

Assisted Reproductive Techniques in Farm Animal - From Artificial Insemination to Nanobiotechnology

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Abstract

It has become evident that advances in farm animal reproduction have become increasingly dependent on advance scientific research in addition to an understanding of the physiological processes involved in reproduction. The use of assisted reproductive techniques (ART) has helped owners to produce offspring from valuable farm animals that were considered infertile using standard breeding techniques. This chapter constitutes an update of recent developments in the field of assisted reproduction includes Artificial insemination, Embryo transfer, in vitro fertilization, embryo cryopreservation, Sexing of semen and embryos, cloning, transgenesis, stem cell technology, embryo genomics, micro and nanotechnology has been included. Recently in some of these fields remarkable progress has been made. None the less, imperfections are remaining and sustained efforts will be required to optimize existing and invent new technologies. Before referring an animal for an ART, the practitioner should be able to identify the underlying cause of subfertility of that animal. Knowing the complexity as well as the risks of these techniques, enables practitioners to refer a sub-fertile animal to the least complex and most appropriate and successful ART that can overcome specific causes of infertility.

KeyWords: Reproductive Techniques, Artificial Insemination, Nanobiotechnology, Embryo Transfer, Cryopreservation, Cloning, Transgenesis, Stem Cell Technology, Embryo Genomics.

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Introduction

Productivity is the key to growth and reproduction is backbone of animal production. Reproductive inefficiency is one of the most important causes of economic losses in animal industries and it is realized throughout the world. Despite of remarkable advancement that has been made in the field of reproductive physiology in recent years, infertility due to low conception rate and high embryonic mortality rate remains a major problem. To meet future needs and to be able to sustain agricultural production, agricultural research and its applications there is a need to use all emerging technologies especially the modern reproductive biotechnologies. Development of reproductive techniques like, estrus synchronization, super ovulation, non-surgical embryo collection, transfer, cryopreservation of embryos, oocytes pick-up from live animals, in vitro maturation, fertilization, embryo development and cloning could not make an impact on quality animal production due to non

availability of low cost embryos from quality animals. There is a scope for further improvement in reproductive status of our farm animals. The recent scientific developments in assisted reproductive techniques have made it possible to manipulate the reproductive processes in many ways to revolutionize world animal agriculture. In present review effort has been made to enrich the knowledge about various recent assisted reproductive techniques which may be helpful for improving the current status of livestock reproduction.

Artificial insemination (AI)

This technology has now become a practical technology in commercial dairy cattle programs in both developed and developmental countries. On historical point of view first successful insemination was performed by Spallanzani, (1784) in a bitch. Pioneering efforts to AI were begun in Russia in 1899 by Ivanow (Ivanoff, 1922). Ivanoff, (1922) had studied AI in domestic farm animals, dogs, foxes, rabbits, and poultry. Later on this technique was

performed by various researchers worldwide in different species. Use of frozen semen (Polge *et al.*, 1949) revolutionized the AI program through worldwide transport of semen. Initially artificial insemination (A.I.) was used to spread improved indigenous breeds which were followed by the introduction of crossbreeding. AI technology maximizes the use of outstanding males, dissemination of superior genetic material, improve the rate and efficiency of genetic selection, introduction of new genetic material by import of semen rather than live animals and thus, reducing the international transport costs, enabling the use of frozen semen even after the donor is dead; and, reduces the risk of spreading sexually transmitted diseases. Mostly AI is performed by utilizing the semen from exotic breeds and used in the expectation of increasing the production of local livestock populations. To a lesser extent, semen from local breeds is also used for this purpose. Various aspects of AI technology now a day have been fairly standardized.

In present scenario a large number of AIs are performed globally, more than 100 million cattle, 40 million pigs, 3.3 million sheep and 0.5 million goats are artificially inseminated every year (Boa-Amponsem and Minozzi, 2006). Regarding to current status of AI in India, the production of frozen semen straw was 44 million and 41 million AI with 35% conception rate was achieved in year 2008 (Annual report, 2007-2008).

The conception rate in field AI programmes in developing countries is very low, and therefore the desired effect in terms of animal improvement has not been achieved. The reasons behind are lack of proper management and technical skill. AI will become more effective only when farmers will have access to considerably better technical and organisational facilities.

