ADVANCES IN REPRODUCTIVE TECHNOLOGIES IN CATTLE: FROM ARTIFICIAL INSEMINATION TO CLONING

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ABSTRACT

The urge for the control of reproductive processes in animals has propelled a great gain in knowledge, also setting off the development of four generations of assisted reproductive technologies (ART) for humans and animals. The use of assisted reproductive techniques has been of great importance in livestock production. In general terms, the main first three generations of ARTs, including 1) artificial insemination (AI) and gamete and embryo freezing, 2) multiple ovulation and embryo transfer (MOET) and 3) *in vitro* fertilization (IVF) procedures, have matured into successful commercial applications, facilitating the increase in production through genetics, the reduction in generation intervals, the control of diseases, and the cutback in production costs. The fourth generation of ART encompasses processes that are still more experimental, comprising cloning by nuclear transfer (NT) of embryonic or somatic cells, transgenesis, and stem cell biology. Such technologies are intertwined with one another and with currently available molecular tools, being completely dependent upon the previous generations of technologies. However, many reproductive challenges still hinder maximal livestock reproductive performance, affecting productivity and profitability. It is clear that the application of such technologies as lucrative activities will remain questionable if not associated with other components of animal production, such as animal health, nutrition and adequate animal husbandry practices.

Key words: Reproductive Technologies, artificial insemination, embryo transfer, cloning, IVF

AVANCES EN BIOTECNOLOGÍA REPRODUCTIVA EN BOVINOS: DE LA INSEMINACIÓN ARTIFICIAL A LA CLONACIÓN

RESUMEN

El afán por controlar los procesos reproductivos en animales ha llevado a una gran ganancia en conocimiento, impulsando el desarrollo de cuatro tecnologías reproductivas asistidas (ARTs) para animales y humanos. El uso de ARTs ha sido de gran importancia en la producción ganadera. En términos generales, las tres principales generaciones de ART, incluyendo 1) inseminación artificial (AI) y congelación de gametos y embriones, 2) superovulación y transferencia de embriones (MOET) y 3) procedimientos de fertilización *in Vitro,* han madurado en aplicaciones comerciales exitosas, facilitando el incremento en la producción a través de la genética, reducción del intervalo generacional, control de enfermedades, y reducción de costos de producción. La cuarta generación de ARTs incluye procesos que aún son muy experimentales, como transferencia de núcleos (NT) de células somáticas, trans-

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génesis, y biología de células madre. Estas tecnologías se intercalan las unas con las otras y con las herramientas moleculares actuales, dependen completamente de las generaciones de tecnologías previas. Sin embargo, hay muchos retos reproductivos que no permiten alcanzar el potencial reproductivo máximo, afectando la productividad y la rentabilidad. Es claro que la aplicación de tales tecnologías como actividades lucrativas se mantendrán cuestionadas si no se asocian a otros componentes de la producción pecuaria, como la salud animal, nutrición, y prácticas de manejo adecuadas.

Palabras clave: Biotecnología de la reproducción, inseminación artificial, transferencia de embriones, clonación, IVF

INTRODUCTION

The biological and technological advances observed during the past six decades have spawned the development of four generations of assisted reproductive technologies (ART). Historically, aside from the mere scientific curiosity, the emergence and development of reproductive technologies have been driven by the economical gain offered by the potential increase in the number of offspring from genetically superior animals or simply to safeguard the genetic pool of infertile or subfertile animals. In other words, reproductive technologies were developed to offer possibilities for wider use of superior germplasm (1). For one to take full advantage of the benefits of assisted reproductive technologies there is a need for the support of long-term and basic research for the understanding of the complex mechanisms that underlie the physiology of the female and male reproductive systems and their reproductive cycles. As novel findings emerge, new perspectives and applications are proposed, tested, refined and, finally, put in practice. However, it is not possible, for example, to separate reproductive and genetic biotechnologies for a successful genetic manipulation program. Consequently, application of biotechnologies to livestock usually falls within four categories: a) management or husbandry, b) herd health, c) nutrition and growth, and (d) reproduction and genetics. In this view, genetic improvement is seldom introduced without other improvements in aspects such as animal management, disease control, nutrition, and reproduction. Therefore, the benefits of ART and genetic improvement can only be expressed if other aspects of livestock management are improved, including the producers' education, in which any implementation of reproductive biotechnology and genetics should be part of a broader program to improve health and nutrition.

