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Assisted Reproductive Technologies in Cattle: Applications in Livestock Production, Biomedical Research and Conservation Biology*

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Abstract

Velazquez MA. Assisted Reproductive Technologies in Cattle: Applications in Livestock Production, Biomedical Research and Conservation Biology. ARBS Annu Rev Biomed Sci 2008;10:36-62. In cattle, assisted reproductive technologies (ART) can be defined as techniques that manipulate reproductive-related events and/or structures to achieve pregnancy with the final goal of producing healthy offspring in bovine females. The present review includes manipulation of female reproductive tract physiology, artificial insemination, multiple ovulation and embryo transfer, *in vitro* production of embryos, *in vitro* assisted fertilization, cloning, transgenesis, xenografting-germ cell transplantation, preimplantation genetic diagnosis and sperm sexing. This review shows that several ART are being currently applied commercially in the cattle industry with acceptable results. On the other hand, others have low efficiency in producing cattle offspring and are predominantly applied in experimental settings. Several of these ART can cause detrimental effects at the prenatal and postnatal period and therefore they need to be improved. However, even if these bovine-related biotechnologies are properly improved, they might be more useful in the conservation of endangered ungulates, production of pharmaceuticals, or as experimental models for human reproduction.

Keywords: assisted reproduction, cattle, conservation biology, biomedicine

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1. Introduction

The way domestic cattle are used for meat and milk production has been changing during the last decades. New biotechnologies have been created and applied to the cattle industry to increase efficiency in both beef and dairy production systems. Among these technologies are those involved in assisted reproduction. The ultimate aim of assisted reproductive technologies (ART) is the birth of healthy offspring. The techniques that are considered ART are usually the ones related to gamete and embryo manipulation (Galli *et al*., 2003; McEvoy *et al*., 2003; Mapletoft & Hasler, 2005). However, for the sake of this review, ART will be defined as any technique that interferes with the normal biological pathways of reproductive-related events and/or structures in order to contribute to the establishment of pregnancy with the final goal of producing healthy offspring in a female bovine. In general, ART manipulate events and/or structures related to ovulation, fertilization and embryo development. The ART considered in this review includes manipulation of female reproductive tract physiology, artificial insemination (AI), multiple ovulation and embryo transfer (MOET), *in vitro* production of embryos, *in vitro* assisted fertilization, cloning, transgenesis, xenografting-germ cell transplantation, preimplantation genetic diagnosis, and sperm sexing. Cryopreservation of gametes and embryos (Curry, 2000; Massip, 2003; Tominaga, 2004; Moore & Bonilla, 2006; Seidel, 2006b) will not be addressed in this review.

Besides its use in livestock production, bovine ART are important for studying reproductive processes. This is reflected by the exponential increase in literature on this subject in recent years (Seidel, 2006a). At present, commercial application of some ART is being compromised by the low production of offspring achieved. The aim of this review is to give a current view on how efficient and useful are the ART in cattle. Besides describing their use in the cattle industry, applied uses and possible applications in the field of biomedical research and conservation biology will be given.

2. Historical Background

2.1. Manipulation of female reproductive tract physiology

In order to achieve pregnancy, the ovarian activity can be controlled by mechanical (*i.e.* ultrasound-guided transvaginal follicular ablation) and/or exogenous hormonal interventions on luteal and follicular development (Diskin *et al*., 2002; Bo *et al*., 2003). These approaches are used for the treatment of conditions such as postpartum acyclicity, repeat breeding and ovarian cysts (Mwaanga & Janowski, 2000; Yavas & Walton, 2000; Wiltbank *et al*., 2002; Bo *et al*., 2003; Macmillan *et al*., 2003; Rhodes *et al*., 2003). Another use of these approaches is the synchronization of the estrous cycle. Synchronization is used in animal production systems where calving patterns are important and as a complement to other ART such as AI and MOET programs (Lowman *et al*., 1994; De Rensis & Peters 1999; Bo *et al*., 2002; Cavalieri *et al*., 2006). Hormonal treatments have been also used to try and improve embryo survival after AI or embryo transfer in an effort to achieve acceptable or high pregnancy rates (Thatcher *et al*., 2001, 2002, 2003). The most common pharmacological approaches include the use of gonadotropin release hormone (GnRH), progestagens (*e.g.,* progesterone releasing intravaginal device [PRID], controlled internal drug release [CIDR]), prostaglandin F2alpha ($PGF_{2\alpha}$), gonadotropins (*e.g.,* human chorionic gonadotropin [hCG], equine chorionic gonadotropin [eCG]), estrogens (*e.g.,* estradiol benzoate, estradiol cypionate) and growth factors (*e.g.,* bovine somatotropin, insulin). These hormonal regimes

are applied alone or in combination, depending on the production system, husbandry facilities (especially in developing countries) and cost.

From hundreds of literature about these methods it can be observed that there is variability in the response of such approaches. For example, the protocol known as "Ovsynch" and its several combinations, which allows a timed insemination without the necessity of estrus detection, has been claimed to be the most successful with pregnancy rates ranging from 50 to 75% (Mapletoft *et al*., 2003; Thatcher *et al*., 2004; Johnson, 2005; Thatcher *et al*., 2006). However, synchronization treatments can increase the incidence of abnormal estrus and the risk of embryo loss (Macmillan *et al*., 2003). No response and variation in the intervals from treatment to estrus and ovulation are in part related to the follicular status at the time of treatment (De Rensis & Peters, 1999). The response to treatment for anovulatory conditions is also affected by age and body condition. In the case of postpartum anestrus, the interval from calving to treatment is important as well (Rhodes *et al*., 2003). Ultrasonography and estrus detection devices are helpful tools in the improvement of the efficiency of these treatments (Ribadu & Nakao, 1999; Diskin & Sreenan, 2000; Fricke, 2002). Treatments with growth factors such as bovine somatotropin appear to be more useful for solving subfertility problems than for improving reproductive efficiency in herds with good fertility (Morales-Roura *et al*., 2001; Selvaraju *et al*., 2002; Oropeza *et al*., 2004; Thatcher *et al*., 2006). Furthermore, in some cases these treatments can infringe detrimental effects on embryo survival with the concurrent negative effect on pregnancy rates (Bilby *et al*., 2004).

In most areas of Europe, because of both year-round calving and ethical or consumer concerns, hormones are mainly used to treat reproductive diseases and not for pharmaceutical control of breeding (van Arendonk & Liinamo, 2003). However, research done in the UK has shown that dairy farmers could have obtained benefits from a planned breeding program, especially in farms with low estrus detection and moderate pregnancy rates (Esslemont & Mawhinney, 1996). Nevertheless, careful analysis regarding cost of labor and hormone administration should be taken into account before considering the application of any reproductive hormonal regime (Rabiee *et al*., 2005).

To obtain precise control of the estrous cycle, it is necessary to understand the hormonal control of ovarian physiology. Knowledge on embryo-maternal interactions is also of pivotal importance for the application of hormonal treatments aimed at correcting situations of subfertility or for the improvement of reproductive efficiency. Detailed characterization of the complex processes of bovine reproductive physiology will be of great importance in the cattle industry, and the knowledge gained will be helpful in understanding clinical reproductive conditions in humans (Campbell *et al*., 2003).

