Embryo technologies in dairy cattle

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Abstract
Embryo technologies are a combination of assisted reproduction, cellular and molecular biology and genomics. Their classical use in animal breeding has been to increase the number of superior genotypes but with advancement in biotechnology and genomics they have become a tool for transgenesis and genotyping. MOET has been well established for many years and still accounts for the majority of the embryos produced worldwide. However, no progress has been made in the last 20 years to increase the number of transferable embryos and to reduce the side effects on the reproductive performance of the donors. In vitro embryo production is a newer and more flexible approach, although it is technically more demanding and requires specific laboratory expertise and equipment that are most important for the quality of the embryos produced. Somatic cell cloning
is a rapidly developing area and a very valuable technique to copy superior genotypes or to generate transgenic animals. More knowledge in oocyte and embryo biology is expected to shed new light
on the early developmental events, including epigenetic changes on embryo survival and their long lasting effect on the newborn.
Embryo technologies are here to stay and their use will increase as advances in the understanding of the mechanisms governing basic biological processes are made.

Key words: MOET, IVP, bovine, cloning, in vitro

INTRODUCTION

The cattle industry is undergoing a major reorganization in Europe, primarily as a result of the BSE and Foot and Mouth disease outbreaks that had a devastating effect in 2001, but also as a consequence of increased competition worldwide. Because of these economic difficulties and the need to advance some research area, the application of advanced reproductive techniques in cattle breeding has stabilised. Research in farm animal reproduction is also shrinking and changing, although the demand worldwide for animal derived products is expected to constantly increase and therefore there is the need to invest in research and technical advancements to sustain such growth. Increasing animal production is certainly not a priority in Europe today and research funds are diverted to more “green areas” such as sustainable agriculture and animal welfare. In this situation the main drivers and users of embryo technologies are not farmers at large but genetic companies
or breeder’s cooperatives that earn their revenues from the sale of genetic stocks (semen, embryos, animals, etc.).

A second problem that Europe is facing is a general negative attitude towards the biotech products, and embryo technologies are identified in this area. It is not uncommon today to find retailers that will distribute, for example, beef that has not been produced by embryo technologies, even by simple MOET, let alone IVP or cloning. An important objective today is to prove more convincingly the role and the value of advanced reproductive technologies in cattle breeding. More research is needed towards this goal.
The scope of this paper is to review the state of the art in the different embryo technologies used by the cattle industry for reproduction and selection, relative advantages and disadvantages, bottlenecks of some technologies and the science needed to overcome them. Some indication will be given for possible future research directions.

MOET

Multiple Ovulation and Embryo Transfer (MOET) is a well established technology and is used to obtain over 80% of the embryos produced for commercial purposes (1). The initial use of undefined superovulation products like eCG has been replaced by pituitary extracts (porcine and ovine) and in some cases by human menopausal gonadotropins (HMG). These latter products have a shorter half-life and require multiple injections but have the advantages of lacking side effects such as over-stimulation, failure of ovulation and persistent follicular cysts. Most of the pituitary extracts available on the market have varying ratios of FSH and LH. They are administered in the mid-luteal phase of the estrus cycle of the donor over a 4-5 days period and are combined with induced luteolysis. At estrus the donor is inseminated, usually with at least two straws of semen 12 h apart, and seven days later the uterus is flushed to recover the embryos. On average 4-6 transferable quality embryos are recovered. The outcome of the treatment is very variable: one third of the donors treated do not respond to superovulation, another third produces an average of 1-3 embryos and only one third actually superovulates giving a large number of embryos (2). This is the main drawback of MOET because breeding companies usually require a few particular sire-dam combinations. Another limitation is the requirement of a donor in perfect gynecological conditions and this is best achieved with donors that have passed their lactation peak since the superovulatory treatment interferes with lactation and induces a drop in milk production. In many cases donors are dried to achieve better results. Repeated superovulatory treatment of heifers or cows has side effects on fertility (development of cystic syndromes, difficulties in getting them pregnant) and on udder ligaments that can relax and downgrade the morphology in show animals. In young heifers, the superovulatory treatment can cause excessive premature udder development that can compromise the future show possibility of the donor.
For a long time several researchers have attempted to improve embryo yield with little success. However these research efforts have resulted in the simplification and rationalization of the procedures involved in MOET (3). For example, dominant follicle ablation and estrogen administration can synchronize follicular wave emergence and the superovulatory treatment can be scheduled at the most convenient time. Because of the BSE threat there is mounting hostility against the use of pituitary extracts since the tissue of origin is now considered Specific Risk Material. Recently, Germany has unilaterally outlawed the use of pituitary extracts for superovulatory treatments leaving de facto only eCG and possibly hMG as economically viable alternatives. Another option would be to use recombinant FSH and LH, that are available for human use, but their high cost is not acceptable for routine use in cattle.