The most consolidated reproductive techniques that have been genetically relevant in the past half a century take part of the first three ART generations, including artificial insemination, cryopreservation of gametes or embryos, induction of multiple ovulations, ultrasonography, embryo transfer and *in vitro* fertilization. Third and fourth generation technologies such as sexed semen or embryos, cloning, transgenesis, stem cell biology and molecular diagnosis have the potential to enhance the influence of superior animals on production, but their commercial applications have been limited. Furthermore, the use of genomics, proteomics, metabolomics and bioinformatics in the study of reproduction will allow a greater understanding of the limitations to efficient reproductive processes. Some relevant aspects of such technologies are discussed below.

FIRST GENERATION OF REPRODUCTIVE TECHNOLOGIES

Artificial Insemination (AI). Artificial insemination is the first generation of

ART, which has been in use for more than 200 years. As a modern technology, AI with fresh or frozen semen has been the most successful and efficient reproductive technology in animal production for the last six decades. The use of AI had a major impact on genetic improvement programs in developed countries, associated with 1,0 to 1,5% annual rates of genetic gains in dairy cattle (2). Through the prospective genetic gain attained by using AI, it is estimated that approximately 50% of the increase in milk production efficiency observed in developed countries during the second half of the 20th century can be attributed only to the genetic gain obtained by the widespread use of AI over conventional breeding, with the other 50% corresponds to significant advancements in the production systems *per se*, including herd health, general management, and nutrition (3). The use of AI has boosted the development of efficient programs involving synchronization of estrus or even ovulation, without requiring heat detection. With the advent of commercially available prostaglandin $F_{2\alpha}$ and its analogues in the 1970s, estrous synchronization systems were developed to assist producers to incorporate AI into their operations by reducing time and labor associated with estrus detection. Recently, with better understanding of endocrine profiles of females throughout the estrous cycle, economical and efficient systems have been developed for the synchronization of ovulation, which allows producers to AI animals at a predetermined fixed-time, eliminating estrus detection. These fixed-time AI systems work well for the synchronization of estrus/ovulation, and also for the induction of cyclicity in anestrous animals. In addition, programs can be scheduled in advance for the AI at the most appropriate period, under each specific circumstance.

Cryopreservation of Gametes and Embryos. It was the attainment of successful protocols for semen cryopreservation that made AI thrive as an accessible reproductive technology that allowed the widespread use of genetically superior sires (3). Frozen semen boosted the dairy industry, for making AI simpler, economical, and successful, with more than 60 percent of dairy cows in the USA bred by AI. Conversely, due to the typical extensive production system, AI accounts for less than 5 percent of inseminations in beef cattle.

Similar to AI, embryo cryopreservation allowed the global commercialization of animals of high genetic merit, as embryos. Embryo freezing has been a successful procedure in cattle for almost three decades and it became of routine use in the field. However, *in vitro*-produced (IVP) bovine embryos are more sensitive to cryopreservation than their *in vivo*-derived counterparts (4). Many efforts have been focused on the adjustment of cryopreservation methods to special requirements of IVP embryos, with vitrification procedures appearing as a promising approach for the cryopreservation of IVP bovine embryos than any other freezing methods (5, 6). The open-pulled straw (OPS) technology has been proven successful not only for the vitrification of bovine oocytes and IVP embryos but also to be combined with the in-straw warming and cryoprotectant dilution method for direct embryo transfer (6).

SECOND GENERATION OF REPRODUCTIVE TECHNOLOGIES

Multiple Ovulation and Embryo Transfer (MOET). Embryo transfer (ET), initiated about four decades ago, is a more advanced reproductive biotechnology that also takes advantage of AI procedures, being consid-

ered the second generation of ART. The progress achieved during the past 25 years has positioned commercial bovine embryo transfer as a large international business (7, 8). Contributing factors that significantly added for the increase in worldwide marketability of embryos were the development of successful cryopreservation and the use of washing procedures to obtain specified pathogen-free embryos (7). In 2005, approximately 130 thousand bovine females were flushed, for more than 600 thousand bovine embryos being transferred, representing a 10% worldwide increase over the previous year, with North and South America and Asia accounting for 45, 21, and 19% of the total worldwide activity, respectively (9).