2.2 Artificial insemination

Worldwide, AI has been the main vehicle for the improvement of genetic quality herds. Risks associated with natural mating such as disease transmission and libido problems can be highly controlled with the use of AI (Vishmanath, 2003; Thibier, 2005). The reproductive potential of valuable males has been maximized by combining AI with semen cryopreservation without limitations by time or distance in such a way that a single bull can produce approximately 50,000 offspring in one year (Funk, 2006). AI also plays an important role in MOET programs (Saacke *et al*., 2000; Kanitz *et al*., 2002; Funk, 2006). Deposition of semen in the uterine body is the conventional form of AI, giving pregnancy rates of 55-60% (Verberckmoes *et al*., 2004). However, other insemination techniques have been developed in an effort to improve pregnancy rates, especially when valuable semen (*i.e.* sexed sperm) is used (Hunter, 2003; Kurikyn *et al*., 2003; Verberckmoes *et al*., 2004). The additional skills needed for such techniques limits their use to special cases (Hunter, 2003).

Variability in fertility among bulls is still one of the problems influencing this biotechnology and an accurate test to predict bull fertility has not been developed (Garner, 1997; Tanghe *et al*., 2002; Flint *et al*., 2003; Rodríguez-Martínez, 2006, 2007). Nevertheless, the main factor affecting the success of AI is the efficiency of estrus detection (Barth, 1993). Radiotelemetric devices and camera systems are becoming useful tools in the accurate prediction of estrus behavior, however visual observation cannot be ruled out completely (Dransfield *et al*., 1998; Peralta *et al*., 2005; Alawneh *et al*., 2006). Timed insemination protocols have been developed with the aim of performing insemination without

necessity of estrus detection, but sometimes when they are applied under field conditions, AI with natural heat detection can be more efficient (Tóth *et al*., 2006). Therefore, besides improvements in estrus detection or hormonal treatments, more important would be to develop a tool capable of predicting the process of ovulation. Several approaches have been tested, including measurement of progesterone concentrations, analysis of estrus behavioral signs and pedometer readings (Velasco-Garcia & Mottram, 2001; Roelofs *et al*., 2005a,b, 2006). However, it is not yet possible to accurately predict ovulation in cattle.

2.3. Multiple ovulation and embryo transfer

The first calf produced by embryo transfer was born more than 50 years ago (see Willet *et al*., 1951). Since then, embryo transfer programs have been implemented with acceptable results into livestock production. The aim of MOET programs in the cattle industry is the production of calves from cattle of high genetic merit (Merton *et al*., 2003). *In vivo* production of embryos by superovulation also offers a safe (*e.g.,* prevention of disease transmission) and economic (*e.g.,* complete herds transported as frozen embryos) way of trading genetic material through cryopreservation (Le Tallec *et al*., 2001). However the variability in the embryo production of donors (Mapletoft *et al*., 2002) and low pregnancy rates (Peterson & Lee, 2003) after transfer are limiting factors affecting MOET programs. In well-organized embryo transfer teams the general mean production of viable embryos is 4 to 8 and 1 to 3 in cows and young heifers, respectively. Approximately 20% of donors do not respond to the superovulatory treatment and do not produce any embryos. Expected pregnancy rates after transfer are between 50-60%, with best results from unfrozen embryos, and heifers are the best recipients (Thibier, 2005; Velazquez *et al*., 2005; Hasler, 2006; Stroud & Hasler, 2006). Intrinsic factors related to the donor (Kafi & McGowan, 1997; Stroud & Hasler, 2006) and the recipient (Broadbent *et al*., 1991; Stroud & Hasler, 2006) need to be taken into account when applying such a technology. In addition, environmental factors also play a pivotal role in the success of this biotechnology (Kafi & McGowan 1997), especially under tropical conditions (Benyei *et al*., 2006).

Despite these problems, most of the embryos produced worldwide for commercial purposes are obtained by this biotechnology (Thibier, 2001, 2004). In recent years, considerable progress has been made in the improvement of the outcome in MOET programs (Kanitz *et al*., 2002; Mapletoft *et al*., 2002; Peterson & Lee, 2003; Baruselli *et al*., 2006; Bo *et al*., 2006; Looney *et al*., 2006; Vasconcelos *et al*., 2006). However, reliable parameters for the prediction of the outcome in terms of embryo viability and pregnancy results are not yet available (Velazquez *et al*., 2005). If this biotechnology is to gain more acceptance in the livestock industry, strategies to identify superior recipients and to improve response and reduce variability in donors have to be developed (Hasler, 2003). Identification of superior recipients is even more important when handling extremely valuable transgenic or cloned embryos.

Another problem affecting MOET efficiency is the evaluation of embryo quality. Embryo transfer teams rely on visual morphological observation for this purpose, which is very subjective; as shown by Aguilar *et al*. (2002), great proportion of embryos classified as good by stereoscopic evaluation showed characteristics of cells in degenerative stage when evaluated by light microscopy and electron microscopy. Given the importance of embryo quality for successful implantation (Mann & Lamming, 2001), it is still necessary to improve the evaluation of embryo competence under field conditions.

Besides the role in livestock production, *Bos taurus* recipients have been used in interspecies embryo transfer for the preservation of endangered species. One example is the live offspring obtained by transferring gaur embryos (*Bos gaurus*) into a dairy cow (Pope *et al*., 1988). In a later study, *in vitro* produced gaur embryos transferred into cattle recipients achieved pregnancy. However, offspring obtained were either stillborn or died soon after birth. Although the authors could not differentiate between problems resulting from *in vitro* procedures and those from interspecies incompatibilities, the model was strongly discouraged (Hammer *et al*., 2001). Nevertheless, with the increasing understanding of the embryo-maternal interactions, interspecies embryo transfer bovine models might achieve acceptable results in the conservation of endangered ungulate species.

Superovulated cattle could also be used as a model for the study of human clinical problems related to the response to gonadotropin stimulation during assisted reproduction cycles. This is supported

by the fact that cattle share similarities with humans in terms of ovarian and embryo physiology (Ménézo & Hérubel, 2002; Campbell *et al*., 2003). Accordingly, patterns of superovulation ranging from low to high response have been reported in cows (De Roover *et al*., 2005; Durocher *et al*., 2006), which is similar to the situation reported in human assisted reproduction, regarded as "low responders" and "the ovarian hyperstimulation syndrome" (Karande & Gleicher, 1999; Whelan & Vlahos, 2000).