IN VITRO EMBRYO PRODUCTION

An alternative system to produce cattle embryos is to use immature oocytes collected from the ovaries of donors of various age and physiological status (4). Reliable procedures allow

maturation and fertilisation of bovine oocytes in vitro and several culture protocols can be used to grow them for about a week up to the stage suitable for transfer or freezing. In vitro embryo production (IVP) was developed initially as a research tool and was applied to rescue follicular oocytes of slaughtered donors. In cattle, besides this use, IVP has become important for the production of embryos from live donors as an alternative to or integrated with MOET because of the advantages and flexibility that it offers (5). As an example of the different activities of an IVP laboratory see Fig. 1.
IVP entails the completion of 3 biological steps that are now relatively well established in cattle: oocyte in vitro maturation, in vitro fertilization and embryo culture (6).

In vitro maturation

Oocytes for in vitro maturation can be collected from different types of donors and by different methods as will be described later. Oocytes are very sensitive to temperature shocks so it is important to monitor carefully the collection procedure temperatures as fluctuations can easily occur. During collection the oocytes are maintained in Dulbecco's PBS or in TCM199 (hepes buffered). The maturation conditions used by the vast majority of the laboratories involve the use of TCM 199 supplemented with 10% fetal calf serum (FCS) and gonadotropins (FSH, LH) in 5% CO2 in air at 38.5 °C. After 20-24 h of incubation the oocytes complete maturation with the extrusion of the first polar body and are ready to be fertilized. If the donors are located far from the main laboratory, maturation can be completed during transport from the site of collection to the laboratory in test tubes in portable battery powered incubators. Under optimal conditions over 90% of the oocytes reach metaphase II. Before fertilization the cumulus cells are partially removed to leave few corona cell layers surrounding the oocyte.

In vitro fertilization

Frozen semen is always used for in vitro fertilization and a Percoll based separation system is the most common method for isolating the motile sperm fraction after thawing (4). Although other systems can be used (swim-up, simple centrifugation) separation through a Percoll gradient offers the consistency, flexibility and reliability that are required in a commercial setting, where new sires are required on a weekly basis by the clients. Two media are generally used for in vitro fertilization (IVF): a TALP-based medium or a SOF-based medium both without glucose and with varying concentration of heparin. The concentration of spermatozoa that is needed for each bull, in order to achieve maximum fertilization with minimal polyspermy, is determined empirically by performing IVF tests with different sperm concentrations. The fertilized oocytes are fixed 18-20 h after co-incubation with the sperm and the chromatin configuration is analyzed following lacmoid staining. The results of this test indicate the optimal sperm concentration for each bull. IVF is completed overnight after 18-20 h of co-incubation of sperm and eggs. At this time the oocytes are completely denuded of the remaining cumulus cells and spermatozoa and are transferred to a culture system suitable for embryo development.

Amongst the factors affecting the success of IVF the bull has an important role to play. Some bulls perform better than others and a small proportion (<5%) performs really poorly. A second factor affecting IVF success rate is the cumulus oocyte complex. In this respect it is important to standardize the procedures for oocytes preparation before IVF, in order to obtain a homogeneous population of oocytes with a cumulus cells mass as similar as possible and consistent with previous replicates. In fact the amount of cumulus is positively related to the sperm concentration required; oocytes with fewer cumulus cells are more susceptible to polyspermy while those with a large cumulus cell mass are less likely to be fertilized. A potential alternative

to IVF is intracytoplasmic sperm injection (ICSI) but it does not work as well in cattle as in other species (see below).

Embryo culture

The culture step is the development of the fertilized oocyte to the blastocyst stage. Several protocols have been developed and applied. They include various coculture and cell-free systems and also the in vivo culture procedure in the surrogate sheep oviduct (4, 7). The latter is the system that yields embryos of quality comparable to MOET embryos especially if they have to be frozen-thawed before transfer. This procedure requires specific surgical expertise and adequate facilities but is justified by the quality of the embryos produced. It is used mainly in research settings and less in commercial settings with the exception of our own laboratory in which most of the commercial IVP embryos are cultured in the sheep oviduct. With this method the embryos are usually allowed to cleave in vitro and 48-72 h post IVF they are transferred in the sheep oviduct. In the case of small numbers of cleaved embryos from different donors they are embedded into agar chips, since this procedure allows the simultaneous transfer of the embryos of a number of donors to the same oviduct (the different number of embryos in each agar chip is the key to identify different batches of embryos at collection). However, from a practical and economical point of view, the simplest option is to continue the culture in vitro following IVF. At present, in vitro culture (IVC) of early cleavage stage bovine embryos is an area that is still the subject of many investigations. Years ago initial success was obtained with the use of coculture either with oviductal cells (8) or with feeder layers of different cell types such as BRL (9) or Vero cells (10). However, this system requires the management of feeder cells that are subjected to biological variations, especially in the case of oviduct cells. As such they are not of practical use in large scale IVP. More recently the use of semi-defined media in the absence of feeder cells with low or no serum added and with low oxygen tension has replaced almost entirely the use of coculture. The SOF aa BSA medium (11, 12) is the most popular but some labs also use medium CR1 (13). Embryo development in vitro is usually assessed on D +6 by looking at the embryos that undergo compaction. This is the first differentiation event taking place during development and the embryos that show a clear process of compaction are those more likely to develop normally (14). The evaluation and selection of embryos for transfer or freezing is conducted on D+7. By this time normally developing embryos should have reached at least the early blastocyst stage, the majority being at the blastocyst stage and some at the expanded blastocyst stage. A number of embryos will reach these stages on D+8 or even on D+9. These embryos are considered of lower quality and are used in a commercial setting only for fresh transfer because results after freezing are poor (9).

