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Bovine embryo technologies

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Abstract

Embryo technologies are a combination of assisted reproduction, cellular and molecular biology and genomic techniques. 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. Multiple ovulation and embryo transfer (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 (IVP) 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 and to produce or copy transgenic animals. More knowledge in oocyte and embryo biology is expected to shed new light on the early developmental events, including epigenetic changes 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.

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1. Introduction

The cattle industry is undergoing a major reorganization especially in Europe, primarily as a result of the BSE and Foot and Mouth disease outbreaks that had a devastating effect in 2001, and also as a consequence of increased competition worldwide. Because of these economic difficulties the application of advanced reproductive techniques in cattle

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breeding has been declining. Research in farm animal reproduction is also shrinking and changing. Increasing animal production is certainly not a priority in Europe today and economic resources 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 but genetic companies or breeder’s co-operatives that earn their revenues from the sale of genetics (for example semen, embryos and animals).

A second problem that Europe is facing is a general negative attitude towards biotechnology 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 multiple ovulation and embryo transfer (MOET), let alone in vitro embryo production (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.

2. MOET

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 to 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 7 days later the uterus is flushed to recover the embryos. On average four to six 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 one to three 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 condition and this is best achieved with donors that have passed their lactation peak since the superovulatory treatment induces a drop in milk production. In many cases donors are dried off 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.

3. 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 fertilization 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. 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 three biological steps that are now relatively well established in cattle: oocyte *in vitro* maturation, *in vitro* fertilization (IVF) and embryo culture [6].

3.1. *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 shock so it is important to monitor carefully the temperature during the collection procedure 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 TCM199 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.

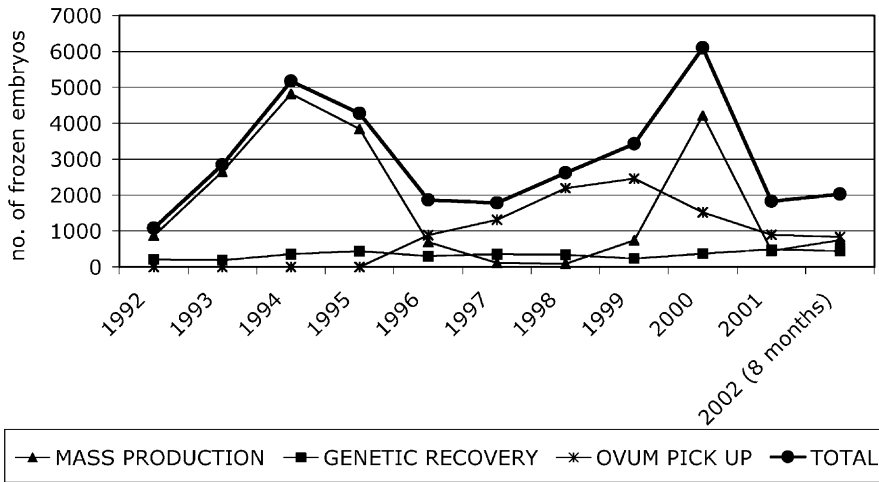


Fig. 1. Overall activity of in vitro embryo production at LTR from 1992 to 2002. The graph shows the commercial production of frozen-thawed embryos at LTR from 1992 to August 2002. In 1993–1995 all the embryos were produced from slaughtered donors as mass production of beef embryos from batches of donors, 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 for commercial use. 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. Bovine spongiform encephalopathy (BSE) especially, but also the Foot and Mouth disease outbreak, were responsible for the contraction in embryo production in 2001. Preliminary data based on the first 8 months of 2002 indicate an overall increase in embryo production.

3.2. In vitro fertilization

Frozen semen is always used for IVF 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 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 oocyte preparation before IVF, in order to obtain a homogeneous population of oocytes with a cumulus cell 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).

3.3. *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 co-culture 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 into 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 co-culture 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 co-culture. The SOF–BSA medium [11,12] is the most popular but some labs also use medium CR1 [13]. Embryo development *in vitro* is usually assessed on Day +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 Day +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 Day +8 or even on Day +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].

Table 1
Production of frozen beef embryos at LTR in the period 1992–2001

Breed	Number		
	Donors	Frozen embryos	Embryos/donor
Piemontese	2118	8989	4.2
Chianina	224	948	4.2
Marchigiana	102	575	5.6
Limousine (heifers only)	2667	6647	2.5
Charolais (heifers only)	129	360	2.8
Total	5240	17519	3.3

3.4. 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 commercialized 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.