The combination of multiple ovulation and embryo transfer (MOET) represents for the female what the AI has been for the male, allowing the production of multiple progeny from genetically superior females. However, ET and AI can be very useful, provided that good production practices (husbandry, nutrition, and management) are in place. One of the limiting factors associated with MOET technology is the variability and lack of predictability in follicular development response and embryo production following a superovulatory treatment (7). The real causes for such problems are likely to be associated with changes in the endocrine profiles of donor females by exogenous hormone treatments (7). In reality, little progress was attained, as the average number of transferable embryos per donor and the side effects on the reproductive performance of the donors remain unchanged in the past two decades (9, 10). As for AI, the use of MOET schemes forced the development of estrus or ovulation synchronization protocols that have facilitated and shortened considerably the whole process. Fixed-time ET and direct ET of frozen embryos are satellite procedures currently in broad use worldwide. However, MOET programs are expensive, mostly due to the cost of labor and hormone treatments. For those reasons, MOET will probably continue to be more intensively used by elite cattle producers.

Ultrasonography. Ultrasonography is among the most important image techniques for a wide number of applications in reproduction in cattle, with interests ranging from scientific to commercial purposes. Since the mid-1980s, many reports have established the accuracy of ultrasonography as a tool for the study of reproductive processes in cattle (11, 12, 13). This noninvasive technology allows the study of the same specimens for the course of gestation without compromising the viability of the conceptus. Using ultrasonography, the phenomenon of follicular dynamics was discovered and well described in cattle, which has allowed a tremendous advance in our ability to understand and manipulate the estrous cycle and, consequently, fertility. Ultrasonography can exceed rectal palpation under field conditions for the assessment, characterization and enhancement of the bovine reproductive status of both the individual animals and the herd as a whole. This method has been effectively used for the a) assessment of ovarian activity and structures; b) diagnosis of pathologies of the genital tract in males and females; c) detection of abnormal pregnancies; d) determination of embryonic and fetal viability; e) identification of twins; f) prediction of fetal gender and; g) for the early diagnosis of pregnancy and embryonic/fetal losses, among other uses (13). Ultrasonography is an early and accurate diagnostic tool used to improve the reproductive efficiency of herds and to enhance bovine reproductive management. Low conception rates and embryonic mortality are common problem in dairy herds, particularly when managed intensively. For the optimal re-utilization of such animals,

the ultrasound scanning can assist with the characterization of the reproductive status of each individual. In that way, the most efficient treatment or management decision can be made in order to reduce the open interval by early re-breeding.

THIRD GENERATION OF REPRODUCTIVE TECHNOLOGIES

The third generation of ART includes gamete and embryo sexing, oocyte recovery and *in vitro* fertilization (IVF). Additional procedures have also evolved, such as gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), and intracytoplasmic sperm injection (ICSI), but still with limited practical applications.

***In vitro* Fertilization (IVF).** The *in vitro* production (IVP) technology evolved from the interest in developing a system to produce embryos completely in the laboratory. The achievements attained by this technology are truly remarkable if one considers that, in the early days, embryos were usually produced using *in vivo*-matured oocytes and, after IVF, zygotes were transiently *in vivo*-cultured into the oviducts of surrogate females (sheep or rabbit) before the definitive embryo transfer. The subsequent development of complete IVP systems not only facilitated the process but also paved the way to studies that resulted in tremendous technological advances and novel knowledge in many related areas. The *in vitro* production (IVP) of embryos from IVF procedures for bovine embryos consists of three steps: first, *in vitro* maturation (IVM) of primary, germinal vesicle-stage oocytes collected directly from the ovaries of donor females; second, IVF by combining *in vitro* matured oocytes with *in vitro* capacitated sperm cells; and third, *in vitro* culture (IVC) of presumptive zygotes to stages of development that allow them to be transferred to female recipients. Today, the IVP of bovine embryos became routinely used for scientific, conservation, and/or commercial purposes. The IVP technology has shown a remarkable increase in efficiency over the years, both qualitatively and quantitatively, which has been manifested by ascending pregnancy rates obtained after the transfer of embryos to host recipients. Many advantages have been identified for the use of this technology over conventional systems for the production of embryos, with cost of production being one of the most important.

