgical recovery apparatus and knowledge of the best time to transfer eggs. My masters, the officials of the Milk Marketing Board, quite rightly wanted assurance that these findings could be applied to field (on farm) conditions with the same success. We were fortunate to use the facilities of a large herd of 2500 cows . Our conclusions were as follows:

- Non-surgical recovery and transfer of bovine ova could be performed successfully in the field.
- An efficient recovery of ova could be obtained from glass collecting vessels if a protein source is included in the recovery medium.
- Using a 3-lumen catheter, recovery of ova was equally efficient from anaesthetised and from sedated standing donors. In heifers, the best results ($p<0.001$) were obtained when the tip of the catheter was placed near the utero-tubal junction. The proportion of ova recovered non-surgically was found to be less than but similar to surgical recovery.
- Donors repeatedly superovulated on up to 10 occasions continued to respond. Ovulation rate was lower after the second than after the initial superovulation ($p<0.05$) but no further reduction was observed.
- In lactating Friesian cows, ovum recovery rate was higher ($p<0.01$) at D7 using the 3-lumen catheter than using a 2-lumen system. At D8, there was no advantage. With neither method did catheter position influence success in cows.
- Fewer unfertilised ova were obtained after insemination with semen from a highly fertile bull than from a bull of average fertility ($p<0.05$). The percentage of unfertilised ova was greater ($p=0.06$) when insemination was only carried out on the first day of oestrus but insemination after the end of oestrus reduced the proportion of ova recovered ($p<0.03$)
- Neither ovarian response nor ovum recovery was influenced by the gonadotrophin used to induce superovulation. However, the administration of hCG at oestrus increased ($p<0.05$) the percentage of ova recovered.
- The proportion of ova recovered tended to be higher when ovulation rate was low. The proportion of viable ova tended to be better from cows in good condition.
- A within animal comparison of surgical and non-surgical transfer methods resulted in a higher ($p<0.005$) foetal survival after the surgical transfer but this did not increase the survival of the non-surgically transferred ovum.
- Transfer to the tip of the ipsilateral horn was more successful ($p<0.25$) than transfer to the base. After bilateral transfer to the tip or base of each horn, foetal survival was also greatest at the tip of the ipsilateral horn.

- In a high proportion (5/9) of recipients carrying twin foetuses after transfer of one ovum to the tip and one to the base of the ipsilateral horn, one of the two had transmigrated.
- More recipients became pregnant after mid-ventral surgical transfer under general anaesthesia than after flank surgery standing ($p<0.05$).
- A high proportion of embryos survived after non-surgical transfer until the normal time of luteolysis. The survival of non-surgically transferred ova was increased when recipients were treated with HCG.
- A between animal comparison of surgical and non-surgical methods failed to demonstrate any difference in success. The age of ovum transferred did not affect results. Eight of ten lactating cows became pregnant after non-surgical transfer with a modified Cassou insemination gun.
- There was a significant effect ($p<0.05$) of the tailhead condition score of recipient heifers on the resulting pregnancy rate. In those animals with a tailhead condition score of 2.0 and <2.0, the pregnancy rate was 40.9% (27/66) whereas recipients with one condition score better, >2.0, the pregnancy rate was 60.9% (25/41)

As a finale to our years at Cambridge, we managed to achieve an in-vitro maturation of oocytes collected from the slaughterhouse, to fertilise them in-vivo and after transfer, to obtain two calves. Working with Tim Rowson had been a great pleasure. The four years that I spent working with Bill Christie was undoubtedly the most enjoyable and productive working relationship of my life. It is with some concern that I am the one to deliver this address when we both worked so closely together and where Bill played such a major role during these years at Cambridge.

I left the Animal Research Station in 1980 after eight happy and eventful years. Some technical staff went into human IVF clinics. Bill Christie and myself went into commercial Embryo Transfer. Apart from brief commercial activity generated by "Mastercalf" the direction of the ARS's research became progressively more fundamental, with the mouse being used as a model. Twink Allen, in his usual uncompromising manner, forecast that if the ARS continued to use the mouse and if large animals disappeared from the agenda, it would close. It is very difficult for the agricultural community to grasp the relevance of fundamental studies but the weight of their opinion is crucial in applying pressure on funding bodies to support research. Unfortunately, Twink's prophecy was fulfilled when, in l986, the staff of the Animal Research Station were moved to the Babraham Institute and a new department of Molecular Embryology was formed under the leadership of Bob Moor. This marked the end of the work at the ARS, as many had known it, during the preceding 50 years. In its closing years, Chris Polge was the director. Chris, who is 80 this year and whose work was so outstanding had, as he put it, "the good fortune to work at the Animal Research Station for 40 years."

For me life after Cambridge involved many trips abroad teaching and advising other veterinarians. I was also involved in a large twin pregnancy study using E.T. at an experimental husbandry farm in Aberystwyth, Wales. Twinning in cattle has never really taken off, but the Aberystwyth experience was that singlet calves grew at 1.3 kg per day whereas each twin grew at 1.1 kg per day, which is a very distinct advantage over single

suckling. Other practical studies involved the successful development of the direct transfer of frozen thawed embryos for the MMB.

The Animal Research Station is now history. According to Wolfgang Jochle (1983), "Knowledge of history allows the conquest of the future." The future is now in the hands of others. I am told that retirement is a very enjoyable experience. I am still trying to find that time but I am most fortunate to be blessed with my own land around me and enough bovines to keep me fascinated by them. Our family of four boys, who all have excellent careers, live close enough that they and the next generation, our two grand-daughters, can come home now and then to enjoy some of their mother's culinary excellence. They are all resigned to my convenient incompetence in the kitchen.

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National Statistical Data of Bovine Embryo Transfer Activity in Europe in 2005

Table 1: Embryo Transfer Activity in 2005

COUNTRY: **AUSTRIA**

A.E.T.E 2006
Data collected by
Dr. Wetchy Gabi

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	39	B / A=	9.5
	Embryos collected	**B**	372	C / A=	5.2
	Embryos transferable	**C**	201	C / B=	54.0%
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 *in vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	201	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	42		
In vivo	Frozen	**I**	102		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	144	H+I+J+K=	
Number of frozen stored embryos		**M**	273		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	70.8%	(I+K) / L=	

Number of E.T. calves born (2005)

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

Table 2: Embryo Transfer Activity in 2005

COUNTRY: BELGIUM ***A.E.T.E 2006***

Data collected by
Dr. Beckers Jean-François

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	451	B / A=
	Embryos collected	**B**		C / A= 4.8
	Embryos transferable	**C**	2166	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 *in vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**	2166	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	600	
In vivo	Frozen	**I**	1519	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	2119	H+I+J+K=
Number of frozen stored embryos		**M**	1273	
% of *in vitro* embryos transferred		**N**		(J+K) / L=
% of frozen embryos transferred		**O**	71.7%	(I+K) / L=

Number of E.T. calves born (2005)

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

Table 3: Embryo Transfer Activity in 2005

COUNTRY: **CROATIA**

A.E.T.E 2006
Data collected by
Dr. Karadjole Martina

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= 4.8
	Embryos transferable	**C**		C / B=
In vitro	Nb of oocyte donors		8	
(OPU)	Nb of OPU sessions		8	
	Nb of transferable embryos	**D**	52	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	52	=(D+E)
Total number of transferable embryos		**G**	52	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	66	
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**		(I+K) / L=

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	6
Number of calves born from *in vitro* embryos	
Total	6

Table 4: Embryo Transfer Activity in 2005

COUNTRY: **CZECH REPUBLIC** ***A.E.T.E 2006***

Data collected by
Dr. Pytloun Jaroslav

Total number of approved E.T. teams in the country	6
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1151	B / A=	10.1
	Embryos collected	**B**	11681	C / A=	4.6
	Embryos transferable	**C**	5322	C / B=	45.6%
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**	113		
Total i*n vitro* embryos		**F**	113	=(D+E)	
Total number of transferable embryos		**G**	5435	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3307		
In vivo	Frozen	**I**	2192	39.9% frozen	
In vitro	Fresh	**J**	22		
In vitro	Frozen	**K**	54	71.1% frozen	
Total embryos transferred		**L**	5575	H+I+J+K=	
Number of frozen stored embryos		**M**	1917		
% of *in vitro* embryos transferred		**N**	1.4%	(J+K) / L=	
% of frozen embryos transferred		**O**	40.3%	(I+K) / L=	

Number of E.T. calves born (2005)

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

Table 5: Embryo Transfer Activity in 2005

COUNTRY: **DENMARK** ***A.E.T.E 2006***

Data collected by
Dr. Callesen Henrik

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	688	B / A=	11.0
	Embryos collected	**B**	7578	C / A=	7.3
	Embryos transferable	**C**	5034	C / B=	66.4%
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**	5034	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2512		
In vivo	Frozen	**I**	1698		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	4210	H+I+J+K=	
Number of frozen stored embryos		**M**	2423		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	40.3%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	1701
Number of calves born from *in vitro* embryos	-
Total	1701

Table 6: Embryo Transfer Activity in 2005

COUNTRY: **ENGLAND** ***A.E.T.E 2006***

Data collected by
Dr. Liddle Alison

Total number of approved E.T. teams in the country	19
Number of teams providing data	9

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)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		(fresh + frozen)
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	2211	H+I+J+K=
Number of frozen stored embryos		**M**	3073	
% of *in vitro* embryos transferred		**N**		(J+K) / L=
% of frozen embryos transferred		**O**		(I+K) / L=

Number of E.T. calves born (2005)

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

Table 7: Embryo Transfer Activity in 2005

COUNTRY: **ESTONIA** ***A.E.T.E 2006***

Data collected by
Dr . Jaakma Ülle

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5	B / A=	4.2
	Embryos collected	**B**	21	C / A=	3.4
	Embryos transferable	**C**	17	C / B=	81.1%
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**	17	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	4		
In vivo	Frozen	**I**			
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	4	H+I+J+K=	
Number of frozen stored embryos		**M**	13		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	0	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	4
Number of calves born from *in vitro* embryos	
Total	4

Table 8: Embryo Transfer Activity in 2005

COUNTRY: **FINLAND** ***A.E.T.E 2006***

Data collected by
Dr. Mikkola Marja

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	478	B / A=	9.7
	Embryos collected	**B**	4647	C / A=	5.6
	Embryos transferable	**C**	2695	C / B=	58.0%
In vitro *(OPU)*	Nb of oocyte donors		8		
	Nb of OPU sessions		31		
	Nb of transferable embryos	**D**	44		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	5		
Total i*n vitro* embryos		**F**	49	=(D+E)	
Total number of transferable embryos		**G**	2744	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	927		
In vivo	Frozen	**I**	1462	61.2% frozen	
In vitro	Fresh	**J**	34		
In vitro	Frozen	**K**	-		
Total embryos transferred		**L**	2423	H+I+J+K=	
Number of frozen stored embryos		**M**	1476		
% of *in vitro* embryos transferred		**N**	2.3%	(J+K) / L=	
% of frozen embryos transferred		**O**	60.3%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	1093
Number of calves born from *in vitro* embryos	
Total	1093

Table 9: Embryo Transfer Activity in 2005

COUNTRY: FRANCE

A.E.T.E 2006
Data collected by
Dr. Guérin Bernard

Total number of approved E.T. teams in the country	28
Number of teams providing data	27

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5988	B / A=	9.2
	Embryos collected	**B**	55253	C / A=	5.3
	Embryos transferable	**C**	31452	C / B=	56.9%
In vitro *(OPU)*	Nb of oocyte donors		48		
	Nb of OPU sessions		90		
	Nb of transferable embryos	**D**	204		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	7082		
Total *in vitro* embryos		**F**	7286	=(D+E)	
Total number of transferable embryos		**G**	38738	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	15007		
In vivo	Frozen	**I**	13460	47.3% frozen	
In vitro	Fresh	**J**	113		
In vitro	Frozen	**K**	18	13.7% frozen	
Total embryos transferred		**L**	28598	H+I+J+K=	
Number of frozen stored embryos		**M**	15601		
% of *in vitro* embryos transferred		**N**	0.5%	(J+K) / L=	
% of frozen embryos transferred		**O**	47.1%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	8162
Number of calves born from *in vitro* embryos	41
Total	8203

Table 10: Embryo Transfer Activity in 2005

COUNTRY: **GERMANY** ***A.E.T.E 2006***

Data collected by
Dr. Cramer Hubert

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	2712	B / A=
	Embryos collected	**B**		C / A= 6.8
	Embryos transferable	**C**	18409	C / B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions		1557	
	Nb of transferable embryos	**D**	3399	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**	3399	=(D+E)
Total number of transferable embryos		**G**	21839	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	6275	
In vivo	Frozen	**I**	7456	54.3% frozen
In vitro	Fresh	**J**	1441	
In vitro	Frozen	**K**	?	
Total embryos transferred		**L**	15172	H+I+J+K=
Number of frozen stored embryos		**M**	2111	
% of *in vitro* embryos transferred		**N**		(J+K) / L=
% of frozen embryos transferred		**O**	49.1%	(I+K) / L=

Number of E.T. calves born (2005)

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

Table 11: Embryo Transfer Activity in 2005

COUNTRY: **GREECE**

A.E.T.E 2006
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**	2	B / A=	6.0
	Embryos collected	**B**	12	C / A=	5.0
	Embryos transferable	**C**	10	C / B=	83.3%
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**	10	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	10		
In vivo	Frozen	**I**			
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	10	H+I+J+K=	
Number of frozen stored embryos		**M**	45		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	100%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	2
Number of calves born from *in vitro* embryos	
Total	2

Table 12: Embryo Transfer Activity in 2005

COUNTRY: HUNGARY ***A.E.T.E 2006***

Data collected by
Dr. Solti Laszlo

Total number of approved E.T. teams in the country	10
Number of teams providing data	8

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	63	B / A=	9.5
	Embryos collected	**B**	596	C / A=	6.4
	Embryos transferable	**C**	402	C / B=	67.4%
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**	402	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	191		
In vivo	Frozen	**I**	1149		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1340	H+I+J+K=	
Number of frozen stored embryos		**M**	125		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	85.7%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	392
Number of calves born from *in vitro* embryos	
Total	392

Table 13: Embryo Transfer Activity in 2005

COUNTRY: IRELAND ***A.E.T.E 2006***

Data collected by
Dr. Lonergan Pat

Total number of approved E.T. teams in the country	6
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	202	B / A=	7.8
	Embryos collected	**B**	1609	C / A=	5.6
	Embryos transferable	**C**	1131	C / B=	70.3%
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**	1131	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	393		
In vivo	Frozen	**I**	720		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1113	H+I+J+K=	
Number of frozen stored embryos		**M**	738		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	64.7%	(I+K) / L=	

Number of E.T. calves born (2005)

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

Table 14: Embryo Transfer Activity in 2005

COUNTRY: ISRAEL ***A.E.T.E 2006***

Data collected by
Dr. Zeron Yoel

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	52	B / A=	3.0
	Embryos collected	**B**	156	C / A=	2.5
	Embryos transferable	**C**	128	C / B=	82.1%
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 *in vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	128	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	128		
In vivo	Frozen	**I**	21		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	149	H+I+J+K=	
Number of frozen stored embryos		**M**	30		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	14.1%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	38
Number of calves born from *in vitro* embryos	
Total	38

Table 15: Embryo Transfer Activity in 2005

COUNTRY: **ITALY**

A.E.T.E 2006
Data collected by
Dr. Lazzari Giovanna

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1120	B / A=	11.5
	Embryos collected	**B**	12880	C / A=	6.4
	Embryos transferable	**C**	7167	C / B=	55.6%
In vitro *(OPU)*	Nb of oocyte donors		116		
	Nb of OPU sessions		226		
	Nb of transferable embryos	**D**	791		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	3008		
Total *in vitro* embryos		**F**	3799	=(D+E)	
Total number of transferable embryos		**G**	10966	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2510		
In vivo	Frozen	**I**	3820	60.3% frozen	
In vitro	Fresh	**J**	200		
In vitro	Frozen	**K**	2032	91.0% frozen	
Total embryos transferred		**L**	8562	H+I+J+K=	
Number of frozen stored embryos		**M**	4380		
% of *in vitro* embryos transferred		**N**	26.1%	(J+K) / L=	
% of frozen embryos transferred		**O**	68.3%	(I+K) / L= %	

Number of E.T. calves born (2005)

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

Table 16: Embryo Transfer Activity in 2005

COUNTRY: **LITHUANIA** ***A.E.T.E 2006***

Data collected by
Dr. Kutra Jonas

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	0	B / A=
	Embryos collected	**B**		C / A=
	Embryos transferable	**C**		C / B=
In vitro *(OPU)*	Nb of oocyte donors		0	
	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**	0	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	0	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 (2005)

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

Table 17: Embryo Transfer Activity in 2005

COUNTRY: THE NETHERLANDS ***A.E.T.E 2006***

Data collected by
Dr. Landman Bas

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2720	B / A=	9.3
	Embryos collected	**B**	25233	C / A=	6.1
	Embryos transferable	**C**	16702	C / B=	66.2%
In vitro *(OPU)*	Nb of oocyte donors		198		
	Nb of OPU sessions		1846		
	Nb of transferable embryos	**D**	2889		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	72		
Total i*n vitro* embryos		**F**	2961	=(D+E)	
Total number of transferable embryos		**G**	19663	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2815		
In vivo	Frozen	**I**	10938	79.5% frozen	
In vitro	Fresh	**J**	852		
In vitro	Frozen	**K**	1003	54.1% frozen	
Total embryos transferred		**L**	15608	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	11.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	76.5%	(I+K) / L=	

Number of E.T. calves born (2005)

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

Table 18: Embryo Transfer Activity in 2005

COUNTRY: **NORWAY** ***A.E.T.E 2006***

Data collected by
Dr. Kummen Eiliv

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	20	B / A=	8.0
	Embryos collected	**B**	160	C / A=	5.3
	Embryos transferable	**C**	105	C / B=	65.6%
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**	105	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	45		
In vivo	Frozen	**I**	35		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	80	H+I+J+K=	
Number of frozen stored embryos		**M**	25		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	43.8%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	45
Number of calves born from *in vitro* embryos	
Total	45

Table 19: Embryo Transfer Activity in 2005

COUNTRY: **POLAND** ***A.E.T.E 2006***

Data collected by
Dr. Jaskowski Jedrzej

Total number of approved E.T. teams in the country	4
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	41	B / A=	9.9
	Embryos collected	**B**	406	C / A=	4.2
	Embryos transferable	**C**	172	C / B=	42.4%
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**	172	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	98		
In vivo	Frozen	**I**	19		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	117	H+I+J+K=	
Number of frozen stored embryos		**M**	79		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	16.2%	(I+K) / L=	

Number of E.T. calves born (2005)

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

Table 20: Embryo Transfer Activity in 2005

COUNTRY: PORTUGAL ***A.E.T.E 2006***

Data collected by
Dr. das Chagas e Silva Joao Nestor

Total number of approved E.T. teams in the country	7
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	75	B / A=	9.2
	Embryos collected	**B**	692	C / A=	5.3
	Embryos transferable	**C**	401	C / B=	57.9%
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**	918		
Total i*n vitro* embryos		**F**	918	=(D+E)	
Total number of transferable embryos		**G**	1319	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	212		
In vivo	Frozen	**I**	180	45.9% frozen	
In vitro	Fresh	**J**	11		
In vitro	Frozen	**K**	5	31.2% frozen	
Total embryos transferred		**L**	408	H+I+J+K=	
Number of frozen stored embryos		**M**	327		
% of *in vitro* embryos transferred		**N**	3.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	45.3%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	124
Number of calves born from *in vitro* embryos	
Total	124

Table 21: Embryo Transfer Activity in 2005

COUNTRY: ROMANIA *A.E.T.E 2006*

Data collected by
Dr. Zamfirescu Stela

Total number of approved E.T. teams in the country	5
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	18	B / A=	7.5
	Embryos collected	**B**	135	C / A=	4.8
	Embryos transferable	**C**	86	C / B=	63.7%
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**	86	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	69		
In vivo	Frozen	**I**			
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	69	H+I+J+K=	
Number of frozen stored embryos		**M**	17		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	0	(I+K) / L=	%

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	21
Number of calves born from *in vitro* embryos	
Total	21

Table 22: Embryo Transfer Activity in 2005

COUNTRY: **SPAIN** ***A.E.T.E 2006***

Data collected by
Dr. De la Fuente Julio

Total number of approved E.T. teams in the country	9
Number of teams providing data	8

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	559	B / A=	5.9
	Embryos collected	**B**	3279	C / A=	2.5
	Embryos transferable	**C**	1398	C / B=	42.6%
In vitro *(OPU)*	Nb of oocyte donors		2		
	Nb of OPU sessions		6		
	Nb of transferable embryos	**D**	20		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	20	=(D+E)	
Total number of transferable embryos		**G**	1418	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	449		
In vivo	Frozen	**I**	1134	71.6% frozen	
In vitro	Fresh	**J**	16		
In vitro	Frozen	**K**	4	20% frozen	
Total embryos transferred		**L**	1603	H+I+J+K=	
Number of frozen stored embryos		**M**	2258		
% of *in vitro* embryos transferred		**N**	1.2%	(J+K) / L=	
% of frozen embryos transferred		**O**	71.0%	(I+K) / L=	

Number of E.T. calves born (2005)

Number of calves born from superovulated embryos	455
Number of calves born from *in vitro* embryos	1
Total	456

Table 23: Embryo Transfer Activity in 2005

COUNTRY: SWEDEN ***A.E.T.E 2006***

Data collected by
Dr. Gustafsson Hans

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	292	B / A=	7.0
	Embryos collected	**B**	2044	C / A=	4.0
	Embryos transferable	**C**	1165	C / B=	57.0%
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**	1165	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	260		
In vivo	Frozen	**I**	978		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1238	H+I+J+K=	
Number of frozen stored embryos		**M**	330		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	79.0%	(I+K) / L=	

Number of E.T. calves born (2005)

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

Table 24: Embryo Transfer Activity in 2005

COUNTRY: **SWITZERLAND** ***A.E.T.E 2006***

Data collected by
Dr. Saner Rainer

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	319	B / A=	10.8
	Embryos collected	**B**	3449	C / A=	7.6
	Embryos transferable	**C**	2418	C / B=	70.1%
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**	2418	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	646		
In vivo	Frozen	**I**	1558	70.7% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	11		
Total embryos transferred		**L**	2215	H+I+J+K=	
Number of frozen stored embryos		**M**	1673		
% of *in vitro* embryos transferred		**N**	0.5%	(J+K) / L=	
% of frozen embryos transferred		**O**	70.8%	(I+K) / L=	

Number of E.T. calves born (2005)

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

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2005

I. EMBRYO PRODUCTION

(Data collected from 24 countries)

In vivo* produced embryos (superovulation)	
- number of flushed donors	16,995
- number of transferable embryos	96,581
- mean number per flushed donor	5.68
***In vitro* produced embryos:**	
From OPU	
- number of OPU sessions	3,764
- number of transferable embryos	7,399
- mean number per session	1.97
From slaughterhouse collected ovaries	
- number of transferable embryos	11,198
Total *in vitro*	18,597
Total number of transferable embryos	115,178

* Data from one country not available and not included.

(S. Merton, AETE Zug, 2006)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2005

II. EMBRYO TRANSFERS

(Data collected from 24 countries)

***In* vivo produced embryos ***	**Number of embryos transferred** 85,007 (36,500 fresh / 48,507 frozen)
***In vitro* produced embryos**	5,816 (2,689 fresh / 3,127 frozen)
Total number of embryos transferred	93,034
Proportion of IVF embryos transferred	6.3%
Proportion of frozen embryos transferred	56.8%

* Data from one country not available and not included.

(S. Merton, AETE Zug, 2006)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2005

Species	Embryo Production	Embryo Transfers	Countries
Sheep	83	61	Greece Hungary Portugal Romania
Swine	271	192	Czech Republic Hungary
Goat	160	76	Portugal Romania Croatia
Horse	509*	711	Czech Republic Finland France Hungary Italy Netherlands Sweden Switzerland

* Data from two countries not available and not included.

(S. Merton, AETE Zug, 2006)

INVITED LECTURES

IMPORTANCE OF THE FALLOPIAN TUBE IN EMBRYO PRODUCTION

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Introduction

Over the past 25 years embryo collection and transfer has, on a worldwide scale, asserted its major position in the field of animal breeding for commercial and scientific application. The synchronization of the reproductive cycle and the induction of multiple ovulations, AI, embryo collection and re-transfer are routinely used to produce an extra number of embryos from females with high genetic merit.

As shown by Thibier (2005), in 2004 about 789,000 bovine embryos were transferred, of which 30% were produced in vitro. These data demonstrate that there is an enormous and constant demand of additional embryos to successfully manage various requests in animal production associated with semen sexing, embryo splitting, cloning, and preimplantation genetic diagnosis. However, most of the embryos are collected at the morula or blastocyst stages either from the uterine horns or through all the steps of in vitro production. This kind of embryo collection does not actually account for the miscellaneous accomplishment of the fallopian tube for early embryo genesis.

There is great evidence that developmental competence of morulae or blastocysts is crucially affected during the first days of embryo formation. Numerous studies have shown that high rates of embryo losses occur during this period, which suggests that these embryonic stages are extremely vulnerable and sensitive to inadequate environmental conditions (King, 1991; Dunne et al., 2000; Thatcher et al., 2001). The use of assisted reproductive technologies intensifies developmental disorders. The proportion of collected oocytes which undergoes successive maturation, fertilization and embryo cleavage until the blastocyst stage in vitro remains low (Meirelles et al., 2004; Merton et al., 2003; Keskintepe and Brackett, 1996; Xu et al., 1992) and many of the transfers miscarry or result in developmental errors of fetuses and placentas (Farin et al., 2006). Although the holistic intermodal actions between the embryo and its environment remain unclear, basic damage during the very early embryo development such as chromosomal abnormalities, incomplete and delayed de- and re-methylation followed by altered expression patterns of a number of genes (Vuiff et al. 1999; Hyttel et al., 2000; Corcoran et al., 2006) provide an exemplary causal insight into the cell machinery factors, which terminate or have detrimental lasting effects on the progression of the embryo and fetal life (King, 1991).

The extent of adequate culture conditions during preimplantation development is likely to be critical for later development of the conceptus; consequently the period where embryos pass through the Fallopian tube seems to be a major bottle-neck situation for entry into life.