IVM/IVF embryo production

The potential for commercial production of genetically superior embryos by *in vitro* fertilization is apparent. In Past few decades there have been unprecedented evolution of technology for *in vitro* embryo production (IVEP) of embryos in farm animals, with the rate of progress getting intensified in the last decade with the characterization of effectively defined and semi defined medium for different species.

In vitro production technologies not only help in production of high genetic merit animals, but also provide an excellent source of embryos for emerging biotechnologies like embryo sexing, cloning, nuclear

transfer, transgenesis etc. Furthermore, it allows analysing developmental potential of embryos, including the pattern of gene expression, epigenetic modifications and cytogenetic disorders during the development (Galli and Lazzari, 2008). Early stages of bovine embryo development show many similarities with human embryos. Therefore, bovine embryos are used as a model organism (Niemann and Wrenzycki, 2000).

Inspite of continuous efforts to improve bovine *in vitro* embryo production (IVP), its efficiency is still low, since only 30 to 40% blastocyst development has been obtained from oocytes after *in vitro* maturation, fertilization and embryo culture (Sirad *et al.*, 2006). Despite of several advantages of *in vitro* embryo production, initial application in both cattle and buffaloes has been limited by the ability to recover oocytes. However, recent development of low invasive ultra sound guided transvaginal oocyte retrieval (TVOR) and oocyte pick up (OPU) has removed these difficulties to a large extent. Through OPU oocytes may be aspirated from live animals. Now OPU-IVEP is used in many countries for the large scale embryo production at commercial level. This repeated recovery permits production of more embryos than might be possible by standard ET practice (Galli and Lazzari, 2008). TVOR also allows repeated collection of oocytes from endangered species of livestock or livestock of high economic importance in order to propagate such genetic resources in much faster way. However, the practical use of IVEP is limited by high production costs and the low overall efficiency under field conditions.

Intra cytoplasmic sperm injection (ICSI)

Development of Intracytoplasmic sperm injection technique furnished a successful treatment for male infertility of different origin and has led to a resurgence of interest in its potential use in farm animal reproduction. In addition to its clinical usefulness, ICSI can be applied for the production of transgenic animal and to study the mechanism of fertilization.

Since the first report of ICSI success in hamster (Uehera and Yanagimachi, 1976) the transfer of embryo produced by ICSI has given rise to live young in rabbits, mice, sheep, humans, horses, cattle and pigs. ICSI is the micro-fertilization technique of the direct injection of a single spermatozoa or sperm head into ooplasm. This technique can further be used to extend the sperm vector system for transgenic animal production. The main indication for ICSI is severe

male infertility due to various abnormalities of ejaculated spermatozoa, epididymal spermatozoa or testicular spermatozoa. ICSI has been performed in domestic species with variable success rate like 70-80% in cattle (Horiuchi *et al.*, 2002), 77% in pig (Martin, 2000), and 48-63% in small ruminants (Catt *et al.*, 1996). In horses, IVF proved to be very complicated and problems-bearing procedure. Thus, intracytoplasmic sperm injection (ICSI) technique is preferably used for this species (Colleoni *et al.* 2007). Although fertilization rates with ICSI may be higher than IVF, pregnancy rates were poor (< 20%) as per various studies and no more effective in terms of clinical pregnancy rates than in vitro fertilization (IVF).

Sexing of semen and embryos

Predetermination of the sex of offspring would provide a greater number of males or females, which will help in selection of individuals with top genetic makeup for improvement in next generation (Plummer and Beckett, 2006). Known sex of embryos produced for use in ET programs can more effectively help to manage producer resources because more heifer calves per ET can be produced. The sexual differentiation of embryo is determined by the presence or absence of elements normally located on the Y chromosome. Some of the techniques employed commercially for the embryo sexing are: i) chromosomal analysis of demi embryos ii) immunological detection of embryonic H- Y antigen iii) use of Y-specific probes iv) Fluorescence in situ hybridization (iv) rapid sexing method for pre-implantation embryos of bovine using Loop-Mediated Isothermal Amplification (LAMP) reaction (Zoheir and Allam, 2010). Another way is the sorting of semen, one sperm at a time, into males and females, using staining procedure and detecting by laser beam with the help of standard flow cytometry equipment (Garner, 2006).