Mass production of embryos

Large numbers of embryos can be produced from the ovaries of slaughtered donors when the female parental origin is not required to be known. In this case, the ovaries from donors of the same breed are pooled and processed as a batch. This procedure significantly simplifies all the steps involved in production, identification and freezing and, as a consequence, embryos are produced at low cost and can be commercialised at very competitive pricing. This type of production is used for premium beef breeds for the commercial production of beef calves (see Table 1) from dairy herds ("beef from dairy") and it is well developed in countries like Italy or Japan where the beef industry relies on high premium local beef breeds. However, mass production can also be used to produce dairy embryos of average genetics for developing countries. Embryos produced in this way are generally frozen for direct transfer to simplify the transfer procedure.

Genetic recovery

When a donor of high genetic value is slaughtered for various reasons (terminal illness, infertility, age, etc.) her ovaries can be collected, processed separately from those of other donors and the oocytes matured and fertilized with the sire required by the client. This procedure, often defined "genetic recovery", allows the production embryos of known parentage (15). The expected results in terms of embryo production are related to the reasons for the slaughter. The outcome is usually poor (1-2 embryos per donor) for what we call "terminal" donors. This definition includes animals that are already dead or are in critical general conditions, such as immediate post calving problems, acute mastitis, displaced abomasus, progressed foot or leg injuries etc.. In the case of healthy donors that are slaughtered because of infertility, end-of-career or for the eradication of infectious diseases, the outcome is much better with an average production of 6 or more embryos per donor (16). We have used this technology in the past to save the genetics of herds infected with leucosis, brucellosis and tuberculosis. More recently, with current sanitary threats, we have been involved in the salvage of the genetics of herds stamped out because one animal tested positive to the rapid BSE test. Current policy in the European Union is to test at slaughter all animals over 24 month of age. When a case is confirmed the farmer has two options: destroy all the animals of the herd or only aged-matched peers (from one year younger to one year older than the positive animal). The Health Authority compensates the farmer for his losses. In the region of Lombardy, in the north of Italy, the veterinary authority allows farmers to collect the ovaries from the best donors providing that the resulting IVP embryos are frozen until all donors are destroyed and the BSE testing is completed. The safety of these procedure derives from the work of Wrathall and collaborators (17) who have demonstrated that BSE is not transmitted through embryo transfer and on the fact that, so far, a second positive animal has never been found in the same herd. In table 2 we report the outcome of such an operation in one herd ranked in the top 100 herds in Italy. The difficulty of this operation, that might to some extent compromises the results, is the high number of donors that had to be processed in one day. In the cases we have treated, because they were a confined slaughtering, the procedure has been concentrated in the shortest time possible. In the case summarized in Table 2 all the cows (66 animals) were slaughtered on one day and a week later the same occurred for the heifers (91 animals). This concentration of donors creates an overload of work in the IVF lab especially in the days on which the oocytes are collected and fertilised and finally on the day of embryo evaluation and freezing. Moreover, it requires a strict and careful data collection procedure to register all the relevant information for each donor/batch of oocytes/embryos. From the point of view of the farmer this operation allows the rescue of the genetics of his herd at a cost certainly lower (at least for top herds) than acquiring new genetics on the market.

Ovum Pick Up

The most flexible and repeatable technique to produce embryos from any given live donor is offered by the technique of ovum pick up (OPU) or ultrasound guided follicular aspiration (5, 9, 18). A scanner with an adequate endovaginal (or adapted for the vaginal use) sector probe with a guided needle is required to perform this procedure. The needle is connected to a test tube and to a vacuum pump to aspirate the follicular fluid and the oocyte contained in it. A scanner with good resolution and with a probe of at least 6 MHz is used to envisage follicles down to 2-3 mm in size and also to view the needle during follicle aspiration. This procedure can be performed either on-farm or in an IVP residential centre. The donor is confined in a crush, mildly sedated and given an epidural anaesthesia just before collection. OPU has virtually no drawbacks for the