2.4. *In vitro* production of embryos

The birth of "Virgil", the first calf produced by *in vitro* fertilization (IVF) (Brackett *et al*., 1982), marked the beginning of IVF as a tool for production in the cattle industry. In the last few years there has been an increment in the *in vitro* production of embryos (IVPE) worldwide (Thibier, 2001, 2004). In some countries it is more expensive to produce embryos with this method than with conventional embryo transfer programs (Hasler, 2003). Despite financial concerns, mass production of *in vitro* embryos has been carried out in some countries (*e.g.,* Japan and Italy) for the commercial production of calves for beef production (Galli & Lazzari, 1996, 2003; Hamano *et al*., 2006). Although the transmission of infection to recipients or offspring has not been demonstrated with *in vitro* embryos, the sanitary risk for IVPE is less conclusive than for *in vivo* embryos and generation of data is required (Le Tallec *et al*., 2001; Hansen, 2006). *In vitro* production of embryos consists of three steps: oocyte *in vitro* maturation (IVM), IVF, and embryo culture. A method for the *in vivo* culture of IVM/IVF embryos has been developed; however, the technical skills required for such a procedure might not popularize its use (Havlicek *et al.*, 2005; Wetscher *et al.*, 2005). Oocytes for IVPE can be recovered from the ovaries of slaughtered donors or from live animals by ultrasound-guided transvaginal follicular aspiration (ovum pick-up) (Galli & Lazzari, 2003). *In vitro* fertilization in conjunction with ovum pick-up (OPU) has become important for the production of embryos from superstimulated donors (Galli *et al*., 2001). It is important to recognize that superstimulatory protocols used for the production of embryos *in vivo* are different than those used to produce embryos *in vitro* with oocytes obtained via OPU. The objective of superovulation in MOET programs is to maximize the number of ovulations without compromising embryo quality, whereas superstimulatory treatments prior to OPU are aimed at increasing the number of follicles suitable for puncture (van Wagtendonk-de Leeuw, 2006), preferentially with a diameter between 5 to 10 mm (Pieterse *et al*., 1988). The efficiency of OPU sessions is affected by several factors, but operator skill is the most single factor influencing efficient oocyte retrieval (Merton *et al*., 2003). Currently, the proportion of presumptive zygotes that become transferable blastocysts during the culture period is 15-40% (Hansen & Block, 2004; Lonergan, 2007). Although high rates of blastocyst production (up to 80%) have been reported using superstimulation protocols in dairy cattle subjected to OPU/IVF programs (Blondin *et al*., 2002), in *Bos indicus* cattle these results could not be reproduced (Barros *et al*., 2005). From the welfare point of view, the general consensus is that donors can tolerate current oocyte collection regimes and resume regular estrous cyclicity shortly after the OPU sessions have ceased (McEvoy *et al*., 2006).

Ovum pick-up is a practical way to obtain oocytes for IVPE in countries where oocyte collection from abattoir material is not possible for religious reasons (Manik *et al*., 2003). Reproductive programs working with OPU/IVF can also be used to produce embryos and calves from valuable cows that are infertile to AI (*i.e.* repeat breeding), that do not respond to superovulation in MOET programs or from animals with blocked oviducts (Galli *et al*., 2001; Faber *et al*., 2003; Hasler, 2003; Imai *et al*., 2006; van Wagtendonk-de Leeuw, 2006). Moreover, OPU/IVP programs cannot only obtain offspring from non-pregnant adult cows but also from prepubertal and pubertal animals and from pregnant cows in the first three months of pregnancy (Armstrong *et al*., 1997; Galli *et al*., 2001; Imai *et al*., 2006). However juvenile embryo production is not used widely, as embryo yield is low compared to adult cows and sometimes involves more invasive procedures (*e.g.,* in calves aged three months) than OPU in adult animals. This can generate animal welfare issues (Armstrong *et al*., 1997; van Wagtendonk-de Leeuw, 2006). Nevertheless, OPU devices have been developed for the collection of oocytes from prepubertal animals as early as six months of age (Oropeza *et al*., 2004). In addition, blastocyst production from young animals can be enhanced to levels found in adult cows with the use of hormonal treatments (Oropeza *et al*., 2004). The use of juvenile animals in OPU/IVF programs has the potential of reducing

the generation interval, but a decrease in selection accuracy might occur as the information on parent performance might not be available at the time of selection (van Arendonk & Bijma, 2003). Although speculative, this could be bypassed by the use of animals cloned from already proved high genetic merit animals. In fact, healthy offspring have been obtained with *in vitro* produced blastocysts from oocytes collected by OPU in cloned heifers (Lucas-Hahn *et al*., 2005).

Another suggested use of IVPE is the production of embryos from dairy cattle of average genetics for developing countries (Galli & Lazzari, 2003), although this might not be entirely applicable in hot climates. Production of hybrid genotypes (*i.e. Bos taurus* x *Bos indicus*) with the potential for better productive performance as compared with local breeds would be a better option for the tropics. This can be achieved in a faster way with IVF than with traditional genetic schemes. Embryo transfer protocols using embryos either produced by IVF or superovulation can also serve as a tool to bypass the effect of heat-stress in lactating dairy cows (Hansen & Block, 2004). Interesting is also the suggestion that the bovine model could be useful for the study of human IVF. This is because the cow and the human share some similarities regarding the final stages of oocyte maturation and the biochemical and intrinsic paternal and maternal regulatory processes in preimplantation embryos (Ménézo & Hérubel, 2002). This has especial relevance in patients undergoing assisted reproductive cycles displaying hormonal imbalances that can be mimicked in cattle (*e.g.,* hyperinsulinemia) (Adamiak *et al.*, 2005).

However, abnormalities in embryonic, fetal and postnatal (*i.e.* large calf syndrome) development have been associated with *in vitro* procedures (Lonergan *et al*., 2003; McEvoy, 2003). These abnormalities are related to the aberrant expression of developmentally important genes imposed by culture conditions (Niemann & Wrenzycki, 2000). For most cattle farmers this technology is an advantage only for extremely valuable cows that are infertile or fail to respond to superovulation. This is likely to change only when the efficiency of *in vitro* production improves significantly and the problems with pregnancies and calves are reduced (Hasler, 2003).

2.5. Cloning

The word clone comes from the Greek, *klon*, meaning a twig or a small branch, and the cloning technique as a scientific procedure might have begun with bacterial cloning (Weiss, 2005). Nowadays, the word cloning is mainly associated with reproductive cloning. Individual separation of embryonic blastomeres up to the fourth cell stage, embryo bisection at the morula or blastocyst stage (embryo splitting), and nuclear transfer (NT) are the three methods carried out so far to get genetically identical individuals in bovine species (Wells, 2003). Live offspring have been obtained in the three methods (Williams *et al*., 1984; Johnson *et al*., 1995; Cibelli *et al*., 1998a). However the first two cloning methods rely on very early embryonic cells and this limits the number of viable embryos and offspring that can be obtained (Wells, 2003). Embryo splitting has been applied to MOET programs, playing an important role in beef production (Gearheart *et al*., 1989). The major commercial advantage of demi-embryos is that more calves result per embryo. This is especially valuable when only one or a few embryos are obtained from high genetic merit donors (Seidel, 1984).

There are approximately 160 NT cloning laboratories, across 37 countries, of which 75% are working with livestock (cattle, pig, sheep, goat and buffalo) cloning (Oback & Wells, 2007). Nearly 50% of these livestock cloning organizations are involved in bovine NT cloning (Oback & Wells, 2007). Somatic cell NT (SCNT) has been suggested as the most efficient technique for obtaining large numbers of genetically identical individuals in farm animals (Kato *et al*., 1998). Although embryonic and fetal cells are also useful for NT cloning, the economic potential of the donor is unknown at the time of the procedure. In contrast, adult somatic cells can be selected from animals already proven to be good milk or meat producers (Kato *et al*., 1998; Bousquet & Blondin, 2004). For instance, SCNT could be used to multiply identical animals of high genetic merit, whether they are founder dams of important families, show cows or progeny-tested sires (Galli *et al*., 2003; Wells, 2003). This could be advantageous for the propagation of valuable F_1 cattle (Oback & Wells, 2007), especially in tropical areas. Moreover, valuable bulls could be cloned to increase the availability of semen for the market (Galli *et al*., 2003). In addition, somatic cell storage from bulls and dams affords the possibility of replacing injured or dead individuals with new identical animals (Galli *et al.*, 2003). In theory, SCNT technology could modify

the normal progeny testing schemes by reducing the cost involved in management and feeding of all the bulls included in the test. After the first semen collections, all bulls could be slaughtered and after a few years when enough cows will be in lactation, the best genotypes could be rescued from the frozen stocks of somatic cells (Galli *et al*., 2003). Another practical application in the cattle industry has been reported recently where a bull with a inherent resistance to bovine brucellosis was cloned using cryopreserved fibroblast from a bull that died 10 years ago (Westhusin *et al*., 2007). Another advantage of cloning would be the generation of more reliable and interpretable data in the field of reproductive biology by reducing the genetic variation in experimental trials (Sreenan, 1983). This suggestion has been proved recently in an OPU/IVF program in monozygotic twin cows (Machado *et al*., 2006).