Oviduct

The oviduct is the physiological site where maternal and paternal gametes meet, interact and where the dialogue between embryonic and maternal cells is obviously initialised. The oviductal activity is characterized by its physiological tasks ensuring:

- Cilia mediated pick-up of the cumulus oocyte complex and forwarding it into the ampullary tubing system;
- the maintenance of sperm viability to bridge the time gap between AI, ovulation and fertilisation;
- the capacitation, suppression as well as hyperactivation of sperm motility, management of the sperm´s energy release;
- sperm reservoir (storage), coordination of sperm transport for an optimised oocyte fertilisation (balance of high fertilisation but reduced polyspermia);
- early embryo development;
- management of embryo migration (delay until the uterus is suitable to accomplish further embryo development) (Hunter, 2005; Töpfer-Petersen et al., 2002; Suarez, 2002).

The oviduct represents a powerful system of regulation harbouring a complex of mucosal topography with regional differences controlled by endocrine signals to guarantee high reproductive performance. Scanning electron microscopy depicts a complex three-dimensional architecture of the oviductal mucosa (Hunter et al., 1991). The ampullary region is characterized by prominent and lower longitudinal folds with oblique running secondary ridges and a well organized system of pockets in the interspaces (Yániz et al., 2000). The appearance of the epithelium depends on the phase of the oestrous cycle. During oestrogen dominance, densely arranged ciliated cells and protruding secretory cells are characteristic, while in the luteal phase the ciliated cells decrease both in number and height (Abe and Oikawa, 1993). The secretory activity of the oviduct epithelium displays its maximum around ovulation (Erikson et al., 1994), resulting in an increase of the oviductal fluid current (Killian et al., 1987; Killian et al., 1989). However, the cilia move the comparatively large COCs slowly, indicating a transient anchorage to the oviductal epithelium (Lam et al., 2000).

The entry of the gametes into the oviduct is performed from opposite directions, thus resulting in a counter-current micro-movement. After mating or insemination, spermatozoa pass the uterine horns, reach the oviduct and bind to the ciliated epithelial cells of the caudal isthmus region (Overstreet and Cooper, 1975; Töpfer-Petersen et al., 2002). Near the time of ovulation there are still unknown signals, which assist in sperm release from the oviductal epithelium. Fertilisation is performed through a mixture of active and passive transport mechanisms occurring between spermatozoa, cumulus oocyte complexes, epithelium and luminal fluid. After fertilization has occurred, the early developing embryos are passively transported to the uterus by a series of closely coordinated mechanical events where activities of cilia and smooth muscle predominate. It is well known, that myosalpinx contractions propagate randomly, producing a backward-forward egg motion over short distances. These mechanisms are thought to be responsible for the contact between the synthesized and secreted molecules acting as signals and nutrients contained in the tubal fluid and the gametes

and early embryos guaranteeing correct fertilisation and development rather than for continuous transportation (Muglia and Motta, 2001; Germanà et a., 2002; Wijayagunawardane et al., 1998). Once the ovum and embryo is captured, ciliary activity seems to be more important than tubal contractility in transporting the ova towards the uterine cavity (Osada et al., 1999). The lumen of the isthmus is extremely narrow and contains viscous secretions, and myosalpingeal contractions are reduced (Hunter, 2005). The three dimensional myoarchitecture of the utero-tubal junction regulates the sperm ascendance towards the ampullary region (Muglia and Motta, 2001) as well as the timed utero-tubal transmission of the embryos.

To date, many studies successfully demonstrated graduated morphologic, metabolic and molecular differences of embryos dependent on their time spent under multifaceted in vitro conditions compared to its in vivo counterparts. Although many in vitro techniques are now available promising to enhance reproductive performance, an advanced optimised procedure allowing higher subsequent embryo development is awaited. There is a plethora of complex factors acting in the life of oocytes and embryos during oviduct passage, which are not fully understood. The more the fallopian tube still remains the environment, which is thought to be optimal for early embryonic nursing. The present paper aims at providing a brief introduction of aspects, showing the use of the oviduct for embryo production exemplary for rabbits, sheep and goats, pigs and cattle.

Rabbits

One of the most cited studies in embryo transfer is that of Heape (1891) who first and successfully performed embryo transfer in rabbits. This technique allowed the recovery and transfer of embryos including an obligate exposition to in vitro culture conditions, to overcome, manipulate or progress embryo development. The rabbit is an exemplary species for demonstration of the necessity of oviductal development. Besides numerous generally accepted in vivo culture demands, known for several mammalian species, the rabbit embryos are enveloped by a mucin layer during tubal migration. Inadequate culture conditions such as in vitro conditions have been suggested as failures of embryo development resulting in fewer cells, retarded development (Carney and Foote, 1990, 1991) and at least in a reduced thickness of the mucin layer. A shortened stay of the embryos in the oviducts results in a deficient mucin shape, which leads to loss of pregnancies after transfer. Asynchronous transfers also significantly resulted in reduced or missing pregnancies (Murakamp and Imai, 1996). Moreover, the extraembryonal matrix including the zona pellucida and the mucin layer gained increasingly interest for studies on investigating the early embryo–maternal signalling by detecting captured residues (Herrler et al., 2002).

Routinely embryos are transferred using the surgical route, which was already established during the fifties (Dowling, 1949). More than one decade ago, we performed high numbers of embryo transfers into rabbit oviducts, which necessitated the development of a new technique to improve access to the oviduct simultaneously minimizing animal manipulation as well as optimising the transfer of early tubal stage into the fallopian tube. In 1993, we described the first transfer of rabbit embryos into the oviduct using a midventral laparoscopy (Besenfelder and Brem, 1993). Rabbits were kept under anaesthesia and fixed in a dorsal recumbent

position. The endoscope (∅ 4 mm, 30° oblique, STORZ, Vienna) was introduced through the abdominal wall about 1 cm cranial to the navel region. The position of the endoscope allowed the inspection of the ovaries, oviducts and uterine horns. Moreover, the topography of the first part of ampulla, located below the ovaries, allowed the straight insertion of an embryo loaded glass capillary: A 2 mm vein catheter was inserted through the abdominal wall near the Fallopian entry, through which the capillary was inserted followed by the deposition of the embryos via infundibulum 3 to 5 cm deep into the ampulla. The tubal transfers of embryos in one rabbit lasted about 1 to 3 minutes. In the first trials we transferred 30 to 50 embryos and 10 to 20 embryos per recipient and all the animals (100 %) and 86 % got pregnant, respectively. Further studies aimed at comparing the effect of endoscopic embryo transfer on reproduction versus artificial insemination in rabbits. For this purpose we chose an experimental design, where rabbit does were assigned to two groups (AI-Group and ET-Group) and used through 4 consecutive reproduction cycles. In the first and third cycle, all animals we inseminated, in the second and fourth cycle the animals were treated according to group assignment. Pregnancies in the ET-Group were similar or higher compared to the AI-Group, although no selection has been done due to insufficient ovarian responses of recipients of the ET-Group. The repetitive use of recipients for ET and AI did not show any negative effect of oviduct manipulation during embryo transfer (Besenfelder et al., 1996). This method has also been proven to evaluate the optimum number of embryos at different stages for transfer as well as to collect repetitively oocytes and embryos at different stages in different rabbit breeds (Bolet et al., 2000, Besenfelder et al., 1998a, 2000).

Regarding several years of application of endoscopic access to the rabbit oviduct, it can be concluded, that this technique provides a minimally invasive access to the reproductive organs, which avoids the exteriorisation of the organs by in situ manipulation. Thus the technique promises a unique use of the Fallopian tube for beneficial application and for associated techniques such as cryobanking (Bolet et al.,2000; Joly et al., 1996) transgenesis (Grosse-Hovest et al., 2004; Aigner et al., 2000) and cloning (Nowshari et al., 2002).

Small ruminants and pigs

For a couple of decades, mostly sheep has been routinely used worldwide for laparoscopy mediated artificial insemination to maximize the use of superior rams and the reproductive potential of superior ewes. Rapid genetic progress of known superior stud rams into the flock is the primary economic benefit of laparoscopic AI (Gourley and Riese, 1990; Ehling et al., 2003). Beside AI the laparoscopic technique served also as a valuable tool for studying follicle aspiration and diagnosis of ovulation and pregnancy (Snyder and Dukelow, 1974).

More recently, studies have focused on minimally invasive techniques to collect and transfer embryos. In 1994, we described the laparoscopic access to the ovine and caprine Fallopian tube (Besenfelder et al., 1994a,b) for a wide range of applications. Animals were positioned on their back and a midventral laparoscopic access cranial to the udder was performed. Atraumatic grasping forceps served for fixation of the ovary on its ligamental origin to confirm the successful synchronisation of the recipient animals. For transfer of tubal embryos, one end of the infundibulum was slightly lifted with the forceps to enable the introduction of the embryo bearing glass capillary for about 3 to 4 cm deep deposition. The first procedures

lasted about 10 minutes and resulted in pregnancy rates of 37 % and 55 % in sheep and goats (Besenfelder et al., 1994a,b). This technique was assessed to be suitable not only for generation of offspring from individual lines and breeds, but also for the collection of embryos at different stages (Lymberopoulos et al., 2001), the sanitary management of a herd (Vainas et al., 2006), in vitro production of embryos (Cseh et al., 1995) as well as the production of transgenic animals (Kuehholzer et al., 1998; Vainas et al., 1999).

Due to the growing pig industry, strict legal conditions and high hygienic requirements there is an increasing interest in handling of swine embryos. Numerous efforts were made to overcome surgical manipulation for a better acceptance on farms (Ratky and Brüssow, 1995; Brüssow and Ratky, 1996; Hazeleger and Kemp, 2001). To date, ET in swine has been practised for more than 50 years (see Hazeleger and Kemp, 2001). Embryos, which were manipulated at an early tubal stage e.g. for gene transfer and for cloning purposes had to be transferred surgically to the oviduct to guarantee maximal developmental rates (Brem et al., 1985; Hoelker et al., 2005). Studies, which attempted to establish an alternative to conventional surgery by minimizing time efforts and traumatic manipulation for embryo transfer into the uterine horns admitted discouraging results when using endoscopy (Wallenhorst and Holtz, 2002; Huang et al., 2002).

Our own studies, which revealed also tubal use in swine, were done according to the treatment of small ruminants. The recipient animals were anaesthetized, put in a dorsal recumbent position, until the area of the abdominal wall was positioned horizontally. The endoscope was introduced midventrally between the last two mammary complexes. The animals were slightly turned to the left and to the right side, to check right and left part of the reproduction organs, respectively. In most cases the ovary and the corresponding oviduct and uterine horn were found at the surface of the abdominal organs. Atraumatic grasping forceps, which were placed about 5 to 8 cm cranial to the endoscope, served for the careful pick up of one half of the infundibulum to release the ovarian surface and to assess the response to hormonal treatment. Fifteen to thirty embryos were loaded into the tip of a flexible catheter or into the glass capillary, which were inserted via metal catheter 8 to 10 and 4 to 5 cm deep into the ampulla. The first transfers resulted in 33 % pregnant animals.

In a more progressed experiment the number of transferred embryos was reduced and the technique was applied under more routine and sophisticated aspects. Thirty recipient gilts were used for unilaterally endoscopic ET into the Fallopian tube in two successive programs: In group I 284 (17.8 ± 3.0 embryos per recipient) embryos were transferred to 16 recipients. Thirteen pregnant recipients (81 %) developed 73 implantation sites until day 21 after ET (implantation rate: 31 %). The average number of implantation sites in pregnant animals was 5.5 (5.6 ± 3.0). In group II all 14 recipients became pregnant. The transfer of 205 embryos resulted in an implantation rate of 61 %. In both groups the distribution of implanted embryos to left or right uterine side was independent of the transfer side. The average time spent for endoscopic ET into the Fallopian tube was ≤ 5 minutes. Three recipients received 14 embryos and 21 days later 8 and 2 x 14 implantation sites were counted.

In a further step we established the collection of tubal stage porcine embryos. We successfully demonstrated, that either unilateral or bilateral flushing of the oviducts resulted in the

collection of high embryo yields compared to the number of corresponding CL and the control group (flushing after slaughter). Two animals of the unilateral flushed pigs, which foetuses were allowed to come to birth, delivered 6 and 8 piglets (Besenfelder et al., 1995, 1997, 1998b). These date emphasis the routine use of this minimal invasive technique to efficiently collect and transfer oviductal procine embryos for multiple reproduction purposes.

Cattle

During the past 25 years in vitro production of bovine embryos (IVP) has come to the forefront of reproductive research and also claims its position in the IETS-statistics (Thibier, 2005). IVP embryos presented a big challenge for scientists, because they were confronted with elementary requirements during early embryo genesis to manage in vitro development at the best possible rate. Numerous different culture media compositions were suggested such as defined media, semisynthetic media, conditioned media and media with co-culture (Rief et al., 2002; de Oliveira et al., 2006; Corcoran et al., 2006) to mimic physiological requirements. In particular, first steps in IVP of embryos were performed using the oviduct of rabbits (Sirard and Lambert, 1986; Wall and Hawk, 1988), sheep (Leibfried-Rutledge et al., 1987; Lu et al., 1987; Westhusin et al., 1989) and cattle (Roschlau et al., 1989; Peura and Aalto, 1989) for temporary in vivo culture. Recent adoption of sheep oviducts (Galli et al., 2003) has increased performance and is still the method of choice, to produce IVP-derived embryos of superior quality. Whereas wholly performed in vitro systems fail to reproduce the nutritive and inductive roles of a countless number of exactly timed, synthesised and released molecules during development.

Only little information is known from studies including the bovine oviduct for production of IVP embryos (Schmidt et al., 1997). However, recent studies support the importance of the Fallopian tube in embryo production within homologous species. A multifactorial and complex acting process is likely to be involved in the early embryo-maternal community, which is crucial for embryonic development, implantation and maintenance of a pregnancy (Wolf et al., 2003). Systematic analysis of local mechanisms, which control the function of the bovine oviduct epithelium in the postovulation period, identified bovine genes of differentially regulated oviductal epithelial cells in ipsilateral and contralateral and during oestrus cycle (Bauersachs et al., 2003; Stojkovic et al., 2003**).**

Most of the work, which has been done at our Institute, was directed towards the access of the bovine oviduct to perform comparative in vivo versus in vitro studies. In 1998 we reported the first successful transfer of IVP-derived bovine embryos into ampulla using a transvaginal endoscopy mediated method (Besenfelder and Brem, 1998). IVM/IVF embryos at the 2- to 4-cell stage were transferred via curved glass capillaries into synchronized heifers. The simple management of the minimal invasive technique, the short duration of the transfer and the number of calves born let us conclude, that the bovine oviduct will be an available tool for routine use. The same approach was used to collect bovine embryos at different stages from single ovulated and superstimulated heifers (Besenfelder et al., 2001; Mosslacher et al., 2001) and finally tubal transfer and flushing were combined for in vivo culture of IVP-derived embryos (Wetscher et al., 2005a,b; Havlicek et al., 2005a,b).

To summarize, the access to the bovine oviduct opens a wide field in the application of reproduction tools. We established the routine handling of the Fallopian tube for in vivo purposes, which was confirmed by the calves born (Besenfelder and Brem, 1998), the demonstration of early embryo kinetics in different hormonal stimulation protocols (Mosslacher et al., 2001), embryo migration pattern in vivo, the assessment of the quality and quantity of embryos cultured in vivo for a different time interval (Wetscher tel., 2005a,b; Havlicek et al., 2005a,b, 2006) and gene expression studies (Hyttel et al., 2001; Tesfaye et al., 2004; Nganvongpanit et al., 2006). Therefore it is recommended to introduce the Fallopian tube to future embryo production.

Conclusion

There are new tools, which provide the means for us to a more comprehensive understanding of the reproductive processes in farm animals. Although many techniques are now available, that aimed at enhancing reproductive performance, additional refinement and optimisation are needed to positively influence developmental competence of embryos. Numerous studies elucidate the superior quality of ex vivo embryos, which have undergone oviduct passage. Hence the use of the Fallopian tube should be a valuable tool to adjust IVP embryos to ex vivo embryos, capable to re-continue embryo-maternal cross talk for passing via normal pregnancies to the birth of healthy calves.

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EMBRYO-MATERNAL INTERACTIONS IN CATTLE

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Establishment and maintenance of pregnancy requires an intact embryo-maternal communication. The endometrium needs to be prepared by endocrine and local signals for implantation of an embryo. The latter emits pregnancy recognition signals, which contribute to the preparation of a receptive endometrium and also prohibit progression into the next estrous cycle. The molecular mechanisms and reactions underlying this complex embryo-maternal cross-talk are only partially understood. In ruminants, interferon tau (IFNT) is the major embryonic pregnancy recognition signal (reviewed in Wolf et al., 2003), however, it is not known whether IFNT is sufficient to maintain pregnancy or whether other signals of the embryo are required.

As a starting point for a systematic analysis of embryo-maternal interactions in cattle, we performed holistic transcriptome and proteome studies of endometrium samples from pregnant vs. non-pregnant animals in the pre-attachment period (Day 18). The workflow of our study is shown in Figure 1.

For the first holistic transcriptome study we used endometrium samples from Day 18 pregnant (after transfer of two in vitro produced embryos on Day 8) vs. non-pregnant twin cows (sham transfer on Day 8; Klein et al., 2006). This genetically defined model system facilitated the identification of specific conceptus-induced changes of the endometrium transcriptome. Using a combination of subtracted cDNA libraries and cDNA array hybridization, 87 different genes were identified as up-regulated in pregnant animals. Almost one half of these genes are known to be stimulated by type I interferons. For the ISG15ylation system, which is assumed to play an important role in IFNT signaling, mRNAs of four potential components (IFITM1, IFITM3, HSXIAPAF1, and DTX3L) were found at increased levels in addition to ISG15 and UBE1L. These results were further substantiated by co-localization of these mRNAs in the endometrium of pregnant animals shown by in situ hybridization. A functional classification of the identified genes revealed several different biological processes involved in the preparation of the endometrium for the attachment and implantation of the embryo. Specifically, elevated transcript levels were found for genes involved in modulation of the maternal immune system as well as genes relevant for cell adhesion and for remodeling of the endometrium.

Endometrium samples from the same monozygotic twin model were processed for holistic proteome studies using two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) analysis (Berendt et al., 2005). Four proteins with significantly higher abundance in each sample derived from the pregnant animals were identified: Rho GDP dissociation

inhibitor beta; 20 alpha-hydroxysteroid dehydrogenase (20 alpha-HSD); soluble NADP1-dependent isocitrate dehydrogenase 1; and acyl-CoA-binding protein. To verify the accuracy of the 2-D DIGE quantification, the abundances of 20 alpha-HSD were quantified by a targeted cleavable isotope-coded affinity tag (cICAT) approach. The mass spectrometry-based ICAT quantification matched perfectly the results obtained by 2-D DIGE quantification, demonstrating the accuracy of our data. These results indicate that our model (monozygotic twins) in combination with the appropriate analytical tools is particularly suitable for the detection of proteins involved in early embryo-maternal communication.

To validate the results obtained from the twin cow model we performed holistic transcriptome analyses of endometrium samples from Day 18 pregnant (after artificial insemination) vs. non-pregnant heifers (Bauersachs et al., 2006). In this model we identified 109 mRNAs with at least twofold higher abundance in endometrium of pregnant animals and 70 mRNAs with higher expression levels in the control group. Among the mRNAs upregulated in the pregnant animals at least 40 are already described as induced by interferons. In addition, transcript levels of many new candidate genes involved in regulation of transcription, cell adhesion, modulation of the maternal immune system, and endometrial remodeling were found as increased (Figure 2). A comparison with similar studies in humans and mice revealed species-specific and common molecular hallmarks of uterine receptivity. Future studies will address dynamic transcriptome and proteome changes in the endometrium at earlier stages of pregnancy as well as position-dependent signaling effects of preimplantation embryos.

In addition to our studies of transcriptome and proteome changes during early pregnancy, we have identified sets of genes, which are regulated during the estrous cycle in the female bovine reproductive tract (Bauersachs et al., 2003; Bauersachs et al., 2004; Bauersachs et al., 2005). The differentially expressed transcripts identified in various screens are currently combined on a “Bovine Oviduct Endometrium (BOE)” cDNA array, which in its current version contains 928 different transcripts, but will be continuously extended. The BOE array is an interesting tool for systematic screening of endometrium biopsies. Potential applications include differential diagnosis of fertility problems or investigations on the association between metabolic condition and uterine receptivity. Such studies provide the basis for understanding and modeling the quantitative biological processes underlying embryo-maternal communication and implantation.

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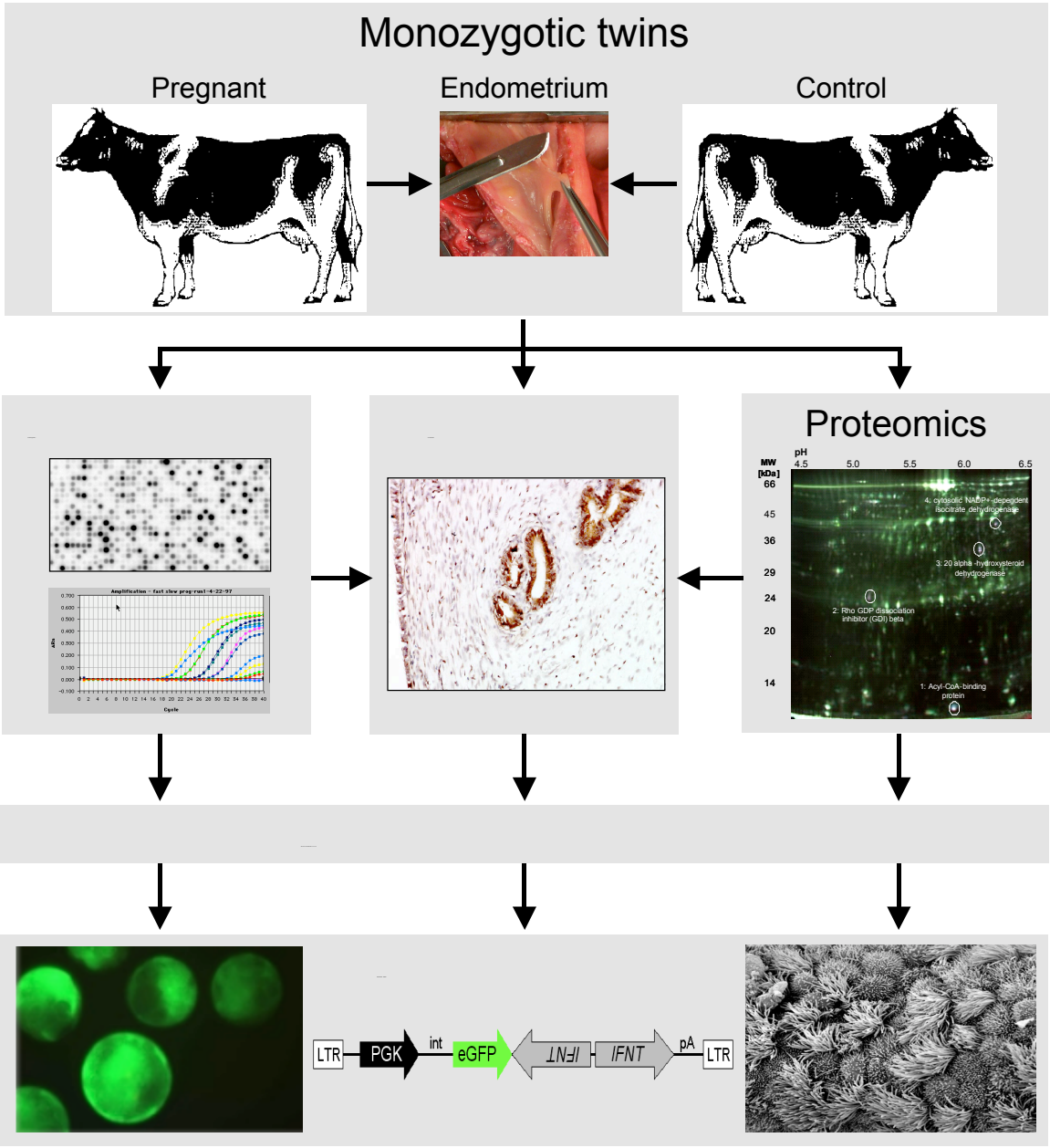

Figure 1. Workflow for systematic analysis of embryo-maternal communication in the pre-implantation period.

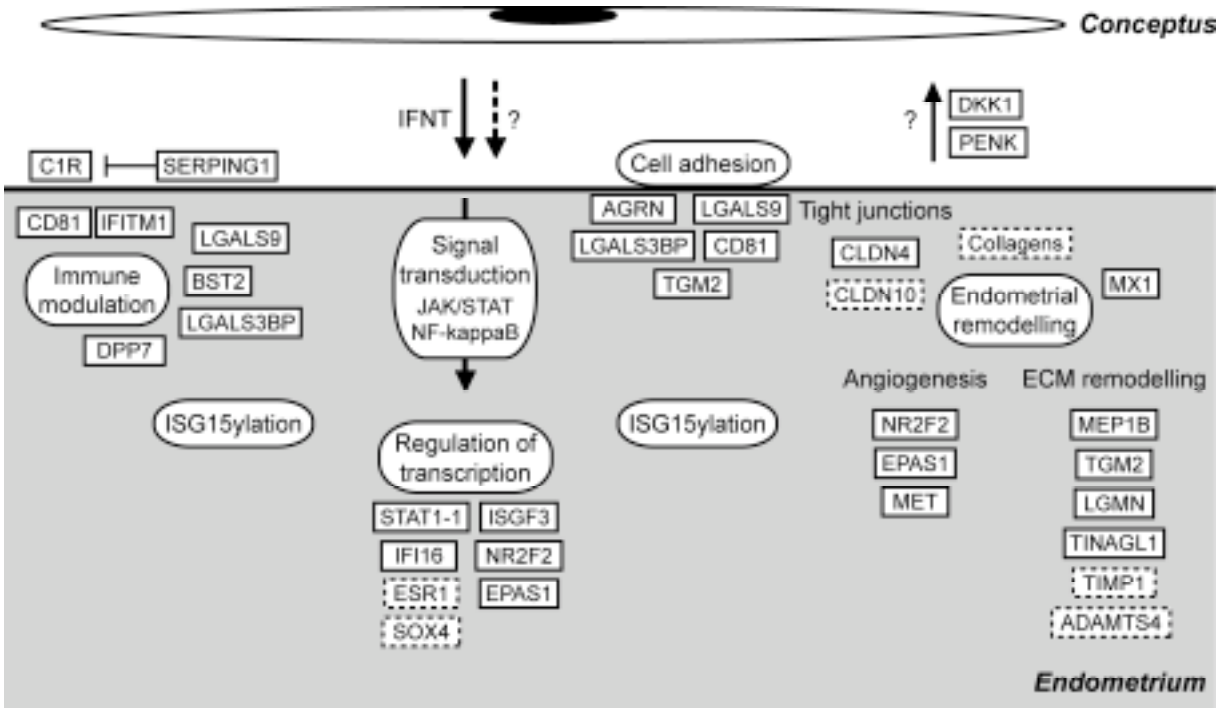

Figure 2. Model of embryo-maternal interactions in bovine in the pre-attachment period (from Bauersachs et al., 2006).