donor and can even have a therapeutic effect in some infertile donors affected by ovarian cystic syndrome
or similar pathologies that compromise reproductive function. Virtually any female starting from 6 months of age up to the third month of pregnancy and also soon after calving (2-3 weeks),
is a suitable donor (9). This makes OPU a very flexible technique that, unlike MOET, does not interfere with the normal reproduction and production cycles of the donor. OPU can be performed sporadically or on a regular basis such as two times a week for many weeks or months. The twice
a week protocol is the one that yields the maximum number of competent oocytes in a given period of time (19, 20, 21). Another advantage of OPU is that it is not necessary to treat the donor with gonadotropins (some people however do this do often because of a poor scanner not suitable for small follicles) with the inevitable side effects. This is a very important advantage especially for young heifers in which gonadotropin-stimulation can cause mammary oedema and ovarian cystic syndrome, and for show cows where repeated superovulation can cause relaxation of the udder ligament. Many of the infertile cows that we treat at our centre are infertile as a consequence of repeated superovulation. Therefore it is desirable to avoid further gonadotropin-treatment of these animals, if a normal reproductive career has to be restored. A final advantage is the possibility of using over a short time, or even on the same collection (when many oocytes are recovered), more sires to achieve in a short time several different dam-sire combinations. The drawbacks of OPU are higher cost compared to MOET and the requirement of specialized laboratory equipment to perform all the steps of embryo production. In Table 3 are shown the results of the OPU work carried out in our laboratory over the years 1997-2004. We achieve 2.1 freezable embryos per cow and 1.2 for heifers.

Juvenile in vitro embryo production

In order to shorten the generation interval several attempts have been made to recover oocytes from young animals well before puberty. Female calves (2-3 months of age) have been used as oocyte donors by OPU conducted via laparotomy. Following in vitro maturation and fertilization,
the embryos are grown to the blastocyst stage and then transferred to recipients (22). With this approach it is possible to achieve a generation interval of 11 months at the risk, though, of breeding progeny from a donor well before she actually begins her lactation, i.e. before she is proven to be
of superior genetics. To have consistent results at this age it is necessary to stimulate the ovaries with gonadotropins (5). The efficiency of the procedure is affected by the type of donor
and by the rearing procedure. Our experience and comparison with results of Australian investigators (23) shows that beef suckling calves are better donors than Holstein Friesian calves that are fed with milk replacements. This difference probably reflects different follicular growth caused
by a different nutritional status, together with a breed effect or also a genetic background effect since some cow families or bloodlines outperform others. In our laboratory we obtain an average of 3.1 transferable embryos from calf donors of dairy breeds (Holstein Friesian and Brown)(5).

Embryo transfer and offspring

Pregnancy rates with IVP embryos can be very variable. This relates to the quality of the embryos as affected by the culture procedure, media used and the subjective evaluation and selection of embryos before freezing and/or transfer. Pregnancy rate is usually acceptable with fresh transfers but becomes more variable amongst different laboratories with frozen embryos.

For successful freezing the culture system used to develop the embryos is very critical. A survival rate equal
to in vivo produced embryos can be obtained when IVM-IVF embryos are cultured in the sheep oviduct (5). Embryos grown in vitro in SOF-BSA, that undergo a clear compaction step on Day 5 and/or 6, blastulate on Day 7 and are classified as grade 1 (according to the IETS manual), survive well to freezing and thawing. By contrast, culture in vitro in the presence of serum reduces the cryotolerance because of lipid accumulation. In this case vitrification could offer an alternative but it is not yet used in practice (24). Once pregnancies are established there is a moderate increase
in the losses in the first trimester that can reach 10 - 12% (5). Reports in the literature indicate that problems may arise at calving. There are reports of extended gestation, dystocia, large calves, increased perinatal mortality, etc, that all together have been termed the Large Offspring Syndrome (25, 26, 27). Most of the calves described in these reports were the result of coculture with granulosa or Buffalo rat liver cells and/or high serum or high BSA and were from a few transfers conducted mainly under uncontrolled conditions by research laboratories. In commercial programmes, where conditions are more controlled, using either the in vivo culture in the sheep oviduct or the SOF system, without high level of serum or BSA, over 95% of the pregnancies are normal and the incidence of LOS is reduced (5, 26, 28, 29). Because the use of IVP is mainly for intensive breeding of the newest genetics, the bulls used are those with little or imprecise information about calving ease, and this can be another factor contributing to the calving problems.