Ovum pick up (OPU). The *in vivo* aspiration of antral follicles (ovum pick up) is a nonsurgical technique adapted from the human in late 1980s that uses an ultrasound-guided needle to aspirate immature oocytes from the ovaries of females of any age (14). Once the immature oocytes have been removed from the ovary, they are submitted to IVP procedures. The application of OPU/IVP has progressed from treating infertile high genetic MOET cows in commercial situations to enhancing breeding scheme designs. Also, the use of prepuberal females for OPU/IVP may increase the genetic gain in up to 22% (2). Potentially, via OPU, a donor European or zebu cow may yield 15-20 oocytes each week (collection of 5-10 twice a week or 15-20 oocytes once a week, respectively). Considering the usual rates of development and losses obtained after IVP and ET, a cow may potentially produce 50 to 100 calves each year. Not surprisingly, a rapid expansion in the commercial application of OPU procedures coupled with the IVP technologies was seen in the past decade. Such phenomenon can be clearly seen in Brazil, where approximately 130,000 IVF-derived embryos, mostly from zebu breeds, were reported as transferred in 2005, accounting for nearly 50% of all

worldwide IVP activity, and surpassing by far the total number of *in vivo*-derived bovine embryos transferred during that same period in that country (9).

Sex Determination of Sperm and Embryos. Naturally, the beef industry prefers male calves for theirs higher body weights and feed efficiency, whereas the dairy industry prefers heifer calves for milk production. Thus, methods to determine the sex of sperm or embryos were developed to control the sex of the livestock's offspring. As the X-bearing sperm in cattle contains about 3,8 percent more DNA than the Y-bearing sperm, sperm cells can be segregated based on their DNA content using a specific dye that binds to DNA and a flow cytometer/ cell sorter. Several companies market sexed semen through that process. Although the process to sort the X and Y bearing sperm is slow, with approximately 10 million live sperm of each sex obtained per hour, which is about the number of live sperm required for one dose of frozen semen for AI, this procedure determines the sex with higher than 95 percent accuracy. Nonetheless, the use of sexed semen technology is associated with a reduced number of sperm that could be separated in a specified time period, lower survival of sorted sperm after cryopreservation, and lower fertility (8). Therefore, producers contemplating the use of sexed semen should understand that the product will be more expensive than conventional semen and fertility may be compromised.

Embryo sexing using molecular tools attained a significant interest in early 1990s, but has lost importance in the past few years. Polymerase chain reaction technology is currently being used for sexing embryos on a small scale. It is likely that this technology will be replaced by sexed semen. However, its importance will be shifted for its use as embryo diagnostics for genetic traits and diseases (7).

FOURTH GENERATION OF REPRODUCTIVE TECHNOLOGIES

The fourth generation of assisted reproductive technologies is now on use and entails embryo cloning, transgenesis, stem cell biology, also including molecular tools that may assist in selection and understanding of physiological processes to increase fertility.

Cloning by Nuclear Transfer (NT). The first livestock animal (sheep) was cloned in 1986 using cells from early embryos (15). Then, the birth of Dolly in July 1996 by transfer of a somatic-cell nucleus of an adult (16) represented the fall of an important biological dogma, i.e., that differentiated somatic cells could not be reprogrammed to a toti- or pluripotent state that would allow development of a new individual. Subsequently, cloning by NT from adult somatic cells, or somatic cell nuclear transfer (SCNT), was repeated and confirmed in an increasing number of animal species. Even if still relatively inefficient, cloning by SCNT, along with IVF, has also contributed to advances and generated great interest in many related field

