Assisted Reproductive Techniques for Cattle Breeding in Developing Countries: A Critical Appraisal of Their Value and Limitations

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Running title: Impact of ARTs for cattle breeding in developing countries

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Commercialisation of animal biotechnologies, including those related to reproduction (also known as Assisted Reproductive Techniques, ARTS) is an increasing reality in developing countries, following the enormous flow of information around us and the increasing global commercial interests in areas where cattle production has its major assets. The present review discusses the achievements of various biotechnological tools for reproduction in cattle including semen handling for artificial insemination (AI), superovulation and embryo transfer (MOET), *in vitro* handling of oocytes and production of embryos, reproductive cloning and emerging technologies (sex selection, gene targeting and nuclear transfer for livestock transgenesis, genomics for marked assisted selection etc). The application of these technologies for cattle breeding is critically discussed in relation to their impact in the improvement of the efficiency of dairy and beef production in developed and –particularly- in developing countries, which ultimately rule the possibilities of a competitive and sound production of food for human consumption. Despite the remarkable progress made and the punctual importance of some of the above-mentioned technologies, AI remains the most important assisted reproductive technology (ART) in developing countries. Any attempt to gain widespread of any other ART under the predominant economical conditions in developing countries ought to match the simplicity and the success of AI as a breeding tool.

Key words: artificial insemination, sperm technology, embryo transfer, *in vitro* ART-methods, cryopreservation, cloning, transgenesis, bovine.

Introduction

Cattle, together with small ruminants, account today for the largest part of the economy of large- medium- or small-farmers in developing countries worldwide and even represent the major economical asset in most countries of the southern hemisphere (Australia, New Zealand, South America) in terms of milk, meat and wool production. As well, ruminants have been domestic animals of choice for biomedical and reproductive research and with the advancements of biotechnology, been chosen for production of foreign proteins in milk (gene farming strategies). Considering this panorama, it seems appropriate to discuss the application of reproductive biotechnologies in cattle and their impact for future achievements, with a focus on developing countries. Reproductive biotechnologies intend to be used routinely to shorten generational intervals and to propagate genetic material among breeding animal populations. To achieve this goal, reproductive technologies have been developed in generations over the years, namely artificial insemination (AI), embryo transfer (ET), manipulation of fertilization and embryo production *in vitro* (IVF) and multiplication techniques (cloning) for the application of transgenesis. These, together with sperm separation techniques (Morrell & Rodriguez-Martinez

2009, 2010), including that of selection of spermatozoa for chromosomal sex (commonly named sex-sorting) all face today a strong wave of increasing commercialization (Faber et al 2003, Gardner & Seidel 2008, Seidel 2009).

Preservation of semen for AI is well advanced and provides semen of good quality for AI on commercial basis, despite we still struggle in trying to understand how spermatozoa lose their capacity to remain fertile upon freezing and thawing. Embryo transfer has benefited from the establishment of newer, more efficient methods for the superovulation of donors, embryo retrieval/transfer by low-invasive methods as well as by the better, simpler and more effective cryopreservation methods made public for freezing and vitrification of embryos. Unfortunately, some of the protocols used for *Bos taurus* do not serve well in *Bos indicus* owing to differences in nutrition and management that constrain reproductive function. Use of AI and ET would aid preventing transmission of undesirable diseases provided more research is carried out to determine risks of sperm- and embryo-pathogen associations. Gradual elimination of animal-derived products during sperm and embryo handling, as well as during the production of *in vitro* developed embryos is increasing. Although *in vitro* techniques for embryo production are well established for cattle, there is as yet a sub-optimal oocyte maturation that limits further developments. Production of offspring combining available reproductive technologies such as trans-vaginal ovum-pick up (OPU), *in vitro* embryo production and vitrification for direct ET appears as a promising combination of good applicability in breeding. Cattle has been successfully used for reproductive cloning and for the production of transgenic clones, yet being affected by low effectiveness owing to epigenetic disarray. Beneficial outcomes of the expanding gene targeting technology combined with nuclear transfer and reproductive cloning are foreseen within "gene pharming" and genetic programmes, including genomic selection of future sires. Many of these technologies aim the creation of ruminants with specific genetic modifications, but they are still shadowed by the well documented adverse effects on the survival and well-being of the offspring. More research in these areas must be carried out to warrant the welfare of the animals produced by these novel reproductive biotechnologies.

The present review summarizes the achievements of the above mentioned ARTs in cattle breeding, albeit being critical with respect to their impact when aiming long lasting improvement of the efficiency of dairy and beef production in developing countries.