Thus, sperms with X or Y chromosome could be used to produce female or male embryos. The bovine Y-chromosome specific sequences are conserved amongst buffalo, Indian zebu and Taurus cattle (Apparao *et al.*, 1993). Thus, the use of bovine Y-chromosome specific primers, demonstrate the sex of buffalo or Indian zebu cattle embryos. Efficient embryo biopsy method has also been developed (Lopatarova *et al.*, 2008). It is a usual practice to extract one cell from an early embryonic stage and with the use of a DNA probe, embryos can be sexed. The high rate of survival and conception rates of

biopsied embryos also reflects the minimal damage of the embryos during the process. In respect to the recent advances in semen sexing, using fluorescence activated cell sorter (FACS) offspring of pre-determined sex have been successfully produced (Garner *et al.*, 2008) using fresh and frozen-thawed spermatozoa in several mammalian species: cattle (Seidel *et al.*, 1999), goat (Parrilla *et al.*, 2004), pigs (Grossfeld *et al.*, 2005) sheep (de Graaf *et al.*, 2007).

Semen and embryo sexing have not been reported in the field in any of the developing countries, except China. At a research level, these technologies are being developed and refined in a number of research institutions in developing countries. The involvement of private companies providing these services is likely to increase their accessibility in developing countries where AI is already established.

Oocyte/embryo cryopreservation

Continuous availability of viable, developmentally competent oocytes has been critical to recent progress in IVEP because of the relatively short fertile life span of mammalian oocytes. Hence, storage of unfertilized oocytes would generate a readily available source, which allow the experiments to be carried out at convenient time and could therefore be of practical importance in the establishment of gamete bank from which particular genetic combinations could be derived. During the past few decades, significant progress in cryopreservation of mammalian oocytes and embryos has been achieved. Live offspring of at least 25 species resulted from transfer of cryopreserved embryos or oocytes (Gajda and Smor¹g, 2009). Preservation of oocytes reduce the risk and expense involved in transport of live animals, hazards of disease transmission and also provides insurance against catastrophes and natural disasters. Preservation of oocytes in endangered species safeguards from danger of extinction. The major problem associated with cryopreservation of germplasm is mechanical as well as osmotic damage during processing steps. With the advent of glycerol as a cryoprotecting agent cryopreservation process became more feasible. Other CPs are also in widespread use, alone or in various combinations. These include permeating CPs such as ethylene glycol (EG) and propylene glycol (Chen *et al.*, 2005, Luz *et al.*, 2009) and non-permeating ones such as sucrose, glucose, or fructose (Barcelo-Fimbres & Seidel, 2007). With the development in traditional cryopreservation methods, vitrification of germplasm was introduced (Rall and Fahy, 1985). Vitrification is a simple, faster, less expensive technology than slow

freezing. Moreover, it was shown to be more effective than slow freezing for material more sensitive to chilling (Vajta *et al.*, 1996). Cryopreservation of oocytes by vitrification was attempted with variable success in bovine (Hochi *et al.*, 2000), swine (Huang and Holtz, 2002), equine (Hurt *et al.*, 2000) and buffalo (Sharma and Loganathasamy, 2006) oocytes. For vitrification various methods like EM grid, OPS, closed pulled straw, nylon mesh etc has been tried with more or less similar results.