CLONING BY SOMATIC CELL NUCLEAR TRANSFER

Cloning technology has attracted the interest of breeders for many years. At the beginning, when an embryo was used as the source of the donor cell, there was no less lottery (genetically speaking), as far as the phenotype of the resulting calves was concerned, than in conventional breeding. For this reason and for the fact that on average only 2-3 offspring could be obtained from each embryo (i.e. a little more than following embryo splitting) embryo cloning has never really entered the commercial arena but it has remained a research tool. The situation changed when it was demonstrated that nuclei taken from an adult animal could be used for cloning (30). Under such circumstances it is possible to make a copy of any given animal whose genotype and phenotype
are well known through milk production, if it is a cow (31), or through progeny testing, if it is a sire (32). For these reasons somatic cell cloning looks very attractive for breeders, but the efficiency
is still too low. It is relatively easy to produce blastocysts by using in vitro matured oocytes from abattoir ovaries and the nuclei taken from somatic tissue of the animal to be cloned. Both recipient oocytes and donor nuclei are virtually in unlimited supply. To make the procedure even simpler, production of cloned embryos can now be conducted without the use of a micromanipulator (33). However, only an average of about 5% of the blastocysts transferred to recipients survive to term. This is much lower than the 40% usually achieved with the transfer of embryos obtained by in vitro fertilisation. After transfer of cloned embryos (either fresh or frozen) the initial pregnancy rate
up to day 35 is not different from that of embryos originating from IVF (32). However, from day 35 to day 60 up to 60% or more of the pregnancies are lost. This is followed by a small but constant loss of the remaining pregnancies up to the 8^{th} month when those that survive go to term. The pregnancy losses can be attributed to a defective placental function, as demonstrated by the lack
of cotyledon development (34) in the initial phase, but later in the pregnancy the principal cause

of abortion seems to be the development of hydrops that is also associated with placenta hypertrophy, enlarged cotyledons and thickening of the umbilical cord (35). By contrast, the fetus
at necroscopy looks relatively normal. The high rate of pregnancy losses and therefore the wastage of recipient heifers is the main cost in the attempt to clone an adult animal. Once the pregnancies have reached term there seems to be a delay in the preparation of the recipients for parturition and,
as a consequence, a delayed calving. To avoid such problems and to reduce the chances of having heavy calves we induce parturition by administering long-acting corticosteroids in the last week
of pregnancy. The calving will take place within 5-10 days after treatment at which time both
the recipient will be prepared and the foetus will have completed lung maturation. In our experience, with proper management the survival of calves is very high both perinatally and in the long-term. Reports in the literature are conflicting regarding calf survival and normality. In a review of published information (36), on average the survival was reported to be in the range of 80%;
this
is 10-15% lower than for normal calving. Epigenetic alterations that derive from the incomplete reprogramming of the somatic nucleus used for the cloning are a cause of possible abnormalities (37). Mammals, however, seem to be tolerant to a leakage in epigenetic imprinting and in any case in mice, epigenetic deviations are not inherited by the progeny (38). Many of the cloned cattle have already reached puberty and proved to be fertile and normal (39, 40, our unpublished observations).

In the cattle breeding industry, cloning can be used to multiply animals of high genetic merit, whether they are founder dams of important families, show cows or progeny-tested sires. Artificial insemination companies, for instance, could find a good use of the cloning technology to support their business once the efficiency is improved. Valuable bulls could be cloned to increase the availability of semen for the market. A widespread policy of somatic cell storage from all bulls affords the possibility of replacing injured or dead bulls with new identical animals. In theory,
the cloning technology could modify/integrate into the normal progeny testing scheme and reduce the costs involved in feeding and caring, for over four years, all the bulls under test. Following
the first semen collections all bulls could be slaughtered and after four years, when enough daughters will be in lactation, the best genotypes could be rescued from the frozen stocks of somatic cells. Another possibility is to make copies of proven bulls to be sold to farmers to be used for natural mating, they could be considered as field artificial insemination stations. This is a real option for extensive breeding herds but also in large intensive breeding herds were management condition make it difficult to have efficient artificial insemination.

A different hurdle that this technology is facing and one that is no less complicated that its biology, is the acceptance by the regulatory authorities. Although cloned animals are a genetic copy of an existing animal, they tend to be assimilated into the category of genetically modified organisms even if they are not transgenic or if their transgenicity is limited to having different mitochondrial DNA (41). At present several AI companies including ours have clones of their top bulls (see Fig. 1) but it is not clear if their semen can be marketed. The regulatory agencies fear that the anti-biotechnology crusades that are sweeping Europe (and other nations) could seriously affect all the cattle industry. On the other hand, there is at present no policy or strategy to follow for the approval of such “new products” even if the potential bio-safety risks are non-

existent. The USA Food and Drug Administration (FDA) has been collecting data on cloned farm animals and their progeny
on a world wide basis to make a risk assessment analysis. Data collected so far would indicate that cloned animals or they products do not pose any health risk to humans. We have also been studying zootechnical and reproductive performanced of cloned cattle and did not find any differences with control animals (42). A second level of discussion is in the breeder organisations. Several Breed Associations have already approved regulations for the registration of cloned animals.
The identification number of the clone is a new number since it is a different individual, but the clone can keep the same name as the animal of which is a copy, followed by the suffix "clone"
with a progressive number indicating how many clones there are. The criticism towards cloning is based on the fear of increasing inbreeding to too high a level, particularly in the Holstein Friesian. However, given the high level of inbreeding already present in the population, this seems of little consequence especially if a clone simply replaces a dead or injured sire. A second criticism
is regarding how identical the clones are; the same genotype in different environmental conditions and nutrition can perform differently but this is nothing new for milking cows and for farm animals in general. Repeating the progeny test for cloned bulls, as has been suggested, does not make much sense unless the test is repeated at the same time for the original sire as well, simply because in 4-5 years the population changes and the comparison will not be realistic. However, a paired progeny test for the original sire and his clone/s, at the same time, would be an interesting and useful study in order to gain approval for the use of the clones for breeding purposes.