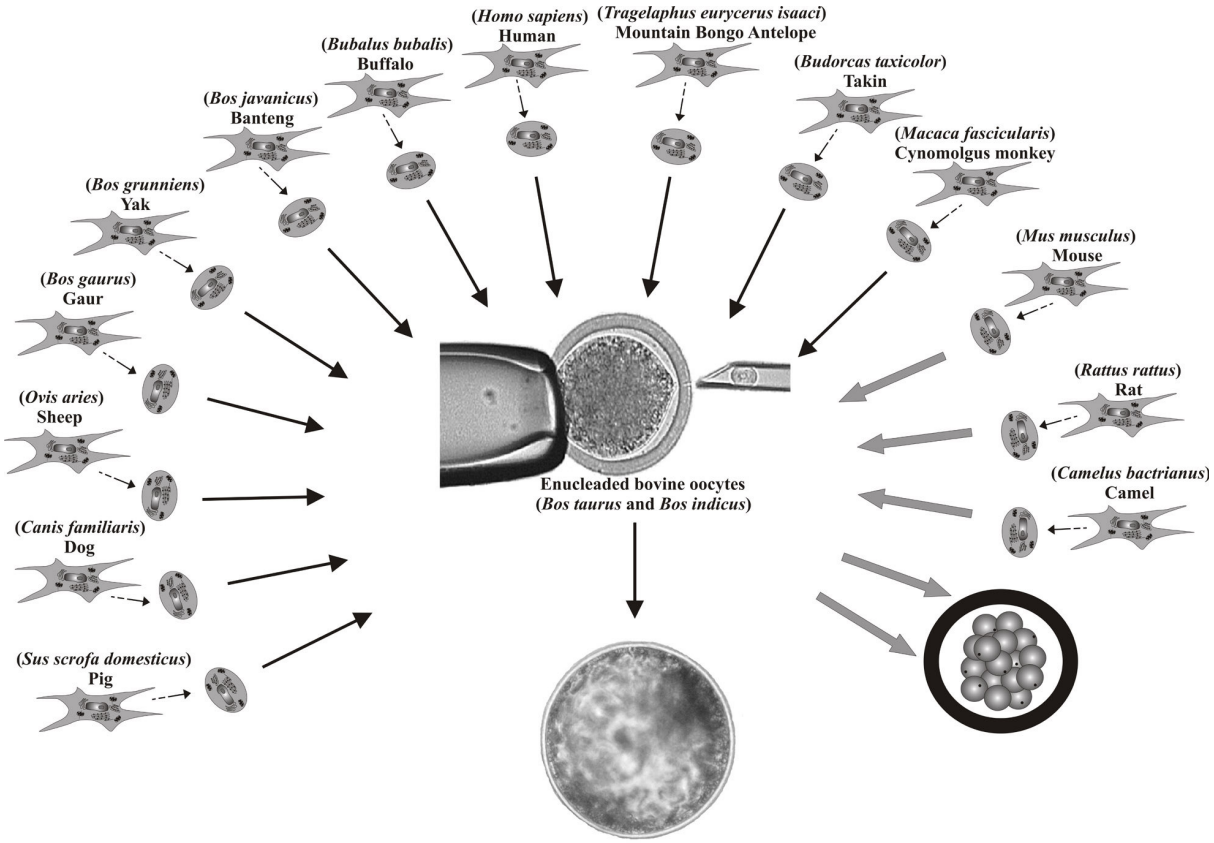

Figure 1. Blastocyst production with interspecies cloning by nuclear transfer using bovine oocytes as recipients. So far only early stage embryos have been produced in the mouse, rat and camel. Based on Dominko *et al*., 1999; Lanza *et al*., 2000; Kitiyanant *et al*., 2001; Saikhun *et al*., 2002; Chang *et al*., 2003, 2004; Lee *et al*., 2003; Atabay *et al*., 2004; Dindot *et al*., 2004; Lu *et al*., 2005; Murakami *et al*., 2005; Sansinena *et al*., 2005; Illmensee *et al*., 2006; Li *et al*., 2006; Zavos & Illmensee, 2006; Zhou & Guo, 2006.

Somatic cell NT may also be used to preserve endangered cattle breeds, especially when no fertile males are available (Wells *et al*., 1998; Cseh & Solti, 2000; McEvoy *et al*., 2003). In addition to the application to animal production and conservation biology, the production of interspecies nuclear transfer embryos using cattle oocytes as recipients can be used as an experimental model to investigate epigenetic modifications and genomic imprinting (Dindot *et al*., 2004). Also, NT studies in cattle might

be helpful to understand the fundamental mechanisms of differentiation, and aging (Tsunoda & Kato, 2000). Moreover, although ethic and legal concerns have to be taken into account, bovine reconstructed oocytes with human fibroblast cells can be useful in the improvement of cloning technology to bypass human infertility (Zavos & Illmensee, 2006) and in the production of embryonic stem cells for therapeutic purposes (Reproductive BioMedicine Online news, 2007). Production of xenogenic nuclear transfer embryonic stem cells using the equine-cow model has been highlighted recently as a putative method to produce material for cell-replacement therapy in horses (Tecirlioglu & Trounson, 2007). Production of pig-bovine blastocysts has been reported in some (Dominko *et al*., 1999) but not all studies (Lagutina *et al*., 2005). Cattle oocytes have been also reconstructed with mouse embryonic fibroblasts, and adult fibroblasts from rat and camel; however, most of the embryos were arrested at early embryonic stages and no blastocyst production was achieved (Dominko *et al*., 1999; Arat *et al*., 2003; Zhou & Guo, 2006). Nevertheless, the above-mentioned ideas are becoming very feasible, as several interspecies blastocysts have been already produced using bovine oocytes as recipients (Fig. 1).

Nevertheless, the current efficiency of NT is low (Wells, 2003; Heyman, 2005). For example, some pregnancies have been achieved with interspecies nuclear transfer embryos, but usually they are lost between days 30 to 90 after transfer (Sansinena *et al*., 2005). In addition, although pregnancy rates for intraspecies cloned embryos can be similar to embryos produced *in vitro* and artificially inseminated up to day 50, there are more continual losses throughout gestation compared to IVF and AI (Wells, 2003). Surviving animals represent only 5-15% of cloned embryos transferred (Oback & Wells, 2003; Wells, 2003; Oback & Wells 2007). Most of the remaining 85-95% die at various stages of development due to placental and fetal abnormalities collectively referred to as the "cloning syndrome" (Tsunoda & Kato, 2002; Oback & Wells, 2003). For large-scale commercial application pregnancy rates of at least 50% per recipient will be required (Lewis *et al*., 1998). To achieve this, efficiency has to be increased and the frequency of abnormalities reduced (Wilmut, 2003). Its degree of utilization in the cattle industry, however, will depend also on social acceptance (Faber *et al*., 2004). In this regard is worthy to mention that current data indicate that there are no major differences in milk and muscle characteristics between cloned and non-cloned cattle (Heyman *et al*., 2007; Yang *et al*., 2007).

