Assisted Reproductive Technology in Sheep  
(A review)  
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Summary

In the last 10-20 years, new biotechnologies have been developed that are on the verge of revolutionizing reproductive processes in humans and animals. In agriculture, modern techniques in assisted reproductive technology (ART) are being used for the introduction, improvement, and preservation of livestock genetics and the enhancement of animal reproductive efficiency. Modern embryology and ART technologies have facilitated the development of methods to transfer desired single genes or, alternatively, the entire genome from desirable individuals or embryos. In addition, rapid advances in techniques to manipulate embryos in the laboratory have permitted screening of embryos for genetic defects or highly desirable quantitative traits using molecular markers.

The ART techniques include artificial insemination, estrus synchronization, estrus induction, synchronization of parturition, superovulation, in vitro fertilization, in vivo and in vitro embryo production, embryo collection, embryo transfer, embryo cryopreservation, embryo splitting, cloning, production of transgenic animals, and preimplantation genetic diagnosis. The sheep is an excellent model for all listed techniques and has been used extensively in basic and applied research. This review describes application of ART in sheep production and research.

Key words: assisted reproduction, biotechnology, sheep

Introduction

Sheep are considered to be one of the first domesticated mammals and are known to have been closely associated with man from a very early date. Sheep offer the potential of making an important contribution to providing food and fiber for a growing world population. The fact that most ewes in the agriculturally productive countries are seasonal breeders and in general produce smaller lamb crops than farmers may wish, has made sheep an obvious target for the reproductive physiologist’s attention (Shelton, 1995).

Reproduction in sheep is seasonal, at least in breeds originating from temperate climates. Over the hundreds and even thousands of years, natural selection presumably has favored sheep that give birth at the most appropriate time of year in terms of food availability and climate. Natural selection has provided sheep with signaling systems which couple certain forms of environmental variation with the appropriate neuroendocrine responses to ensure that reproductive activity occurs at the most favorable time of year, depending on the length of gestation. Such neuroendocrine responses continue to operate in the sheep despite the selection practices by man over several thousands years of domestication (Gordon, 1997).

The development of commercially acceptable controlled reproduction techniques in sheep started in the 1950’s. At that time, the role of progesterone in facilitating the induction of coincident estrus and ovulation had been explained (Dutt, 1952; Robinson, 1952) and the highly potent progesterone analogues had been developed (Robinson, 1965).
Controlled reproduction in sheep covers several areas of reproduction, including:
- breeding sheep toward the end of the normal anestrous period (early-lamb production),
- breeding ewes to permit an extremely compact spring lambing period in small flocks,
- breeding ewes to top quality rams by artificial insemination,
- rapid build-up of stocks of certain breeds of sheep by embryo transfer,
- sperm collection and storage,
- estrus and parturition synchronization and induction,
- superovulation,
- in vivo and in vitro embryo production,
- embryo transfer and storage
- cloning and production of transgenic animals (Gordon 1997; Cognie et al., 2003).

For sheep producers, the technology for the control of reproduction, including control of estrus, fixed-time artificial insemination (AI), early pregnancy diagnosis and synchronization of lambing are now available and offers possibilities in allowing lamb production to be planned in a way which is not feasible under nature.

One of the goals of assisted reproduction is genetic improvement of sheep as a species. It leads to a substitution of genetically superior animals for those of a little genetic merit. Cross breeding has helped in increasing milk, meat and fiber production to a great extent, but in cross breeding the benefit has been primarily from male germ plasm, ignoring the potential of the untouched female (Ishwar and Memon, 1996). The procedure of superovulation followed by recovery of embryos and transfer to appropriately synchronized recipients has proven to be an effective means of increasing the contribution of superior females to the gene pool of the population.

Nearly all the technologies related to embryo production and manipulation in domestic ruminants, from artificially controlled breeding to the production of Dolly, the first cloned mammal, have been developed in sheep, before being transferred to its more economically important counterpart, the cow (Loi at al., 1998).

The first embryo transfer in sheep was reported by Warwick et al. (1934). When Hunter (1955) transferred 19, 2-16 cell embryos to 18 recipient ewes, eight lambs were born. Their procedure remains the basis for surgical embryo transfer in sheep today. The success of embryo transfer depends on management of donor and recipient animals, synchronization of estrus in donors and recipients, superovulation of donors, embryo collection and evaluation, transfer of embryos, and factors affecting survival of transferred embryos.