TRANSGENIC ANIMALS

Besides animal breeding, the cloning technology could find its main application for the production of transgenic animals both for agricultural (43; 44) and biomedical application because the desired genetic modification can be produced in cultured cells and the value of a transgenic animal can be substantial. Efficiency of this approach is mainly limited by the efficiency of nuclear transfer,
but has significant potential given the high prospective value of transgenic animals and future optimisation in nuclear transfer efficiency. The side effects described above with nuclear transfer together with problems caused by random integration (e.g. the interruption of a normal gene due to random integration of the gene construct), and limited control of amount and tissue specificity of gene expression raise animal welfare issues. However, offspring of the transgenic animal should inherit the genetic modification without suffering from the side effects of nuclear transfer, homologous recombination allows site specific integration and increased understanding
of functional genomics will lead to the development of a broader range of tissue specific promoters, which can be activated or regulated via external stimuli. Transgenic technologies ranging from gene enrichment (to add additional copies of species specific genes functional genes) to cross-species transgenic routes, can lead to significant changes in any production trait. For example Brophy et al, introduced additional copies of the casein gene to produce a milk with better caseification qualities and Wall et al, introduced lysostaphin (an antibmicrobial protein produced by bacteria) to have
it expressed in the mammary gland to give protection against mastitis.

SEMEN SEXING

The year 2003 has seen the beginning of commercialisation of bovine sexed semen in Europe. Flowcytometrically sorted semen can be frozen in straws containing two million sperms and

used for artificial insemination. This technology demonstrates that it is possible to significantly shift
the sex ratio in cattle however the technology is still far from being robust for a wide scale application both on technical and economical grounds (45; 46).
Sex-sorting of mammalian spermatozoa has applications for genetic improvement of farm animals, in humans for the control of sex-linked disease, and in wildlife as a captive management strategy and for the re-population of endangered species. Considerable research has been undertaken worldwide on the Beltsville sperm sexing technology, the only effective method for pre-selection
of sex of offspring. The combination of this method with assisted reproductive technologies has resulted in the birth of offspring in a wide range of animals, including cattle, the only livestock species in which sperm sexing is used commercially. Major improvements in the efficiency
of sorting, in particular the development of high speed sorting (15 million X and Y spermatozoa per hour) have led to the production of offspring using conventional and low dose AI and the successful cryopreservation of sorted spermatozoa in cattle, sheep, horses and elk. A major limitation remains the short viable lifespan of sorted spermatozoa in the female genital tract, in most species necessitating sperm deposition deep in the uterus, and close to the expected time of ovulation,
for acceptable fertility after in vivo insemination.

SPERMATOGONIAL STEM CELLS

Recent developments in in vivo and in vitro culture of spermatogonial stem cells has opened new possibilities in assisted male reproduction. This is a rather new technology wich has been developed in mice (47; 48) and is now being adapted to livestock. The techniques covers the collection and
in vitro culture of spermatogonial stem cells for further transplantation to the testes of recipient males previously denuded from their own stem cells. Prolonged storage of these spermatogonial stem cells is feasible creating the possibility of reconstituting in vivodelivery systems of elite germplasm after genetic evaluation and selection.

PROBLEMS TO BE SOLVED AND FUTURE PERSPECTIVES

The technology of embryo production in cattle is widely used both in research and for applied purposes. As a research tool, the technology is of great value to address fundamental questions
on the endocrine control, the molecular switches and the metabolic pathways that regulate early development. As an applied technology it provides the opportunity to increase the number of offspring from superior genotypes. However, in order to make the technology accessible to a wider base of breeders or even to commercial farmers, there are several aspects that need to be addressed at the clinical, cellular and molecular level to increase the efficiency and to reduce the costs involved.

The Ovum Pick Up technique tends to be more consistent than MOET and it allows repeatable and safe embryo production without interfering with the reproductive cycle or milk production of dairy donors. However, only a minority of the collected oocytes develop into viable embryos. Several factors have a role to play in this context. The nutritional status of the donor (49) together with intraovarian factors such as the stage of the oocyte within the follicular wave, are likely

to substantially affect embryo development. There is evidence that oocytes collected in the presence of a dominant follicle are of lesser quality than those collected during follicular growth (19, 20).
In addition, comparing in vitro with in vivo maturation, recent studies (50) have made it clear that the developmental potential of in vitro matured oocytes is generally lower than that of in vivo matured oocytes. This finding is also an obvious consequence of the collection of a heterogenous population of oocytes with different developmental potential, most of which would never ovulate
or are already in an advanced stage of atresia. The same authors (50) have shown that the quality of the embryos developing following IVM-IVF is very much influenced by the culture conditions from zygote to blastocyst. Therefore, improved oocyte maturation remains perhaps the most fundamental step to increase embryo production while embryo culture is the step that affects
the quality/viability of the embryos.