2.6. *In vitro* assisted fertilization

In vitro fertilization is normally accomplished by incubating oocytes and sperm cells together in fertilization medium. However, microinsemination techniques have been developed to bypass the hurdles imposed by the zona pellucida during fertilization, which is especially useful in situations of infertility (Gwatkin, 1993). These techniques have a major relevance in treating human infertility, but its use has been explored also in productive animals, including cattle. Bovine oocytes have been fertilized using zona pellucida drilling (ZD), partial zona pellucida dissection (PZD), subzonal injection (SUZI) and intracytoplasmic sperm injection (ICSI) (Figs. 2 and 3).

Schutze *et al*. (1994) attempted to fertilize cattle oocytes by drilling the zona pellucida with an ultraviolet-laser microbeam and by inserting, directly through the laser drilled hole, one sperm with optical tweezers into the perivitelline space (PVE). The tweezer trap consisted of a single strongly focused laser beam that could be used to capture, move, and position a wide variety of cells. In that study no fertilization was observed, but the same group in a later experiment achieved fertilization by inserting in the PVE three to five sperms instead of one (Clement-Sengewald *et al*., 1996). Laser-drilled openings have been also used in an effort to increase pregnancy rates after transfer of embryos produced by standard IVF (Schmoll *et al*., 2003).

Fertilization by means of PZD has been also reported in cattle (Basovskii, 1999). Zona pellucida dissection is carried out usually with a fine needle and sometimes acidified solutions (partial zona digestion) can also be used for this purpose (Gwatkin, 1993), but this latter procedure has not been reported in bovine species. Subzonal injection has been reported in cattle using both bovine and equine spermatozoa (Heuwieser *et al*., 1991; Li *et al*., 2003). In the bovine-equine SUZI model the maximum development of embryos was only to the 8-cell stage (Li *et al*., 2003). Although its use in bovine oocyte microfertilization might not be practical, SUZI is important for other ART such as transgenesis and cloning by NT (Liu *et al*., 2000; Hofmann *et al*., 2003). The only microinsemination technique that has

produced live offspring in cattle is ICSI (Goto *et al*., 1990). In bovine oocytes, this technique is usually accomplished by inserting a needle carrying a single male gamete through the ZP into the oocyte cytoplasm. Although laser-assisted ICSI in which part of the ZP is removed to introduce the needle has been carried out in humans and other mammals, no reports in cattle were found in the present work. Despite several works reporting production of calves with this technique, its effectiveness remains unsatisfactory for commercial application (Horiuchi & Numabe, 1999; Horiuchi, 2006). The main underlying cause of the reduced efficiency of ICSI is the lack of protocols able to induce proper oocyte activation and decondensation of the sperm nucleus (Horiuchi & Numabe, 1999). ICSI may be used in circumstances in which natural mating and conventional IVF is not an option for the production of calves (McEvoy *et al*., 2003).

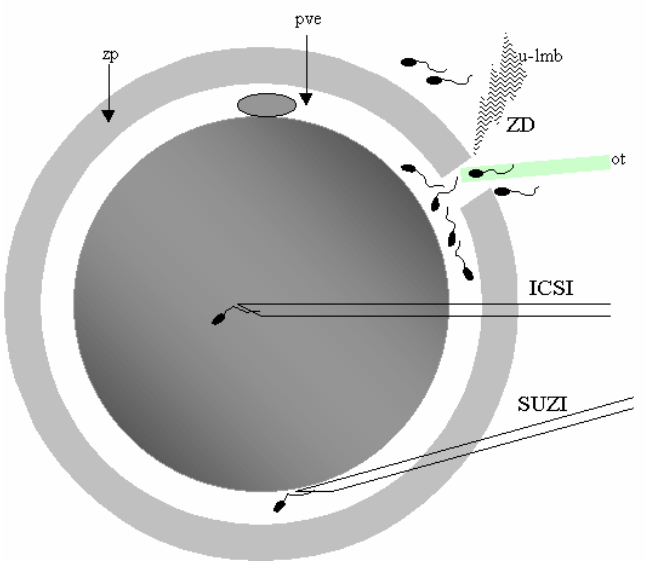

Figure 2. Microinsemination procedures carried out in cattle. Fertilisation was achieved by drilling (zona drilling [ZD]) a hole in the zona pellucida (zp) with an ultraviolet-laser microbeam (u-lmb) and introducing 3-5 sperms into the perivitelline space (pve) with optical tweezers (ot). Bovine oocytes have been fertilized by subzonal injection (SUZI); however, live offspring have been obtained only with intracytoplasmic sperm injection (ICSI). Data based on Goto *et al.* (1990), Heuwieser *et al.* (1991), and Clement-Sengewald *et al*. (1996).

For McEvoy *et al*. (2003), ICSI is unlikely to be used for commercial purposes in the cattle industry, as it is a technically demanding and costly procedure. Instead, its use for conservation biology of non-productive ungulates might be more applicable (Cseh & Solti, 2000; McEvoy *et al*., 2003). This seems very promising especially when bovine embryos have been already produced with ICSI using freeze-dried spermatozoa, heat-dried sperm heads or frozen-thawed oocytes (Keskintepe *et al*., 2002, Rho *et al*., 2004; Lee & Niwa, 2006). Interspecies microfertilization using bovine oocytes can also be used to study key processes during sperm-oocyte fusion, oocyte activation and fertilization (Li *et al*., 2003; Kobayashi *et al*., 2006). This has a special relevance in human fertility, as bovine ICSI has been suggested to be an appropriate model to assess human sperm oocyte activation ability (Terada *et al*., 2004). Considering animal production, Horiuchi *et al*. (2002) suggested that ICSI could be used to maximize the use of costly semen. Because sperm motility is not essential in this technique, if cheaper and efficient procedures capable of maintaining the nuclear integrity of the spermatozoa are developed, ICSI might be acceptable (McEvoy *et al*., 2003). However, its effectiveness has to be improved and acceptable before this can be considered a viable proposition.

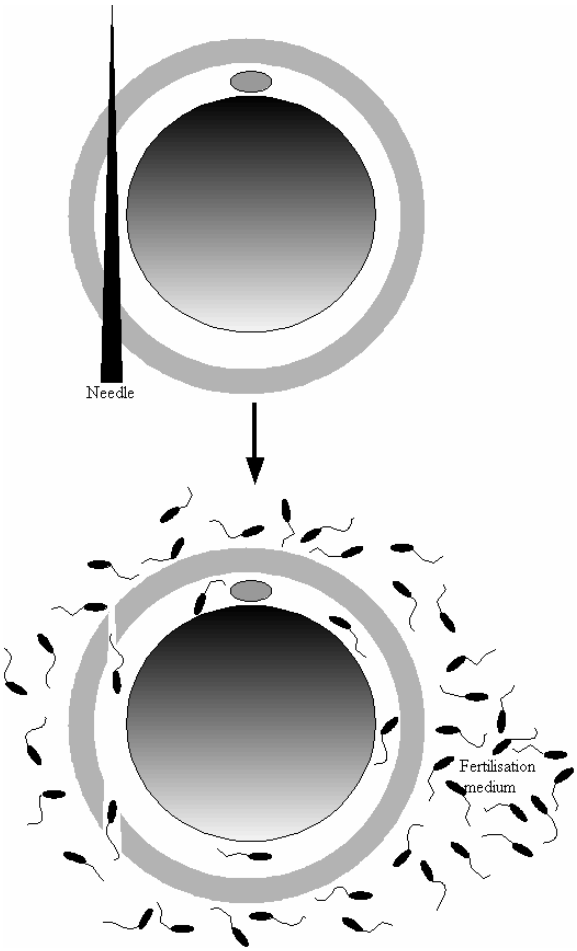

Figure 3. Fertilization has been achieved in cattle with partial zona dissection (PZD). Based on Basovskii (1999).

