ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (2): 371-384, 2008
ISSN 1682-296X

A Review of Reproductive Biotechnologies and Their Application in Goat

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Abstract: Tremendous developments have been achieved in the field of assisted reproductive technology (ART) both in human and animals in the last two decades. In livestock including goats, the modern ARTs are being used for the improvement and preservation of livestock genetics and the enhancement of reproductive efficiency. The ARTs together with embryo technologies have facilitated the development of methods to transfer desired single genes or the entire genome from desirable individuals or embryos. Rapid advances in ARTs to manipulate embryos in the laboratory have permitted screening of embryos for genetic defects or highly desirable quantitative traits using molecular markers. The ARTs include artificial insemination, embryo transfer, estrus synchronization and superovulation, multiple ovulation embryo transfer, laparoscopic ovum pick-up, *in vitro* production of embryos, intracytoplasmic sperm injection, cryopreservation of sperm, cryopreservation of oocytes and embryos, sexing of sperm and embryos, embryo splitting, cloning and gene transfer and marker-assisted selection. The goat is an excellent model for all these ARTs and has been used extensively in both basic and applied research. The applications of these ARTs in goat enable to increase the rate of genetic progress, reduce generation interval, enhance production, help utilizing genetically important but biologically inferior individual, improve the management of infertile/sub-fertile buck/doe and eliminate reproductive diseases. This review describes different ARTs and their application in goat production and research.

Key words: Goat, assisted reproductive technology (ART), artificial insemination (AI), laparoscopic ovum pick-up (LOPU), *in vitro* production (IVP), intracytoplasmic sperm injection (ICSI)

INTRODUCTION

Biotechnology is one of the most fast growing scientific disciplines of the twenty-first century. Recent advances in technology, nuclear biology and genetic engineering have made possible a wide range of new technologies and products which provide scientists with a spectacular vision of the design and function of living organisms and provide technologists, including reproductive biotechnologist, with the tools to implement exciting commercial applications. Over the next decade, these may revolutionize commercial livestock production as well as other animal-related sectors. Like humans, livestock animals including goats, suffer from infertility or sub-fertility, which lowers their lifetime productivity and reduces the number of offspring that could be obtained from a sire or dam. The prevalence of this problem coupled with the desire of people to understand and subsequently control the reproductive processes has led to the development of novel reproductive biotechnologies, which are also known as assisted reproductive techniques (ARTs). These ARTs include artificial insemination (AI); embryo transfer (ET); multiple ovulation embryo transfer (MOET); estrus synchronization and superovulation; laparoscopic ovum pick-up (LOPU), *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) collectively known as *in vitro* production (IVP), intracytoplasmic sperm injection (ICSI), cryopreservation of sperm, cryopreservation of oocytes and embryos, sperm and embryo sexing, embryo splitting, embryo cloning, nuclear transfer (NT), gene transfer and marker-assisted selection (MAS). The application of these biotechnologies enables to increase the rate of genetic progress (Nicholas, 1996). Some of these techniques increase the selection differential (e.g., AI, ET) while others accelerate progress by shortening the generation interval (e.g., juvenile *in vitro* embryo technology or JIVET) (Baldassarre and Karatzas, 2004). The ARTs allow animals of high genetic merit to produce more offspring than would be possible by natural breeding. The goat is a convenient domestic species for biological investigation and application as it

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has diversified products of commercial value (Amoah and Gelaye, 1997). Besides, it has a relatively short generation interval compared with cow, the predominant livestock species. Biotechnological alterations could make significant impact on the quality of goat meat. It has been reported that broiled goat meat has lower total lipid, phosphorus and vitamin B_{12}, but higher calcium, potassium and thiamin than composite values for beef (Johnson *et al*., 1995). The current paper will discuss various ARTs for goats in general and highlight the scopes and limitations of these techniques for application for the improvement of goat population.

WHY BIOTECHNOLOGIES IN GOAT REPRODUCTION?

Researchers working with goat reproduction have devoted themselves to develop and apply new reproductive biotechnologies for increasing reproductive efficiency. There are a number of reasons for developing ARTs in goat reproduction. These are as follows:

- Reproductive biotechnologies could be an approach to generate transgenic goat for the propagation of useful genetics (Wang *et al*., 2002) or gender pre-selection (Hamano *et al*., 1999). These goats could be used as founder animals for the production of recombinant protein in their milk such as pharmaceutical proteins for the treatment or prevention of human diseases or biomaterials for medical use (Keefer, 2004; Niemann and Kues, 2003). Therefore, engaging in goat reproductive biotechnologies appears to be profitable.
- Breeding of goats in some regions of the world (e.g., cold and temperate regions) are limited to a specific period of the year and therefore, development of appropriate ARTs would enable greater flexibility to produce offspring that would be economically viable to produce high quality milk, meat, skin and wool all year round.
- The application of ARTs would help to use genetically important but biologically inferior male gametes for procreating domestic and wild goat species (Keskintepe *et al*., 1997) and thereby facilitate the production of a large number of embryos and offspring from a single genetically valuable animal.
- Development of new and appropriate ARTs would also improve the management of infertile/sub-fertile buck and will eliminate reproductive diseases.
- It is well known that the use of human oocytes for research purposes is severely limited. Therefore, due to their convenient size and management, goat can be considered as unique laboratory animal models to study the reproductive processes in humans, necessitating a better understanding of the mechanisms underlying the biology of the reproductive process.