Somatic-cell cloning may be accomplished for reproductive purposes, i.e., to produce a genetically identical copy of the individual that supplied the donor cell, or for therapeutic purposes, i.e., to produce cells or tissue for transplantation back to the individual that supplied the donor cell. Somatic-cell cloning is a rapidly developing area and a valuable technique to copy superior genotypes and to produce or copy transgenic animals. The promises behind SCNT technology include the possibility to duplicate a number of copies of animals using somatic cells and the feasibility of

cloning a valuable animal. However, the efficiency of the cloning technology remains low. Along with IVF procedures, SCNT is associated with increased rates of pregnancy losses, placental and fetal alterations, dystocia and birth of large calves with lower postnatal survival. A relatively low number of cloned embryos survive to term (1 to 5%), with approximately a third of cloned calves not surviving to weaning, and more than 8% dying before reaching 4 years of age (17). Clearly, more research is needed for the understanding of the process involving the failures in pre- and postnatal development.

In the USA, the Food and Drug Administration (FDA) recently considered milk and meat from cloned animals safe for human consumption, which will boost the use of such reproductive technology in that country. Most likely, other countries will follow, causing the same phenomenon. Despite that, many breed associations may not approve the registration of cloned cattle.

Stem Cell Biology: Embryonic Stem (ES) cells and Embryonic Germ (EG) cells. Embryonic stem cells are characterized by their self-renewal capacity for indefinite proliferation *in vitro* in an undifferentiated, pluripotent state. Embryonic stem cells possess the *in vitro* and *in vivo* capacity to differentiate into any specialized cell type, from *in vitro* formation of embryoid bodies to *in vivo* differentiation into somatic and germ cell lineages (18). Since the first isolation of mouse ES cells (19, 20), they have been extensively used both as a model for the study of cell lineage and regulation of gene expression during mammalian embryogenesis and as a vehicle for genetic engineering, functional genomics, and cell-therapy studies. Primordial germ cells are embryonic cells that migrate from extra-embryonic origins to the sexually undifferentiated gonad and ultimately give rise to gametes. Culture of PGC can yield pluripotent EG cells that display characteristics similar to ES cells, but much less research has been conducted with PGC culture and EG cells compared with research involving ES cells (18).

Despite potential uses for ES and EG cells in biomedical research and application, documented isolation of ES and EG cells having proven *in vivo* developmental capacity in the homologous species (i.e., chimera production) is limited to the laboratory mouse, the rabbit and the pig. Since the pigs share many anatomical and physiological features with humans, the isolation of porcine ES cells from nuclear-transfer cloned blastocysts offers an enormous opportunity to create an animal model for therapeutic cloning and resolve a critical issue in most cell and tissue transplants, i.e., graft rejection by the host. Once created, such animal models could be used in clinical trials following therapeutic cloning from wild-type stem cells of the same origin.

Transgenic Animals. A transgenic animal is defined by having a genetic material from another species added to its genome. Genetic engineering allows us to bypass species restraints altogether to produce genetically modified animals with beneficial traits and transmission to future generations in a Mendelian fashion (21). Transgenic mice, pigs, goats, sheep and cattle were produced by the pronuclear microinjection of a foreign gene. Nevertheless, the efficiency of such procedures is still very low, i.e., in general, less than 5% carries the transgene (22). Alternatively, cloning by SCNT offers the possibility of genetic manipulations of somatic cells in culture prior to cloning, rendering offspring that are transgene carriers. This technology has been proven effective for the production of transgenic animals by random integration of the foreign DNA into cells (23, 24). With further modifications, gene insertions and deletions are possible

through gene targeting, albeit still technically challenging and rather inefficient.

At present, no transgenic animal was incorporated into breeding programs. However, the use of transgenic animals to produce therapeutic proteins in the milk has been heavily invested and the shortage of human cells, tissues and organs and therapeutic proteins has increased the pressure for the creation of transgenic herds. Another application for genetically modified animals has been directed at engineering animals to render their organs immunologically compatible for human transplantation (xenotransplantation), with the pig being currently the species of choice for this research (25).