2.7. Transgenesis

Transgenic technology provides a method to rapidly introduce "new" or modified genes and DNA fragments into cattle and other livestock species without crossbreeding (Wheeler, 2003, 2007). Approximately 25 animal species are currently involved in the developing of transgenic lines for basic biomedical research and applied purposes (Houdebine, 2005). The first transgenic farm animals (*i.e.* rabbits, pigs and sheep) were produced by Hammer *et al*. (1985). Roschlau *et al.* (1989) were the first to report a successful production of transgenic cattle. Since then, steady progress has been made with cattle transgenesis and several research groups have managed to produce transgenic bovine offspring (Table 1). Possible applications of gene transfer in cattle include the production of valuable proteins in milk and serum for therapeutic purposes in humans ("biopharming"), which is currently the most advance state of bovine transgenesis (Piedrahita, 2000; Kuroiwa *et al*., 2002; van Berkel *et al*., 2002;; Keefer, 2004; Robl *et al*., 2006). In fact, several therapeutic proteins (*i.e.* growth hormone, albumin, fibrinogen, collagen, and lactoferrin) produced in the milk of transgenic cows are currently under preclinical trial for future commercialization (Niemann & Kues, 2007). Research in New Zealand is also being carried

Table 1. Examples of production of transgenic bovine offspring.

Gene inserted	Gene transfer method	Possible application	Reference
•*hLF*	Microinjection	Therapy for infectious and inflammatory diseases, and production of infant formulas	•Krimpenfort *et al*., 1991
•*Chicken c-ski*	Microinjection	No therapeutic or productive purpose[1]	•Bowen *et al*., 1994
•*hEpo*	Microinjection	No therapeutic or productive purpose[1]	•Hyttinen *et al*., 1994
•*HbsAg*	Retroviral infection	No therapeutic or productive purpose[1]	•Chang *et al*., 1998
•*β-galactosidase*	Microinjection and nuclear transfer	No therapeutic or productive purpose[1]	•Cibelli *et al*., 1998b
•*hα-LA*	Microinjection	Therapy for PKU[2] and production of infant formulas	•Eyestone *et al*., 1998
•*hSA*	Microinjection	Therapy for restoration and maintenance of blood volume	•Behboodi *et al*., 2001
•*Prochymosin*	Nuclear transfer	No therapeutic or productive purpose[1]	•Zakhartchenko *et al*., 2001
•*BSSL*	Nuclear transfer	Therapy for pancreatic insufficiency and production of infant formulas	•Chen *et al*., 2002
•*hIg*	Nuclear transfer	Therapy for immuno-related diseases	•Kuroiwa *et al*., 2002
•*β- and κ-casein*	Nuclear transfer	Improvement of nutritional and processing properties of milk	•Brophy *et al*., 2003
•*EGFP*	SMGT	No therapeutic or productive purpose[1]	•Shemesh *et al*., 2000
	Nuclear transfer		··Bordignon *et al*., 2003
	Nuclear transfer		··Gong *et al*., 2004
	Lentiviral infection		··Hofmann *et al*., 2004
•*r28M**	Nuclear transfer	Tumor therapy	•Grosse-Hovest *et al*., 2004
•*Lysostaphin*	Nuclear transfer	Resistance to mastitis[3]	•Wall *et al*., 2005
•*hGH*	Nuclear transfer	Therapy for growth-related disorders	•Salamone *et al*., 2006

hLF= Human lactoferrin *hEpo*= Human erythropoietin *HbsAg*= hepatitis B surface antigen gene *hα-LA*= Human α-lactalbumin
hSA= Human serum albumin *BSSL*= Bile salt-stimulated lipase *hIg*= Human immunoglobulin *EGFP*= Enhanced green fluorescent protein
hGH= Human growth hormone SMGT= Sperm-mediated gene transfer
*A recombinant bispecific single-chain antibody directed against T-cell surface-associated costimulatory molecule CD28 and a melanoma-associated proteoglycan (MAPG)
[1]Studies were conducted to increase the efficiency of transgenic animal production [2]Phenylketonuria [3]In mastitis caused by *Staphylococcus aureus*

out with transgenic cows that can produce human myelin basic protein with the goal of extracting and purifying this protein for the treatment of multiple sclerosis (Rutter, 2006).

Improvement of milk composition and disease resistance are examples of agricultural applications of this biotechnology in cattle (Paape *et al*., 2002). Transgenic dairy cows capable of producing high levels of casein (Brophy *et al*., 2003) and resistant to mastitis caused by *Staphylococcus aureus* (Wall *et al.*, 2005) are now available. Other economically important traits in livestock production like growth rate and feed conversion have not yet been explored in cattle using transgenic technology.

Due to inefficient outcomes obtained from pronuclear DNA microinjection, NT became the most feasible technique to generate transgenic livestock (McEvoy *et al*., 2003; Niemann & Kues, 2003; Thomson *et al*., 2003; Niemann *et al*., 2005). Other approaches to generate transgenic cattle include the production of chimeric embryos with DNA-modified stem-like cells and through microinsemination (notably ICSI) of oocytes with DNA integrated into bovine spermatozoa (sperm-mediated gene transfer) (Cibelli *et al*., 1998a,b; Gandolfi, 1998; Shemesh *et al*., 2000; Celebi *et al*., 2003; Lavitrano *et al*., 2006). In addition, transgenic cattle embryos have been produced delivering genes into the oocyte and embryo with recombinant viruses (viral transgenesis). However, only lentiviral vectors injected to the oocyte generated viable offspring (Hofmann *et al*., 2003, 2004). Nevertheless, abnormalities have been reported in transgenic calves during pregnancy and only 1-10% of transgenic embryos produced resulted in the birth of healthy offspring. These abnormalities have been suggested to be more related to the cloning techniques and *in vitro* culture conditions than to the gene targeting *per se* (Cibelli *et al*., 1998a,b; Hill *et al*., 1999; Zakhartchenko *et al*., 2001; McEvoy *et al*., 2003; Thomson *et al*., 2003). Animal transgenesis relies heavily in some ART used to generate non-transgenic offspring. Therefore, improvements in reproductive biotechnologies, such as cloning and ICSI, would give major benefits to the production of transgenic cattle.