IN VITRO PRODUCTION OF CATTLE EMBRYOS

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Introduction

In vitro fertilization (IVF) literally means fertilization in glass and has been adopted as a generic phrase that often includes the procedures of in vitro maturation (IVM) and culture (IVC). When all three procedures are employed, in vitro production (IVP) of embryos is achieved. Today, the Latin term *in vitro*, meaning in glass, could more accurately be termed *in polystyrene*, since virtually all in vitro procedures today are conducted in inert, polystyrene, disposable plastic ware sterilized with gamma irradiation.

"A repeatable procedure for fertilization of bovine ova in vitro is described." (Brackett et al.,1982). This quote is taken from Brackett's description of the facilitation of sperm capacitation by exposure of sperm to high ionic strength (380 mOsm) medium to achieve IVF, which led to the birth of the first calf resulting from IVF. Although only a single calf was produced, the Brackett study demonstrated that IVF could be achieved in the bovine. The high ionic strength approach to capacitation was subsequently replaced by the use of heparin (Parrish et al.,1985).

Another significant breakthrough during this period involved the discovery that IVF proceeds much more efficiently at 39°C than at the 37°C temperature that works so well in mice and is universally used in most tissue culture systems (Lenz et al.,1983).

The first repeatable, efficient technique involving transvaginal ultrasound-guided aspiration of cattle oocytes was developed in 1988 (Pieterse et al., 1988) and has become widely known as ovum pick-up (OPU). Prior to this, in vitro procedures were limited to the research laboratory, utilizing oocytes collected from slaughterhouse-derived ovaries. The development of OPU paved the way for the commercial application of in vitro procedures utilizing oocytes from living cattle.

Current Status of Commercial bovine IVP Worldwide

Moving the clock ahead 20 years after Brackett's announcement of successful IVF, Thibier (2002) reported the following regarding the 2001 worldwide embryo transfer statistics: "It is reasonable to assume that in vitro produced embryos represent about 10% or even slightly more of the total number of bovine embryos transferred". In Thibier's report, a total of more than 450,000 in vivo-derived embryos were transferred world wide. Thus, in the 20

years following the first reported successful bovine IVF, the new technologies of OPU and IVP had gained a significant foothold in the field of bovine embryo transfer. The numbers of IVP embryos transferred annually between the years 1999 and 2004 in several key areas of the world are shown in Figure 1. These data are taken from the annual reports by the IETS Data Retrieval Committee, chaired by Michel Thibier and published in the IETS 'Embryo Transfer Newsletter'.

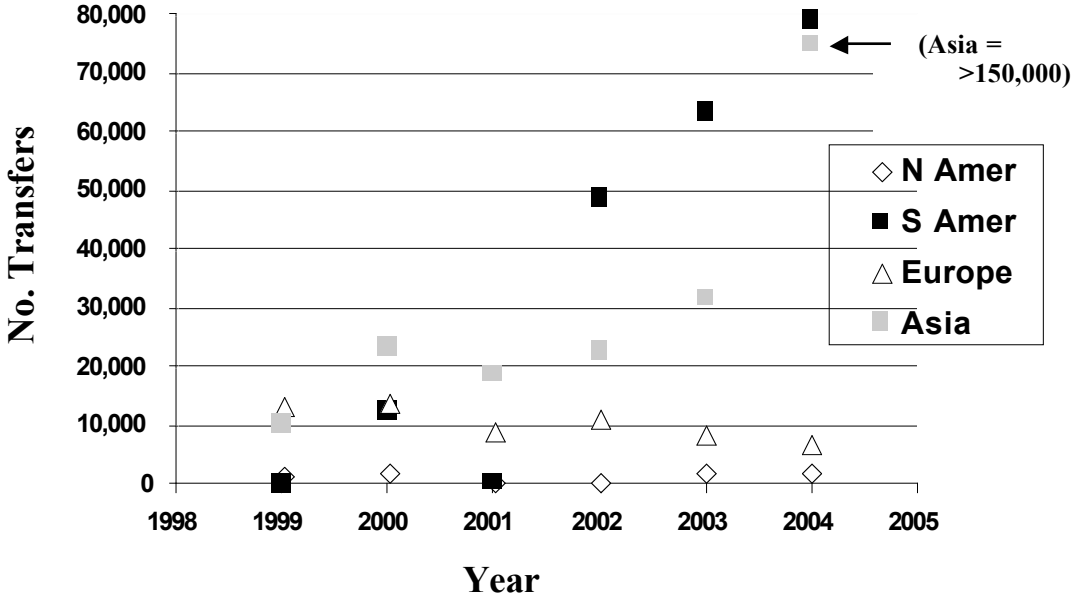

Figure 1. Yearly changes in the number of bovine IVP embryos transferred in major parts of the world between 1999 and 2004.

In 2002, more than 22,000 IVP embryos were transferred in Asia, of which approximately 9,000 (41%) were previously cryopreserved (Figure 1), while in South America (SA) more than 48,000 were transferred; however, only approximately 2,000 had been cryopreserved (Thibier, 2003). Although hard data on the number of cryopreserved IVP embryos in SA are lacking, it is evident that very few IVP embryos are cryopreserved. This may reflect both cultural differences between Asia and SA and the fact that embryos from Bos indicus cattle, whether they are in vivo- or in vitro-derived, do not survive cryopreservation as well as embryos from Bos taurus cattle.

Moving ahead to 2004, there was enormous growth in the numbers of IVP embryos transferred in Asia (>150,000), of which 72% were cryopreserved, and in South America (>80,000), none of which were reported as having been cryopreserved (Thibier, 2005). In contrast to the enormous growth in Asia and SA, there were only minor yearly differences from 1999 through 2004 in the number of IVP embryos transferred in Europe and North America.

There are a variety of applications of IVP commercially, some of which are listed in Table 1.

Table 1. Some commercial applications of bovine IVP.

OPU
Substitute for superovulation in healthy donors
Prepuberal heifers
Pregnant donors
Infertile donors
Slaughterhouse-derived ovaries
Inexpensive embryos for dairy infertility
Identified donors

Initially, OPU and IVP often were utilized in attempts to salvage genetics from cattle with serious infertility problems. In one program, IVP embryos were produced from cattle with blocked oviducts, fimbrial adhesions, chronic cystic ovaries, failure to achieve fertilization upon AI and/or superovulation and, in one case, absence of a uterus due to surgery (Hasler, et al.,1995). During the course of this program, at least one pregnancy was produced from each of more than 95% of nearly 300 donors that were entered into the OPU-IVP program (unpublished data). There continues to be a demand for applying this technology to infertile donors. However, the inefficiency and expense of working with this type of donor animal has decreased the appeal to embryo transfer practitioners. For example, in the program described by Hasler, et al. (1995), an average of only 4.1 usable oocytes were recovered per OPU attempt. This is in contrast to much larger average numbers described frequently in the literature for reproductively healthy, and in many cases, younger donors. In Brazil, much of the in vitro work involves Nelore cattle, of Bos indicus ancestry. Mean oocyte numbers in the range of 18-20 per OPU session are recovered from these animals (Personal communication), making it a much more productive and inexpensive procedure in cattle averaging very small oocyte numbers.

In Japan, there is a long-standing industry of producing cryopreserved IVP embryos from Japanese Black Cattle (Kuwayama et al.,1996). These embryos are selectively produced from donors identified after slaughter as having high marbling scores for the longissimus muscle area. The cryopreserved embryos are transferred primarily into dairy cattle.

Very large numbers of dairy embryos are being produced in North America from slaughter-house ovaries for export to China (personal communication). Various estrous synchronization systems have been investigated to maximize the utilization and conception rates in native Chinese recipient cattle, with the use of eCG showing promise (Remillard et al., 2006).

In a 1998 review of in vitro procedures in cattle and other livestock (Hasler, 1998), the author stated “Currently, most of the commercial in vitro production of bovine embryos involves the use of co-culture systems that include a monolayer of somatic cells”. This is clearly not the case today involving commercial bovine IVC. Embryo culture media fall into three general categories: non-defined, semi-defined and defined. Defined culture media are primarily used in research, and BSA is usually replaced with a surfactant such as PVA. BSA is not precisely consistent from batch to batch, and some practitioners wish to eliminate if from

IVP procedures. Nevertheless, BSA has proven to be extremely efficacious in bovine IVC and as a consequence, semi-defined media containing BSA are necessary if one does not want to turn to co-culture.

An informal survey of commercial bovine IVP systems in various countries revealed a surprisingly wide variety of IVC media currently being utilized. Although co-culture is still being used in some programs, it is not as widely-used as are a number of different semi-defined media listed in Table 2.

It is not meaningful to attempt a comparison of production efficiencies among various commercial enterprises based on the IVC system in use. It is clear that many different media are working at an acceptable level and there are a number of considerations that probably come into play regarding the choice of IVC system for individual practitioners.

Table 2. Some of the culture media currently in use among commercial bovine in vitro production programs.

IVC system	Reference
Menezo's B2* with BRL co-culture	Durocher et al.,2006
Menezo's B2* with Vero cell co-culture	Ponsart et al.,2006
Sequential media G1.2/G2.2* ('Gardner's media')	Gardner and Lane, 1999; Lane, et al.,2003
Sequential media CDM1/2 ('Colorado defined media')	De La Torre-Sanchez et al., 2006a
SOFaa	Holm et al.,1999
CR1	Rosencranz and First,1994
KSOM*	Erbach et al.,1994

(*Commercially available)

Three of the media listed in Table 2 are commercially available, which probably makes them more attractive to some practitioners. Also, although co-culture systems include the added complexity of needing a cellular component, they tend to be less sensitive to slight media imbalances and also to problems such as oxidative stress and heavy metal contamination. Another advantage is that they operate with 20% atmospheric oxygen, eliminating the need for the complicated gas system (5% O_2) required for semi-defined media.

Three of the media in Table 2 are not commercially available and must be made by the individual wishing to use them. This requires access to highly purified water and a number of accurate, regularly-calibrated scientific instruments. Most laboratories are reluctant to produce and then maintain and use IVC media beyond a few weeks of storage. Two very successful commercial operations, however, indicated that they successfully freeze and thaw batches of SOFaa and an inhouse-formulated version of G1.2/G2.2 (personal communication).

Conception rates following transfer of cryopreserved embryos are lower for both in vivo-produced and IVP embryos. However, conception rates for cryopreserved, in vivo-produced embryos are within 10 percentage points of fresh embryos. The conception-rate gap between cryopreserved and fresh IVP embryos appears to be larger and a good deal of

attention has been directed at improving crypreservation methods for IVP embryos. There is no consensus, however, as to which method is best, and a listing of cryopreservation approaches currently used commercially is shown in Table 3.

Table 3. Some of methods currently used commercially for cryopreservation of bovine IVP embryos.

Method	Reference
Slow Freezing – glycerol	Rall, 2001
Slow Freezing – EG	Voekel and Hu, 1992
Vitrification – 0.25ml straw	Campos-Chillòn et al., 2006
Vitrification - OPS	Vajta et al., 1998
Vitrification–CLV™ (CryoLogic)	Lindemans et al., 2004

Conception rates reported for commercially-cryopreserved IVP embryos range from 20 to 50%, and none of the above systems has conclusively proven to be optimal. Embryo survival is not the only consideration for commercial programs; convenience of use is also very important.

There is an increased degree of attention to OPU-IVF procedures from an ethical standpoint. Although no country specifically prohibits the utilization of OPU-IVP procedures, Denmark strongly discourages it. The Danish Advisory Committee for Animal Ethics announced (paraphrased) "It is unacceptable to use biotechnologies that are a burden for animals, if the purpose is not vital, e.g., as part of a more effective production of animal products. Therefore, the use of the two technologies of OPU and IVF are not acceptable ---". Research in these technologies is not prohibited, but there is no commercial application of OPU-IVF in Denmark.

McEvoy et al. (2006) provided a very comprehensive review of ethical issues related to the health consequences of both donors undergoing OPU procedures and offspring resulting from the transfer of IVP embryos. Lassen et al. (2006) provided an overview of public and philosophical concerns regarding the application of various biotechnology procedures. The authors made the point that in the long run, the usefulness of biotechnologies must outweigh public moral concerns.

A random sampling of recent improvements involving in vitro technology

The following is a brief review of some recently published technologies that may have immediate application to commercial IVP programs. The list is not comprehensive but represents this author's arbitrary choice of a sampling of subjects.

Cysteamine

The inclusion of 0.1mM cysteamine during IVM of both slaughterhouse-derived oocytes (Merton et al., 2004) and oocytes obtained by OPU (Merton et al., 2006) resulted in a higher embryo production rate during subsequent IVC compared to IVM without cysteamine. With oocytes obtained by OPU, embryo production on day 7 (morulae + blastocysts) following IVM with cysteamine was 34.4% compared to 23.4% for controls. The improvement in production (47% more embryos) following IVM in cysteamine was primarily due to an increase in the number of blastocysts. Pregnancy rates for fresh or frozen embryos

were not affected, however, by this treatment. The benefit of cysteamine was attributed to its facilitation of the synthesis of glutathione by the oocyte, leading to a reduction of reactive oxygen species.

Decreased oxygen atmosphere

Although it long has been known that oxygen concentration clearly affects the efficacy of IVC when co-culture is not used (Thompson et al., 1990), it has continued to receive attention in IVC research. The increased use of semi-defined media is undoubtedly responsible for the continued examination of decreasing oxygen concentrations in incubator gas atmospheres. When G1.2/G2.2 media were used for IVC of bovine zygotes, nearly three times as many blastocysts developed in 5% O_2 compared to 20% O_2 (Lane et al., 2003). Similarly, Olson and Seidel (2000) reported that blastocyst production more than doubled in 5% O_2 compared to 20% with CDM media.

Sexed Semen

Sex-selected (sorted) semen is available in some areas of the world, but only on a limited basis and straws contain much lower numbers of sorted sperm than normal. Pregnancy rates following AI with sorted sperm have been only 70-80% of controls in heifers and considerably lower in cows (for review see Garner, 2006). This potentially makes the use of sorted sperm in IVP procedures very attractive and a number of trials have been reported. Lu et al. (1999) reported that cleavage rates were similar for sorted versus unsorted frozen sperm, but they were lower for fresh sorted sperm versus unsorted sperm. The percentage of blastocysts produced from oocytes was significantly lower for both fresh and frozen sorted sperm compared to unsorted. In addition, the rate of development to blastocysts was lower for sorted sperm.

Other studies have also documented similar cleavage rates for oocytes fertilized in vitro with sorted versus unsorted semen, while blastocyst development rates were lower for sorted sperm (Wilson et al., 06); Cleavage rates were similar between sorted and unsorted sperm when heparin and sperm concentrations were optimized for each bull (Lu and Seidel, 04).

Using IVF sperm concentrations between 1 and $2x10^6$, Ferre et al. (2004) showed that sperm sex-selected by flow cytometry produced cleavage and blastocyst rates comparable to unsorted sperm in some bulls. However, sexed sperm resulted in poor cleavage and embryo development rates in other bulls.

CODA incubator gas filtration

The use of a commercially available intra-incubator carbon-activated air filtration system (CODA) was shown to significantly improve pregnancy rates following transfer of both fresh and frozen IVP bovine embryos (Merton, et al., In Press). Pregnancy rates following transfer of embryos cultured in a filtered atmosphere versus controls were 46% versus 41% for fresh and 41% versus 36% for frozen IVP embryos. No differences were detected in embryo production efficiency or in the quality of embryos cultured in filtered versus control incubators.

Fructose

Replacement of glucose with fructose during IVF and IVC resulted in a higher production of morulae and blastocysts (Chung et al., 2000; Barcel-Fimbres and Seidel, 2006)

Production of embryos that contain less lipid

Bovine embryos cultured in an in vitro medium containing phenazine ethosulfate were shown to take up less lipid during development to the blastocyst stage (De La Torre-Sanchez, et al.,2006b). This was attributed to the fact that PES is an electron acceptor that oxidizes NADPH to DADP, which increases flux of glucose through the pentose phosphate pathway and decreases lipid accumulation in embryos. However, although "PES" embryos accumulated less lipid during IVC, pregnancy rates following transfer of embryos cultured in PES were similar to control embryos (Barcelo-Fimbres et al.,2006)

IVP Embryo-Recipient synchrony

It is conventional to designate the day of estrus in cattle as day 0. Consequently, ovulation and fertilization normally occur on day 1. However, in most of the in vitro literature, the day of IVF is designated as day 0. This leads to a one day discrepancy between determination of synchrony involving IVP embryo age and recipient estrus. Hasler (1998) showed that pregnancy rate was highest when day 7 IVP embryos were transferred to day 8 recipients and that pregnancy rates were significantly lower when day 6 or 7 recipients were utilized. Very similar results were reported by Aoki et al. (2004). Thus, what might be conventionally called '0' synchrony (a day 7 recipient) is actually already minus one day when IVP embryos are transferred. Therefore, a day 8 recipient is actually perfectly synchronized for a day 7 IVP embryo.

Molecular Biology

At the most recent annual conference of the IETS, it perhaps was noteworthy that more abstracts were listed under the categories of Developmental Biology and Gene Expression than under traditional subject titles such as Superovulation and Embryo Transfer. The convenience of obtaining oocytes from slaughterhouse-derived ovaries and the continued evolution of more powerful molecular biology tools have provided a new level of sophistication to the study of oocyte maturation, fertilization and development.

It is often difficult for the commercial practitioner, with an interest in improving IVP, to sift through the increasingly numerous and sophisticated studies involving molecular techniques. There is, however, real interest in research projects such as the large cooperative program involving the use of microarray chips to study mRNA levels during embryonic development (Sirard et al., 2005).

It has been clearly demonstrated that there is a large gap in the efficiency of embryos developing from IVM oocytes compared to oocytes ovulated singly or as a result of superovulation (reviewed in van Wagtendonk-de Leeuw, 2006). Current knowledge of

development during the IVM to IVC period, based on molecular studies of gene expression was reviewed by Lonergan, et al. (2006). The authors' summarized the subject as follows: "The challenge for the future is to use this knowledge to establish culture conditions that take into account the changing needs of the developing embryo and allow the genes present to be expressed in a manner similar to how they are expressed in vivo".

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SHORT COMMUNICATIONS

SURVIVAL RATES AND MORPHOLOGY OF CLONED BOVINE EMBRYOS ON DAYS 14 AND 21

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A majority of early embryonic mortality in cattle occurs within the initial three weeks of gestation. The aim of this study was to compare survival rates and morphology of embryos produced by either in vitro production (IVP), cloning by subzonal injection of the karyoplast (SUZI) or handmade cloning (HMC) at Days 14 (D14) and 21 (D21) of gestation. Embryos were transferred at D7 to synchronized donors. At D14 and D21, recipients were slaughtered and embryos recovered. For collection on D14, 120 embryos were transferred into 6 recipients, 102 SUZI embryos into 5 recipients and 213 HMC embryos into 11 recipients. For collection on D21, 134 IVP embryos were transferred into 7 recipients, 98 SUZI embryos into 5 recipients and 97 HMC embryos into 4 recipients. Embryos recovered were processed for molecular analysis, immunohistochemistry (IHC) or transmission electron microscopy (TEM). At D14, all 6 IVP recipients yielded embryos, while 3 of 5 SUZI and 6 of 11 HMC recipients did so. On D21, 6 of 7 IVP recipients, and 5 of 5 SUZI , but only one of 4 HMC recipient(s) yielded embryos. D14 embryos sectioned for IHC or TEM were considered normal if they presented epiblast (E), hypoblast (H) and trophectoderm (T). Simularly, D21 embryos were considered normal if they presented differentiation into the germ layers (ectoderm, mesoderm and endoderm). Such embryos ranged from neural groove (NG) formation to development of the neural tube and somites (S). Table 1 summarizes the number of embryos seen with these morphological features.

Table 1. Morphological features observed at D14 and D21 for IVP, SUZI and HMC embryos.

Embryo type	# embryos recovered	# embryos molecular analysis	# embryos IHC,TEM	# embryos with abnormal morphology	# embryos with normal morphology
D14 IVF	30/120 (25%)	9	21	T= 2/21 H= 9/21	10/21 (48%)*
D14 SUZI	15/102 (15%)	4	11	T= 4/11 H= 2/4	5/11 (45%)*
D14 HMC	14/213 (7%)	6	8	T= 1/8 H= 4/8	3/8 (37%)*
D21 IVF	31/134 (23%)	14	17	nil	NG= 8/17 (47%)** S= 9/17 (53%)**
D21 SUZI	15/98 (15%)	6	9	T,H= 2/9 (22%)	NG= 4/9 (44%)** S=3/9 (33%)**
D21 HMC	1/97 (1%)	0	1		NG= 1/1 (100%)**

T= trophectoderm only; H= hypoblast only *= trophectoderm, hypoblalst and epiblast; **= three germ layers

At D14, recovery rate for IVP embryos was much higher than that for SUZI and HMC embryos. However, the difference in embryos that appear normal was only slightly higher for IVP embryos compared to clones. At D21, a higher proportion of IVP embryos than SUZI or HMC survived. IVP embryos also appear more developmentally advanced at this stage. This may help to explain the higher success rate to term of IVP embryos.

Notes

IMPROVEMENT OF THE CRYOTOLERANCE OF SHEEP BLASTOCYSTS BY USING ALBUMIN AND HYALURONAN

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Our study was conducted to examine the viability, after transfer, of in vitro produced frozen blastocysts derived from oocytes cultured in medium with bovine serum albumin (BSA) with hyaluronan (HA), and without HA, or with fetal bovine serum (FBS). Ovaries of sheep of the Sarda breed were collected from the slaughterhouse. Recovered oocytes were matured in TCM199 supplemented with 4mg/mL BSA, with 100µM cysteamine, 0.3 mM Na pyruvate and with 0.1 IU/mL r-FSH (Gonal-F® 75), with 0.1 IU/mL r-LH (Gonal-F® 75), 1µg/ estradiol-17_. Matured oocytes were fertilized in SOF with 20% heat inactivated estrous sheep serum. All zygotes were cultured for 48h in SOF supplemented with 1% BME and 1% MEM with 1mM glutamine and 8mg/ml fatty acid free BSA.

On the third and fifth day they were divided into three groups: Group BSA, Group BSA+HA, control group FBS, and the culture medium was supplemented with 8mg/mL BSA_{FF} with 6 mg/mL hyaluronan in group BSA+HA or without HA in group BSA, while the control group was supplemented with 5% charcoal stripped FBS. Expanded blastocysts (Day 6-7) were vitrified and warmed according to Dattena et al. (2004). They were then transferred to synchronised recipient ewes. Pregnancies were confirmed by ultrasonography at 40, 60, and 80 days. Cleavage, blastocyst development, pregnancy rate, lambing rate, twinning rate and body weight and sex ratio are presented in Tables 1,2. There were no significant differences among the three groups in cleavage rate, blastocyst rate or pregnancy rate at 40d, while there were significant differences among the three groups in pregnancy at 60d and 80d and lambing rate. From these results we could conclude that BSA and HA improved cryotolerance of sheep blastocysts.

Table 1. Percentage of cleavage, blastocyst, pregnancy derived from embryos produced by different culture medium

Treat.	No. oocytes	Cleavage rate	Blastocyt rate	Pregnancy 40d	Pregnancy 60d**	Pregnancy 80d*
BSA	267	235/267 (88.0)	118/267 (44.1)	21/23 (91.3)	21/23 ***a*** (91.3)	19/23 ***a*** (82.6)
BSA+HA	202	173/202 (85.6)	92/202 (45.5)	20/22 (90.9)	19/22 ***ab*** (86.3)	17/22 ***ab*** (77.2)
Control	180	147/180 (81.6)	72/180 (40.0)	14/20 (70.0)	12/20 ***b*** (60.0)	11/20 ***b*** (55.0)

**a-b* Values in a row of column with different letters differ (P<0.05).
** *a-b* Values in a row of column with different letters differ (P<0.01).

Table 2. Percentage of lambing, twining, body weight, male, female derived from embryos produced by different culture medium

Treat.	Recipient	Lambs born / E.T %*	Twining %	Body weight (Kg)			M %	F %
				<1.8	*1.9- 4.4*	*≥4.5*		
BSA	23	23/49 ***a*** (46.9)	5/18 (27.7)	2/23 (8.6)	12/23 (52.1)	9/23 (39.1)	12/23 (52.1)	11/23 (47.8)
BSA+HA	22	19/48 ***ab*** (39.5)	4/14 (28.5)	5/19 (25.3)	13/19 (68.4)	3/19 (15.7)	12/19 (63.1)	7/19 (36.8)
Control	20	11/41 ***b*** (26.8)	1/10 (10.0)	2/11 (18.1)	7/11 (63.6)	2/11 (18.1)	6/11 (54.5)	5/11 (45.4)

**a-b* Values in a row of column with different letters differ (P<0.05).

Notes

INFLUENCE OF CUMULUS OOCYTE COMPLEX (COC) MORPHOLOGY AND OOCYTE DIAMETER ON APOPTOSIS OF PREPUBERTAL GOAT OOCYTES

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Slicing of slaughtered goat ovaries allows the recovery of a high number of COCs, but the oocyte population obtained is very heterogeneous, with different degrees of maturation and atresia. The use of atretic COCs could impair in vitro embryo development. Our aim was to study the incidence of apoptosis in oocytes at collection time depending on COC morphology and oocyte diameter. Prepubertal goats COCs were recovered by slicing and classified as: Healthy (H: compact cumulus and homogeneous cytoplasm) and Early Atretic (EA: heterogeneous cytoplasm and/or initial cumulus expansion). Oocytes from each COC group were denuded and classified by diameter: 110-125 µm; 125-135 µm; >135 µm. Early and late stages of apoptosis in denuded oocytes were detected by Annexin-V staining (*Annexin-V-FLUOS Staining ki*t, Roche) and TUNEL assay (*In Situ Cell Death Detection Kit*, Roche), respectively.