**Synchronization of estrus in donors and recipients**

Synchronization of estrus may be achieved through the use of prostaglandin F$_{2a}$ and progesterone, repeated progesterone injections for 16-17 days, intravaginal sponge [30-40 mg of fluorogestone acetate (FGA) for 11-18 days or 50-60 mg of medroxy progesterone acetate (MAP) for 15-18 days], or by using subcutaneous ear implants with a dose rate of 2-6 mg of progesterone for 9-17 days (Ishwar and Pandey, 1990; Nuti at al., 1987; East and Rowe, 1989; Stenbak et al., 2001, 2003). It is possible to synchronize estrus through luteolysis. Prostaglandin F$_{2a}$ and its analogues have luteolytic action and two injections administered 11 days apart in cycling females gives satisfactory results (Trounson 1976; Ishwar and Memon 1996). Progesterone may be considered as a drug of choice, because it is easily applied at low cost.
Superovulation

Principles of inducing superovulation in sheep are the same as in cattle. A follicle-stimulating gonadotropin is administered either near the end of the luteal phase of the cycle (Days 11-13) or around 1 or 2 days before the end of the synchronizing treatments (Jablonka-Shariff et al., 1993, 1994; Stenbak et al., 2001, Grazul-Bilska et al., 2003; Ishwar and Memon 1996). A high degree of ovulatory response is observed in sheep during superovulatory treatment which hampers the process of fertilization. This fertilization failure appears to be due to faulty transport of spermatozoa through the cervix whether bred naturally or inseminated artificially. This problem can be overcome by direct deposition of semen into the uterus (Trounson and Moore, 1974).

An enormous amount of literature concerning superovulation in sheep has been produced and from its analysis it is evident that an accurate control of ovulation has never been achieved. Embryo yield after superovulation is dependent upon many factors that can be grouped as follows (Loi et al., 1998):

1. Factors inherently variable and difficult to modify (breed, season, management). It is easy to understand that very little improvement can be expected from factors like breed and consequently flock management techniques as well as nutrition. Reproductive biologists made a major effort in the past to fit suitable superovulation protocols into a large number of domestic breeds under a broad environmental range (Gordon, 1997).

2. Factors susceptible on improvement (gonadotropin, knowledge of ovarian physiology). The two most widely used gonadotropin preparations for superovulation are pregnant mare serum gonadotropin (PMSG) and pituitary follicle stimulating hormone (FSH-P). Pregnant mare serum gonadotropin is administered as a single subcutaneous or intramuscular injection given 1 day prior to the last synchronization treatment. FSH-P is given at 12h intervals in decreasing doses for about 3 days on Days12-16 of the estrous cycle. Prostaglandin F$_{2\alpha}$ is administered i.m. at the time of the fifth FSH injection (Senn and Richardson, 1992).

Exogenous gonadotropin interplay with somatic and germinal compartments of the follicle leads to greater than normal ovulation rates. Additional negative effects can occur during early embryonic development as a result of unbalanced hormonal profiles. Several strategies have been suggested for optimizing the yield of transferable embryos from superovulated donors including the administration of anti-PMSG antibodies, pituitary follicle stimulating hormone-FSH instead of PMSG, association of these two gonadotropins, single versus multiple injections, or inclusion of GnRH or growth hormone in the treatments (Bindon and Piper, 1977; Ryan et al., 1991; Meinecke-Tillman, 1993; Walker et al., 1986). However, the well-known side-effects of the superovulatory treatment such as unovulated follicles, low fertilization and recovery rates, were still not fully solved (Loi et al., 1998).