A recent series of studies have attempted to improve oocyte quality by mimicking the last phase of in vivo oocyte growth with a period of prematuration in vitro (24-48h) before final maturation. Several compounds have been used to maintain the oocyte in meiotic arrest during prematuration. Notably butyrolactone-I and roscovitine, two protein kinase inhibitors, alone (51, 52) or in combination (53) can maintain the oocytes in a fully reversible meiotic block for at least 24 h without interfering with the following embryo and foetal development (54). However, all these studies have failed to show any improvement versus controls without prematuration. This is likely to limit any progress in the use of juvenile breeding as it was expected to get the most benefit from a prematuration step. From an applied perspective, though, these results are very important because they provide a method to increase the flexibility of the whole procedure of embryo production
in vitro by allowing the adjustment of the time of prematuration-maturation to the needs of the IVF lab or for cloning work (55).

Embryo quality and viability is mainly affected by the culture system following IVF (50, 5). This means that the viability of an in vitro produced embryo can vary considerably from lab to lab and amongst different protocols in the same lab. In our experience, embryo observation during culture especially at the compaction stage (Day 5 and 6) is crucial for selecting good quality embryos that survive cryopreservation. Even better results can be obtained by the in vivo culture of IVM-IVF embryos in the sheep oviduct. This system is still in use in our laboratory for most of the commercial embryo production. Under the conditions of a good and reliable in vitro culture system or in vivo culture in the sheep oviduct, the pregnancy rate of frozen-thawed IVM-IVF bovine embryos is close to that of in vivo produced embryos following superovulation (5). Therefore, further improvements of culture media that allows a more physiological growth of the embryos, including a clear and long compaction stage, will be of great benefit. Besides the defined chemical composition of the medium, an important role is played by the type of macromolecules present during in vitro culture. The presence of serum is usually associated with accumulations of lipids and a poor survival after freezing. The supplementation of media with albumin instead of serum, alleviates this problem but in both cases the expression of several genes is different to that
of embryos cultured in the presence of polyvinyl alcohol. PVA supplementation produces embryos with expression patterns similar to those grown in vivo (56). The culture system not only affects
the viability of the embryos and their freezability, but also the characteristics of the newborn calf.
It has been shown that in vitro culture in the presence of high level of serum or BSA can induce

a significant increase in birth-weight (57) and the occurrence of the complex condition known as Large Offspring Syndrome (25). A recent study has highlighted several cellular and molecular deviations occurring during the preimplantation development of IVM-IVF embryos cultured in vitro versus in vivo. Interestingly, this study has shown a clear correlation between these deviations
and the occurrence of LOS in the resulting calves (57). This knowledge will be very useful for monitoring the effect of different culture media on early embryo development and eventually on the characteristics of the calves at birth. In addition, it could provide defined criteria to select the culture systems that are more prone to induce LOS and hence the study on the molecular mechanisms that underlie this syndrome.

In vitro fertilisation with frozen semen is an efficient procedure in bovine. However, there are some situations where the use of intra-cytoplasmic sperm injection (ICSI) could be of advantage. For instance, in the case of the few sires or dam-sire combinations which result in no fertilisation or massive polyspermy. The use of sexed semen for IVF could also increase if an efficient ICSI procedure was available, given that IVF results with frozen-thawed sexed semen are poor. Interestingly, while bovine ICSI results are inconsistent and only a few calves have been born (58), this technique works very well in humans (59) and horses (60, 61) - so well in fact that is replacing IVF in humans and in the horse is the only option at present. Sperm injection can be easily executed with the use of a piezo-electric manipulator (62) and high rates of activation and pronuclear formation can be obtained in several species. In the bovine, however, cleavage and embryo development is retarded and reduced. Sperm pre-treatment (63), oocyte activation (64) and methods of injection have been studied but the efficiency remains low.

Somatic cell cloning could become a useful tool for the commercial breeder, as it is for plant breeding, to guarantee product quality, uniformity and consistency. For this to occur, higher efficiency and lower costs are required. Superior genotypes could be spread by cloning and combined with transgenesis could become important to introduce traits (if identified) related
to secondary characteristics such as disease resistance (mastitis), fertility, etc. These traits are not currently taken into consideration in selection schemes but are now becoming more important, partly for animal welfare reasons. The main limit of this technology is the high rate of pregnancy losses (on average 5% of the cloned embryos develop to term). Blastomere cloning can produce reduced losses (65) indicating that the main cause of failure is the donor cell used. This is true irrespective of the type of donor cell, the cell cycle stage of the nucleus, the technique used for embryo reconstruction and the activation protocol (66, 67). Further improvement will come from the understanding of the basic biological processes underlying DNA reprogramming, of the epigenetic control mechanisms that govern gene expression (37) and probably from more physiological methods to induce embryo activation. Addressing these issues is expected to improve not only the survival of the cloned embryos but also to reduce some of the problems described
in late pregnancies and in some of the calves born.