Table 1. Apoptosis detection assessed by Annexin V and TUNEL assay in different COC morphology and oocyte diameters.

0 h	Ø (µm)	ANNEXIN V					TUNEL	
		N	**VIABLE**	**EA**	**LA**	**NECR**	**N**	**APOP**
Healthy	**110-125**	79	56 (70.88)	4 (5.06)	14 (17.72) ^{1}	5 (6.32)	64	33 (51.56) ^{a1}
	125-135	125	94 (75.20)	7 (5.60)	21 (16.80)	3 (2.40)	104	45 (43.27) ^{a1}
	>135	69	50 (72.43)	7 (10.14)	8 (11.59)	4 (5.79)	66	8 (12.12) ^{b}
Early atretic	**110-125**	68	39 (57.35) ^{a}	2 (2.94)	27 (39.70) ^{a2}	0	60	7 (11.67) ^{2}
	125-135	109	76 (69.72) ^{ab}	6 (5.50)	25 (22.93) ^{b}	2 (1.83)	91	19 (19.78) ^{2}
	>135	62	49 (79.03) ^{b}	1 (1.61)	9 (14.52) ^{b}	3 (4.84)	55	14 (25.45)

EA: Early apoptotic; LA: Late apoptotic; Necr: Necrotic; Apop: Apoptotic
Different letters ($^{a, b}$) in the same column and COC morphology group differ significantly (F, $P<0.05$). Different numbers ($^{1, 2}$) in the same column and oocyte diameter, and different COC morphology differ significantly (F, $P<0.05$).

In conclusion, late apoptosis rate, detected by TUNEL and Annexin assay, decreased with increasing oocyte diameter in H and EA groups, respectively. These results could explain the highest blastocyst rate obtained in previous work from oocytes of the largest diameter.

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Notes

THE ABILITY OF FIVE RADIOIMMUNOASSAY SYSTEMS TO DETECT EARLY PREGNANCY-ASSOCIATED GLYCOPROTEIN IN BOVINE PLASMA

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Pregnancy-associated glycoproteins (PAG) constitute a large family of molecules specifically expressed in the outer epithelial cell layer of the placenta in eutherian species (Green et al., Biol. Reprod., 2000, 62, 1642-1631). Radioimmunoassay for PAG detection in serum or plasma is currently used as a specific serological method for pregnancy diagnosis in cattle from Day 28 (Zoli et al., Biol. Reprod., 1992, 46, 83-92), and also allow the diagnosis of embryonic or fetal mortality (Szenci et al., Vet. J., 2000, 159, 287-290).

This study was conducted to compare different pregnancy-associated glycoprotein radioimmunoassay (PAG-RIA) systems: RIA-497, RIA-706, RIA-780, RIA-809 and RIA-Pool. Their ability to distinguish between non-pregnant (n=10) and pregnant (n=40) females at Day 30 after artificial insemination (AI) was investigated. In all RIA systems, 67 kDa PAG preparation was used as tracer (labeled with ^{125}I according to the Chloramine T method) (Greenwood et al., Biochemistry, 1963, 89, 114-123) and as standard. Five antisera were raised in rabbits against different PAG preparations according to the technique of Vaitukaitis **(**Vaitukaitis et al., J. Clin. Endorinol. Metab., 1971, 33, 988-991): R#497 ($boPAG_{67}$), R#706 ($caPAG_{55+60}$), R#780 ($ovPAG_{57+59}$) and R#809 ($ovPAG_{55}$). These four antisera were mixed (R#497 one part; R#706 one part; R#708 two parts; and R#809 two parts) and used as an additional antiserum (Pool). Plasmatic PAG concentration was measured by radioimmunoassay with some modifications (Perényi et al., Repro. Dom. Anim., 2002, 37, 100-104). Statistical analyses were carried in STATA/SE 8 (StataCorp. 2003). A binomial exact distribution was used to calculate confidence interval for sensitivity, specificity, positive and negative predictive values. The PAG concentrations in pregnant females at Day 30 after AI were shown to be higher by using antisera R#706, R#780, R#809 and Pool when compared with antiserum R#497. All the RIA systems gave 100% sensitivity and negative predictive values. On the other hand, the use of antisera R#780 and R#809 resulted in lower specificity (95.6 % and 99.7 %, respectively) and positive predictive values (75% and 98%, respectively).

The present study clearly shows that the ability of PAG-RIA systems to diagnose specifically pregnancy at Day 30 after AI can be improved by selecting the antiserum and by using a combination of antisera raised against different forms of PAG.

Notes

PROGESTERONE, LUTEINIZING HORMONE, PROLACTIN AND PREGNANCY-ASSOCIATED GLYCOPROTEINS DURING THE FIRST TRIMESTER OF PREGNANCY IN CATTLE

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During gestation, several hormones and proteins are secreted by the maternal and fetal endocrine systems. The dialogue between corpus luteum and the conceptus-placenta is very important for a successful outcome of gestation. The hormones originating from the corpus luteum (e.g. progesterone) and ante-pituitary gland (e.g. LH and PRL) are suspected to be directly or indirectly influenced by molecules expressed in the endometrium (e.g. $PGF_{2\alpha}$) and trophoblast (e.g. interferon-tau, placental lactogen and pregnancy-associated glycoproteins). Bovine PAGs are expressed in the trophoblastic binucleate cells and released into the maternal circulation as early as Day 25 after conception (Perényi et al., Repro. Dom. Anim. 2002).

The aim of this study was to compare LH, PRL, PAG profiles in cows by analyzing the levels of P4 measured at Day 21 after AI. The experiment was carried out in Holstein Friesian females (n=37) of mixed age and parity. The herd was retrospectively split into two groups: females presenting P4 levels lower than the mean (8.74 ± 3.48 ng/mL) and females having concentrations higher than the mean (Group 1 and 2, respectively) during early pregnancy. Blood samples were collected from the coccygeal vein into tubes containing EDTA at Days 21, 30, 45, 60 and 80 after AI. Plasma was obtained by centrifugation (1,500 x g for 15 min) immediately after collection and was stored at -20 °C until assay. Plasma progesterone, LH, PRL and PAG were determinated by RIA techniques. Results are summarized in Table 1:

Table 1. Mean (± SD) hormone and protein concentrations from Day 21 to Day 80 after AI.

Group 1

Day after AI	Concentration (mean ± SD) (ng/mL)				
	P4	LH	PRL	PAG-497	PAG-Pool
Day 21	6.26 ± 1.70	4.16 ± 1.26	27.24 ± 16.18	0.26 ± 0.16	0.19 ± 0.22
Day 30	8.18 ± 2.15	3.43 ± 1.00	24.18 ± 14.44	1.70 ± 0.63	4.37 ± 2.13
Day 45	6.57 ± 1.99	3.34 ± 1.27	26.89 ± 14.83	2.49 ± 0.92	4.02 ± 1.99
Day 60	5.60 ± 2.20	3.62 ± 1.33	27.31 ± 10.20	4.06 ± 1.88	6.10 ± 3.37
Day 80	5.86 ± 2.44	3.84 ± 1.29	24.38 ± 14.72	6.90 ± 3.75	16.26 ± 9.67

Group 2

Day after AI	Concentration (mean ± SD) (ng/mL)				
	P4	LH	PRL	PAG-497	PAG-Pool
Day 21	11.05 ± 3.76	3.80 ± 1.09	18.10 ± 4.17	0.20 ± 0.00	0.16 ± 0.02
Day 30	8.58 ± 2.44	3.45 ± 0.90	15.39 ± 9.98	1.99 ± 1.60	5.14 ±3.29
Day 45	6.97 ± 2.80	3.09 ± 0.66	17.49 ± 10.54	3.67 ± 1.42	6.68 ± 4.94
Day 60	8.79 ± 2.34	2.83 ± 0.64	25.26 ± 9.89	5.40 ± 2.78	9.70 ± 6.37
Day 80	8.00 ± 4.12	3.28 ± 0.85	25.56 ± 12.99	10.21 ± 8.73	19.41 ± 12.13

In conclusion, our preliminary results indicate that the levels of P4 at Day 21 after AI can be related to clear differences in concentrations of analyzed molecules (P4, LH, PRL, and PAG) during the subsequent period (until Day 80). However, in our herd no pregnancy loss was observed in either the low P4 group or the higher one.

Notes

OOCYTE SELECTION BY MEANS OF BRILLIANT CRESYL BLUE STAIN ENHANCES BLASTOCYST RATE AFTER BOVINE NUCLEAR TRANSFER

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Nuclear transfer (NT) has been successfully carried out in different species of animals using a variety of somatic cell types as nuclear donors, albeit with low efficiencies. Apart from other factors, the low level of efficiency of NT or in-vitro embryo production, manifested by the frequent failure of up to 60% of recovered oocytes to reach the blastocyst stage after fertilization, has been proposed to be related to the quality of the oocyte at the beginning of maturation. The aim of the present investigation was to study the effect of oocyte selection on the efficiency of bovine nuclear transfer in terms of increased blastocyst production. For this purpose, prior to in-vitro maturation (IVM), oocytes were selected for their developmental competence on the basis of glucose-6-phosphate dehydrogenase (G6PDH) activity indicated by brilliant cresyl blue (BCB) staining. The G6PDH enzyme is active in the growing oocyte but has decreased activity in oocytes that have finished their growth phase. BCB test is based on the capability of the G6PDH to convert the BCB stain from blue to colourless.

For our experiments, compact cumulus oocyte complexes (COCs) were recovered from slaughterhouse-collected bovine ovaries by slicing the surface of the ovary. Oocytes were classified either as control group, which were placed immediately into culture without exposure to BCB stain (irrespective of their G6PDH activity), or treatment group, which were stained with BCB for 90 min before maturation in vitro. Treated oocytes were then divided into BCB_ (colourless cytoplasm, increased G6PDH) and BCB+ (coloured cytoplasm, low G6PDH) based on their ability to metabolize the stain. After IVM, oocytes were subjected to nuclear transfer procedure (Handmade Cloning, HMC^{TM}) for the production of cloned embryos which were then cultured for a period of 8 days to determine the blastocyst rate. The data were evaluated by one-way analysis of variance (ANOVA). All results with $P < 0.05$ were considered statistically significant.

Whereas the BCB+ group exhibited a 78% cleavage rate, the BCB_ group showed a significant reduction (51%) in terms of the proportion of cleaved embryos. No significant differences in terms of the cleavage rate were recorded between the control group (73%) and the BCB+ group (78%). However, the BCB+ oocytes yielded a significantly higher blastocyst rate (39%) than the control (21%) or BCB_ oocytes (4%) and also showed significantly higher cell numbers (Hoechst stain). These results show that the staining of bovine cumulus oocyte complexes with BCB before in vitro maturation could be used to select developmentally competent oocytes for nuclear transfer. In addition, G6PDH activity could prove to be a useful marker for determining the developmentally competent oocyte.

Notes

AUTOFLUORESCENCE CHARACTERISTICS OF PREOVULATORY PORCINE FOLLICLES

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Maturation of ovarian follicles and of oocytes is characterized mainly by size, hormone content of follicular fluid, state of cumulus-oocyte-complexes (COCs) morphology and nuclear maturation. Although a relation between COC morphology and nuclear maturation during preovulatory follicle maturation has been shown (Torner et al., Theriogenology 1998), to predict the stage of follicle maturation on the basis of size only and without extracting follicular fluid and/or COCs is impossible. The aim of the present study was to characterize preovulatory follicles based on autofluorescence. It is well known that several biological products excited with light waves (e.g. at 370 nm) reveal emission in a spectrum of 400-600 nm. NADH (nicotinamid-adenin-dinucleotide-hydroeen) and FAD (flavin-adenin-dinucleotide) are important substances of cellular respiration with autofluorescence; and its ratio of emission intensity (FAD/FAD + NADH), called redox-ratio (RR), will characterize metabolic status. High redox-ratio indicates low cellular metabolism.

In the present study, autofluorescence of preovulatory follicles (n = 148) was measured 10 or 34 h post hCG at laparotomy in 12 oestrus synchronized German Landrace gilts. Size of each follicle was measured and its position recorded on a digital photo. Fluorescence emission was measured using a CCD spectrometer (USB 2000, Ocean Optics, USA) adapted with a backscattering probe. As excitation lightsource we used a pulsed Xe-flashlamp with a bandpass filter of maximum transmission at 370 nm.

After fluorescence recording gilts were ovarioectomized, and COCs were gently aspirated from individual follicles. Thereafter, their morphology (compact, expanded or denuded COC) and nuclear maturation (immature: GV; meiosis resumed: diakinesis to anaphase 1; mature: telophase 1 to metaphase 2) was recorded. Spectra of individual follicles were standardized and transformed into a derivative. The emission intensities of NADH at 460 nm and of FAD at 520 nm were selected and the RR calculated. The calculated RR was classified by cluster analysis. Altogether, six significantly different RR classes ($P < 0.05$) were determined (Fig. 1). Cumulus morphology is related to RR classes 1-5. The percent of compact COCs decreased and that of expanded COCs rose with increasing cellular metabolism (Fig. 2). Nuclear maturation is less related to RR classes (Fig. 3).

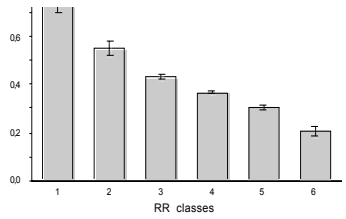

Fig 1. Redox-ratio (RR) classes

Fig 2. Distrubution of COCs regarding their morphology to RR classes

Fig 3. Distrubution of oocytes regarding their nuclear maturation to RR classes

Based on these first studies, autofluorescence characterization of follicles could be a future, additional tool of predetermination of follicles for in vivo and in vitro techniques.

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Notes

ANALYSIS OF DNA FRAGMENTATION OF IN VITRO CULTURED AND IN VIVO DERIVED PORCINE BLASTOCYST USING TUNEL

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Introduction. Apoptosis, an active and programmed form of cell death has been observed in preimplantation embryos of many mammalian species, such as human, mouse, rat, cattle, horse, sheep and pig. Programmed cell death (PCD) seems to play an important role in mammalian reproduction and development. A very important function of apoptotic processes during preimplantation development is the elimination of the minority of cells with abnormal potential and the control of embryo cell number. Possible causes of apoptosis in preimplantation embryos are chromosomal and nuclear abnormalities, such as multiploidy or mosaicium and inappropriate development potential, such as the presence of ICM (inner mass cell) cells with the potential to form trophoectoderm. Cell death was confirmed in preimplantation porcine embryos using the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling- TUNEL assay.

Material and methods. In this study we analyzed two groups of in vivo and in vitro produced porcine embryos.The embryos were fixed in 4% paraformaldehyde solution for 1 h and then membranes were permeabilized in 0.1% Triton X-100. The total number of cells per embryo and the number of cells with fragmented nuclei per embryo was assessed in selected blastocysts by (TUNEL) and DAPI. Fixed embryos were incubated in a TUNEL reaction mixture for 1h at 38.5° in the dark. The embryos were mounted on slides and analyzed under a fluorescence microscope.

Results. In total, 100 expanded blastocysts were analyzed in this experiment. Significant differences in the percentage of TUNEL stained nuclei were observed in in vivo derived blastocysts (7±1.4) and in vitro cultured blastocysts (32±5.3).

Conclusions. Most of the in vitro produced porcine blastocysts in this study contained more TUNEL stained nuclei than in vivo derived blastocysts. The TUNEL assay is a valuable tool to evaluate preimplantation porcine embryos.

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Notes

SUCCESSFUL VITRIFICATION OF PARTHENOGENETIC AND CLONED PORCINE BLASTOCYSTS PRODUCED FROM DELIPATED OOCYTES

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In vitro produced (IVP) porcine embryos are more sensitive to cryopreservation than their in vivo derived counterparts since higher levels of intracellular lipids are accumulated under suboptimal culture conditions (Sturmey *et al*. Reproduction 2003;126:197-7). Recently, a non-invasive method for delipation based on partial zona pellucida digestion and subsequent centrifugation has been published (Esaki *et al*. Biol Reprod. 2004, 71:432-6). The purpose of the present study was to investigate how oocyte delipation influences subsequent embryo development after parthenogenetic activation (PA) or somatic cell nuclear transfer (SCNT). The results were evaluated by Chi-square analysis and student's *t*-test. In Experiment 1, 44 hours after the start of in vitro maturation (IVM), cumulus cells were removed and oocytes were randomly distributed into two groups. In the test group, oocytes were digested with 1mg/ml pronase in the presence of 50% cattle serum (CS) for 2 min, and washed in Hepes-buffered TCM-199 medium supplemented with 20% CS. Subsequently 40-50 oocytes were centrifuged (12000 rpm, 20 min) in Hepes-buffered TCM-199 medium supplemented with 2% CS, 3 mg/ml PVA and 7.5 ug/ml cytochalasin B. Both centrifuged and intact (control) oocytes were completely freed from zona pellucida with 5mg/ml pronase solution. A single direct current of 85kV/cm for 80 _s was applied to both groups, followed by 4 h treatment with cytochalasin B and cycloheximide in cultured medium. All embryos were then cultured in the modified NCSU37 medium as described elsewhere (Kikuchi et al. Biol. Reprod. 1999, 60:336-5). Day 7 blastocysts were then vitrified and warmed by using the Cryotop technique (Kuwayama et al., RBM Online.2005, 11:300-8). Survival of blastocysts was determined according to re-expansion rates after 24 h recovery in culture medium with 10% CS. Delipation was successfully performed in 90% (173/192) of oocytes. Parthenogenetic activation resulted in similar blastocyst rates in delipated vs. control oocytes (28±7% vs. 28±5%). Subsequent vitrification of produced blastocysts with the Cryotop technique resulted in higher survival rates in the delipated group compared to control blastocysts (85±6% vs. 32±7% respectively; $P<0.01$). In Experiment 2, delipated oocytes were used for handmade cloning (HMC) with normal oocytes as control system. Delipated oocytes were bisected manually. HMC was performed as described previously (Du et al, Cloning and Stem Cells. 2005;7:199-6.). A similar blastocyst rate was obtained in the HMC group derived from delipated oocytes (21±6%) as in the control group (23±6%). A significantly higher cryosurvival rate (79±6% vs. 32±8%; $P<0.01$) with similar cell number was observed in the delipated group compared with control blastocysts (39±5 vs. 41±7). Our results prove that porcine embryos produced from delipated oocytes by parthenogenetic activation or handmade cloning can be cryopreserved effectively by ultrarapid vitrification. Further experiments are required to assess the in vivo developmental competence of the cloned-vitrified embryos.

Notes

COMPARISON OF CELLULAR INTEGRITY OF EMBRYOS IN HOLSTEIN AND JERSEY COWS AFTER FREEZING AND THAWING

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The aim of this experiment was to compare the cellular integrity of embryos after freezing and thawing in Holstein and Jersey cows. A total of 25 Holstein embryos from 11 donor cows and 25 Jersey embryos from 8 donor cows were used. Embryos were harvested and handled according to the standard procedures of the IETS and submitted to a combined staining, Hoechst 33342 and propidium iodide (Sigma-Aldrich Canada; Toronto, Canada). The combined staining with these dyes makes possible analysis of cell death. Propidium iodide is specific to dead cells, whereas Hoechst stains all of the cells. Embryos were put in a PBS solution containing 20_g/ml of Hoechst 33342 and propidium iodide for 15 minutes and placed between a slide and coverslip. Fluorescent microscopy was then used to assess the proportion of blue nuclei (live cell) and red nuclei (dead cell) in each embryo. The averages of live and dead cells were 84.92 ± 16.36 and 7.52 ± 10.33, respectively, for Holstein embryos, and 83 0 ± 14.93 and 8.08 ± 9.81, respectively, for Jersey embryos. The t-test was used to compare the two groups. There was no significant difference in the number of live or dead cells between Holstein and Jersey embryos ($p > 0.05$). In conclusion, freezing and thawing affects Holstein and Jersey embryos in the same way.

Notes

THE USE OF AGAROSE IN THE FREEZING OF SEXED BOVINE EMBYOS

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The objective of this experiment was to develop an effective method of freezing biopsied and sexed bovine embryos. The embryos (in vivo) were collected and handled according to the conventional approach approved by IETS. Biopsies were performed on class 4 to 7 grade 1 embryos (IETS classification). Biopsies and sexing protocols were those developed by A.B. Technology, (Theriogenology 1991;35:45-54). After the biopsies, the embryos underwent three consecutive baths in an embryo culture medium (Emcare holding media, ICPbio Limited, New Zealand). They were then placed in a 1.0% solution of agarose (L.M.P. Agarose, Life Technologies; Burlington, Canada). The agarose was diluted in Dulbecco's phosphate-buffered-saline (DPBS; Life Technologies, Burlington, Canada) containing 1000 mg/ml D-glucose and 35 mg/ml sodium pyruvate. The biopsied embryos were then put in a 1.5 M solution of ethylene glycol containing 0.1 M sucrose for 5 minutes. They were finally chilled at - 6.5°C and seeded 5 minutes later. After 15 minutes, the temperature was lowered to -32°C at a cooling rate of 0.5°C per minute. They were then immersed in liquid nitrogen. The frozen sexed embryos were thawed then transferred into synchronized recipient heifers. They were distributed to 50 different farms.

In addition, 3018 intact non-sexed embryos were frozen according to the procedures described above and were also thawed and transferred to recipients on the same farms. To thaw embryos, straws were exposed to air for 5 sec and then immersed in water at 22°C for 15 seconds. The embryos were transferred within 5 minutes to the recipients. Pregnancy diagnoses were performed sixty days after transfer. The experiment was conducted over a 9 years period, extending from 1996 to 2005.

During the course of this experiment, 3484 embryos were transferred. Recipients of biopsied and sexed embryos (466 heifers) had a pregnancy rate of 63.73 %. Among the 3018 recipients carrying intact non-sexed embryos, 1945 were pregnant, which represents a pregnancy rate of 65.08 %. These data demonstrate that there is no significant difference ($P>0.05$, chi-square test) between the pregnancy rates of intact non-sexed embryos versus biopsied and sexed embryos after freezing and thawing. This method of freezing could be used successfully for freezing sexed bovine embryos.

Notes

IDENTIFICATION OF DIRECT CANDIDATE GENES RELATED TO PREGNANCY SUCCESS IN BOVINE EMBRYOS THROUGH TRANSCRIPTIONAL ANALYSES OF BLASTOCYST BIOPSIES

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Early embryonic mortality is a recognized cause of reproductive failure in cattle leading to the loss of a large number of potential calves, retarded genetic progress, and significant loss of money and time in rebreeding cows. The purpose of this work was to address the relationship between transcriptional profile of embryos and the success of pregnancy based on blastocyst biopsies taken prior to transfer to recipients using microarray analysis. Biopsies (30-40% of the intact embryo) were taken from IVP Day 7 blastocysts (n=98) and the 60-70% portions were transferred to recipients after re-expansion. Based on the success of pregnancy, biopsies were pooled in three groups namely: those resulting in no pregnancy (G1), embryo resorption (G2) and those resulting in calf delivery (G3). Gene expression analysis of these groups of biopsies was performed using home made bovine preimplantation specific array (with 219 clones) and BlueChip bovine cDNA array (with ~2000 clones). Approximately 2µg of amplified RNA from triplicate pools of biopsies (each 10 biopsies) used as a template in reverse transcription reactions incorporating amino allyl-dUTP into cDNA using CyScribe post-labelling kit (Amersham Bioscience, Freiburg, Germany). The synthesized cDNAs from G1 or G2 and G3 groups were alternatively labeled using N-hydroxysuccinate-derived Cy3 or Cy5 dyes for dye-swap hybridization. Images were analysed using GenePix Pro Version 4.0 software (Axon Instruments, CA, USA). Data were normalized using GPROCESSOR freeware (http://bioinformatics.med.yale.edu/softwarelist.html) and finally analysed using Significant Analysis for Microarray (SAM) software. Quantitative real-time PCR was used to validate the results of microarray experiments. Data analysis revealed that a total of 52 genes were differentially regulated between G1 and G3 and 58 genes differentially regulated between G2 and G3. Biopsies resulting in calf delivery were found to be enriched with genes necessary for implantation (Cox2 and Cdx2), signal transduction (PLAU), poly amine biosynthesis (ODC1), response to oxidative stress (Thioredoxin), carbohydrate metabolism (ALOX15), growth factor (BMP15), and placenta-specific 8 (PLAC8). Biopsies of embryos resulting in resorption are enriched with transcripts involved protein phosphorylation (Cytokeratin A), plasma membrane (occludin) and glucose metabolism (PGK and aldose reductase). Biopsies of embryos which resulted in no pregnancy were enriched with transcripts involved ininflammatory cytokines (TNF1_), protein amino acid binding (EEF1A1), transcription factors (MSX1 and PTTG1), glucose metabolism (PGK and aldose reductase) and CD9 which is inhibitor of implantation. In conclusion, we generated direct candidates of blastocyst specific genes which may determine the fate of the embryo after transfer.