Semen handling for artificial insemination (AI)

Genetic progress in cattle can be increased up to 50% through the application of AI, the first generation biotechnology, using either extended semen that has been preserved in liquid form (fresh, or cooled to 5ºC), or deep-frozen (Vishwanath 2003). During the past 50 years, the development and application of cattle AI with preserved (either chilled or frozen) semen have been growing exponentially on a global scale (Thibier & Wagner, 2000). The number of produced semen doses is >250 million worldwide (FAO), using standardized methods for extension, cooling, freezing and thawing basically all over the world, with only subtle differences between *Bos Taurus, Bos indicus, Bubalus bubalis* or *Bos javanicus* (Rodriguez-Martinez 2001, 2003, 2006, 2007, Rodriguez-Martinez & Barth 2007). Sires delivering semen for AI are to be housed in strictly protected semen collection centres to avoid association of pathogens to the germplasm, a situation still not fully established in many developing countries. Artificial insemination is usually performed intra-uterine (transcervical) with routine placement in the uterine body, but with increasing attempts for an AI deeper intra-cornual (deep intrauterine AI). Fertility after AI of fresh- liquid conserved semen is as good as natural mating (above 80%), while that of frozen semen is somewhat lower (yet usually above 60%). Large variation is still observed among sperm numbers for AI, with an overall tendency to reduce them per straw,

following the innate optimal fertility level achieved by the individual sires. The AI of low-sperm doses, including those containing sex-sorted semen by flow cytometry (Garner & Seidel 2008) is usually done more or less deep into the uterine horn with acceptable results (Andersson et al 2004,Verberckmoes et al 2004, Ballester et al 2007, Seidel & Schenk 2008, Schenk et al 2009).

The extensive and safe use of AI with preserved bull semen has led, incorporating the use of milk recording and of effective evaluation systems (BLUP for instance), to the establishment of progeny testing systems for both dairy and beef cattle, a matter of utmost importance for developing countries in order to avoid dependency upon trans-national breeding companies (Normal et al 2003). It is important to remember that the globalization of genetics for dairy led to terrible consequences regarding fertility in Holstein-dominated herds, particularly in those holding high producing cows, whose health and fertility dramatically deteriorated. Inappropriate use of sires, selected without taking enough consideration to reproductive traits and focusing mainly for increased milk production, in countries that heavily used American Holsteins, led to a documented decline in reproduction success. In some cases, the impairment of fertility and health was so heavy that it was considered a major obstacle for milk production management, as in the UK, for instance. There, pregnancy rate to first service dropped from 56% in 1972-1982 to about 40% in 1995-1998, a rate of about 1% per year, and undisputedly related to a genetic deterioration in this breed (see Rodriguez-Martinez et al 2008 and references therein). Other associated disarrays, such as less intense oestrous signs leading to wrong timing for AI have also been detected (García et al 2011a,b). Considering that improvements of the genetic basis of a herd takes about 7-8 years when selected semen is used for AI, a full restoration of the troubles mentioned above is yet to come.

Embryo transfer

The embryo transfer (ET) methodology is a suitable, more integrated approach for genetic distribution than AI, leading to improvement of genetic basis within 5 years. Moreover, as for AI, allows movement of material worldwide and reduces the risk of transmitting specific diseases, provided the embryos are free from contamination. Although MOET (multiple ovulation and embryo transfer) would be considered advantageous as a methodology for genetic improvement, and up to 80% of embryos have been commercially transferred, the technology has not reached optimality due to the variability of the ovarian response to the superovulatory gonadotrophin treatment used so far (Mapletoft et al 2002, Betteridge 2006, Lonergan 2007). Despite its superiority respect to *Bos taurus* under tropical or subtropical environments, where stressors like high humidity, high temperature, parasitic pressure and low quality pastures are less likely to affect *Bos indicus*, the latter presents smaller preovulatory follicles, a lowed LH secretion capacity and a shorter oestrous duration all of which difficult oestrus detection, and make responses to oestrus synchronization, management of follicle development and superovulation highly variable (Bo et al 2003). Another matter of concern with the manipulation of ovulation is the asynchrony between time of AI and occurrence of ovulation, and the decreased sperm transport registered after a superovulation treatment, both of which lead to low fertilization rates in cattle. As a method, ET basically requires synchronization of the donor and the recipient females so that the embryos are recovered and transferred in synchrony in order to warrant a proper embryo elongation and the recognition of pregnancy by the recipient cow (Rodriguez-Martinez et al 1999). ET does not have be done immediately, and bovine embryos can be frozen, either conventionally (slow freezing using ethylene-glycol) or by vitrification (high concentrations of cryoprotectants and plunging into LN_2), ensuring safe storage and better management of the genetic resources (Saragusty & Arav 2011). ET of *in vivo* (or in vitro) produced embryos to the uterus of a recipient cow is easy and reliably done by transcervical intrauterine deposition, with >60% of pregnancy rates. Use of MOET for selection purposes