Recently, high-security vitrification device (Camus *et al.* 2006), pipette tip (Sun *et al.*, 2008), fiber plug (Muthukumar *et al.* 2008), vitrification spatula (Tsang & Chow, 2009), Cryo-E (Petyim *et al.*, 2009), sealed pulled straw (Yavin *et al.*, 2009), plastic blade (Sugiyama *et al.*, 2010), Vitri-Inga (Almodin *et al.*, 2010), Cryopette (Portmann *et al.* 2010), and Rapid-i (Larman & Gardner 2010) has been introduced with more convenient and better results. Freezing of embryo is an established commercial practice and the major aim of it is to preserve the embryo in a viable condition from which it may be revived to continue a normal development. Embryo freezing is an essential component in the commercial ET programmes as there is an obvious need to store the embryos on temporary basis until they are transferred, as the embryo viability starts to decline after 12 h of storage in holding media. With regard to the embryo sexing, naturally, both male and female embryos should be stored to ensure representation of both sexes and wide genetic diversity. It is a useful conservation strategy for endangered breeds. For these species, cryobanking of embryos could be helpful in establishing founder populations with the aim of eventual reintroduction into the wild (Ptak *et al.*, 2002). Recent advancement in cryopreservation of mammalian oocytes and embryos has almost exclusively been achieved with the new vitrification techniques and may offer new perspectives to routine alternative for breeding programmes in most domestic animal species and commercial embryology.

Embryo transfer

Embryo transfer technology is an important tool to improve livestock at faster rate as well as gives an opportunity to utilize the genetic contribution of both male and female at the same time. With the help of ET (embryo transfer) or MOET (multiple ovulation embryo transfer) techniques (Nicholas and Smith, 1983) faster improve of livestock, rapid expansion of elite animals, genetic gain, accelerated herd development and conservation of rare genetic stocks could be achieved. Embryo transfer and other associated

reproductive technologies facilitate genetic improvement and have been successively used for rapidly multiplying the population of elite breeds of cattle (Thibier, 1996), buffalo (Madan *et al.*, 1993), sheep (Holm *et al.*, 1996), goat (Pawshe *et al.*, 1994), horse (Squires *et al.*, 1999) and pig (Hazeleger and Kemp, 1999). In 2002, approximately 539680 ETs were performed worldwide, mainly in dairy cattle, with 62% being transferred in North America and Europe, 16% in South America and 11% in Asia (Madan, 2005). Moreover, the latest report of the International Embryo Transfer Society Data Retrieval Committee, 800 000 embryos in cattle (Thibier, 2009), 25000 in sheep, 7000 in goat, 30000 in pig and 12000 in horses (Thibier, 2006) were transferred worldwide (two thirds as in vivo derived embryos and one third as in vitro produced) with 55-70% conception rate (Thibier, 2009).

Embryo genomics

The emerging array of new methods in biology such as differential display reverse transcription-Polymerase chain reaction (DDRT-PCR), subtractive cDNA libraries and quantitative real time Polymerase chain reaction (RT-PCR) will significantly extend the possibilities of revealing differences in mRNA expression patterns throughout the preimplantation development. The expression pattern of specific genes critically involved in preimplantation development will aid in selecting markers for determining quality embryo and acts as a useful tool to assess the normality of the embryos and a tool to optimize the reproductive technologies. Using qualitative and quantitative RT-PCR assays, expression pattern of several classes of genes have been studied in preimplantation stages of murine and bovine embryos (Niemann *et al.*, 2000) covering more than 32 physiological functions involving the expression of about 250 different genes in the murine embryos and about its physiological functions and expression of 60-70 genes in farm animal embryos (Wrenzycki *et al.*, 2005).

Cloning

Cloning is a powerful technique and potentially it could be used for multiplication of elite animals and minimize the genetic variation in experimental animals. It can be used for the conservation as well as propagation of endangered species. It may be used as a tool for the production of stem cells for therapeutic purposes, as therapeutic cloning. Cloning using somatic cells offers opportunities to select and multiply animals of specific merits (Das *et al.*, 2003). Numerous types

of somatic cells are used as donors in somatic cloning: foetal fibroblasts, adult fibroblasts, granulosa cells, hepatocytes, lymphocytes etc (Campbell *et al.* 2007). First animal obtained by somatic cloning was a sheep, “Dolly” (Willmut *et al.* 1997). Since then, SCNT was used successfully for cloning cattle (Cibelli *et al.*, 1998), pig (Polejaeva *et al.*, 2000), goat (Baguisi *et al.*, 1999) and horse (Galli *et al.*, 2003). Cloning procedure using embryonic stem cells (ESCs) is called Nuclear Transfer-derived Embryonic Stem Cell (NTESC). However, although ESCs were derived for humans and some laboratory animals, derivation of farm animal embryonic stem cells (faESCs) is still unsuccessful (Beyhan *et al.* 2007). The alternative to faESCs could be embryonic germ cells (EGCs) and spermatogonia stem cells (Brevini *et al.* 2008).