Embryo genotyping is another tool that could be used in selection programmes. Besides sexing embryos via the use of sexed sperm, approaches have already being used for the identification
of embryos carriers of genetic diseases such as BLAD (bovine leukocyte adhesion deficiency)(68), and will be extended to CVM (complex vertebral malformation) (69) and others conditions. Another possibility being explored at present is to screen embryos for loci of breeding value (70). Because of the small amount of DNA available from an embryo biopsy (usually 5-8 cells)

it is necessary to have a reliable total DNA amplification procedure and then to screen for the loci of interest (71).

CONCLUSIONS

Embryo technologies applied to animal breeding have the important role of increasing the impact of superior genotypes in the population. However, a more widespread and competent use of the available techniques is required in order to gain the most benefit from their application. Future developments, linked to the newest area of research such as somatic cloning and embryo genotyping, are expected to find a role in advanced animal breeding.
Together with the requirement for continuous scientific progress there is also the need to address public concerns over new biotechnologies. In this respect, more knowledge is needed to demonstrate the safety of embryo biotechnologies and the suitability of the derived products to enter the food chain.

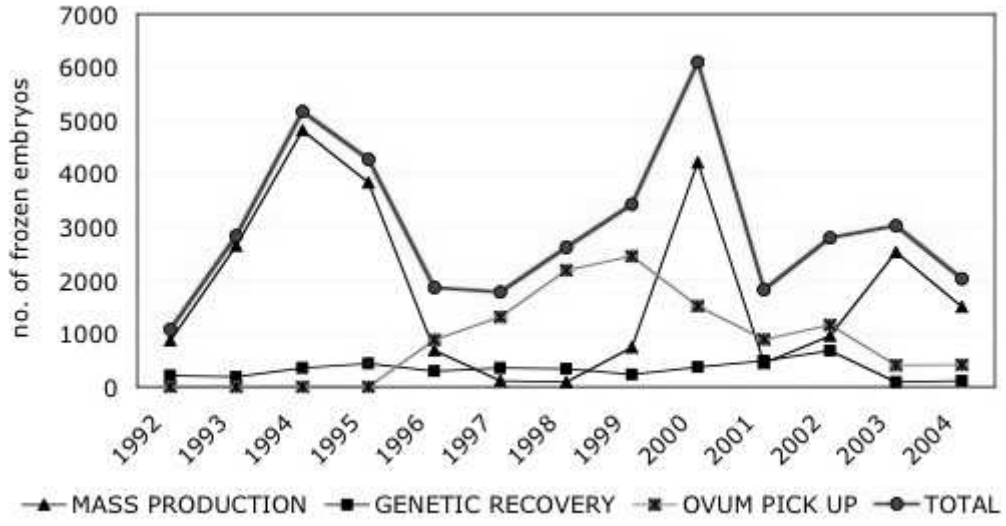

Fig. 1. Overall activity of in vitro embryo production at LTR from 1992 to 2004.
The above graph shows the commercial production of frozen-thawed embryos at LTR from 1992 to 2004. In 1993 to 1995 all the embryos were produced from slaughtered donors as mass production of beef embryos from batches of donors, or as genetic recovery from valuable donors. From 1996 onwards OPU activity began from live donors, and reached a peak in 1999 with 2457 embryos frozen and commercialised. The decline of OPU in 2000 was balanced, however, by an increase in the mass produced embryos. Overall, over 6000 embryos were frozen in the year 2000.

Table 1: Production of frozen beef embryos at LTR in the period 1992-2004.

breed	no. donors	no. frozen embryos	no. embryos/donor
Piemontese	3505	12269	3,5
Chianina	730	1831	2,5
Marchigiana	255	961	3,8
Limousine (heifers only)	2813	6947	2,5
Charolais (heifers only)	167	435	2,6
total	7470	22443	

The table shows the production of frozen-thawed embryos from a BSE infected herd by genetic recovery. All donor cows were slaughtered in one day and after a few days the same was done for the heifers. The high number of donors/oocytes, concentrated in two days, requested a specific organisation of the lab work. Eight technicians were involved in oocytes collection that was completed in three hours. Following IVM the oocytes were split in two groups according to the time of collection and fertilised in two subsequent rounds. Most of the cleaved embryos were transferred in the sheep oviduct and a minority was cultured in vitro in medium SOF-BSA. No difference in development was observed. On Day 7 the embryos at the blastocyst stage evaluated as grade 1 (according to the IETS manual) were frozen conventionally in 10% glycerol.

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