The fact that the generation of transgenic livestock is expensive and inefficient, joined by the reality that most agriculturally relevant traits are complex and controlled by more than one gene, has limited the use and application of transgenic technology. The sequencing of the bovine genome, in conjunction with new methods for modifying the genome, is encouraging a resurgence of research using transgenic livestock.

Marker-assisted selection (MAS). Superior phenotypes have been the focus for selective breeding in livestock improvement for centuries, which has proven to be very successful. However, in recent years, information on the organization and functioning of genomes of livestock are becoming available, which can be used in breeding programs to improve certain traits. Many traits are controlled by several loci, where each can contribute to the variation in the trait and hence are called quantitative trait loci (QTL). These molecular markers are used to identify loci or chromosomal regions that affect single gene traits and also QTLs. The idea behind this is to use these genetic markers rather than the phenotypes in a process called marker-assisted selection, or MAS (26). A substantial number of genetic markers are available to study the genetic structure of traits and its use in MAS. By genotyping, accurate detection of specific DNA variations that have been associated with measurable effects on complex traits can be made. By this way, combining phenotypes with genetic markers seems to be a promising approach for improving health and welfare traits in farm animals. These traits are often difficult to define since they bear low heritabilities and a corresponding lack of genetic gain in conventional selection and breeding programs (27). Several QTLs have been identified for farm animals and MAS is currently been used in commercial livestock breeding. As an example, markers associated with marbling and tenderness in cattle are currently available in the market. It is anticipated that MAS schemes will eventually be more widely implemented (14).

CONCLUSIONS AND FINAL CONSIDERATIONS

The control of reproductive processes in animals offers numerous advantages, serving as an essential instrument for the application of biotechnology to livestock production. The use of farm animals and their products have significantly contributed to the quality of human health and for the establishment of civilization. It is within this scope that the first three generations of ART have contributed tremendously to the satisfaction of the increasing demands of the modern society (28). Many producers are now familiar with more advanced methods to enhance reproductive efficiency, which may further add potential economic efficiency to cattle operations. Artificial insemination, estrous synchronization, embryo transfer, *in vitro* fertilization, sexed-semen, and cloning are all procedures that have already influenced or will further in-

fluence livestock industry in the near future. With the development of the forth generation of ART, the potential of farm animals for improving human health is growing and many areas remain to be explored and, when associated with modern molecular tools, will further advance progress in animal breeding and genetics (28). In this regard, the use of molecular markers will favor an even faster pace in our ability to manipulate animals genetically, either through breeding or by means of genetic engineering. Still, as genome resource banking continues to arise, the most applied reproductive technology remains artificial insemination (29). In this reality, reproductive biotechnology cannot be dealt as the solution for poor management. Indeed, genetic advancement can only be attained when good practices in livestock management are improved. To be successful, the application of biotechnologies must include good practices in animal husbandry, animal health and nutrition, and reproduction.

In addition to the focus on economical growth of individual groups, and as there are negative and positive impacts of reproductive technologies on animal genetic resources, reproductive technologies should also be more intensively used for other noble purposes. The use of reproductive technologies to introduce foreign or exotic genetic material by AI or ET, either for crossing with local breeds or as purebreds, has created negative impacts in certain regions of the world. Not surprisingly, with few mammalian species being well studied, the limited resources and the complexity of management and conservation of endangered species, along with differences observed between species and insufficient knowledge on basic reproduction, a delay in development in reproductive technologies for non-domestic species is expected (29, 30).

Advances in animal reproductive technology promise new possibilities, but many ethical challenges have emerged with the development of the fourth generation technologies, including therapeutic and reproductive cloning, stem cell biology, cell and gene therapy and transgenesis. The religious, ethical and political turmoil caused by these reproductive technologies is important and absolutely necessary, and should serve as an alert for responsibility and accountability to professionals in the field and related areas. Since reproductive technologies can benefit mankind, any emerging new development should be extensively and fully tested in laboratory and domestic animals before any attempts are made to make them available for humans. Consequently, more restraint and thoughtful consideration of each technological advance should be made before its application to human medicine (31).

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Recibido 11-09-2007 y aprobado 7-09-2009