Cloning holds the promise of bypassing conventional breeding procedures to allow creation of thousands of precise duplicates of genetically engineered animals. In remote areas, where sampling and storage of adequate samples of semen and embryos is not practical, one could use clone samples from diverse animals for conservation of the available genetic diversity. The local breeds may contain valuable genes that confer adaptation, especially to heat tolerance or disease resistance, and there is an urgent need to prevent their extinction which can be achieved by cloning techniques. In the future, cloning may be used in xenotransplantation, as it would allow multiplication of humanized pigs, the organs of which could be transplanted to humans (Duszewska and Reklewski 2007).

In regards to the status of cloning technique, a survey conducted in 2005 by the OIE (MacKenzie, 2005), in which 91 countries participated (60 percent from developing countries), 4 percent of the respondents in Africa and 23 percent of the respondents in Asia, reported having cloning capabilities. Recently in the list of cloned animal, first cloned camel, “Injaz”, a female, (2009) and second cloned camel, Bin Soughan, a male, (2010) were born at the Camel Reproduction Center in Dubai, United Arab Emirates. Through introducing a new technique, “Hand guided Cloning Technique” world's first buffalo calf GARIMA-I (2009) was born at NDRI, Karnal (India). Later on by utilizing same technique, GARIMA-II (2009), and a male buffalo calf, Shresth (2010) have been produced in same institute.

Transgenesis

Since the initial demonstration in 1980s that a transgenic animal can be generated harboring a

transgene from a different species, genetic engineering has revolutionized all aspects of fundamental biological and biomedical research. Since then much has been accomplished in the generation of various types of first transgenic animals like mouse (Gurdon and Ruddle, 1981) pig (Hammer *et al.*, 1985) sheep (Simon *et al.*, 1998) goat (Ebert *et al.*, 1991) and cattle (Cibelli *et al.*, 1998). Several biotechnological techniques viz., pro-nuclear micro-injection, cytoplasmic micro-injection, retrovirus based vectors, transferring DNA to embryos or embryonic stem cells via retroviral vectors, sperm mediated gene transfer of lentivectors and RNA interference, are presently being used to produce transgenic animals.

Transgenic farm animals can be used both in breeding and biomedicine (Robl *et al.*, 2007; Wells, 2010). In breeding, transgenic individuals produced are equipped with disease resistance and improved quantitative and qualitative traits. For example transgenic cows producing milk of increased α -casein and β -casein content, pigs with high body weight gain or fat to muscle tissue, expressing human growth hormone and human haemoglobin (Niemann *et al.* 2005), sheep with integrated keratin-IGF-I gene and higher production of wool (Kues and Niemann 2004), sheep and goat with antitrombin III and α -antitrypsin in milk (Kues and Niemann 2004). An important achievement was production of transgenic cows resistant to *mastitis* (Wall *et al.* 2005). There is a high interest in using transgenic farm animals as bioreactors producing human recombinant proteins in mammary gland (Kues and Niemann, 2004; Redwan, 2009). Transgenic domestic pigs are used in studies on xenotransplants, *i.e.* transplantation of animal body parts into humans (Niemann *et al.*, 2005). Researches are going on for production of environment-friendly transgenic individuals or using such animals in basic studies as a model to understand various physiological processes in farm animals and humans (Niemann *et al.*, 2005).