2.8. Xenografting-germ cell transplantation

Xenografting in bovine species is being carried out mainly with ovarian and testis tissue. Germ cell transplantation is also being actively investigated in cattle, but mainly in bulls. The bovine model has been used for the implementation of protocols for fertility preservation of cancer patients. For example, spermatogonial proliferation has been observed with bovine male germ cells transplanted into mice recipients (Dobrinski *et al.*, 2000). Although no further differentiation was accomplished in this model, the results have encouraged more research into the understanding of testis function in order to preserve male fertility, including humans. Cross-species ovarian tissue transplantation has been also achieved using cattle-mice models. Herrera *et al.* (2002) transplanted fresh or frozen-thawed bovine ovarian cortex grafts into mice under the kidney capsule and subcutaneously. They observed follicular development up to the antral stage in subcutaneously transplanted ovarian tissue but not in tissue transplanted under the kidney capsule (Herrera *et al*., 2002). In other studies, newborn and adult bovine ovarian cortical pieces were transplanted into male severe combined immunodeficient (SCID) mice, and after treatment with gonadotropins recovery of oocytes was possible (Hernandez-Fonseca *et al*., 2004, 2005). Furthermore, some experiments have demonstrated that xenografting of bovine ovarian follicles under the kidney capsules of female SCID mice can develop until the antral stage. These follicles contained oocytes that were capable of resuming meiosis, achieving fertilization, cleavage and develop until the 5- to 8-cell embryonic stage, (Senbon *et al*., 2003, 2004, 2005). Due to the similarities between humans and cattle in terms of ovarian physiology (Campbell *et al*., 2003), bovine models for xenografting could be very valuable in biomedical research.

Male germ cell transplantation in the same species has been carried out in cattle to elucidate basic biological aspects of testis function. For instance, experimental models have been developed in bulls to study the possibility of restoring spermatogenesis in individuals with azoospermia (Schlatt *et al*., 1999; Dobrinski, 2005a). In fact, regeneration of spermatogenesis has been achieved in cattle by autologous and heterologous male germ cell transplantation (Izadyar *et al*., 2003; Herrid *et al*., 2006). It has been suggested that transplantation of germ cells could be also used to restore bull fertility after an insult to the testis or to preserve genetic material from valuable bovine males that are lost before reaching puberty (Dobrinski, 2005b). Another option includes studies regarding spermatogenesis *in vitro* with

male bovine animals in conjunction with germ cell transplantation in order to get new insights concerning male gamete biology (Parks *et al*., 2003). Since germ cell transplantation protocols are relatively well established in cattle, the propagation of endangered ungulates could be achieved through bovine surrogate recipients (Dobrinski & Travis, 2007). Besides germ cells, testis xenografts from several species (including cattle) could also be used to study toxicants or drugs with the potential to reduce or improve male fertility without the necessity of performing experiments in the target species (Dobrinski, 2005b). In addition, application of testicular xenografting and germ cell transplantation techniques can be useful in shortening the interval to produce transgenic bulls (Dobrinski, 2006, 2007). Accordingly, transgenic spermatogonia were obtained with bovine testicular tissue transducted with β-galactosidase and subsequently grafted onto the backs of castrated immunodeficient nude mice (Oatley *et al*., 2004). At present, offspring have been obtained only in goats with germ cell transfer protocols (Hill & Dobrinski, 2006).

Grafting of ovarian and testicular tissue and male germ cell transplantation are unlikely to be used for breeding purposes in the cattle industry. This is because other ART, such as cloning, might offer a more practical approach. Instead, its usefulness will be more applicable in experimental models to develop strategies aimed at restoring fertility in human patients subjected to gonadotoxic therapy and for the conservation of endangered bovid species.

2.9. Preimplantation genetic diagnosis

Currently, the most applicable use of preimplantation genetic diagnosis (PGD) in cattle is the sex determination of embryos. In several countries embryo sexing is being applied at commercial level in companies and farms working with embryo transfer technology. Knowing the sex of embryos produced for the use in an embryo transfer program can assist the producer in managing resources more effectively by choosing future replacement heifers and sires (Shea, 1999). Several attempts have been carried out to sex embryos; however, the polymerase chain reaction (PCR) technique seems to be the more efficacious with an accuracy of 90 to 100% even under field conditions. Pregnancy rates with sexed embryos produced *in vivo* are comparable to those achieved with intact embryos in MOET programs. On the contrary, sexed IVF embryos have a reduced capability to attain pregnancy than *in vivo*-produced embryos (van Vliet *et al*., 1989; Thibier & Nibart, 1995; Shea, 1999; Lopes *et al*., 2001; Hasler *et al*., 2002; Alves *et al*., 2003). Still, normal calves have been born using vitrified-thawed sexed embryos produced *in vitro* (Agca *et al*., 1998). Embryo sexing with PCR implies embryo biopsies, with possible concurrent damage that can influence the probability of pregnancy. Nevertheless, non-invasive techniques are available, such as the method based on the detection of the H-Y sex-specific male antigen with 80% accuracy and with similar pregnancy rates to sexing using PCR (Ramalho *et al*., 2004).

Embryo sexing is only one of the advantages of PGD in cattle. Identification of genetic abnormalities in preimplantation embryos prior to embryo transfer may improve the likelihood of a successful pregnancy and/or viable offspring. Preimplantation GD had played a pivotal role in improving the outcome of assisted reproduction technologies in humans (Kuliev & Verlinski, 2005). In cattle, assays have been developed for the simultaneous detection of embryo sex and genes of importance for the bovine industry, including some relevant diseases and production traits (Table 2).

This biotechnology will be pivotal in the identification of genes of reproductive importance, as shown recently by El-Sayed *et al*. (2006). Using microarrays to analyse bovine embryo biopsies, these authors revealed differential gene expression between biopsies derived from embryos that resulted in no pregnancy, resorption or calf delivery, thus providing candidate genes of embryo developmental competence (El-Sayed *et al*., 2006). PGD has been also used to assess transgenic integration in bovine embryos (Bowen *et al*., 1994; Chen *et al*., 2002; Forsyth *et al*., 2005). Besides its usefulness in animal breeding, PGD in bovine species has been used to develop a training protocol aimed at improving the performance of professionals working with human embryos (Almodin *et al*., 2005). Another major commercial application of PGD may be the analysis of selected genetic markers. Such marker-assisted selection (MAS) can be applied at the pre-elongation embryo stage. Selection of embryos carrying genes of economic importance would revolutionise the cattle industry (Bodo *et al*., 2001; Mapletoft & Hasler, 2005; Moore & Thatcher, 2006). Cloning procedures could be advantageous in the multiplication

of these embryos (Oback & Wells, 2007). However, this will not be possible until sufficiently valuable markers are identified.

Table 2. Detection of genes related to productive traits and genetic diseases via PGD in cattle.

Gene	Reference
Kappa-casein	Schellander *et al*., 1993
Growth Hormone (GH)	Chrenek *et al*., 2001
Prolacting (PRL)	Chrenek *et al*., 2001
Growth Hormone Receptor (GHR)	Peippo *et al*., 2007
Prolacting Receptor (PRLR)	Peippo *et al*., 2007
Bovine Leukocyte Adhesion Deficiency (BLAD)	Hochman *et al*., 1996
Claudin-16 Deficiency	Hirayama *et al*., 2004
Band 3 deficiency	Kageyama *et al*., 2006

2.10. Sperm sexing

Embryo sexing is identification rather than selection of sex (Seidel & Johnson, 1999). Hence, sex pre-selection is more advantageous for productive purposes than embryo sexing. Hundreds of thousands of calves have been born from sexed sperm (SS). Most of these calves were produced in USA, UK, Argentina, Brazil, and Mexico, with lesser numbers in several other countries. Currently, sexed bovine sperm can be purchased from companies in the UK, Canada, USA, Mexico, Argentina, Brazil and China. In several countries licensing and commercialization of this biotechnology is in various phases of development (Garner, 2006). Companies have to decide exactly what product might be provided (*e.g.,* fresh and/or frozen sperm, number of sperms/dose, which class of bull, etc.) (Seidel, 2003a). Predetermination of sex of offspring with SS could increase efficiency of producing meat and milk and improve cattle welfare. An example of the influence on welfare of SS is a decrease of calving difficulty in primiparous heifers by selecting for female calves (Seidel, 2003b).