Notes

LONG TERM ALTERATIONS IN MICE PRODUCED BY ICSI WITH DNA-FRAGMENTED SPERM

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Genetic and environmental factors produce different levels of DNA damage in human and animal spermatozoa. DNA-fragmented spermatozoa (DFS) are usually used in intracytoplasmic sperm injection (ICSI) treatments in human and are still a matter of concern. The purpose of the present study was to investigate the long-term consequences on development and behaviour when using DFS in ICSI. Using the CD1 and B6D2 mouse strains, oocytes were injected with spermatozoa frozen-thawed without cryoprotectors (which produces ≈30% of TUNEL positive sperm) or with fresh spermatozoa (which ≈6.3% of TUNEL positive sperm, control group). Embryos were cultured for 24 h in KSOM, and 2-cell embryos were transferred into CD1 females. ICSI with DFS did not produce any effect on the percentage of embryos that survived micro-injection or that cleaved to the 2-4 cell stage. However, the proportion of transferred embryos that gave rise to live offspring was twice as high when fertilization was done with fresh sperm cells (26%), than when fertilization was done with DFS (13%). At week 20, ICSI produced animals and controls were submitted to behavioural tests: locomotor activity (open field), exploratory/anxiety behaviour (elevated plus maze, open field), and spatial memory (free-choice exploration paradigm in Y maze). Males produced by ICSI with DFS showed more anxiety and lower locomotion in the p-maze and the Y-maze tests (Z-test, $P < 0.05$), but no significant differences were found in the open-field test. Also, no differences were found in spatial memory or in the habituation pattern. Postnatal weight gain of mice produced by ICSI was heavier than those from their control counterparts from 10 weeks on ($P < 0.01$). Anatomopathological analysis of animals at 16 months of age showed some emlarged organs (heart, lung, and liver; $P<0.01$) and an increase in pathologies (33% of CD1 females produced by ICSI presented some solid tumours in lung, dermis of back, or neck). We suggest that, depending on the level of DFS, oocytes may partially repair fragmented DNA, producing blastocysts capable of implanting and producing live offspring. However the incomplete repair may lead to long term phenotypes. Our data indicate that, the use of DFS in ICSI can generate effects that only emerge in later life, such as, aberrant growth, aging, behaviour changes, and mesenchymatical tumours.

Notes

THE COMPARISON OF TWO SUPEROVULATORY PROTOCOLS IN TERMS OF THE OVARIAN RESPONSE AND QUALITY OF OOCYTES IN BEEF CATTLE

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This study aimed to observe the ovarian response and the quality of oocytes obtained after different hormonal treatment in beef cattle. Different hormonal treatment were performed in 26 crossbred dams separated into 2 groups. Both groups were initially treated twice with PGF2alpha (Oestrophan, Praha) at an 11 day interval. Successively the first group was treated with p-FSH (120 I.U.) and the second group received Pregnyl (1500 I.U.). A group of 12 untreated dams of the same breed were used as control. The folowing parameters were considered: the size of ovary, the size and number of follicles, the total number of oocytes and the number of usable oocytes. Cumulus oocytes complexes (COC) recovered from ovaries of slaughtered females were evaluated under a stereomicroscope using the following criteria:

Category 1	Compact multilayered cumulus investment; homogenous ooplasm; total COC light and transparent
Category 2	Compact multilayered cumulus investment, homogenous ooplasm but with a coarse appearance and a darker zone at the periphery of the oocyte; total COC slightly darker and less transparent
Category 3	Less compact cumulus investment; ooplasm irregular with dark clusters; total COC darker than 2 described above
Category 4	Expanded cumulus investment; cumulus cells scattered in dark clumps in jelly matrix; ooplasm irregular with dark clusters; total COC darker and irregular

The first three groups of COC are considered for IVP. The largest ovary (4.21 cm) was observed in the first group. We detected the highest number of follicles 0.5 – 1 cm in the second group (10.00 fol./head), and of follicles 0.2 – 0.5 cm in control group (10.67 fol./head). The highest total number of oocytes was isolated in the first group (19.00 ooc./head) and the lowest number was isolated from control group (10.08 ooc./head). The same number of usable oocytes were obtained after in both hormonal treatments (p-FSH 4,00, resp. Pregnyl 3.90 ooc./head). The best quality oocytes were detected after application of Pregnyl; on the other hand the worst quality of oocytes were detect in control group.

Due to a small number of animals in both groups, the results were not statistical significant; however, our results indicate that treatment with p-FSH induced better response to stimultion. Further studies are in progress to confirm these preliminary data.

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Notes

EFFECT OF DISEASE ON REPRODUCTIVE PERFORMANCE IN DAIRY COWS

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Effects on reproduction of anoestrus, acyclicity, atretic ovaries, hypoplasis, atrophic ovaries, cystic ovaries, permanent oestrus, scars on the cervix, coinage on ovary, endometritis, mastitis, and locomotor disorders were reviewed. Czech spotted cattle (C) and Holstein (H) cows from agricultural cooperative _ichlínek were observed in this experiment during 1993 – 2003. Data were collected from 4086 cows in 14 herds, including 3 free-stall and 11 tie-stall barns. Herd size ranged from 50 – 250 milking cows. We used 7 different types of therapy and effectiveness of particular therapy (the main parameter was interval from first treatment to conception) was monitored.

Therapies :

1.	Prostaglandin F_2_ at a dose of 0.5 mg cloprostenol in preparation Oestrophan, or Remophan
2.	Lecirelin at a dose of 0.05 mg , it is 2 ml Supergestran
3.	Lecirelin in tested developmental form in preparation Polygestran
4.	Prostaglandin F_2_ at a dose of 0.5 mg cloprostenol in tested developmental form in preparation Suiphan
5.	flushing the uterus
6.	control in prearranged term
7.	massage in prearranged term

Paper was considered if they provided quantitative estimates of diseases on between-calvings interval (BCI), service period (SP), insemination interval (II), days to first service, days from first service to conception, number of services per cow, and number of treatment per cow. Next we monitored the impact of breed on these parameter. Both observed breeds achieved values in SP, II, number of services per cow, and number of treatment per cow which signalized poor level of reproduction (SP over 120 days; II to 100 days; number of treatment per cow 2 and more; number of services per cow over 1,6). The interval from first treatment to conception was better in C breed then in H breed (30,81 days vs. 45,86 days). Overall the H breed assing to badly reproduction parameters opposite C breed(II 98,48 vs. 89,30 days; SP 140,13 vs. 123,31days; number of treatment per cow 2,4 vs. 1,9; number of services per cow 1,8 vs. 1,6).

In conclusion, we can note that the occurrence of these diseases in Holstein cows was more frequent and more severe, the reproduction parameters were worse in correlation to situation in C breed. The subsequent treatments had a lower effectiveness compared to Czech spotted cattle.

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Notes

EFFECT OF VITAMIN-E ON DEVELOPMENT AND QUALITY OF PORCINE EMBRYOS CULTURED IN VITRO

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Porcine embryos contain a considerably higher concentration of fatty acids than other mammalian embryos (McEvoy et al., Human Fertil Camb 2000; 3:238-46) and they should be more susceptible to lipid peroxidation. Peroxidation of fatty acids is known to inhibit the function of cells and might induce cell death (Spiteller, Mech Ageing Dev 2001; 122:617-57). Vitamin-E is a fat-soluble antioxidant that suppresses peroxidation of membrane lipids (Tappel, Ann. N Y Acad Sci 1980; 355:98-108).

The aim of the experiment was to investigate the effect of vitamin E on the in vitro development and quality of porcine embryos. The experiment was done on 2-4 cell embryos obtained from superovulated sows, slaughtered 48 hours after insemination. The embryos were collected by flushing the fallopian tubes with PBS supplemented with FCS at 38°C. The embryos were in vitro cultured in defined chemical medium NCSU-23 containing: 0 (control), 25 μM (group 1), 50 μM (group 2) and 100 μM vitamin E (group 3). The culture was performed at 39°C, with 5% CO_2 in air for 69 to 120 hours. The main evaluation criterion was embryological development up to the blastocyst stage. Furthermore, in selected blastocysts the number of cells per embryo and the degree of apoptosis were assessed by terminal transferase-mediated DNA end labeling (TUNEL) technique and Hoechst 33342 staining. Results are shown in Table 1 and 2.

Table 1. Effect of vitamin-E on porcine embryo development

Treatment group	No. of 2-4 cell embryos	No. of morulae (%)	No. of expanded blastocysts (%)
Control	20	19 (95.0)	17 (85.0)
1	25	23 (92.0)	20 (80.0)
2	27	23 (85.2)	21 (77.7)
3	24	22 (91.7)	21 (87.5)

Table 2. The quality of porcine expanded blastocysts

Treatment group	No of blastocysts	No. of cell/embryo	No. apoptotic nuclei	% TUNEL positive
Control	10	33.8	8.2	24.2
1	12	24.3	1.2	4.9
2	13	25.8	1.45	5.6
3	12	24.3	1.65	6.8

This experiment indicated that there was no difference between the control and treated groups (supplementation of NCSU-23 with vitamin-E) in percentage of embryos that developed to morulae and expanded blastocysts during culture in vitro. We have also shown that the average number of cells per blastocyst was even lower in embryos cultured with 25-100 μM vitamin E compared to the control. However, both the lower number of apoptotic cells per embryo and percentage nuclei containing fragmented DNA were observed in the group of embryos cultured in NCSU-23 with addition of vitamin-E.

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Notes

IN VITRO MATURATION OF PIG OOCYTES IN MEDIUM DMEM/F12 VERSUS TCM199 AFFECTS NUCLEAR MATURATION RATE AND SUBSEQUENT DEVELOPMENT TO THE BLASTOCYST STAGE

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Several culture media have been reported in the literature for the in vitro maturation of pig oocytes. They include defined serum-free media, media supplemented with follicular fluid, follicle shells, foetal calf serum or also conditioned media of oviductal epithelia cells. Other common supplements include growth factors, glutathione, cysteine and cysteamine. In most cases TCM199 is used as the basic medium to which supplements are added.

The aim of this study was to compare the maturation and embryo development rate of pig oocytes matured in TCM199 or in DMEM/F12 both supplemented with 10% foetal calf serum, ITS (Sigma), cysteine (0.4mM), cysteamine (0.6mM), Long-IGF-I (100ng/ml, Sigma), Long-EGF (50ng/ml, Sigma), bFGF (5ng/ml, Peprotech), FSH and LH (0.05 IU/ml each). The oocytes were collected by aspiration of the ovarian follicles of cycling females and matured for 42h at 38.5°C in 5% CO2. After maturation the oocytes were either denuded of the surrounding cumulus cells and scored for the presence of the first polar body or fertilised in vitro and allowed to develop to the blastocyst stage. In vitro fertilization was carried out in medium TALP supplemented with 1µg/ml heparin and PHE containing 0.1 million frozen-thawed-sperm per ml. The cleaved embryos were surgically transferred in the sheep oviduct for further development up to Day 7. Our results indicate a significantly higher maturation rate of pig oocytes matured in DMEM/F12 as compared to TCM199: 75.0 vs 67.6% (851/1135 vs 759/1123) (Chi square-test, $P<0.05$). Similar positive effect was observed on embryo development as shown in Table 1.

Table 1. Effect of maturation medium on the development of pig oocytes fertilized in vitro and cultured in the sheep oviduct from cleavage to the blastocyst stage.

Maturation medium	N oocytes fertilized	N cleaved and transf. in sheep ovid. (% of oocytes)	N recovered from sheep ovid. (% of cleaved)	Bl D7 (% of recovered)
DMEM/F12	385	178 (46.2)	139 (78.1)	69 (49.6) b
TCM 199	366	145 (39.6)	117 (80.7)	38 (32.5) a

Number of replicates = 3.
Values within columns with different letters differ significantly. Chi square-test, $P<0.05$.
Bl D7 – blastocysts on Day 7.

In conclusion our data indicate that in vitro maturation of pig oocytes in medium DMEM/F12 as compared to TCM199 promotes higher maturation and blastocyst rates.

Notes

EXPRESSION OF APOPTOSIS REGULATORY GENES AND THE INCIDENCE OF APOPTOSIS IN DIFFERENT MORPHOLOGICAL QUALITY GROUPS OF IN VITRO-PRODUCED BOVINE PREIMPLANTATION EMBRYOS

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Despite the fact that apoptosis plays an important role in preimplantation embryo development, the correlation among morphological embryo quality, cell numbers and the expression of apoptosis regulatory genes in bovine embryos has not been established. The objectives of this study were, therefore, to investigate stage-specific expression profiles of apoptosis regulatory genes in three quality groups of in vitro-produced bovine preimplantation embryos and to analyze the relationship between DNA fragmentation and morphological quality of embryos. The relative abundance of mRNA of 6 pro- (Bax, caspase-9, Bcl-xs, P53, Caspase-3, Fas) and 3 anti- (Bcl-w, Bcl-2 and Mcl-1) apoptotic genes was analyzed by using real time PCR. In addition, differential cell staining and TUNEL labelling were done to analyze the variation in cell numbers and detect apoptotic nuclei, respectively. The expression of Bax, Caspase-3 and Caspase-9 genes was found to be significantly ($P<0.05$) higher in poor quality preimplantation embryos as compared to morphologically good embryos of the same stage of development. Moreover, the anti-apoptosis Mcl-1 expression was significantly higher in good quality groups of immature oocytes, 8-cell and Morula stage embryos than that of their poor quality counterparts. A higher DNA fragmentation and Bax protein expression was evidenced in morphologically poor quality blastocysts. In conclusion, our study demonstrates a high degree incidence of apoptosis in morphologically poor quality blastocysts. Moreover, Bax, Caspase-9, Caspase-3 and Mcl-1 can be used as potential markers of embryo quality to evaluate in vitro produced bovine embryos.

Notes

DIFFERENTIAL REGULATION OF GENES IN BOVINE OOCYTES COLLECTED AT GROWTH AND DOMINANCE PHASES OF FOLLICULAR DEVELOPMENT

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The developmental competence of bovine oocytes collected from different phases of folliculogenesis is known to vary. Previous studies have shown that large proportion of oocytes aspirated at growth phase reached blastocyst stage (24-70%) than those aspirated at dominance phase (11-40%). However, the molecular mechanisms underlying this variability are not well elucidated. Here we aimed to investigate transcriptional activity of bovine oocytes derived from growth versus dominance phases of the first follicular wave. For this, thirty oestrus-synchronized cyclic Simmental heifers were used, and the onset of oestrus was considered as day 0. Ultrasonography-guided ovum pick up (OPU) was performed to collect oocytes from small follicles (3–5 mm) at growth (day 3) and dominance (day 7) phases of the first follicular wave. Triplicate pools of oocytes (each with 20 oocytes) from each developmental phase were used for transcriptional analysis using BlueChip bovine cDNA array (with ~2000 clones). Images were analyzed using GenePix® Pro 6.0 (Axon Instruments, CA, USA) and then the data were LOESS normalized with GPROCESSOR 2.0a (http://bioinformatics.med.yale.edu/softwarelist.html). Microarray data were analyzed using Significance Analysis for Microarray (SAM) followed by validation of representative number of transcripts by quantitative Real-time PCR. Data analysis revealed a total of 51 transcripts to be differentially regulated in the two oocyte groups. All differentially regulated genes were classified functionally based on the criteria of Gene Ontology Consortium classifications (http://www.geneontology.org). According to this classification, out of these 51, 36 are of known function, 6 are ESTs and 9 are novel transcripts. The quantitative real time PCR has validated the expression profile of 8 out of 10 to be in agreement with microarray results. Oocytes at Day 3 were found to be enriched with transcripts involved in protein biosynthesis (RPLP0, RPL8, RPL24, ARL6IP, RpS14, RpS15, RpS4x and RPS3A) or as translation elongation (EF1A), energy production as mitochondrial clones (ATP5A1, FL396 mitochondrion and FL405 mitochondrion), cytoskeleton or chromosome organization (Actin, beta-Actin, H2AZ and KRT8), calcium ion binding (S100A10 and ANXA2), signal transduction (G-beta like protein) and thiol-disulfide exchange intermediate (TXN). Oocytes of day 7 were enriched with genes involved in cell cycle (CCNB1, CKS2, UBE2D3 and CDC31), transcription factors (MSX1, PTTG1, FANK1 and PWP1), Aldehyde reductase activity (AKR1B1), nucleotide binding (TUBA6 and K-ALPHA-1), growth factor (BMP15), and fertilization (ZP4). In conclusion, differences in developmental competence of oocytes from growth and dominant phase of follicular development are also accompanied by differences in transcriptional activity.

Notes

DIFFERENTIAL REGULATION OF APOPTOSIS RELATED GENES BY RETINOID RECEPTOR SPECIFIC LIGANDS

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The p53 tumour suppressor activates apoptosis via transcription dependent and independent pathways that converge at the mitochondria. Endogenous p53 physically interacts with the anti-apoptotic outer mitochondrial membrane stabilizer Bcl-2 and activates Bax, an apoptosis inductor. All-trans-retinoic acid (ATRA) modulates the expression of p53, Bcl-2 and Bax in cancer and somatic cells, and we have demonstrated that ATRA regulates apoptotic distribution in the bovine blastocyst and increases p53 expression after hatching (Rodríguez et al, Hum. Reprod. In press). In the present work, together with ATRA, an agonist for the Retinoic Acid-Receptor (RAR), we use the specific Retinoid X Receptor (RXR) agonist LG100268 (LG, a gift of Ligand Laboratories), to analyze the expression of p53, Bcl-2 and Bax in bovine blastocysts.

Cumulus-oocyte complexes from slaughterhouse ovaries were matured and fertilized in vitro. Presumptive zygotes were cultured in B2 medium with Vero cells until 139 h post-insemination, time at which embryos (morulae [~90%] + early blastocysts) were cultured for 48 h in SOFaaci + 5% FCS containing: 1) ATRA 0.7 μM; 2) LG 0.1 μM; 3) LG 10 μM; and 4) no additives. Day-8 hatched blastocysts were pooled in groups of 4 to 6 embryos and stored at -80ºC. mRNA was extracted, reverse transcribed and analyzed by RT-PCR. Primers used and conditions can be seen in table 1. Expression was normalized by the DDCT method to the geometric average value of Lamin B, Ubiquitin and Succinate dehydrogenase flavoprotein subunit A. These genes were selected by their low variability in the present experimental conditions among 7 putative housekeeping genes by using the GeNorm method (unpublished data) as described by Vandesompele et al (Genom. Biol. 18;3(7):RESEARCH0034). Data from 4 replicates were analyzed by GLM and Duncan´s test, and expressed as arbitrary units (LSM±SE).

Expression of p53 was dependent only on RAR, as ATRA 0.7 μM showed values significantly higher (p=0.01) than controls and LG 0.1 μM and LG 10 μM (43.25±10.77 vs. 4.66±8.86, 4.96±9.36 and 12.28±10.32, respectively). Bax expression did not change between controls and both receptor agonists (p>0.05; data not shown). Interestingly, Bcl-2 expression was significantly increased (p=0.01) by stimulation of both RAR and RXR receptors (3.78±0.82, 3.75±0.78 and 5.94±0.80 vs. 1.00±0.80, for ATRA, LG 0.1 μM, LG 10 μM and controls, respectively). Although transcriptional upregulation of p53, Bax and Bcl-2 is not strictly coupled to apoptosis, our findings provide evidence of a retinoid control of genes involved in the apoptotic response.

Table 1.- Details of primers used for quantitative PCR

Gene	Forward and Reverse Primers 5´-3´	Amplicon size	Annealing Temperature	Accession Number
p53	CTCAGTCCTCTGCCATACTA (0,2 _M) GGATCCAGGATAAGGTGAGC (0,2 _M)	364 bp	55ºC	U74486
Bax	TGAGCGAGTGTCTGAAGC (0,1 _M) GCCTTGAGCACCAGTTTG (0,1 _M)	196 bp	60ºC	U92569
Bcl-2	TTTGCTTCAGGGTTTCATCC (0,2 _M) TGTTACTGTCCAATTCATCTCC (0,2 _M)	146 bp	60ºC	NM_173894

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Notes

DOES THE TIME NEEDED TO PASS DIFFERENT TRANSFER GUNS THROUGH THE CERVIX AND UTERINE BODY INFLUENCE THE PREGNANCY RESULTS IN RECIPIENT HEIFERS?

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The influence of the time needed to the pass the embryo transfer gun through the uterine cervix and body on pregnancy results after embryo transfer in recipient heifers was recorded. The experiment was carried out on 226 heifers of 15-18 months age. Oestrous cycles were synchronized using one or double prostaglandin injections. Shortly before embryo transfer, epidural anesthesia was carried out with 1% polocaine. Embryos (81 fresh and/or 88 and 57 frozen resp. in glycerol and ethylene glycol) were transferred to ipsilateral uterus horn on Day 7. For the transfer, two instruments were used – metal tip for transfer with a gold plated and transfer gun with sheaths and metal tip. The time of the manipulation into the uterus tract, – that is, the passage of instruments through cervix and uterine body – to the location of embryos in the uterus horn, was measured. In order to randomize the mistake risk, all manipulations were carried out by one experienced operator. The average time needed to the insertion of embryos into the uterus was 50.1s and it was longer for transfer gun with sheaths than for metal tip for transfer (60.9 and 40.8s resp.). A longer time to insert embryos in the uterus horn was noticed if the epidural anesthesia was performed before embryo transfer in comparison to cases without anesthesia (42.1 and 58.6s resp.). No significant differences with respect to the time of embryo location in the left or right uterine horn were noticed The average conception rate was 53.9%. If the time needed to insert embryos into the uterus was 10 – 60 s, the conception rate was 56.7% (up to 20, 21-30, 31-40, 41-50 and 51-60s – 100, 50, 54,1, 65,1 and 50% resp.). In contrast, if the time needed to insert the embryo in the uterus horn was longer than 60 s, the conception rate was 20.5% (61-70, 71-80, 81-90, > 90 – 26.7, 22, 0, and 22.2% resp.). Results that were achieved, indicate the essential influence of the manipulation time within the uterus and cervix during the embryo transfer on pregnancy results in recipients. Taking into consideration time relations, it seems that the type of instrument used during the transfer as well as epidural anesthesia preceding the manipulation are quite significant.

Notes

INTERACTIONS AMONG OOCYTE SIZE, FERTILISATION PROCEDURE AND EMBRYO BIOPSY ON GOAT BLASTOCYST QUALITY.

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The aim of this study was to study the relationship among oocyte size, fertilisation procedure (ICSI vs. IVF), embryo biopsy and their interactions on blastocyst quality expressed as cell number and apoptotic grade. Prepubertal goat oocytes were recovered from a local slaughterhouse and matured in a conventional IVM medium (TCM199 with serum, hormones and cysteamine) for 27h. Spermatozoa were selected by swim-up and capacitated with heparin (10 μg/ml)plus ionomycin (200 nM) for 15 min. A total of 930 IVM-oocytes were divided into four groups depending on size (GI: 110-125μm and GII: >125μm) and fertilisation procedure: ICS or IVF. Zygotes were cultured in TALP fertilisation medium. At 24 hours postinsemination (hpi), zygotes were cultured in SOF. At 48 hpi, oocyte cleavage was recorded. Four days after insemination, 6-8 cell embryos were biopsied by removal off one blastomere. After biopsy, embryos were cultured in SOF for a further 4 days. Blastocyst cell number and their apoptotic grade was assessed by TUNEL. Analysis of variance was done using SAS and Tukey post-test among oocyte size, fertilisation procedure (ICSI vs. IVF) and non-biopsied vs. biosied embryos from a total of 101 observations. We observed a positive correlation between oocyte size and both ICM and TE cell number ($P<0.001$), but no correlation was found with apoptotic cell number. Non biopsied embryos had a higher TE (not ICM) cell number than the biopsied ones ($P< 0.002$), but no differences were found in apoptosis. No differences were found in the rest of the interactions.

Goat blastocysts were also divided into three groups depending on their apoptotic grade (0 to 4.9; 5 to 9.9 and >10%). It was observed that blastocysts with less than 4.9% of apoptosis had a mean cell number of 249.2±11.1 cells; while blastocysts from 5- 9.9% of apoptosis had 116.4±18.6 cells; and blastocysts with more than 10% of apoptosis had a mean of 87.3 ±21.1 cells.

In conclusion, there is a positive correlation between oocyte size and ICM and TE cell number; and a negative relationship between embryo biopsy procedure and TE cell number. No parameter studied significantly affected blastocyst apoptotic grade. However, independent of the parameters studied, bigger blastocysts had a lower apoptotic grade.

Notes

COMPARISON OF TWO SPERM SELECTION METHODS AND TWO MEDIA FOR IN VITRO FERTILIZATION IN BOVINE

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The swim-up procedure is a commonly employed technique for sperm selection in the vitro fertilization (IVF) laboratory. It is a simple, fast and physiological method based on the spermatic motility. This work was designed to compare two swim-up methods (A, B) and two media (STL, SR®) in an IVF programme.

Bovine cumulus-oocyte complexes (COCs) were matured in TCM 199, 25mM $NaHCO_3$, 10 % FCS, LH (5 μg/ml), FSH (1 μg/ml), 17 β-Estradiol (1 μg/ml) and cysteamine (100μM). After maturation, two selection methods were performed. For the procedure Swim-up A (Parrish et al., 1986; Theriogenology 25, 591-600), 180 μl of thawed semen was layered under 1 ml of Sperm TALP medium (STL) or Sperm Rinse (SR®) medium, in conical tubes. They were incubated for 60 min at 39ºC and 5% CO_2 in air. Then, 0.8 ml aliquots from the top of each tube were pooled into a 15-ml conical centrifuge tube and centrifuged at 200 xg for 10 min. After discarding the supernatant, the volume needed to achieve a concentration of $1x10^6$ espermatozoa/ml was added to each fertilization well. For the procedure Swim-up B (Solvas et al., 2002, Asebir 7, 28-32), 2 ml of STL or SR® and 2 ml of thawed semen was mixed in a 15 ml tube and centrifuged at 363 g for 7 min. After discarding the supernatant, 0.8 ml of STL or SR® were carefully layered over the pellet. The tubes were held at a 45° angle and incubated for 60 min at 39ºC and 5% CO_2 in air. Then, the top 0.4 ml of the supernatant was recovered, analysed and used for fertilization. Frozen semen from the same bull was used for all the experiments. After IVF, presumptive zygotes were cultured in synthetic oviductal fluid (SOF) (Holm et al., 1999; Theriogenology 52, 683-700). Culture drops were supplemented with 5% FCS on Days 3 and 6. Embryo development was recorded on days 3, and 6 to 8. Data from 4 replicates were evaluated by χ^2 and expressed as percentages (a,b,c: $p<0.05$). The results are shown in the table below.

Table 1. Embryo development after sperm separation using two (A and B) swim-up procedures and two different media (SR® and STL).