Stem cell technology

Stem cells are cells found in all multicellular organisms. They retain the ability to self-renew through mitotic cell division and can differentiate into a diverse range of specialized cell types. Embryonic stem cells, cord blood cells, adult or somatic stem cells and spermatogonial stem cells are the major types used for various purposes. Stem cells are having various applications like, model for developmental biology, organ transplantation, gene therapy, drug development, chimera production and in the field of regenerative

medicines (Bajada *et al.*, 2008). Regarding its application in farm animals reproduction, embryonic stem cells are important to provide a method to introduce precise genetic modification into animals by homologous recombination of ES cells (Lombardo *et al.*, 2007) followed by blastocyst injection for chimera derivation and breeding, or by somatic cell nuclear transfer. A second important application is to provide large animal models in which the ES cell technology can be tested for tissue-specific differentiation (Brown *et al.*, 2007) and cell therapy of various tissues and organs. ES cell mediated gene transfer has some distinct advantage over other transgenic methods. First, the efficiency of producing transgenic animal could be significantly increased with this technology. Secondly, these cells can be transformed in vitro with foreign DNA (Robertson, 1991). This provides the opportunity to establish the stable incorporation of the desired gene before chimera production. For the production of chimera several lines of ES cells were obtained from (1) inner cell mass of blastocysts, (2) single blastomeres isolated from embryos at earlier stages of development, or even from (3) one-cell stage embryos (Hwang *et al.*, 2004, Klimanskaya *et al.*, 2006).

Spermatogonial stem cells (SSCs) transfer is commonly used in rodents to study the control of spermatogenesis with the ultimate goal to enhance or suppress the male infertility. The successful transplant of testicular tissue containing SSCs used in goat and pig is readily adapted in cattle (Honaramooz *et al.*, 2003, Joerg *et al.*, 2003). In cattle germ line transfer has the potential use in commercial breeding systems. By transplanting SSCs from elite bulls into lesser males followed by natural service, elite genetics could be disseminated more widely (Herrid *et al.*, 2006). This system could create an alternative to artificial insemination for the use in elite sires in the cattle industry in areas where AI is not practicable (Hill and Dobrinski, 2006). Herrid *et al.* (2006) demonstrated that male germ cell transplantation between the unrelated bull calves and between cattle breeds could also be successful. Current knowledge on the biology of stem cells is limited regarding to their potential applications in various research field and farm animal reproduction but researches are going on for betterment.

Nanotechnology

Nanotechnology is recent advancement in cellular and molecular biotechnology. It has an enormous potential to revolutionize agriculture and livestock sector. This technology allows researchers to

handle biological materials and media in minute quantities usually nanoliters or picoliters. In addition to its applications in cellular biology, biotechnology, therapeutic medicine and genetics, it might be useful technique in farm animal breeding and reproduction. Microfluidic and nanofluidic (Schuster *et al.*, 2003; Eijkel *et al.*, 2005) are recent tools to simplify traditional procedures of in vitro fertilization (IVF) and in vitro embryo production (Suh *et al.*, 2006). More recent reports have demonstrated the utility of microfluidics in isolation of motile sperm without centrifugation (Schuster *et al.*, 2003). Oocyte manipulation under in vitro condition can also become feasible with advent of this technique (Beebe *et al.*, 2002). Glasgow *et al.*, (2001) first established that manipulation and movement of an embryo in a microfluidic environment. It can also be used in sorting of sperm and eggs. These systems control the flow of liquids or gases through a series of micro and nanoscale channels and valves, which is assisted with computer circuit for data analysis. Nanotechnology has its application in genome mapping and sequencing furnishes a hope to identify gene sequences that relate to commercially valuable traits such as disease resistance and leanness of meat. By including probes for these traits on biochips, breeders will be able to speedily identify elite breeders and screen out genetic diseases. In farm animal breeding heat detection can be done by implanting a nanotube (O'Connell *et al.*, 2002) under the skin to detect the changes in the level of estradiol in the blood. The signal from this sensor will be incorporated as a part of a central monitoring and control system to actuate breeding.

Conclusion

By way of conclusion it may be stated that, after a hiatus following the introduction of artificial insemination during the 1980s, recently assisted reproduction in farm animals is receiving renewed attention. This growing interest is largely driven by the desire to breed transgenic founder animals as well as cloned animal providing substances suitable for the pharmaceutical industry. Embryo genomics are a valuable tool for studying responsible cause of various defects at cellular level (early embryonic death due to defective expression of genes etc). Now a days introduction of stem cells and Nanotechnology made acceleration to the field of assisted reproductive techniques create a hope for such animals which may contains all desired characters. In the long run, the results of these ongoing efforts will benefit the entire farm animal industry worldwide.

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