3.5. 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 as “genetic recovery,” allows the production of 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 (one to two embryos per donor) for “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 six or more transferable 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 eradicated 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 months of age. When a case is confirmed the

Table 2
Genetic recovery from culled donors of one BSE infected herd

	Number				
	Donors	Oocytes (per donor)	Cleaved (% of oocytes)	Freezable embryos (% of oocytes per donor)	Transferable embryos (% of oocytes per donor)
Cows	66	1413 (21.4)	970 (68.6)	206 (14.6, 3.1)	308 (21.8, 4.7)
Heifers	91	1831 (20.1)	1148 (62.7)	187 (10.2, 2.1)	285 (15.6, 3.1)
Total	157	3244	2118	393	593

The above table shows the production of frozen-thawed embryos from a BSE infected herd by genetic recovery. All donor cows were slaughtered in 1 day and after a few days the same was done for the heifers. The high number of donors/oocytes, concentrated in 2 days, requested a specific organisation of the lab work. Eight technicians were involved in oocytes collection that was completed in 3 h. Following IVM the oocytes were split in two groups according to the time of collection and fertilized 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.

farmer has two options: destroy all the animals of the herd or only aged-matched peers (from 1 year younger to 1 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 procedures derives from the research of Wrathall et al. [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 1 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 1 day and a week later the same occurred for the heifers (91 animals). This concentration of donors creates an overload of work especially in the days on which the oocytes are collected and fertilized 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.

3.6. 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 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 center. 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 [19–21]. Another advantage of OPU is that it is not necessary to treat the donor with gonadotropins (some laboratories however do this, 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 treated at our center 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 1997–2001. We achieve around 2.5 freezable/transferable embryos per cow and about 1.4 for heifers.

3.7. 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*

Table 3
Embryo production by OPU at LTR in the period 1997–2001

	Number					
	OPUs	Oocytes (per OPU)	Cleaved (per OPU)	Cleavage (%)	Freezable embryos (per OPU)	Transferable embryos (per OPU)
Cows	2641	26532 (10.0)	18822 (7.1)	70.9	5945 (2.3)	7403 (2.8)
Heifers	2143	16786 (7.8)	11231 (5.2)	67.0	2593 (1.2)	3284 (1.5)
Total	4784	43318	30053		8538	10687

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].

3.8. 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 freezing and thawing well. 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 [25–27]. Most of the calves described in these reports were the result of co-culture 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 programs, where conditions are more controlled, using either *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.

3.9. 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 two to three 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 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 fertilization. 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 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 months 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, in some studies the fetus at necropsy 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 fetus 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 4 years, all the bulls under test. Following the first semen collections all bulls could be slaughtered and after 4 years, when enough daughters will be in lactation, the best genotypes could be rescued from the frozen stocks of somatic cells.

A different hurdle that this technology is facing and one that is no less complicated than 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. 2) 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. A second level of discussion is in the breeder organizations. 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



Fig. 2. Three clones produced at LTR, of the Holstein Friesian bull Carol Prelude Mtoto. At present Mtoto is ranked second in the Top 100 TPI International Bull list.

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.

Besides animal breeding, the cloning technology could find its main application for the production of transgenic animals for biomedical application because the desired genetic modification can be produced in cultured cells and the value of a transgenic animal can be substantial.

3.10. 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 OPU 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 [42] 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 [43] 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 [43] 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 pre-maturation *in vitro* (24–48 h) before final maturation. Several compounds have been used to maintain the oocyte in meiotic arrest during pre-maturation. Notably butyrolactone I and roscovitine, two protein kinase inhibitors, alone [44,45] or in combination [46] can maintain the oocytes in a fully reversible meiotic block for at least 24 h without interfering with the following embryo and fetal development [47]. However, all these studies have failed to show any improvement versus controls without pre-maturation. This is likely to limit any progress in the use of juvenile breeding as it was expected to get the most benefit from a pre-maturation 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 pre-maturation–maturation to the needs of the IVF laboratory or for cloning work [48].

Embryo quality and viability is mainly affected by the culture system following IVF [43,5]. This means that the viability of an *in vitro* produced embryo can vary considerably from laboratory to laboratory and amongst different protocols in the same laboratory. 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 IVC 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 IVC. 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* [49]. 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 IVC in the presence of high level of serum or BSA can induce a significant increase in birth-weight [50] 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 pre-implantation 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 [50]. 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 fertilization with frozen semen is an efficient procedure in bovine. However, there are some situations where the use of ICSI could be of advantage. For instance, in the

case of the few sires or dam–sire combinations which result in no fertilization 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 [51], this technique works very well in humans [52] and horses [53,54]—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 [55] 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 [56], oocyte activation [57] 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), and fertility. 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 [58] 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 [59,60]. 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 programs. Besides sexing embryos via the use of sexed sperm (see Seidel in this volume), approaches have already being used for the identification of embryo carriers of genetic diseases such as bovine leukocyte adhesion deficiency (BLAD) [61], and will be extended to complex vertebral malformation (CVM) [62] and others conditions. Another possibility being explored at present is to screen embryos for loci of breeding value [63]. Because of the small amount of DNA available from an embryo biopsy (usually five to eight cells) it is necessary to have a reliable total DNA amplification procedure and then to screen for the loci of interest [64]. This has been conducted for single trait loci like casein (Michele Blasi, personal communication).

4. 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.

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