Swim Up	Medium	Nº CCOs	Cleavage % (day 3)	Morulae % (day 6)	Blastocysts % (day 6)	Blastocysts % (day 7)	Expanded Blastocysts (%)
A	SR®	166	72.46a	19.75a	16.84a	18.40a	23.88a
A	STL	209	77.52a	13.03b	11.90b	11.90b	14.59b
B	SR®	192	75.81a	24.95c	12.37b	12.37b	23.30a
B	STL	125	62.64b	8.74d	2.25c	0c	2.71c

These results indicate that SR® commercial medium provides the best quality of spermatozoa, because the subsequent early embryo development is better than with STL medium. Therefore, SR® can be used as an alternative to STL for bovine sperm separation.

Notes

TRANSCRIPTIONAL PROFILE OF SPERMATOZOA AND OOCYTES IN PIG

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It has been reported that spermatozoa contribute in fertilization of the oocyte more than just their DNA. They also deliver a suite of novel mRNAs which may encode proteins crucial for early zygotic and embryonic development. Microarray analysis has revealed a complex population of transcripts that are common to both testis and mammalian mature spermatozoa. The structure of spermatozoal mRNAs is well defined but the role of these transcripts in spermatogenesis or early embryogenesis remains unclear. It has been suggested that the paternal contribution may be responsible for proper embryonic development and also exerts effect on a health of mammals in postnatal life. Ostermeier et al. (2004) revealed that several messenger RNAs can be delivered to the oocyte at fertilization.

Using reverse transcription and real-time quantitative PCR analysis (RQ-PCR), we decided to search spermatozoal mRNA, which could be delivered to the pig ovum during fertilisation. Spermatozoal mRNAs of clusterin (CLU), cAMP-response element modulator protein (CREM), linker histone 1 (H1), CYP19A1 (P450 aromatase), protamine 1 and 2 (PRM1, PRM2), TATA-associated factor 1 (TAF1) and TATA-binding protein (TBP), were screened in pig spermatozoa and oocyte.

Prepuberal gilt ovaries were collected from a local slaughterhouse and transported to the laboratory at 35C. Cummulus-oocyte complex (COC) were isolated after scarification of ovary surface under a stereoscopic microscope. These isolated COC were washed with NCSU37.

Pig ejaculated spermatozoa were purified by centrifugation thought discontinuous Percoll density gradient (80:40, vol/vol) and swim-up technique. Total RNA was isolated according to the method of Chomczy_ski and Sacchi. RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm. RNA samples were treated with DNase I, and reverse-transcribed into cDNA using oligo-dT primers. RQ-PCR was conducted in a Light Cycler real-time PCR detection system Roche Diagnostics GmbH, (Mannheim, Germany) using SYBR® Green I as detection dye and target cDNA was quantified using relative quantification method. The quantity of investigated transcript in each sample, was standardized by either glyceraldehyde-3-phosphate dehydrogenase or calculated per million cells.

In pig spermatozoa and oocytes, we identified CREM, H1, CYP19A1, PRM1, PRM2, TAF1 and TBP mRNAs. However CLU transcript was only present in spermatozoa. Our observation suggests that pig spermatozoa may delivery CLU mRNA into the oocyte, which may serve for protein biosynthesis and contribute to zygotic and embryonic development.

Notes

DETECTION OF SPECIFIC TRANSCRIPTS IN PIG OOCYTES, ZYGOTES AND 4 – CELL STAGE

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Maternal mRNAs play an important role to control of zygotic and embryonic development. During oogenesis in mature oocytes three classes of transcript are accumulated. In fertilized eggs these mRNAs are used as a template for protein biosynthesis that are relevant for normal zygote and embryo growth. First class of oocyte transcripts are immediately translated in fertilized female germ cells. Translation of protein corresponding to transcripts of class one also triggers transcription of these transcripts in zygotes. Second class of oocyte transcripts are used as template for protein biosynthesis during latter stages of embryogenesis. However, third class of transcripts are not used for translation after fertilization of oocyte.

Determination of quality and number of transcripts in the oocytes may serve as markers of oocyte maturation, which is responsible after fertilization for successful embryogenesis. We attempted to find transcripts of pig ovum, which can be used as the markers of normal egg development as well as proper embryogenesis. Using reverse transcription and real-time quantitative PCR analysis (RQ-PCR), we searched transcripts of calmegin (CLGN), clusterin (CLU), cAMP-response element modulator protein (CREM), linker histone H1, CYP19A1 (P450 aromatase), protamine 1 and 2 (PRM1, PRM2), TATA-associated factor 1 (TAF1) and TATA-binding protein (TBP) in mature oocytes, zygotes and 4-blastomers embryos.

Prepuberal gilt ovaries were collected from a local slaughterhouse and transported to the laboratory at 35ºC. Cumulus-oocyte complex (COC) were isolated after scarification of ovary surface under a stereoscopic microscope. These isolated COC were washed with NCSU37. Selected crossbread Landrace prepuberal gilts between 80 – 90 kg were superovulated by intramuscular injection of 1500 iu. PMSG (equine chorionic gonadotropin) followed 72 hours later by 500 iu hCG (human chorionic gonadotropin) and artificially inseminated 24 and 38 hours later after hCG. Gilts were slaughtered 48 hours after the hCG treatment. Reproductive tracts wererecovered, and embryos flushed with DPBS from the oviduct. After flushing, embryos were isolated under a stereoscopic microscope.

We detected in mature oocytes cAMP-response element modulator protein (CREM), linker histone H1, CYP19A1 (P450 aromatase), protamine 1 and 2 (PRM1, PRM2), TATA-associated factor 1 (TAF1) and TATA-binding protein (TBP) transcripts. We did not detect mRNA for CLGN in pig zygotes and CYP19A1, PRM1, PRM2 in 4-cell embryos. The presence of TAF1 and TBP transcripts might be associated with intensive transcription regulation mechanisms that take place during oocyte maturation. We also observed a two-fold higher presence of CYP19A1 mRNA in pig oocytes, probably associated with aromatization of androgens to estrogens that regulate oogenesis. Lack of CLGN and CLU transcripts can be resulted from needlessness of these mRNAs for maturation of oocytes. The lack of CLGN mRNA in pig zygotes and CYP19A1, PRM1, PRM2 in 4-blastomers embryos might be a reason of specific transcripts degradation mechanisms soon after fertilization.

We found several specific transcripts in pig oocytes, zygotes and 4-cell embryos; however, the relevance of them as markers of mature oocytes and normal embryogenesis requires more detailed investigation.

Notes

OPTIMIZATION OF FLUSHING PROCEDURES FOR EMBRYO RECOVERY IN DAIRY CATTLE

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The aim of our study was to invest the effect of extra flushing media on embryo recovery rate compared to the standard (control) flushing procedure in the same animal. Donors were both nulliparous Holstein heifers (11-15 months old) and non pregnant lactating primiparous Holstein Friesian cows (30-60 months old). Between day 8-13 of estrus cycle, donors were treated for superovulation with FSH. On the 3th day of superovulation luteolysis was induced subsequently followed by artificial insemination between 12 and 24 hrs after detection of first estrus behavior. In the control flushing procedure, the uterus was flushed horn by horn with 500 ml (per horn) of PBS. In experiment 1, the procedure was continued according Neto et al (Theriogenology 2005. 63(5):1249-1255) by recovering 150 ml to 200 ml of PBS left in the uterine body for 30 min. directly after the first flushing, with or without movement during these 30 min. In order to evaluate the effect of movement during the second flushing, half of the group was tied up and the other half was allowed to move. In experiment 2, adapted from Sartori et al (J. Dairy Sci. 2002. 85(11):2803-2812), by increasing the volume of flushing from 500 ml to 1000 ml per horn, the connecting tube and the collecting bottle were changed after the first 500 ml was infused. Subsequently an extra 500 ml of PBS was infused in the same horn and collected in a new bottle. Once the horn was totally flushed with 1l (500 ml Control + 500 ml extra), the catheter was taken out and passed again through the cervix to flush the remaining horn (500 ml initially and then 500 ml extra). Retrieved embryos were evaluated for number, quality and stage according IETS guidelines (1998). Data were analysed by using the Genstat version 8.01 computer programme by Ch-square and generalised linear models.

The average number of transferable embryos (class 1 and 2) collected in experiment 1 was 4.1. From these embryo's, 91% (3,7 transferable embryo) were recovered during the normal flushing procedure and 9% (0,4 transferable embryo) with a second flushing after a period of 30 min. The effect of moving during the waiting period had a positive significant effect on the recovery of total embryos (0,48 vs 0,18 extra transferable embryo, $p < 0,05$). In experiment 2, the average number of transferable embryos collected was 4.8 (class 1 and 2). From those embryos 81% were recovered during the initial volume of 500 ml (per horn) and 19% after the additional 500 ml.

Table 1: Results (mean ± SEM) from superovulated Holstein heifers/cows (n=40) with normal flushing (control, 500 ml) and additional volume (500 ml extra)

Embryos	Total Mean ±S.E.M	Control Mean ±S.E.M (% of total)	Additional Volume Mean ±S.E.M (% of total)
Class 1	4.0 ±0,31	3.3 ±0.28 (81)	0.7 ±0.13 (18)
Class 2	0.8 ±0.14	0.6 ±0.12 (75)	0.2 ±0.07 (25)
Class 3	0.4 ±0.1	0.3 ±0.09 (75)	0.1±0.02 (25)
Class 4	1.3 ±0.18	1.2 ±0.17 (92)	0.1 ±0.05 (8)
Total	6.6 ±0.4	5.5 ±0.37 (84)	1.1 ±0.16 (16)
Total unfertilized oocytes	2.9±0.27	2.3 ±0.24 (79)	0.6 ±0.12 (21)

Results from the first experiment showed that additional embryos/ova can be recovered by using an additional second flushing. It seems that mechanical factors (walking) could influence positively the number of embryos recovered during the second flushing. Results from the second experiment indicate that the use of 500 ml extra of PBS per horn is an efficient method to enhance the total embryo recovery by a factor 1,2. These findings confirm the hypothesis that not all embryos are recovered during the normal flushing procedure (Neto et al, 2005). Although the mechanism causing this increase is not fully defined it is suggested that increasing the flow rates might acts as a mechanical factor capable of increasing embryo recovery **(**Sartori et al, 2002**).**

Notes

ATTEMPTS TO IMPROVE NUCLEAR REMODELING IN BOVINE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS BY USING OOCYTE TREATED WITH MG-132.

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Complete reprogramming of somatic cell nuclei after nuclear transfert (NT) depends on extensive remodeling of chromatin structural components by factors present in the recipient cytoplast. When these nuclei are introduced into non-activated oocytes, the nuclear membrane breaks down rapidly and premature chromosome condensation (PCC) occurs. However, our previous experiments in bovine nuclear transfer demonstated that only 20% of somatic nuclei undergo PCC. This might be related to the recipient oocytes which exhibited an interphase-like stage after enucleation before fusion (Vignon et al, 2002. 18th AETE meeting). In rat species, it has been shown that the proteasome inhibitor MG-132 could reversibly prevent meiosis resumption in MII oocytes (Zhou et al, 2003. Science 302:1179). The aim of the present study was to maintain the bovine recipient cytoplast at the metaphase II cell stage during the fusion procedure and to assess the consequences on nuclear remodeling and developmental potential up to the blastocyst stage.

Bovine cumulus-oocytes complexes were recovered from slaughterhouse ovaries and matured in vitro for 23 h according to our standard protocol. Matured oocytes were denuded and then incubated for 45 mn and enucleated in the presence of 5µM MG-132. The control group was incubated and enucleated in TCM199. After rinsing, embryos were reconstructed by fusion with bovine adult fibroblasts and activated in 10µg/ml cycloheximide and 5µg/ml cytochalasin B. In experiment 1, embryos were fixed 1h after fusion in cold methanol. Microtubules and chromatin were then revealed by immunofluorescense with anti-tubulin antibody and staining with 10µg/ml propidium iodide and then observed using a confocal laser scanning microscope. In experiment 2, embryos were activated for 5h after fusion and co-cultured on Vero cells for 7 days in microdrops of B2 medium. In vitro development of both group (MG-132 and control) were recorded and cell counting were done at blastocyst stage (D7).

Results from experiment 1 revealed a higher rate of PCC in the MG-132 originated embryos than in control embryos: 96.0% (23/24) versus 24.0% (7/29). Moroever, multipolar microtubules close to chromatin were detected in MG-132 originated embryos attesting a metaphase state whereas cytoplasmic microtubules were observed in control embryos. In experiment 2, the reconstructed embryos fused at the same rate in treated and control groups. Cleavage, morula and blastocyst rates were not significantly different between the two groups. However the cell number in MG-132 treated embryos was significantly higher than in control (Table 1).

Table 1. In vitro development of NT embryos obtained with MG132 treated or control cytoplasts.

Recipient oocytes	Reconstructed embryos	Fused (%)	Cleaved (%) of fused	Morula (%) of fused	Blastocysts (%) of fused	Cell number X±SEM (n)
MG-132	93	70 (75.3)	58 (82.9)	40 (57.0)	39 (55.7)	134±25 (19)[a]
Control	106	85 (80.2)	64 (75.3)	43 (50.6)	43 (50.6)	109±43 (15)[b]

[a,b] : P<0.05, Student 's t-test

This study suggests that MG-132 could maintain the bovine recipient oocytes in a metaphase stage and that the induction of PCC occuring accordingly may be important for blastocyst quality and nuclear reprogramming in NT embryos. The full term development of MG-132 derived embryos is currently under investigation.

Notes

BOS TAURUS INDICUS PREGNANCY RATES FROM OPU/IVF: EMBRYO CULTURE IN SOFAACI WITH BSA OR BSA+FCS

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Bovine programs based on ovum pick-up associated with *in vitro* production of embryos become more developed in the last five years Brazil. Following this growth, new commercial perspectives were opened as well as the need for constant improvement. Pregnancies results in *Bos taurus indicus* derived from OPU/IVP programs with embryos cultured in BSA+FCS are few. In this study, immature *Cumulus*-oocytes complexes (COC; n=1745) recovered by follicular aspiration (OPU) from *Bos taurus indicus* donors of commercial programs were randomly assigned in two groups according their number and quality to evaluate the effect of fetal calf serum (FCS) on the *in vitro* culture (IVC). Standard bovine IVM procedure was carried out in modified TCM-199 + FSH, LH, Oestradiol, EGF, insulin and 10% bovine fetal serum (BFS) in an incubator at 39ºC with satured humidity and 5% CO_2, for 24h. Frozen semen was selected by Percoll gradient (90, 60 and 30%). The insemination was performed with $2x10^6$ spermatozoa/mL in Fert-Talp with heparin and PHE, for 18 to 22h. Presumptive zygotes were cultured (IVC) in SOFaaci (Holm *et* al., 1999) during 6 days with 5%CO_2, 5%O_2 and 90% N_2 an incubator at 39ºC with satured humidity. The SOFaaci was added of 4mg/mL bovine serum albumin (BSA=control) or 4mg/mL BSA+2% bovine fetal serum (BSA+FCS). The viability was accessed by morphological evaluation in D2 (cleavage), D7 (blastocysts and quality I category), and D60 (pregnancy rates), considering day zero (D0) as the fertilization day. Data were analyzed by an ANOVA (GLM procedure, SAS). The blastocyst rates (51%) and quality I blastocysts (41%) in D7, were higher ($P<0.05$) in BSA+FCS group compared to the BSA group (42 and 30%), respectively. The pregnancy rates from embryos cultured in BSA (41.3%) or BSA+FCS (41.0%) groups were similar ($P>0.05$; Chi-Square). Considering pregnancy rates corresponding only to IVM COC, the rates were higher ($P<0.05$) for the BSA+FCS group (16%) than the BSA group (12%). The IVC of bovine embryos with BSA+FCS to the SOFaa medium increased blastocyst quality and pregnancy rates.

Notes

PRODUCTION OF EMBRYOS FROM TOP BREEDING DAIRY CATTLE IN A FIELD MOET

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A field MOET called "HAKA project, Top Breeding Animals from Northern Savo" was organized by local and national breeding authorities in the Northern Savo, Finland between years 2000 and 2006. This project established 12 embryo pools including 121 dairy farms distributing the recovered fresh and frozen-thawed embryos inside and between the pools. Only top breeding dairy cattle were selected as embryo donors.

During the period of six years, a total number of 375 embryo collections were performed. The donors were either Finnish Ayshire (ay, 57%) or Holstein-Friesian (fr, 43%) dairy cattle. Of these, 51.5% were heifers and the remaining 48.5% were cows that had calved on average 2 to 3 times before flushing. The majority of the donors were treated with FSH (Folltropin-V) to induce superovulation and inseminated two to three times with frozen-thawed semen of top breeding bulls. The donors were flushed on Day 7 by five embryo transfer veterinarians. An average total number of all embryos (transferable, degenerated and unfertilized oocytes) was 11.4 (ay) and 9.9 (fr) and an average total number of transferable embryos was 8.0 (ay) and 6.8 (fr) per donor. Seven percent of the flushings resulted in no transferable embryos and no embryos were recovered in 7% of the flushings. Of the recovered transferable embryos (n= 2,699), majority (72%) were at the blastocyst stage (ranged from early to hatched blastocysts). Of all transferable embryos, 72%, 17%, 10% and 1% were of Grade I, Grade II, Grade III or embryos which were not graded, respectively. In principal, Grade I and Grade II embryos were either transferred fresh or cryopreserved but grade III embryos were only transferred fresh. The embryos were cryopreserved using 1.5M ethyleneglycol as a cryoprotectant and a standard slow freezing protocol in a LN_2 or a methanol bath freezer.

One fifth of the embryos (mainly transferred fresh) were biopsied, sexed and 52% were of female and 48% of male gender. To date, 82 percent of the transferable fresh and frozen-thawed embryos have been transferred into the recipients with an average rate of success of 47% (Table 1.). The remaining 18% include embryos stored in LN_2 and male embryos that were discarded. Success after transfer was recorded by born calves, abortions or re-inseminations within 60 days after transfer.

Table 1. Results of success after transfer of embryos in a field MOET "HAKA" during the years 2000-2006.

Transferred embryos	Born calves and abortions	Inseminated within 60 days after transfer (% of transferred embryos)	Success after transfer not known	Total
Fresh	650 (52)	413 (33)	187 (15)	1250 (100)
Frozen-thawed	393 (41)	399 (41)	172 (18)	964 (100)
Total	1043 (47)	812 (37)	359 (16)	2214 (100)

In conclusion, excellent embryo yields and good pregnancy results encourage embryo pools to continue flushing donors of top genetic merit. "HAKA" brand contributes to a high quality of the embryos.

Notes

PREDICTION OF IN VITRO FERTILIZING ABILITY OF SPERMATOZOA IN BULLS

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The bull's effect on efficiency of in vitro embryo production has been well documented. The aim of this work was to characterize fertilization in bulls with good outcomes in field fertility and different efficiency in embryo production to discover some parameters suitable for a prompt and valuable prediction of in vitro fertilizing ability of bulls.

The frozen semen of nine two-year-old bulls of the Czech pied breed obtained from one AI-center with 60.2-66.4 % non-return rates was used. At the beginning of the experiments, Day 8 embryos were prepared from each bull by a standard protocol in triplicate. Oocytes were matured in TCM-199 medium with 5% estrus cow serum, gonadotropins (P.G.600), and antibiotics for 24 h, and they were fertilized by spermatozoa isolated by the swim-up method from frozen-thawed sperm. Fertilization was carried out in IVF-TALP medium containing $1x10^6$/ml spermatozoa and 10 μg /ml heparin. Presumptive zygotes were transferred to a BRL cell line monolayer 24 h after fertilization and co-cultured at 38.5ºC in a humidified atmosphere of air with 5% CO_2. According to the embryo development rate, the bulls were categorized as those with high (n=3), medium (n=3) or low (n=3) production of embryos (mean ± SD %: 25.2 ± 0.7, 20.5 ± 2.1 and 13.8 ± 1.1 respectively). To characterize fertilization in each bull, adequate numbers of inseminated oocytes were denuded, fixed in 2.5% glutaraldehyde water solution (v/v), stained in bisbenzimide-33258 Hoechst solution (10 μl/ml in citrate buffer), and examined by fluorescence microscopy. The rates of penetrated and fertilized oocytes were evaluated at 6 h and 18 h, respectively, and the rate of syngamy-stage oocytes at 18 h after fertilization. The rate of cleaved oocytes from inseminated oocytes and that of 4-cell embryos from cleaved oocytes were checked at 24 h after co-culture. Data were analyzed by the Chi-square test and Pearson's correlation test.

The rates of both of penetrated oocytes at 8 h (mean ± SD %: 65.2 ± 10.6, 49.0 ± 10.1 and 33.3 ± 7.4) and syngamy-stage oocytes at 18 h (mean ± SD %: 18.7 ± 6.1, 9.4 ± 0.6 and 5.6 ± 1.5) differed significantly ($p \leq 0.01$) among bulls with high, medium or low embryo production, respectively. On the other hand no differences in fertilization rate at 18 h were found among bulls with high, medium and low embryo production (mean %± SD: 88.3 ± 5.5, 91.0 ± 1.9 % and 85.4 ± 2.9 %, respectively). The cleavage rate of oocytes and rate of 4-cell embryos from cleaved oocytes were significantly higher ($p \leq 0.01$) in the high than in the low-embryo production bulls.(mean ± SD %: 83.4 ± 2.9 vs. 72.1 ± 10.1 and 82.2 ± 1.8 vs. 73.2 ± 4.3 respectively), but not when compared with the medium-embryo production bulls. A correlation was determined between embryo production and each of the parameters, i.e., penetration ($r = .803$, $p \leq 0.01$), syngamy ($r = .826$, $p \leq 0.01$), cleavage ($r = .635$) and 4-cell embryos ($r = .709$, $p \leq 0.05$). In conclusion, the parameters evaluated varied in their value for predicting in vitro fertility of bulls, the most important being early penetration and syngamy stage.

Notes

EFFECT OF TAXOL ON THE SPINDLE CONFIGURATION AFTER CRYOPRESERVATION OF BOVINE OOCYTES IN OPEN PULLED STRAWS: A CONFOCAL STUDY

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The meiotic spindle of matured oocytes is vulnerable to cryoinjury with depolymerization of its microtubules at lower temperatures. Stabilizing the cytoskeleton system during vitrification could be beneficial for improving post-thawed survival and subsequent development of vitrified oocytes. Taxol™, paclitaxel, is a microtubule stabilizer and is currently being used as an anticancer drug. It increases the rate of polymerization by reducing the critical concentration that is needed for polymerization. Taxol has been used in cryopreservation of embryos [1], and it has been found that Taxol pre-treatment can improve development competence of vitrified mouse [2] and human [3] oocytes. Our objective was to evaluate the incidence of spindle abnormalities on bovine matured oocytes vitrified and thawed when Taxol™ was added to the vitrification solution. Matured oocytes were vitrified by the open pulled straw (OPS) method. Once in vitro matured, oocytes were divided into 4 experimental groups: 1) left untreated (control); 2) cryopreserved by the open pulled straw method (OPS); 3) exposed to 1μM Taxol™ (Taxol); 4) vitrified by OPS including 1μM Taxol™ to the vitrification solution (Taxol+OPS). After thawing, oocytes were fixed, stained using specific fluorescent probes and examined under a confocal microscope.

Table 1. Spindle morphology and chromosomes alignment in MII stage cow oocytes after vitrification by OPS method with or without Taxol™.

			Spindle configuration, *n* (%)*			Chromosome alignment, *n* (%)*		
	n	MII Total *N* (%)	Normal	Abnormal	Absent	Dispersed	Decondensed	Absents
Control	193	172 (89,12) a	132 (76,74) a	39 (22,67) a	1 (0,58) a	39 (22,67) a	1 (0,58) a	0
OPS	145	118 (81,38) ab	55 (46,61) b	52 (44,07) b	11 (9,32) b	48 (40,68) b	15 (12,71) b	0
Taxol	66	54 (81,82) ab	42 (77,77) a	12 (22,22) a	0 c	8 (14,81) a	4 (7,41) b	0
Taxol + OPS	107	86 (80,37) ab	43 (50,00) b	39 (45,35) b	4 (4,65) ab	37 (43,02) b	6 (6,98) b	0

Values with different superscripts within each column are significantly different, $P<0.05$.* Values calculated over the number of oocytes reaching the MII stage after 24 h of culture Percentages of normal spindle and chromosome configurations were significantly lower in OPS oocytes (46.6%) and Taxol+OPS (50%) compared to control (76.7%) or Taxol (77.8%). Vitrification by OPS or Taxol+OPS led to increased percentages of oocytes with abnormal spindles structures associated with disorganized or decondensed microtubules or chromosomes compared to control oocytes or oocytes exposed to Taxol. These results indicate that stabilizing the cytoskeleton system in mature cow oocytes with Taxol™ did not maintain normal spindle and chromosome configuration after vitrification.

[1]Dobrinsky et al. (2000) *Biology of Reproduction* 62, 564-70; [2]Park et al. (2001) *Fertility and Sterility* 75, 1177-1184; [3]Fuchinoue et al. (2004) *Journal of Assisted Reproduction and Genetics* 21, 307-309

Notes

TRANSGENERATIONAL EPIGENETIC ALTERATIONS PRODUCED BY ART PROCEDURES IN MICE

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In mammals, there are two major epigenetic reprogramming events which, occur during primordial germ cell differentiation and preimplantation development. Transgenerational effects in response to particular embryonic environments are well recognised and have been associated with the abnormal establishment of epigenetic regulation in the foetus, however, no relationship has been establish between alterations in the epigenetic reprogramming during preimplantation and transgenerational responses. Recently, it has been found in the mammalian genome, several parasitic segments of DNA with the ability to move from one place to another with high epigenetic variability at specific insertion sites, capable of originating substantial phenotypic variability, retaining the capacity for epigenetic inheritance. The objective of our study was, to evaluate the ability of particular assisted reproductive technology (ART) procedures to induce epigenetic alteration in these, also called, metastable epialleles. For this, B6D2 mice suffering from kinky tail phenotype, which is known to be a consequence of an intracisternal-A particle (IAP) metastable epiallele insertion in the intron 6 of the mouse axin gene, were submitted to several ART procedures and the methylation frequency in the IAP promoter region in the blastocysts produced after matting with normal mice, and the kinky tail phenotype in the offspring was evaluated. The ART procedures tested included, superovulation, in vitro culture, in vitro culture with 10% fetal calf serum (FCS), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), and were used alone or in combination. Relatively to in vivo controls, a significant increase ($P<0.05$; Z-test) in IAP promoter region methylation, after in vitro culture with 10% FCS, or superovulation followed by IVF (or ICSI) and in vitro culture, was found. Concomitant with this, in relation to natural matting, ICSI significantly increased ($P<0.05$; Z-test) the frequency of kinky tail in the offspring of male mice carrying this phenotype. These results confirm that particular ART procedures are capable, either alone or in combination, of inducing epigenetic alteration in metastable epialleles; in other words, transgenerational inheritance of epigenetic alteration.