makes possible to gather information on sibs to estimate breeding values rather quickly (compared to conventional progeny testing of AI-sires) alongside decreasing the number of selected dams for the next generation provided the superovulatory response is maximized to bring MOET to its highest efficiency. Use of transvaginal, ultrasound-guided follicular puncture for oocyte retrieval (commonly named ovum-pick-up, OPU) may make MOET more effective since it waives superovulation and AI treatments, by the collection of oocytes (up to 1000 oocytes can be collected from a heifer/cow per year) and following in vitro embryo production (up to 300 in vitro produced, IVP, embryos can be obtained per year)(Presicce et al 2011). Moreover, oocytes can be OPU retrieved from pre-pubertal heifers and pregnant cows thus maximizing harvesting possibilities, although with a marked ethical component.

In vitro embryo production (IVP)

Methods for *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) are available for cattle, proved by the birth of innumerable calves worldwide (Galli et al 2003). However, methods are still sub-optimal respect to oocyte maturation when using offal COCs and even when using OPU (Merton et al 2003, Lonergan 2007). Another problem has been the low oocyte yield per ovary when aspiration is continuously used to increase the number of oocytes per session or cow (Machado et al 2005). Optimized OPU schedules respecting half of the oestrous cycle and spontaneous ovulation have proved more effective, since they do not affect the physiology of the ovary and the expression of normal oestrous signs (Båge et al 2003, van Wagtendonk-de Leeuw 2006). Velogenesis (e.g. IVM/IVF/IVC of prepubertal oocytes) has also been carried out successfully aiming the shortening of the generational interval. However, its eventual application has caused, obviously, major ethical concerns. Although no major differences have been seen comparing *Bos taurus* and *Bos indicus*, the efficiency of IVP is much lower in buffalo than in cattle, covering both IVM, IVF and IVC (Nandi et al 2002). It seems clear that more efforts have to be made to optimise both repeated OPU retrieval and, particularly, the current *in vitro* maturation procedures, which appears to be the major limiting factor for a satisfactory IVP at present. The sub-optimality and the costs related make these techniques of little application in cattle breeding, particularly in developing countries.

Embryo splitting, bisection and reproductive cloning by nuclear transfer

Cloning, as a multiplication technique, has been used in small ruminants since the late 1970´s. Splitting of cattle embryos can be used to increase the number of embryos available from selected females and to produce genetically identical animals for biomedical research. Both separation of blastomeres in 2-4 cell-embryos and embryo (morula or blastocyst) bisection have proven efficient to yield monocygotic twins after quick laparoscopic transfer to recipient cows. Pregnancy rates achieved were similar to when transferring whole embryos, and twinning reached 50% after pair transfer. The overall efficiency of cow embryo splitting (number of calves born per embryos bisected and transferred) can reach almost 60%.

Nuclear transfer has been attempted and succeeded in small and large ruminants using blastomeres from 8-16 cells embryos, 32 cell embryos (goats) or sheep ICM-cells. Somatic cloning has, as everyone is aware, succeeded both in small and large ruminants and today it has proven successful in up to 23 species. Sheep was the first mammal to be cloned from an adult somatic cell (Dolly, 1997) and some other sheep and innumerable calves (above 4,000 reported) followed, using variants of the original technique (Vajta & Gjerris 2006). Calves have been successfully cloned via somatic nuclear transfer, both using adult as well as foetal cells (mainly fibroblasts) as nuclear donors. Most of the work done so far seems directed to, using foetal cells and gene transfer, to produce transgenic animals for production of specific substances in milk

(such as α-1-antitrypsin or Factor IX). The effectiveness reached is still very low (in terms of living clones per number of ooplasts used), the reasons behind being related to the type of cell used as nuclear donor as well as the problems related to large offspring size, foetal death and congenital abnormalities registered so far, related failure in the reprogramming of the donor nucleus leading to epigenetic disorders in the founder conceptus (Lee et al 2004). The healthy clones or the offspring of cloned bovine produced by AI do not show transmissible defects (Heyman et al 2004, Zhang et al 2004). The NT-technology has, however, not impact on cattle breeding and deemed by several instances, including the European Food Safety Authority, EFSA, 2010. Other applications of NT involve production of "parthenotes" using male diploid cells to repopulate a genome-emptied oocyte, resumption of meiosis to metaphase II and ICSI of a sperm on this "male haploid" cell. Although feasible, it has not rationale for breeding unless elite bulls are donors for both the diploid cell and the spermatozoa.