It seems that progress in the effectiveness of superovulation will be associated with development and availability of systems for controlling follicular recruitment and selection. A detailed understanding of the processes involved in growth and differentiation of ovulatory follicles has been achieved in cattle and leads to the development of strategies for the control of the follicular wave (Bo et al., 1995). For sheep, several successful superovulation protocols have been reported (Gordon, 1997).
Repeated superovulation

Once a suitable superovulation protocol is established, the next step is to verify responsiveness of the same donor to repeated treatments. Multiple superovulations can be induced in sheep at a 1-year interval without a significant reduction in ovarian response (Loi et al., 1998). Whether immunological responses induced by gonadotropins used for superovulation can reduce the ovarian response still remains an open question. However, side-effects of repeated treatment with gonadotropins are not the major factors limiting multiple superovulation in sheep. The major problems related to repeated superovulation and frequent laparoscopic procedures of oocyte or embryo collections are adhesions caused by protrusions of the endometrium at the puncture site in laparoscopic recovery. The occurrence of adhesions may reduce the number of flushings obtainable from one donor (Nelleshulte and Nieman, 1992).

Artificial insemination

Regardless of the type of superovulation treatment, fertilization often fails, particularly in ewes showing a high ovulatory response. Fertilization failure is equally frequent in ewes bred naturally or artificially inseminated, and appears to be due to faulty transport of spermatozoa through the cervix (Boland et al., 1983). This problem can be overcome by intra-uterine deposition of semen through surgical procedure in superovulated ewes or by laparoscopic insemination (Ishwar et al., 1996). Intra-uterine insemination is especially effective in overcoming the fertilization failure of donors exhibiting high ovulation rates. In sheep, intra-uterine laparoscopic insemination should be carried out in the middle of the estrous period (Walker et al., 1986). However, if fertile rams are available, natural mating should be used in the superovulation program, and ewes should be mated every 6 h during standing estrus (Ishwar and Memon, 1996).

Embryo collection

Collection and transfer procedures for the ewe are similar and have changed little from those described by Hunter in 1955. Sheep embryos are usually surgically recovered and surgery is carried out under general anesthesia. All donor animals are taken off feed for 24 hours and water for 12 hours prior to surgery. During surgical embryo collection the uterus and oviducts are exposed to a midventral laparotomy and the reproductive tract is flushed using different shaped catheters with sterile medium. Flushings are collected in sterile dishes for immediate examination under a dissecting microscope.

Laparoscopic techniques were introduced for embryo recovery to reduce the extent of surgical intrusion (McKelvey et al., 1986). Despite lowering the level of surgical intrusion, laparoscopic techniques, like surgical collection, may lead to adhesions of the reproductive tract and ovaries. This limits the number of times a sheep can be used as a donor. Attempts are made for cervical collection after ripening the cervix with hormones like prostaglandin E2 and estradiol (Barry et al., 1990). Although some successes have been achieved using cervical collection of embryos, more work is necessary to make it a reliable option in sheep.

The embryos are usually recovered at the morula-blastocyst stage, 6-7 days after fertilization, evaluated under a dissecting microscope before being frozen or immediately
transferred, either by laparotomy or laparoscopy, into suitable synchronous recipients (Loi et al., 1998).

**Embryo freezing**

Successful methods for freezing sheep embryos have been available since the 1970's, when the first lamb was born following a transfer of frozen-thawed embryo (Willadsen et al., 1976). The embryos were incubated in an appropriate concentration of permeating cryoprotectors, cooled 3-7 C below freezing point, then ice nucleation was induced in the medium by touching the vial with a pair of precooled forceps. The cryoprotectant was removed step-wise after thawing and the embryo was ready for transfer. Research in the area of embryo freezing has led to the increase of embryo survival through the adoption of low toxicity cryoprotectants such as ethylene glycol and to incorporation of non-permeating osmotic buffer, sucrose, into the freezing medium, which allows direct transfer after thawing (reviewed by Loi et al., 1998). Further research allowed the development of cryopreservation protocols where embryos were cooled very rapidly in a high concentration of cryoprotectants which formed a glass structure without the formation of ice crystals. This procedure, called vitrification, has been adapted for many species including sheep, and the results in terms of survival rate and lambs born are continuously progressing (Szell and Windsor, 1994; Dobrinsky, 2001, 2002).

**Embryo transfer**

Embryos are transferred to the uterus or oviduct of recipients by laparotomy or using a laparoscopic technique. Comparison of the laparoscopic and surgical transfer of embryos showed that the laparoscopic method can achieve high pregnancy rates (Stefani et al., 1990). It appears, that laparoscopic transfer is a safe, minimally invasive surgical procedure and it should be recommended for transfer of embryos in small ruminants. Successful transcervical embryo transfers in small ruminants also have been reported, but only limited studies have been carried out on this matter (Flores-Foxworth et al., 1992).