Notes

IN THE HORSE, N-ACETYLGLUCOSAMINE RESIDUES ON OOCYTE ZONA PELLUCIDA ARE NOT NECESSARY FOR SPERM-ZONA PELLUCIDA BINDING

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In the horse, in vitro fertilization (IVF) rates are low. Spermatozoa bind to the zona pellucida (ZP) but do not penetrate. To improve IVF rates, the mechanism of equine sperm-zona binding must be elucidated. In the mouse, sperm binding is mediated by a sperm surface galactosyltransferase (GalTase) that recognizes N-Acetylglucosamine (N-AcGlc) residues on the ZP glycoprotein ZPC. In the horse, the mechanism of sperm-zona interaction remains unknown. We demonstrated previously the presence of N-AcGlc residues on the equine ZP (Kölle et al., 2006, Mol. Reprod. Dev., in press). Our aim was to analyse the role of N-AcGlc residues in sperm-zona binding in the horse.

Equine oocytes were collected from slaughtered mares and in vitro matured for 30 hours. N-AcGlc residues were then blocked. In experiment 1, oocytes were incubated for 1 hour with or without the enzyme GalTase; in experiment 2, oocytes were incubated for 30 minutes with or without the lectin wheat germ agglutinin (WGA) or the lectin WGA linked with a fluorochrome FITC.

The oocytes were then placed in the IVF medium (Synthetic Oviductal Fluid supplemented with 15% Fetal Calf Serum and 40µg/ml gentamicin). Semen was collected, filtered, diluted and treated with calcium ionophore A23187. Gametes were co-incubated for 20 minutes. Then, the number of sperm cells bound to the ZP was counted.

In experiment 1, we ascertained the Galtase binding on the ZP using an anti-Galtase antibody. The number of spermatozoa bound to the ZP was 99 ± 37 in the presence of GalTase (n=43 oocytes) and 104 ± 71 in its absence (n=42). In experiment 2, we ascertained the WGA binding on the ZP using the lectin linked to the fluorochrome. The number of spermatozoa bound to the ZP was 78 ± 46 in the presence of WGA (n=37), 72 ± 33 in the presence of WGA linked to the fluorochrome (n=36) and 64 ± 38 with no additives (n=35). The number of spermatozoa bound to the ZP was not significantly influenced by the Galtase or the WGA.

In conclusion, in our conditions, blocking N-AcGlc does not affect equine sperm-zona binding. This residue may not be involved in the mechanism of sperm-zona interaction.

Notes

GENE EXPRESSION IN THE PRE-IMPLANTATION BOVINE EMBRYO

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The main goal of this study was to identify mRNA transcripts which may play a role in the minor and major activation of the embryonic genome. Bovine embryos were obtained after in vitro maturation of oocytes and their subsequent fertilization and culture in vitro (COOK media). We compared the gene expression profile between 4-cell stage embryos and bovine MII oocytes using the technique of supression subtractive hybridisation (Clontech PCR-Select cDNA Subtraction kit, BD Biosciences Clontech). Differentially expressed amplicons were subcloned (TA cloning kit, Invitrogen) and 60 of them sequenced (ABI Prism 310, PerkinElmer). Analysis of these sequences with the GenBank database resulted in identification of 41 known cDNAs and 19 novel sequences. From known cDNAs, 3 exist more then one time in our library. The expression of seven differently expressed genes with apparent function in cell proliferation, chromatin remodelling, regulation of transcription or translation and protein ubiquitination was further characterized during pre-implantation bovine development (MII oocyte, 2, 4, early 8, late 8-cell stage, morula, blastocyst, hatched blastocyst) by real-time RT-PCR (RotorGene 3000, Corbett Research). The data were obtained from three independent real-time RT-PCRs from three different batches of embryos. The significance of differences between stages was evaluated using a t-test (SigmaStat).

Table of studied genes

Identity	**Abbreviation**	**Homology**	**Gene Bank Accession N^{o}**
Homo sapiens nuclear receptor co-repressor 1	NCOR1	88%	NM_006311
Bos taurus thymosine beta4	Tmsb4	99%	AY_192438
Mus musculus ring finger protein 4	Rnf4	92%	NM_011278
Bos taurus poly(rC) binding protein 1	PCBP1	98%	NM_001015565
Bos taurus D-glucuronyl C5 epimerase	Glce	99%	AF003927
Homo sapiens 26S proteasome, subunit 9		96%	AF001212
Bos taurus plasma membrane Calcium transporting ATPase	PMCA	95%	AF 332982

We observed an increase in the relative abundance of NCOR1, tmsb4, Rnf4, PCBP1 and Glce both at the 2-4-cell stage and late 8-cell stage. The transcription of NCOR1 and tmsb4 is _-amanitin sensitive in 2-4-cell and late 8-cell embryos. It means that both genes are expressed even during the period of minor gene activation. The transcription of Rnf4, PCBP1 and Glce is _-amanitin sensitive only from the late 8-cell stage. The mRNAs for 26S proteasome and PMCA show the highest abundance in MII oocytes; their content decreased gradually thereafter until the early 8-cell stage. The increase of expression at the late 8-cell stage can be inhibited by _-amanitin. All described genes can play an important role in embryonic genome activation and during pre-implantation development of bovine embryos.

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Notes

EXPRESSION OF APOPTOTIC PROTEINS IN BOVINE IMMATURE OOCYTES WITH DIFFERENTIATED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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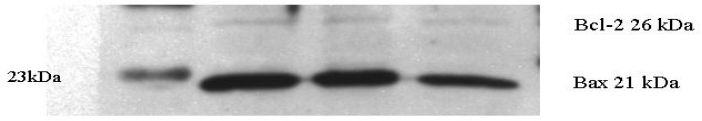
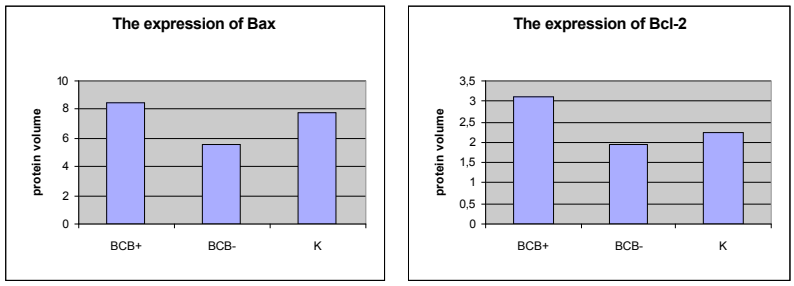
It has been demonstrated that brilliant cresyl blue (BCB) can be used for the selection of competent oocytes of prepubertal pigs, goats and cattle. Since the ovaries from the slaughtered animal contain heterogeneous population of oocytes the BCB test was used by Alm et al. (Theriogenology 2005, 63:2194-2205) to select bovine oocytes developmentally competent for embryo production. BCB staining demonstrates the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD); the high concentration of G6PD reduces a blue compound in BCB to a colorless one (BCB-). The grown oocytes are blue (BCB+), because the G6PD activity is too small to reduce the staining. However, there are no data indicating the correlation between activity of G6PD and the degree of apoptosis. Therefore the aim of our study was to estimate the level of apoptosis in immature bovine oocytes on the basis of Bcl-2 and Bax proteins expression and assess whether there is a correlation between the level of G6PD activity and the apoptotic process.

Bovine immature cumulus-oocyte complexes (COC) recovered from ovaries of slaughtered cattle 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 (K). The oocytes of all groups (n= 140 each) were stripped off cumulus cells and stored at –80°C for immunostaining (Bcl-2 and Bax).



M BCB+ BCB- K



Looking at the correlation between Bcl-2 and Bax proteins expression in oocytes BCB+, BCB- and control it is not obvious which oocytes are the best. The level of Bax protein expression is even lower in BCB- group when compared to BCB+ and K, however the Bcl-2 expression is also lower in BCB- group. The BCB+ group has the highest overall protein expression (as estimated by Bcl-2 and Bax) when compared to BCB- and K groups. This may imply that BCB+ oocytes are more competent in aspect of cytoplasmic maturation. The overexpression of Bax protein doesn't necessarily mean that the fate of the oocytes is already directed towards programmed cell death, as the results presented above do not include the complicity of relations between all pro- and anti-apoptotic proteins.

[1] *Research was supported by the State Committee for Scientific Research as a project 2PO6D 00728.*

Notes

APOPTOSIS AS A QUALITY MARKER IN BOVINE IMMATURE AND MATURE OOCYTES WITH DIFFERENTIATED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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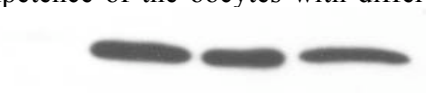
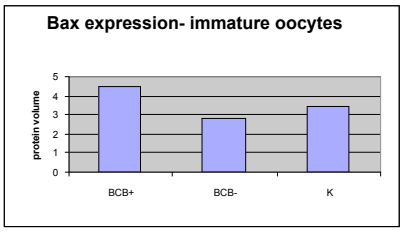
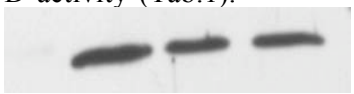
Brilliant cresyl blue (BCB) staining can be used to select oocytes developmentally competent for embryo production. BCB staining demonstrates the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD); the high concentration of G6PD reduces a blue compound in BCB to a colorless one (BCB-). However, there are no data indicating the correlation between activity of G6PD and the degree of apoptosis. Therefore the aim of our study was to estimate the level of apoptosis in immature and mature bovine oocytes on the basis of expression of death promoting protein (Bax) and to assess whether there is a correlation between the level of G6PD activity and the apoptotic process.

After careful morphological selection, bovine immature cumulus-oocyte complexes (COC) recovered from slaughtered ovaries were stained with 26 μM BCB, then separated according to the cytoplasm coloration (BCB+ and BCB-). COCs not subjected to BCB staining served as a control (K). A pool of 110 immature oocytes and 30 in vitro mature oocytes for each group (BCB+, BCB- and K; and 3 replications) were used for immunostaining (Bax). The in vitro fertilization and embryo culturing were carried out with the mature oocytes of all groups to establish developmental competence of the oocytes with differentiated G6PD activity (Tab.1).







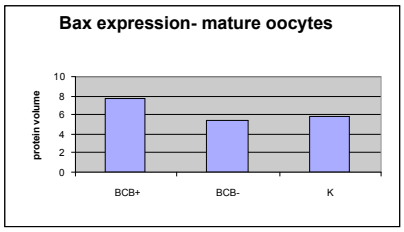


Table 1. Developmental competence of oocytes with differentiated activity of G6PD

Type of oocytes	N of oocytes taken to IVF	N (%) of cleaving oocytes	N (%) developing blastocysts
BCB+	732	609 (83.2%)*	68 (11.2%)
BCB-	317	204 (64.4 %)*	18 (8.8%)
K	81	63 (77.8%)*	6 (9.5%)

*Significantly different from other values within column (chi-square, P<0.1).

Bax expression in immature and mature oocytes was smaller in BCB- oocytes when compared to BCB+ and K. On the other hand the cleavage rates of BCB- oocytes as well as the blastocyst rates indicate that these oocytes showed the lowest developmental competence. Presented results may suggest that lowered developmental competence of BCB- oocytes was not connected with apoptosis, and the low Bax expression in BCB- oocytes might be a result of smaller overall protein amount in growing oocytes when compared to grown ones. To precisely assess correlation between the G6PD activity and the apoptotic process in the oocytes several pro- and anti-apoptotic proteins should be estimated.

[1] *Research was supported by the State Committee for Scientific Research as a project 2PO6D 00728.*

Notes

EMBRYONIC DEVELOPMENT IN THE BITCH DURING DAYS 10 TO 19 AFTER OVULATION

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The knowledge about embryonic development in canine species is clearly lagging behind most other domestic species. The time course of maturation, fertilization and early embryonic development *in vivo* has been investigated just recently (Reynaud et al., 2005, Biol. Reprod. 130, 193-201). Obviously there is a certain asynchrony in the early embryonic development, so that 2-cell, 4-cell and 8-cell embryos can be found within one particular bitch. Entrance into the uterus seems to occur between the 16-cell stage and the early blastocyst stage (Holst and Phemister, 1971_Biol Reprod 5: 771-779; Bysted et al., 2001 Reprod Fert, Suppl 57: 181-186). The aim of this study was to describe the development of canine embryos during the time from the morula stage to hatching and implantation.

A total of 25 female dogs, from which at least one embryo could be recovered, were included in this study. The bitches had been either mated or inseminated with freshly recovered semen. The day of ovulation was determined by a combination of vaginoscopy, vaginal cytology, and concentration of progesterone in the peripheral blood. Ten to 19 days after ovulation ovariohysterectomy was performed under general anesthesia. The experiments have been conducted under the appropriate legal authority. The excised uterus was trimmed free of connective tissue and the uterine horns were flushed with 10 to 20 ml warm PBS to which 10% (v/v) fetal calf serum had been added. Embryos were searched under a dissecting microscope and evaluated morphologically.

A total of 106 embryos (morula or blasotcysts) were recovered from 25 dogs. Only 8 unfertilized oocytes and 15 retarded embryos (2- to 4-cell) were found along with normal embryos. Embryos in the morula stage were recovered on day 11, 12, 14. Non-expanded blastocysts could be found from day 12 until day 15 after ovulation. Expanded blastocysts with a thinning zona pellucida were recovered as early as day 12 until day 17. A zona-free blastocyst was detected on day 16. In one of those dogs, 5 expanded blastocysts were collected, from which the biggest embryo had a diameter of nearly 1 mm. In these embryos the trophectoderm and the inner cell mass was somewhat collapsed so that the embryonic coat was clearly visible. The nature of this embryonic coat could not be verified. A total of 32 empty embryonic coats could be isolated from the flushing media in 5 dogs. Due to their shrunken appearance, their size and nature could not be identified. In three dogs neither embryos nor an empty zona pellucida could be found on day 18 or 19 after ovulation but the uterus contained clearly visible implantation sites.

The results of this study suggest that an asynchronous development can be observed also during the period of 12 to 15 days of embryonic development in the bitch. Thinning of the zona pellucida in expanding embryos could be frequently observed but none of the embryos could be seen during the process of hatching and only one hatched blastocyst could be recovered. A very important observation is that well expanded blastocysts on day 16 after

ovulation are contained in a clearly visible embryonic coat. The morphology of these embryos had a striking similiarity to equine day-8 blastocysts with the so-called capsule.

Notes

FACTORS RELEASED FROM DEATH SPERMATOZOA INDUCE SPERM DNA FRAGMENTATION AND REDUCE IMPLANTATION AFTER ICSI

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The role of sperm chromatin integrity during intracytoplasmic sperm injection (ICSI) is of critical importance, since it bypasses multiple mechanisms, apparently redundant, which have evolved to ensure selection of high quality sperm cells for fertilization. When ICSI is employed, even spermatozoa with severe DNA damage may undergo pronucleus formation. We have analyzed in B6D2 mouse strain, the proportion of DNA-fragmented spermatozoa (DFS) produced by incubation in condicionated medium from fragmented sperm (CM), and the subsequent development, implantation, and offspring obtained after ICSI of incubated spermatozoa. The condicionated medium used in this study was generated by freeze-thawing sperm in the absence of cryoprotection. When fresh sperm cells were incubated for 90 min in this medium, a significant increase in the DFS was detected by TUNEL assay (30% vs 7% in fresh sperm cells and 14% in sperm incubated for 90 min in M2 medium). After ICSI of fresh and incubated spermatozoa, embryos were cultured in vitro either until 2-cell or blastocyst stage, before transfer into pseudopregnant CD1 females. At day 14, recipients were sacrificed and the implantation rate, number of foetuses, and reabsorptions was evaluated. Although differences in embryo development and morphology were not detected during the in vitro culture period, it was observed that independently of sperm pre-treatment, the implantation rate was significantly reduced (Z-test; $P<0.05$) when blastocysts were transferred. In addition, a negative correlation was found between the percentage of DFS (higher in incubated sperm) and the implantation rate. Interestingly, no difference was found in development to term between sperm pre-treatments. These results demonstrate that there are factors released from fragmented spermatozoa (most likely endonucleases) capable of inducing DNA fragmentation in intact sperm cells. They illustrate, as well, that embryo development and morphology can not be used as predictors for ICSI outcomes, whereas the evaluation of the DFS may prove to be useful.

Table 1. Effect of the sperm pretreatment on embryo development after ICSI.

Sperm Pre-treatment	Injected Oocytes (Sessions)	Surviving Oocytes (%)	2-cell Embryos (%)	Bl (%)	2-cell or Bl Transferred	Implantation (%)	Day 14 Foetuses/ Reabsorptions
No incubation	59 (2)	49 (83%)	47 (96%)	-	47	32 (68%)[a]	19(40%)/ 13 (28%)
90 min in CM	75 (2)	54 (72%)	49 (91%)	-	49	24 (49%)[b]	15 (31%)/ 9 (18%)
No incubation	57 (2)	57 (100%)	53 (93%)	26 (49%)	26	14 (54%)[ab]	4 (15%)/ 10 (38%)
90 min in CM	85 (2)	70 (82%)	64 (91%)	28 (44%)	28	6 (21%)[c]	4 (14%)/ 2 (7%)

[a,b,c] Significant differences ($P<0.05$) are indicated

Notes

CRYOPRESERVATION OF IN-VIVO DAY 7 QUALITY 2 AND 3 BOVINE EMBRYOS CULTURE FOR 24 HOURS BEFORE FREEZING IN THREE DIFFERENT CRYOPROTECTANTS

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The objective of this study was to test whether Day 7 quality 2 and 3 (IETS manual) in-vivo derived bovine embryos can be successfully frozen after additional in-vitro culture for 24 h. Embryos were collected post-mortem and cultured in TCM-199 medium containing 0.3% BSA incubated for 24 h under paraffin oil in humidified atmosphere of 5% CO_2 in air at 39^0C. Only embryos that developed to the blastocyst stage were then allocated at random to one of 3 different cryoprotectants solutions; ethylene glycol, 1,2 propanediol or glycerol. Embryos were equilibrated in each cryoprotectant in a single step at room temperature for 10 min during which five to 10 embryos were loaded into 0.25 ml straws (IMV, L'Aigle, France. Straws were placed directly into the freezing chamber of an alcohol freezer (Bio-Cool 3; FTS-Systems, Inc., Stone Ridge, NY, U.S.A.) at -5^0C, and after 1 min were seeded with cotton Q-tip, held for 10 min, cooled at 0.5^0C/min to -35^0C, and then plunged into liquid nitrogen. Embryos were thawed for 10 sec in the air and then in a 30^0C water bath without stirring until ice was melted (~10 sec). Embryos were expelled into TCM-199 with 0.2M sucrose and held for 3 min at 25^0C. After two washes in Hepes and once in TCM-199 embryos were cultured as described above. Embryo morphology and development were evaluated under the stereomicroscope. Embryos that re-expanded within 2 h after thawing and progressed further in development were considered as viable.

Table 1. Post-thaw survival of Day 7 quality 2 and 3 in-vivo bovine embryos previously cultured for 24 hr and frozen in 1.5 M of three different cryoprotectants and rehydrated directly in PBS after thawing.

Cryoprotectant (1.5 M)	Total No. of embryos	Survival rate after thawing (%)	Survival rate after 48 hr in culture (%)
Glycerol	85	85.8	21.2 [a]
Propanediol	95	70.5	51.7 [b]
Ethylene glycol	104	79.8	59.6 [b]

(ab - $P<0.05$)

Although there was no difference in embryos survival/expansion after 2 hr after thawing and culture in any tested cryoprotectant, post-thaw embryo survival frozen in glycerol and rehydrated directly after thawing was significantly lower ($P<0.05$) than embryos frozen in propanediol and ethylene glycol that were no different.

Our results indicate that bovine embryos that in many instances are considered not of transferable quality maybe successfully frozen after a 24 h in-vitro culture and propanediol and ethylene glycol are equally efficacious for direct cryoprotectant removal after freezing/thawing.

Notes

BACTERIAL CONTAMINATION IN LIQUID NITROGEN CONTAINERS: PATHOGEN AND DISINFECTION[1]

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The use of liquid nitrogen containers to store frozen semen, embryos or both is common in many countries. Some of them are for private use while others are for commercial reasons, particularly for bovine, buffalo, sheep, goat, swine or horse herds. Inadequate and poor handling of the straws, racks, canes and also liquid nitrogen may occur and lead to containers becoming contaminated. This kind of manipulation can reduce the herd productivity by lower pregnancy rates with important economic impact. Knowledge about the sanitary conditions of the containers is important to established a preventative program and also to improve pregnancy rates. In this study swabs were collected from two canes still under the liquid N_2 and from the bottom of containers (n=12) used for storage of semen and embryos. The samples were identified and sent in a modified Stuart's transport medium to the Microbiology Laboratory of the Federal University of Santa Maria to be cultured for aerobic bacteria. Afterwards the canisters and containers were washed with water, then with water and neutral Extran®-MA02 soap (Merck) following rinsed and disinfected. Group A (n=8): rinsed with 2% glutaraldeid (Glutaron II®- Rioquimica) and after that with 70% ethanol. Group B (n=4): rinsed only with 70% ethanol. After they were left to dry at room temperature and canisters were dried in Pasteur oven at 60ºC. A second sample was taken from each container and canisters to verified the efficiency of the procedure. The *Bacillus cereus* was isolated from eleven (91.6%) containers as well as from corresponding canisters. Additionally, *Bacillus gram negative oxidize negative, Staphylococcus sp., Streptococcus sp.* and *Escherichia coli* were isolated from two of twelve containers. In one (8.4%) container it was found *Proteus mirabilis*. After disinfection none of the containers showed evidence of bacterial contamination. This study emphasizes the importance of routine disinfection of the containers to avoid bacterial contamination.

[1] *This work was supported by a grant from Fapergs/RS. Proade 3 Project: 0523100/2006*

Notes

PUBERTY AND FERTILITY OF SOMATIC CLONED HEIFERS COMPARED TO CONTROL AI FEMALES

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Cattle produced by adult somatic cloning have proved to be able to develop, mature sexually and reproduce by natural mating or AI. However limited information has been published on the reproductive characteristics of cloned females. Lanza et al. (2001) reported that cloned animals exhibited puberty at expected age and body weight, but Enright et al. (2002) who investigated the endocrine status of cloned heifers observed a delayed onset of puberty in a set of 4 females.

The aim of the present work was to evaluate sexual maturity in a group of cloned females in comparison with control females born and raised under the same conditions at the experimental farm of INRA. Clones (n=10) were derived from cultured skin fibroblasts of 3 different Holstein adult donors. Control heifers (n=11) were contemporary born Holstein calves derived from AI. All the calves were carefully checked for health and development from birth to weaning, housed in the same barns and fed on the same regimen. Growth rate was evaluated by monthly weighing. In order to detect onset of cyclicity by rapid plasma progesterone analysis, blood samples were taken by venipuncture on all females every 10 days between 8 and 14 month of age. Heat detection was carried out twice a day by visual observation. Age at puberty was evaluated by estimation of the first ovulation before plasma progesterone increase, associated with estrus behaviour. At 15-16 month of age, cyclic cloned and control heifers were bred by AI to assess fertility. Frozen semen from the same bull was used for insemination at estrus induced by progestagen implant synchronisation treatment.

Table 1. Onset of puberty in clone and control heifers

Group	Clones	Controls
Nb of heifers	N= 10	N=11
Mean birth weight(kg ± SE)	45.8±3.6[a]	39.6±4.0[b]
Mean age at puberty (days ± SE)	419.3±42.5[a]	356.5±50.5[b]
Mean body weight at puberty (kg ± SE)	359.0±38.9[a]	303.0 ±22.3[b]
Daily gain up to 15 months (kg/day ± SE)	0.746±0.069	0.779±0.085
Mean estrus cycle length before breeding (days)	20.9±0.9	20.1±1.0

Student test : [a,b] values within line with different superscript are significantly different P< 0.05

As shown in table 1, in our conditions, clones reached puberty significantly later (+ 62 days) than controls and at a higher body weight (+ 56 kg). This was not due to difference in growth rate as daily gain was similar between groups. After first ovulation, however, the mean estrus cycle length was similar in both groups. At first insemination, 5 out of 8 females became pregnant in the clones group and 4 already calved normally. This was similar to the control group in which 7/10 females were pregnant at 1rst AI and subsequently calved.