Several approaches to sexing sperm have been proposed (Seidel & Garner, 2002). However, the sorting of sperm by flow cytometric has been found to be the more efficacious so far (Johnson, 2000). Accuracy of the process is about 90% for either sex, and resulting calves appear to be no different from non-sexed controls in birth weight, mortality, rate of body weight gain, and incidence of abnormalities (Seidel, 2003a). Sorted sperm can also be used for *in vitro* production of embryos and large-scale production is on the way to practical application (Wheeler *et al*., 2006). Encouraging results have been obtained in China, where healthy offspring was achieved with sexed IVF embryos. Moreover, there were no differences in pregnancy rates when compared to nonsexed IVF and *in vivo*-derived embryos (Xu *et al*., 2006). Sexed sperm has been also used in ICSI procedures where normal calves have been obtained (Hamano *et al*., 1999). Although SS tends to degenerate faster than normal sperm, cryopreservation overcomes this problem allowing its use anywhere in the world. Furthermore, pregnancies have been reported with sperm that was frozen-thawed before being sorted and re-frozen-thawed after sexing (Underwood *et al*., 2007). All applications of SS required strict management practices and the use of AI or IVF, which is a limitation. However, sorting speed is the primary limitation to the technology. In addition, when superovulated animals are inseminated with SS, the production of transferable embryos is low compared with non-sexed controls (Schenk *et al*., 2006). Another disadvantage is that when SS is used in lactating cows (Seidel, 2003a,b; Garner, 2006) and in heifers subjected to timed AI (Seidel, 2007), pregnancy rates are usually low. Despite these constrains, sorted sperm is already at commercial level and as efficiency improves and cost declines sperm sexing will be more widely used (Seidel & Garner, 2002).

3. Concluding Remarks

Several ART have been applied in the cattle industry (Fig. 4). Some of them have provided acceptable results; but others have low efficiency, which limits their use for cattle breeding. This is because they do not always achieve pregnancy and in some cases have prejudicial affects in the prenatal and postnatal period. Hormonal treatments to correct reproductive problems or to improve reproductive performance have produced variable results and sometimes might infringe negative effects. Currently, the most cost-effective way to disseminate genes is with conventional AI. Among the ET technologies, MOET is the most efficient so far. Improvements in estrus detection and prediction of superior recipients will benefit AI (especially with SS) and embryo transfer-related technologies. Improvements in OPU/IVF programs would have a great economical impact in the cattle industry and could overtake the traditional MOET programs. Embryo and sperm sexing are already at commercial level with acceptable results. Improvements in the sorting of sperm will make this biotechnology more acceptable and might overtake embryo sexing.

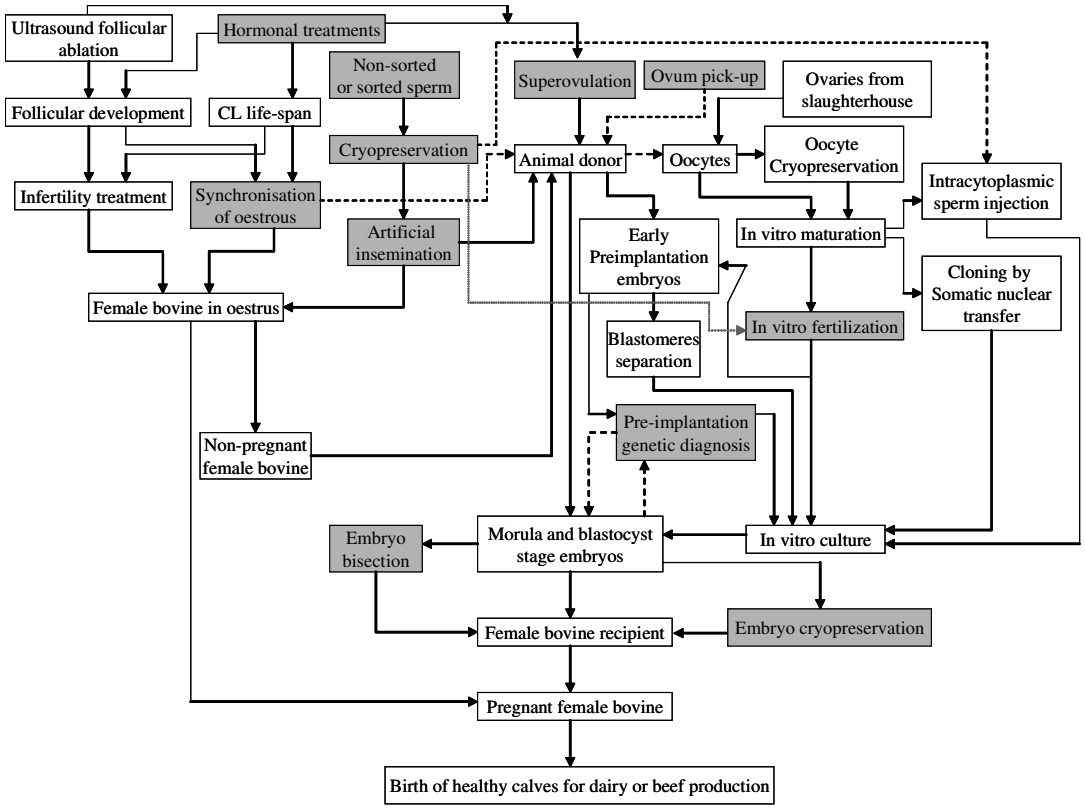

Figure 4. Application of ART in cattle. In grey are the ART with current commercial application. Dotted and grey lines are intended to clarify the pathway.

Transgenesis, xenografting-germ cell transplantation, cloning and microinsemination procedures need to be seriously improved before they can be considered for implementation into the cattle industry at commercial level. However, even if there is an acceptable improvement in such technologies, some of them might not be applicable to cattle production. Instead, their use in conservation biology, production of pharmaceuticals and as a model to study human reproduction will be more useful. Here it is important to recognize that data generated from bovine ART models can not be directly extrapolated to humans. Instead, the information obtained will be important to build conceptual models that will help to create hypothesis that should be ultimately tested on the human itself. Apart from concerns regarding cost and efficiency, implications for animal welfare have to be taken into account when applying any ART. In

addition, it is important to take into account the repercussions that can occur using ART to overcome fertility problems, as there is a risk of disseminating infertile genotypes. Although generation of information is of pivotal importance, it is worthy to consider the possibility that a concise understanding of the current knowledge on reproductive physiology would give us palpable improvements. As highlighted by Lucy (2005) and Seidel (2006a), our current capacity to generate information is great, however our skill to understand it properly is low. It is curious how information generated more than 20 years ago (Linares *et al*., 1982) is being just recently (Wathes *et al*., 2003) put into perspective (*i.e.* the early "window" of progesterone rise is very important for preimplantation embryo development). Perhaps it is time to recapitulate a little bit.

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