The developmental stages at which embryos or oocytes were transferred have ranged from follicular oocytes (transferred to mated recipients) and zygotes to elongated blastocysts on day 12 of the cycle (Gordon, 1997). Number of embryos transferred ranged from one to more than five per ewe, but experimental results indicate, that transferring two embryos gives acceptable results and yields with increased number of twin pregnancies. More than 80% of recipients of two embryos carried pregnancies to term, with approximately two-thirds of these giving birth to twins (Ishwar and Memon, 1996).

The occurrence of estrus in donor and recipient ewes must be closely synchronized if the survival rate of transferred embryos is to be optimized. Optimum results in terms of embryo survival, were found among recipients in heat 12 hours before to 12 hours after donors (Shelton and Moore, 1966). Among various problems in sheep embryo transfer is the need for more efficient selection of ewes as potential recipients; such selection might be one means of improving the success rate of transfers (Gordon, 1997).
Production of embryos by \textit{in vitro} fertilization (IVF)

Recovery of sheep oocytes for the production of embryos may be of interest to those involved in conventional sheep embryo transfer applications for breeding improvement purposes. As with cattle, the repeated recovery of oocytes from live sheep may increase commercial interest in the development of effective breeding improvement programs. Oocyte recovery from live sheep by the way of laparoscopic follicular aspiration procedures shows that such oocytes are capable of maturation and fertilization. In Texas, Flores-Foxworth et al. (1995) found the laparoscopic oocyte aspiration technique to be relatively simple and effective, as well as being less traumatic than normal embryo recovery procedures. By using laparoscopic oocyte recovery, \textit{in vitro} maturation of oocytes, \textit{in vitro} fertilization followed by embryo transfer, researchers have managed to successfully deal with donor Red sheep (\textit{Ovis orientalis gmelini}) in work aimed at producing Red sheep lambs from domestic sheep (\textit{Ovis aries}; Flores-Foxworth et al., 1995).

\textit{In vitro} fertilization (IVF) of ovulated oocytes in sheep goes back to the 1950's, however, it was little more than a decade ago that the first IVF lamb was born in Japan (Gordon, 1997). After oocytes have been recovered from large or small follicles, three steps are required to produce \textit{in vitro} embryos:

1. Oocytes are allowed to mature \textit{in vitro} for about 24 hours in medium containing hormones;
2. Oocytes are fertilized \textit{in vitro}, then 24-48 h later the embryos are placed in culture medium;
3. After a few days in a culture medium, normally developing embryos, which have reached the blastocyst stage, are transferred into recipients or frozen for later transfer (Wang et al., 1998; Stenbak et al., 2001; Grazul-Bilska et al., 2003)

After embryo transfer, these blastocysts yield pregnancy rates approaching those of the blastocysts collected \textit{in vivo} (Gordon, 1997).

\textit{In vitro} production of embryos is used in cattle, pigs, sheep and goats to produce offspring from subfertile males and females, increase the number of progenies from selected mature or juvenile females, and salvage oocytes or sperm from valuable dead or dying animals. One very important issue to be solved in \textit{in vitro} production of embryos remains the survival rate of frozen sheep embryos. Although cryopreservation of bovine embryos has made great progress in recent years, little achievement was obtained in ovine embryo freezing, especially \textit{in vitro} produced embryos (Zhu et al., 2001).

The development of \textit{in vitro} production of embryos has led to the next generation of reproductive techniques, including intracytoplasmic sperm injection (ICSI), production of transgenic animals and cloning. With intracytoplasmic sperm injection, only one sperm is needed to fertilize an egg, and motility of that sperm is not necessarily required for fertilization. Interestingly, when cloning techniques are used, sperm is no longer needed at all.