Emerging reproductive technologies to be seriously considered for animal breeding

Several new reproductive technologies are foreseen developing further in a near future, with obvious advantages for breeding. One of them is sexing spermatozoa for directed production of offspring of a desirable sex by use of modified flow cytometric cell sorting of fluorescent dye-loaded living spermatozoa. Cattle present about 3.8% differences in DNA contents between their X- and Y-chromosome-bearing spermatozoa, a difference large enough to allow successful sorting (Garner & Seidel 2008). Although the numbers of sorted spermatozoa per hour reach at present larger figures that for a decade ago (50-100 million compared to 350,000), these numbers imply few sperm doses for AI, impairing their application for conventional breeding. The technology is, however, very promising and provides opportunities for sex-selection of IVP-embryos, surpassing the need for sex diagnosis of the embryos (which is reliably done today by DNA probing, specific for the Y chromosome, but still time-consuming and –perhaps- not risk-free)(Blondin et al 2009, Carvalho ett al 2010). Moreover, it appears to be the only fully validated technology for pre-selecting offspring for sex available at present, although new applications are incoming. Sex-sorting, albeit interesting for animal breeding strategies, is too costly (a flow sorter costs above 300,000 U$S), slow, and yields weak spermatozoa with reduced lifespan (Lonergan 2007, Gosalvez et al 2011). Nevertheless, the products (male- or female-sorted spermatozoa) are available and becoming more competitive by the day (Pontes et al 2009, Hayakawa et al 2009, Underwood et al 2010).

Technologies based on research in functional genomics, proteomics and NT-cloning have significant potential, but considerable research effort will be required before they can be utilized for cattle breeding and production. Preimplantation genetic diagnosis (PGD) for well-defined selection targets would allow for the selection of embryos for valuable production traits using marker-assisted selection (MAS). PGD can detect a good allelic profile and/or the insertion of a transgene thus helping the application of the information gathered by whole genome scan aiming the detection of chromosomal regions affecting multiple traits (health, production and fertility) in cattle (Schrooten et al 2004).

Experimental generation of transgenic cows via somatic NT have paved the way to increase the availability of transgenes for use in livestock breeding. *In vitro* transfection of intended nuclear donors, followed by transgene integration screening and further transfer to enucleated oocytes accelerates the production rates of transgenic embryos, thus warranting 100% of transgenic offspring (Niemann & Cues 2003, Wheeler 2007). Obtaining specific gene-carriers (eg carrying high prolificity genes) or transgenic individuals multiplied by somatic cloning increases the availability of populations that can be incorporated into breeding nuclei for commercial purposes. Generation of loss-of-function transgenic livestock can be reality by combining gene

targeting in somatic cells and their use in nuclear transfer, a combination of techniques used for the production of calves since few years ago. A similar bright future is foreseen when the use of established bovine stem cell lines, particularly those of induced pluripotent stem cells (iPSCs) would be widely used (Yu et al 2007) . However, until the problems of survival of offspring are solved or largely ameliorated, a combination of recent advancements in reproductive technologies with the tools available in molecular biology and informatics shall not be accepted by the public, no matter how positive officers and their Agencies would be. The welfare of eventual transgenic animals must be secured and a level of Zero-tolerance should be present when considering eventual animal suffering due to manipulations of the animal genome.

Can reduced fertility be ameliorated by use of ARTs?

Since ARTs require to be applied on healthy individuals (particularly reproductively healthy), it is difficult to see how reduced fertility can be ameliorated beyond the treated individual per se. Poor animal health status, malnutrition and mis-management lie behind a reduction of cattle fertility throughout the world. Increases in milk yield associated to improved breeding by use of AI or ET, which is not accompanied by improved health and proper nutrition can be realised at the expense of reduced fertility in dairy cows. Periods of stress due to inadequate nutrition or high milk yield reduce the intensity of oestrous signs by affecting the endocrinology of behaviour and ovarian function and jeopardize the outcome of AI or ET (Rodriguez-Martinez et al 2008). An "easy alternative" has been the use of "hormone treatment remedies" a practice overruled owing to consumer concerns and their low effectiveness when trying to establish MOET (Santos et al 2004, Tenhagen et al 2004, 2005). Undernutrition at the time of AI or during early pregnancy leads to repeat breeding, low fetal weight and later adverse health, and can not be remedied by using the best possible semen for AI or the use of the best strategy for MOET (Sheldon & Dobson 2003, Chebel et al 2004). The challenge for any ART to attain widespread use in either developed or, particularly, in developing countries is to match AI, i.e. being simple, economical and successful. Such development is far from visible.

Acknowledgments

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Conflicts of interest

Nothing to declare.

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