Lanza et al., (2001) Science 294:1893-94 ; Enright et al. (2002) Biol.Reprod. 66:291-296

Notes

EFFECT OF MATURATION STAGE OF BOVINE OOCYTES ON SEX RATIO AND SPEED OF EMBRYO DEVELOPMENT

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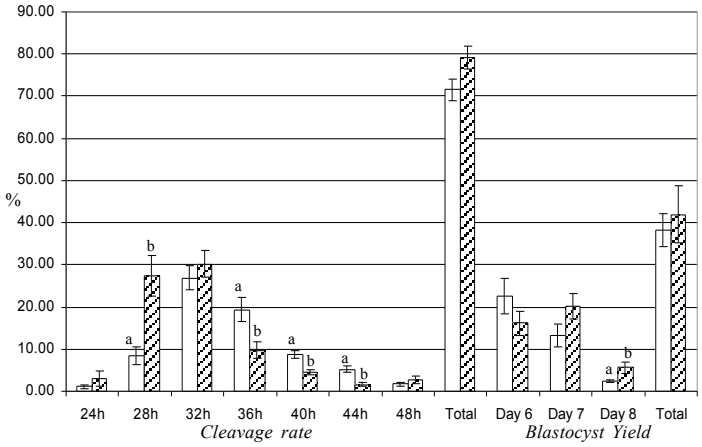
Evidence from several species indicates that embryos produced in vitro that reach the blastocyst stage earliest are more likely to be males than females. Furthermore, the faster-developing blastocysts in in vitro culture systems are generally considered more viable, and better able to survive cryopreservation or embryo transfer than those that develop more slowly. The aim of this study was to determine if the maturation stage of the oocyte at the time of insemination could affect early embryo development (kinetics of cleavage and blastocyst yield) and the sex ratio (male:female) of in vitro derived bovine embryos. Oocytes were collected from ovaries of slaughtered heifers and randomly divided in two groups for maturation. One group was matured for 16 h (n=804) and the other for 24 h (n=791) before insemination. Presumptive zygotes from each group were examined every 4 hours between 24-48 h post-insemination (hpi) and cultured up to Day 8 to assess blastocyst development in synthetic oviduct fluid (8 replicates). Blastocysts on Day 6, 7 and 8 from both groups were snap frozen for sexing. Sexing was performed with a single PCR using a specific primer BRY (Reed et al., 1989). There was a significantly lower number of cleaved embryos at 28, 36, 40 and 44 hpi from 16 h compared to 24 h maturation group ($P \leq 0.05$). However, the overall cleavage rate at 48 hpi was similar for both groups (Figure 1). The overall blastocyst yield on Day 8 was similar for both groups (16 h: 38.24 ± 4.04 and 24 h: 41.93 ± 6.79). In the 24 h maturation group, a higher proportion of Day 6 blastocysts were male than in the 16 h group (67.2 vs 51.3% respectively, $P \leq 0.05$). However, the total number of male blastocysts obtained after 8 days of culture was not different between groups (16 h: 49.7% and 24 h: 58.1%). These results show that the maturational stage of the oocyte at the time of fertilization has an effect on the kinetics of early cleavage divisions but not on the overall cleavage rate or blastocyst yield. Furthermore, while there was no difference in the overall sex ratio amongst the blastocysts obtained, the distributrion of male and female blastocysts between Days 6 and 8 was affected by the duration of maturation.

Figure 1. Effect of duration of maturation (16 h: white bars vs 24 h: dashed bars) on the kinetics of cleavage and blastosyst yield.



Notes

AN ASSESSMENT OF BOVIPURE® AND SWIM UP SEPARATION OF BULL SPERMATOZOA FOR *IN VITRO* FERTILIZATION

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The aim of our research was to examine the ability of density gradient separation BoviPure® and swim up method on bull sperm separation and in vitro embryo production (IVP). Frozen/thawed semen from six Simmental bulls was pooled and treated using both methods. The sperm motility, concentration, membrane activity, membrane integrity and acrosomal status were evaluated and compared before and after sperm processing using BoviPure® and swim up methods. A total of 641 oocytes were matured and fertilized in vitro and cultured in SOFaaBSA. The cleavage rates on Day 2, the total number of morulas (M) and blastocysts (Bl) on Day 7 and the numbers of hatched blastocysts (hBl) on Day 9 were recorded. Differential staining of the inner cell mass (ICM) and trophectoderm cells were performed on Day 7 blastocysts. The percentage of cleavage and the percentage of hatched embryos were similar for both methods, 77.25 ± 2.02%;14.88 ± 2.38% for BoviPure® and 72.63 ± 3.98%;12.11 ± 0.69% for swim up. However, embryo production rate at Day 7 was significantly higher using BoviPure® method (P<0.05) vs. swim up, 31.79 ± 0.71% and 21.91 ± 2.49%, respectively. Also, total cell number and embryo differential staining of day 7 blastocysts showed that BoviPure® treated sperm displayed better quality embryos compared to swim up method (P<0.05).

Table 1. Sperm parameters results (means ± S.E.M.)

Sperm separation method	Progressive motility (%)	Concentration (10^6spz/mL)	HOS % active	SYBR-14/PI % live	EthD1/FITC-PSA % live with intact acrosome
Initial (n = 6)	50.00 ± 8.16[a]	82.75 ± 5.25[a]	39.94 ± 8.98[a]	43.34 ± 6.88[a]	46.04 ± 12.56[a]
BoviPure®(n=6)	70.00 ± 3.54[b]	27.25 ± 1.70[b]	54.35 ± 2.75[b]	72.68 ± 2.79[b]	75.93 ± 0.91[b]
Swim up (n = 6)	53.75 ± 3.15[a]	20.00 ± 5.34[b]	45.90 ± 1.84[a]	50.99 ± 2.18[a]	59.24 ± 2.42[a]

Values with different superscripts within the same columns differ significantly (P<0.05; ANOVA)

Table 2. Effect of BoviPure® and swim up methods on total cell number and number of inner cell mass cells in day 7 blastocysts (mean ± S.E.M.)

Sperm separation method	Total cells N	ICM N	ICM Proportion (%)
BoviPure® (n=12)	141.83 ± 6.14[a]	39.92 ± 2.83[a]	28,15[a]
Swim up (n= 12)	121.92 ± 7.32[b]	31.83 ± 2.13[b]	26,11[a]

Values with different superscripts within the same columns differ significantly (P<0.05; ANOVA)

Therefore, our results indicate that BoviPure® method has an enhanced capacity in sperm selection for in vitro embryo production when compared with swim up method. So, we concluded that BoviPure® could be considered as a better alternative to swim up method for separating bull spermatozoa from frozen/thawed semen for IVP of bovine embryos.

Notes

METABOLISM OF IVF CALVES COMPARED WITH AI CALVES BORN BY CAESAREAN SECTION

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A number of in vitro produced (IVF) calves are delivered by caesarean section. The present study compared neonatal parameters such as blood chemistry, organ weights, thermoregulation and raising behaviour in IVF calves born by caesarean section with those of half siblings produced by artificial insemination (AI).

Dairy heifers were pregnant either after receiving an IVF embryo (in vitro fertilized with a dairy bull and cultured to blastocyst stage in SOF (n=8) or after artificial insemination (n=6) with the same bull. At gestation day 278, the calves were delivered by caesarean section. Instantly after parturition, permanent catheters were inserted into the jugular veins of the newborns and blood samples as well as body temperature were taken at 5 min, 2h, 1h, 12h, 2h, 22h, 3h, 6h, 9h, 12h, 19h, 21h, 22_h, 24h. The calves were fed with 40 ml colostrum/kg at 2h, 6h, 12h, 21h and euthanized at 24h for collection and weighing of internal organs. Whole blood samples were analysed for pH, pCO_2, pO_2, hemoglobin, glucose, Na^+ and Cl^-. The data were analysed by Fishers Exact test and are presented as LS means ± SEM values with a significance level of $P<0.05$.

Within 24 h after birth, the IVF calves compared with the AI calves showed lower mean blood pH (7.29±0.02 vs. 7.34±0.02), lower Na^+ (140.5±0.6 vs.142.1 ± 0.7mM) higher cortisol (129±8 vs 32± 8 ng/ml) lower relative weights of liver and colon, higher relative weights of heart and thymus, lower body temperature (38.11± 0.22 vs 38.52± 0.17 ^oC) and retarded raising behaviour.

With maturity, the weight of the thymus decreases and the heavier thymus in the present study was therefore likely due to immaturity of the IVF calves. The lower blood pH, thermoregulation and delayed raising behaviour reflected a slower adaptation to postnatal life and the lower weight of the liver and colon may a consequence of a slower developmental growth of the gastrointestinal organs in the IVF calves. The higher cortisol level could be a result of a higher level of postnatal stress, since immaturity usually is associated with lack of cortisol.

The study documented that neonatal IVP calves delivered by caesarean section were more compromised and more immature during the first 24 h than their half siblings produced by artificial insemination.

Notes

VITRIFICATION OF BOVINE BLASTOCYSTS PRODUCED AFTER FERTILIZATION OF OOCYTES MATURED IN FATTY ACIDS CONTAINING MEDIA

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Non-esterified fatty acids (NEFA) such as palmitic acid (PA), stearic acid (SA) and oleic acid (OA) are the predominant fatty acids in the follicular fluid during the period of negative energy balance (NEB) of high yielding dairy cows. We showed previously that high PA and SA concentrations during maturation had a negative effect on oocyte developmental competence (Leroy et al., 2005), which may be due to changes in membrane properties of the oocytes. It was the aim of the present study to evaluate whether high PA or OA concentrations present during maturation could have a carry-over effect on the embryo and subsequently could affect embryo freezability, by using open pulled straw (OPS) vitrification.

Cumulus oocytes complexes (COCs) were matured for 24 hours at 39 °C in a humidified atmosphere of 5% CO_2 in air. The basic maturation medium consisted of serum-free TCM199 supplemented with EGF 20 ng/ml (negative control) and this was supplemented with ethanol alone (positive control) or with NEFA (0.133 mmol/l PA or 0.200 mmol/l OA) solved in ethanol. Both NEFAs were tested in separate experiments in 4-5 replicates. Fertilized oocytes (n = 2776) were cultured for 7 days in SOF medium. At day 8 post insemination, the embryos were divided according developmental stage to expanded blastocyst (ExB) and hatching or hatched blastocyst (HB). Then each group was cryopreserved by OPS-vitrification using ethylene glycol and dimethyl sulfoxide as cryoprotectants. Data were analysed using logistic regression analyses and considered statistically significant when $P < 0.05$.

Addition of OA to *in vitro* maturation media had no significant effects on post thawing viability of vitrified embryos. However, addition of PA to *in vitro* maturation media (Table 1) significantly ($P < 0.05$) decreased the freezability of bovine embryos from 66.7% in positive control to 51.3% in PA. Moreover, ExB had significantly higher survival rate than HB in all studied groups.

Table 1: Percentage of survival (Mean ± SD) of vitrified bovine blastocysts matured in PA

Treatment		*ExB*	*HB*	**Total**
Palmitic acid	No. Vitrified	63	17	80
	% Survived	57.14 ± 2.9	29.4 ± 4.6[a]	51.3 ± 6.1[a]
Positive control	No. Vitrified	62	22	84
	% Survived	79.0 ± 2.5	31.8 ± 5.2[b]	66.7 ± 7.4[b]
Negative control	No. Vitrified	41	30	71
	% Survived	61.0 ± 2.7	30.0 ± 2.1[a]	47.9 ± 4.1[a]

[a,b] Values in the same column with different superscripts differ significantly ($P < 0.05$).

We concluded that the addition of PA to *in vitro* maturation media affected the freezability of in vitro produced bovine embryos. We also suggest that the absence of the zona pellucida significantly affected embryo survival post-vitrification in a negative way.

Notes

PREIMPLANTATION DEVELOPMENT OF PORCINE TRANSGENIC CLONED EMBRYOS DERIVED FROM NUCLEOFECTED FOETAL FIBROBLAST CELLS EXPRESSING ENHANCED GREEN FLUORESCENT PROTEIN

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The purpose of our study was to determine the *in vitro* developmental potential of porcine nuclear transfer-derived embryos reconstituted with foetal fibroblast cells, which had been subjected to nucleofection with *pWAPhGH-GFPBsd* gene construct. This method of transfection as a combination of lipofection and electroporation enables targeted transduction of the gene construct directly into the cell nucleus of somatic cell, which is mediated through liposome carriers. Moreover, compared to the standard transfection methods of somatic cells such as lipofection or electroporation, nucleofection technique allows considerable shortening the verification timing for transgenesis efficiency, which is performed through live-eGFP reporter gene expression control, from 24 to 48 h up to even 4 to 6 h after transfection procedure. The nucleofection samples consisting of 0.5-1 × 10^6 cells per 100 μL Dermal Fibroblast Nucleofector™ Solution (Amaxa Biosystems) with the 3.5 μg highly purified linear DNA in the 2 μL Tris/HCl-EDTA buffer were transferred into the amaxa certified cuvettes. The cuvettes were inserted into the Nucleofector™ apparatus, in which AC pulses were generated using U-20 program for high transfection efficiency. Positively-selected transgenic foetal fibroblast cells, which had been evaluated by live-eGFP fluorescence excitation, were *in vitro* cultured up to a total confluence state and then used for the somatic cell nuclear transfer (SCNT). *In vitro*-matured oocytes were the source of recipient cells. Maternal chromosomes were removed by a modified chemically assisted microsurgical technique. Single nuclear donor cells were injected into a perivitelline space of previously enucleated oocytes. Fibroblast cell-ooplast couplets were simultaneously fused and activated with two consecutive DC pulses of 1.2 kV/cm for 60 _sec. Reconstructed embryos were *in vitro* cultured at 39.0°C in a humidified atmosphere of 5% CO_2 and 95% air in 50-μL drops of NCSU-23 supplemented with 0.4% BSA-V for 4 days, followed by NCSU-23 with addition of 10% FBS for 2 to 3 days. The rates of cleavage and development to morula/blastocyst stages were examined on Days 2 and 6/7, respectively. A total of 98/177 (55.4%) enucleated oocytes were successfully fused with transgenic nuclear donor cells and intended to be *in vitro* cultured. Out of 98 reconstructed oocytes, 82 (83.7%) SCNT embryos were cleaved. The frequencies of cloned embryos, that reached the morula and blastocyst stages, were 31/98 (31.6%) and 22/98 (22.5%), respectively. Nucleofection efficiency of *in vitro* cultured porcine foetal fibroblasts as estimated by nuclear donor live-fluorescent evaluation based on expression index of eGFP reporter transgene was nearly 100%. It was also found that porcine SCNT-derived morulae and blastocysts exhibited approximately 100% index of xenogeneic eGFP gene transcriptional activity, which revealed the live diagnostics of emission intensity for green fluorescent protein-derived biochemiluminescence.

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Notes

A NEW AND SIMPLE METHOD TO EVALUATE EARLY MEMBRANE CHANGES IN FRESH AND STORED BOAR SEMEN

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Introduction. Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. It is a well-characterized mechanism that allows eukaryotes to eliminate unneeded, senescent, or aberrant cells. While apoptosis in somatic cells and in spermatocytes and spermatids *in vivo* is well established, the presence and significance of apoptosis in ejaculated animal sperm is still unresolved. The basal apoptotic cell population in ejaculated sperm might result from abortive apoptosis of sperm that escaped the elimination mechanism operating during spermatogenesis. In the present study we designed a new YO-PRO-1/PI assay to detect changes in boar spermatozoa, based on the slight increase of sperm membrane permeability.

Material and methods. Three ejaculates from three boars were used in the experiment. After collection, separation of gel, the semen was diluted in Biosolwens plus extender and stored for three days at 15°C. Every day of experiment the sperm pellet was resuspended in TALP to the concentration of 2 X 10^6sperm/ml. 1µl YO-PRO-1 stock solution and 1µl of PI stock solution were added to the samples. The tubes were gently mixed and incubated for 20 minutes at room temperature in the dark. After the incubation period the sperm cell suspensions were analyzed under a fluorescence microscope at 40X magnification.

Results. We observed three groups of sperm in fresh semen under a fluorescence microscope: apoptotic sperm showed green fluorescence (2-13%), necrotic sperm showed red and green fluorescence (9-26%) and live sperm showed no fluorescence (64-89%).

Boar No.1			
	Ejaculate		
Semen	I	II	III
Fresh semen (%)	A=2 a,b	A=2 a,b	A=4 a
	N=9 a	N=19 a	N=19 a
	L=89 b	L=79	L=77
First day of storage (%)	A=5 a	A=2.5 a,b	A=3
	N=14 a,b	N=21 a	N=20
	L=81 a,b	L=76.5	L=77
Second day of storage (%)	A= 3 b	A=1 a,b	A=5.5 a
	N=22	N=27	N=26.5
	L=81 b	L=72	L=68
Third day of storage (%)	A=7	A=4	A=6
	N=25	N=31.5	N=27
	L=68	L=64.5	L=67

Boar No.2			
	Ejaculate		
Semen	I	II	III
Fresh semen (%)	A=3 a	A=8 b	A=9 a,b
	N=17	N=23	N=24
	L=80 b	L=69	L=67
First day of storage (%)	A=6.5	A=8.5 b	A=9 b
	N=18	N=24	N=28
	L=75.5 a	L=67.5	L=63 a,b
Second day of storage (%)	A=9 b	A=9 b	A=12 b
	N=20 b	N=25.5	N=33
	L=71	L=65.5	L=62.5
Third day of storage (%)	A=9	A=10.5	A=11
	N=23 a,b	N=25.5	N=38.5 a
	L=86 a,b	L=64 a	L=50.5 a

Boar No.3			
	Ejaculate		
Semen	I	II	III
Fresh semen (%)	A=9	A=13	A=6
	N=18	N=23	N=26
	L=73	L=64 b	L=68
First day of storage (%)	A=8 a,b	A=14 a,b	A=1 a,b
	N=20	N=23	N=37 b
	L=72	L=63 b	L=62 b
Second day of storage (%)	A=9.5 a,b	A=10 a,b	A=1.5 a,b
	N=24 a	N=34	N=42 a,b
	L=66.5	L=56 b	L=56.5 b
Third day of storage (%)	A=6.5 a	A=12 a	A=3 a
	N=30 a	N=39	N=43 a,b
	L=63.5 a	L=49 a,b	L=54

A=apoptotic sperm (%), N= necrotic sperm (%), L= live sperm (%)

[a]=significant differences in the percentage of apoptotic, necrotic and live sperm among the ejaculates of the same boar

[b]= significant differences in the percentage of apoptotic, necrotic and live sperm among the boars

The results were compared by _^{2}test. Statistical differences were considered to be significant at $P<0.05$

Conclusions. As storage time increased, the number of necrotic spermatozoa in semen increased. In some ejaculates, both increased and decreased numbers of apoptotic spermatozoa were observed during storage at 15°C. Significant differences in the percentage of apoptotic sperm among the boars and among the ejaculates of the same boar were observed.

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Notes

DIPLOIDY AND ANEUPLOIDY IN PORCINE OOCYTES MATURED IN VITRO IN RELATION TO OOCYTE DONOR (GILT VS SOW)

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Early embryonic loss is a very well known phenomenon in the pig, since 25-40% of embryos die within the first 30 days after insemination. A large proportion of porcine IVM oocytes (17% - 30%) and IVF embryos (39.1%) are chromosomally abnormal. Diploidy accounts for the most often observed anomaly in porcine secondary oocytes *in vitro* (15% – 27.7%) whereas the frequency of aneuploidy is lower (5%-14%). The number of analyzable spreads however is usually low due to chromosome clumping, overlapping and also chromosome loss. Fluorescence in situ hybridization (FISH) shows several advantages over the conventional Giemza staining, since metaphase spreads of reduced quality as well as interphase nuclei can be successfully analyzed. Sow oocytes are characterized by better morphology, cleavage and blastocyst rates when compared to gilts. Moreover, gilts ovulated more aneuploid cells and their oocytes require longer time to mature *in vitro*. Since prepubertal ovaries dominate as oocyte source in slaughterhouse material, it is likely that elevated levels of chromosome unbalance observed among pig oocytes when compared to other species of domestic animals is partly due to this factor.

The aims of the project are i) to find out whether the incidence of chromosomally unbalanced oocytes (diploid, aneuploid) differs between cells derived from sow and gilt ovaries, ii) to compare effectiveness of conventional analysis and FISH in terms of chromosome investigation of pig secondary oocytes.

Cumulus-oocyte complexes of good morphology were matured *in vitro* (TCM199+FCS+17β-estradiol+hCG+antibiotics) for 44h at 39°C in 5% CO_2 humidified atmosphere. After maturation, oocytes were subjected for slide preparation (air-dry method). Some slides were stained in 5% Giemza whereas the remaining slides were stored at -20°C for FISH. BAC probes specific for two porcine chromosomes (pairs 1 and 10) were applied.

Up to now, 296 oocytes collected from 34 females (23 gilts, 11 sows) were analyzed. Ovaries of older sows of known reproductive history were collected after slaughter. This group was characterized by the following parameters: average age - 3.5 years, mean number of litters – 5.1, mean litter size – 10.6. On average 20-30% of all collected COCs were suitable for IVM. A successful analysis was possible for 64.5% of oocytes (191/296). Conventional analysis: A correct, haploid set of chromosomes was found in 81% cells (47/58), whereas in the remaining 19% (11/58) a diploid set was observed. FISH: Altogether 133 mature oocytes were analyzed. The majority of cells 71.4% (95/133) was haploid, whereas the remaining 28.6% (38/133) comprised unbalanced chromosome set: 31 were diploid (23.3%) and 7 aneuploid (5.2%; 4 with disomy: 2 of chr. 1 and 2 of chr. 10; 3 with nullisomy of chr. 10). The frequency of aberrant oocytes differed between sows (diploidy 19.4%, aneuploidy 1.6%) and gilts (26.8%, 8.4% respectively). Interestingly, aneuploid oocytes were found in 1 sow

(12.5%) and 6 gilts (50.0%). Basing on the preliminary results one may suggest that sows and gilts may differ with regard to aberrant oocytes after maturation in vitro.

Notes

DIELECTROPHORETIC BEHAVIOUR OF BOVINE ZYGOTES AND ITS RELATION TO DEVELOPMENTAL COMPETENCE AND MRNA EXPRESSION

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This experiment was conducted to validate the use of dielectrophoresis as a non invasive technique to select developmentally competent bovine zygotes by evaluating the dielectrophoretic behaviour of bovine zygotes in relation to the developmental competence and transcriptional abundance. To achieve this, two consecutive experiments were conducted. In the 1st experiment, bovine zygotes were subjected to dielectrophoresis at 4MHz Alternating Current field frequency, 450 µm electrode distance and medium conductivity of 80 µs/cm. Depending on their speed in the electric field, zygotes were classified into very fast (1-6 s), fast (7-15 s), slow (16-20 s) and very slow (≥21 s). These dielectrophoretic categories were further cultured to assess the rate of embryonic development. The result shows that the blastocyst rate at 7 days post insemination (dpi) was significantly ($p<0.05$) higher in very fast (16.1 ± 2.7) than slow (9.1 ± 2.7) and very slow (10.6 ± 2.7). However significance difference was not observed at 8 and 9 dpi between the groups revealing that dielectrophoretically separated zygotes different in the rate of development otherwise the total blastocyst yield was the same. Investigating the molecular mechanisms for the difference in dielectrophoretic behaviour and developmental rate between the very fast and very slow groups was the challenge to be answered. Therefore, in the 2nd experiment transcriptional analysis was performed between the very fast and very slow dielectrophoretic categories to investigate the mRNA transcription abundance level using Blue chip cDNA bovine microarray. The result shows that 845 genes were equally expressed and 42 genes were differentially regulated at False Discovery Rate (FDR) of 3.2%. A total of 25 genes were up-regulated and 17 genes were found down regulated in the very fast dielectrophoretic categories of zygotes compared to the very slow. Among the up regulated genes, ZNF85, ZNF519, NANOS1, DNMT1, and ANXA2 are involving in ion binding, DDX10 is thought to involve in embryogenesis, IQGAP1 involves in calmodulin binding, and RGS2, ARL6IP and RALA are signal regulators. Additionally, STK6, SMARCA5 and NASP are needed for cell cycle. On the other hand, RPL8, RPLPO and RPS8 were among the down regulated genes and involve in protein biosynthesis and RNA binding. Hence, from this experiment we can conclude that the difference in rate of embryonic development at 7dpi and transcriptional abundances of dielectrophoretically separated zygote is an indicator of the tendency of dielectrophoresis procedure to discriminate zygotes according to the developmental competence.

Notes

IN VITRO MATURATION OF OOCYTES AND DEVELOPMENT OF ACTIVATED OR CLONED PORCINE EMBRYOS IN A CHEMICALLY DEFINED MEDIUM

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Optimization of pig oocyte maturation and embryo culture systems may have a crucial value to fulfill the increasing demand for numerous good quality oocytes and embryos in agricultural, medical and basic research. Chemically defined media are of great importance to determine the actual role of supplements, such as growth factors, cytokines, and hormones on maturation of oocytes and preimplantation development of embryos. In the present study, we evaluated the effect of PZM4 (PZM+3mg/mL PVA, Yoshioka et al, Bio. Reprod.2002,66:112-119) on the nuclear maturation of gilt cumulus-oocytes complexes (COCs) and their subsequent development after parthenogenetic activation. In addition, we investigated the effect of PZM4 on in vitro development of somatic cloned embryos derived from sow oocytes. Data were analyzed using chi-square and student's *t*-test. In Experiment 1, abattoir-derived prepubertal gilt COCs were matured in BSA-free NCSU23 (Petters & Wells, J. Reprod. Fertil. Suppl.1993,48:61-73) supplemented with 10 iu/mL eCG,10 iu/mL hCG, 10ng/mL EGF and 10% porcine follicular fluid (pFF); or PZM4 completed with the same supplements except pFF; for 40-44h at 39°C, 100% humidity and 5% CO_2 in air. Nuclear maturation was evaluated by the presence of the 1st polar body. For parthenogenetic activation, denuded oocytes with polar body were selected and stimulated with a single 2.0kV/cm, 30μs direct pulse followed by culture in NCSU23. The rate of nuclear maturation was higher for COCs matured in NCSU23 than for those matured in PZM4 (88.6% vs. 74.2%; $p<0.05$). For the cleavage rate, no significant difference was observed between parthenogenetic activated embryos derived from the two groups (60.4% vs 50.9%, $p>0.05$); while the blastocyst rate was significant higher in NCSU23 group than that in PZM4 group (23.4% vs 11.4%, $p<0.05$). No significant difference in total cell number of blastocysts was observed between the two groups. In Experiment 2, we used in vitro matured abattoir-derived sow oocytes as recipients, and ear skin fibroblasts from a Göttingen miniature pig as somatic cell donors to produce cloned embryos. Following reconstruction, activated embryos were cultured in either PZM4, PZM3 (PZM+3mg/mL BSA) or PZM5 (PZM+5% cattle serum),. Similar cleavage (70.1%, 82.7% and 80.2%, respectively, $p>0.05$) and blastocyst rates (19.3%, 26.8% and 30.8% respectively, $p>0.05$) were observed in the three groups. Again, no significant differences between the three groups in the total cell number of blastocysts were observed. Our results demonstrated that chemically defined PZM4 could support in vitro maturation of gilt oocytes and development of these oocytes to the blastocyst stage after activation. Additionally, in vitro development of somatic cell cloned pig embryos did not differ in the chemically defined PZM4 from that obtained in PZM3 and PZM5, with BSA and serum supplementation, respectively. This model may be helpful to investigate the exact role of certain supplements of culture media on early development of pig embryos.

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