Cloning and production of transgenic animals

In nature, clones or genetically identical animals are produced when early embryos split into halves, creating identical twins. This has been simulated in the laboratory by cutting embryos in half with microtools. Recently, mammals have been cloned through nuclear transfer. During this procedure, a nucleus is transferred from one cell to another cell that has had its nucleus removed. Nuclear transfer has been used for embryo cloning in sheep and other species.
The first cloned sheep, named Dolly, has been produced through nuclear transfer using a mammary gland cell from a 4-year old sheep as a cell "donating" genetic material to the oocyte from another sheep. In addition to producing Dolly from an adult mammary gland cell, the same researchers produced lambs from fetal cells (Wilmut et al., 1997). Research efforts have been intensified toward improving the efficiency of reprogramming cells such as adult mammary gland cells and fibroblasts for starting life all over again.

Cultured embryonic cells when injected into unnucleated oocytes have resulted in live calves and lambs (Wilmut et al., 1997, Wells et al., 1997). However, a high incidence of abortion around Day 40 has been seen with the cell-cloned embryos, accompanied by poorly developed placentomes (Stice et al., 1996). Among all the reproductive technologies, cloning with embryonic and fetal cells has progressed dramatically in sheep and nuclear transfer has been used to produce transgenic animals as an alternative to pronuclear injection (Loi et al., 1998). The ability to produce live offspring by nuclear transfer from cells that can be propagated and maintained in culture offers many advantages, including the production of many identical offspring over an extended period (Eyestone and Campbell, 1999). An ever-expanding variety of cell types have been successfully used as donors to create the clones. Both cell fusion and microinjection are successfully being used to create these animals. However, it is still not clear which stage of the cell cycle for donor and recipient cells yield the greatest degree of development (Kuhholzer and Prather, 2000).

Cloned sheep, cattle, goats, pigs and mice have now been produced using somatic cells for nuclear transplantation (Polejaeva and Campbell, 2000; Westhusin et al. 2001). Despite the rapid progress in cloning technologies, animal cloning still remains inefficient with an average less than 10% of the cloned embryos transferred resulting in a live offspring. The overall success rate for cloning sheep and cattle cells is very low. Dolly, for example, was the only live lamb produced out of about 300 cloning attempts. In addition, cloned animals frequently express numerous defects (Cibelli et al., 2002). Cloning techniques, however, once perfected, will allow rapid multiplication of performance-proven animals, i.e. sheep more efficient in wool production (Damak et al., 1996), and of animals possessing unique characteristics, such as disease resistance (Piedrahita, 2000).

Cloning techniques are a great hope to improve transgenic animal production (McCreath et al., 2000; Westhusin et al., 2001). Genes of these animals are altered, so the animals can secrete rare and expensive drugs in their milk (i.e., transgenic sheep which produced human anti-hemophylic factor IX in milk; Niemann et al., 1999; McCreath et al., 2000). Sheep-derived protein has now entered clinical trials for cystic fibrosis (UK and USA) and congenital emphysema (UK), and the utilization of cloning and transgenic technology is making inroads into more traditional ways of making biopharmaceuticals (Colman 1999).

Utilization of cloning technologies and animal models, including sheep, in biomedical research may significantly contribute to vascular research and our understanding of the mechanisms of atherosclerosis and restenosis formation and the evaluation of therapeutic options. Another approach to altering genes may lead to changes in the immunity, so animal organs may be used as donor organs in people without the risk of rejection (Piedrahita, 2000; Wolf et al., 2000).

The issue of cloning animals has captured the attention of popular media, provoking countless discussions whether adult animals should be cloned. However, the real issue here is what it could mean for future research. Research into reprogramming cells and manipulating cell
Differentiation will help to understand uncontrolled cell division, as seen in cancer, and lack of cell division, as seen in damaged tissues such as nerves, muscle and tendons.

The last two decades have dramatically changed the management of sheep breeding, through improved nutrition and veterinary assistance. However, despite these improvements, artificial insemination is the only reproductive technology applied in selection programs. Among the technologies described here, some have a limited perspectives for improvement, particularly the surgical or laparoscopic procedures. The principal ones, however, like superovulation and all in vitro technologies, including cloning, still have a large margin for improvement. Technologies like cloning or transgenesis, which are expensive and technically complex, are already being adopted by biotechnological companies, and the production of pharmaceutical proteins from transgenic animals is very close to reaching commercial applications. Nevertheless, before a large-scale application of these technologies can be made, it is essential to solve the problems related to the present poor efficiency of these technologies and to some problems with fetal development.

Literature Cited