DIFFERENT ART FOR GOAT

Nowadays, a number of ARTs are being used for enhancing the number of good quality goat, mostly in the developed countries of the world. However, not all techniques are efficient and widely used; some are still under the laboratory condition. Among these, AI, superovulation and estrus synchronization and ET are the most widely utilized biotechnologies for genetic improvement programs in goats, due to their simplicity, relatively low cost and proven efficiencies. AI and superovulation and oestrus synchronization are the key technologies for managing production systems, allowing the concentration of mating and parturition and production of meat and milk during specific times of the year for strategic marketing and other purposes. Recent advances in ARTs in small ruminants, especially in goat, include improvement of methods for IVP of embryos and attempts at spermatogonial stem cell transplantation (Tibary *et al*., 2005). *In vitro* production of embryos by IVM/IVF, ICSI and NT has been made possible by improvements in oocyte collection and maturation techniques and early embryo culture systems. Although IVP systems have great potential for more efficient propagation of valuable animals, however, application of these technologies is restricted by the need for more demanding laboratory conditions and several limiting factors affecting the outcome of each step of the process. In the present study some of the important reproductive biotechnologies are described in brief:

Artificial insemination: Of all the reproductive biotechnologies have been discovered, artificial insemination (AI) is the one that has, so far, made the greatest impact on animal production (Wilmut *et al*., 2000). AI may be regarded as a first generation ART that is the most widely used and the one that has made the most significant contribution to genetic improvement worldwide (Evans and Maxwell, 1987; Chemineau and Cognié, 1991; Leboeuf *et al*., 2000). AI offers a low cost and relatively simple method for dissemination of valuable genes. Three types of insemination techniques e.g., vaginal, cervical and intrauterine, are currently used in goats all over the world (Evans and Maxwell, 1987; Chemineau and Cognié, 1991; Leboeuf *et al*., 2000). AI was the first technique that was developed and used in

farm animals since 1900s (Abdullah *et al*., 2001). During the past 50 years, application of AI has contributed much more in both the control of diseases and genetic improvement (Wilmut *et al*., 2000). The discovery of semen cryopreservation (freezing) methods (1950s) revolutionized AI in livestock breeding. Like cattle and sheep, the use of sex-sorted sperm for AI has been promoted as a means of increasing reproductive efficiency in goats, especially in the dairy industry where males have less commercial value (Baldassarre and Karatzas, 2004). Like other countries, AI in goat is now successfully using in Malaysia (Abdullah *et al*., 2002).

Embryo transfer: After the success of AI, research concentrated upon the development of methods for embryo transfer (ET), to offer the opportunity for genetic selection in female reproduction (Wilmut *et al*., 2000). The first embryo transfer in goat was reported by Warwick *et al*. (1934). In early sixties, fundamental and physiological studies related to ET in small ruminants were performed by several famous scientists. However, it was not until mid-1970s when ET emerged as an industry in cattle, the development of ET technology in small ruminants was begun. In the early 1980s, due to the huge demand for Angora goat and Mohair in the world market, the application of ET for commercial purposes in goats became widely accepted (Thibier and Guerin, 2000). Three methods of ET, namely, laparoscopic ET, Surgical ET and transcervical ET have been reported in small ruminants (McKelvy *et al*., 1985) of which laparoscopic ET is more successful (Abdullah *et al*., 1995; Ishwar and Memon, 1996). Anyway, considerable technical progress in the methods of induction of superovulation and estrus synchronization, recovery, storage, sorting, transfer and implanting embryos in several countries of the world have made ET in goats more successful. The success of ET depends on several factors including management of donor and recipient does, estrus synchronization in donors and recipients, superovulation of donors, breeding (natural or AI), embryo collection and evaluation, transfer of embryos and factors affecting survival of transferred embryos (Ishwar and Memon, 1996).

Oestrus synchronization and superovulation

Oestrus synchronization: Oestrus synchronization is a key element of all the ART-protocols and has a major influence to increase the overall efficiencies of these programmes (Baldassarre and Karatzas, 2004). Oestrus synchronization plays a major role in fixed time breeding, AI, LOPU for oocyte or embryo collection and ET. The value of estrus synchronization is vital in goats as the duration of both estrous cycle and estrus is variable and estrus detection cannot be accomplished safely without a buck (Chemineau and Cognié, 1991; Jainudeen *et al*., 2000). This technique has been developed in the early 1960 s and since then a number of synchronizing methods has been developed for goats (Chemineau and Cognié, 1991). However, the most widely used method in goat is the treatment of progesterone/progestogen for 9-11 days followed by a luteolytic dose of prostaglandin (or an analogue) administered in the period 36 h prior to removal of intravaginal sponge (Baldassarre and Karatzas, 2004). The progesterone/progestogen treatment is delivered through an intravaginal sponge, a CIDR (controlled internal drug release) device or a subcutaneous implant (Evans and Maxwell, 1987; Freitas *et al*., 1997). However, subcutaneous implants or CIDR are preferable because sponges frequently cause discomfort and may adhere to the vaginal wall causing problems with removal (Holtz, 2005). Anyway, ovulation in goat can be synchronized more precisely by administering GnRH around the time of estrus (Pierson *et al*., 2003), which improves the success of fixed-time AI and the collection of oocytes/embryos at a controlled stage of development for specific applications such as oocytes for IVP, ICSI or SCNT and zygotes for pronuclear microinjection (Baldassarre and Karatzas, 2004). Although, in the past, a considerable attention was focused in estrus synchronization, however, there is an urgent need for additional research conducted in a well-organized and systematic fashion to help establish guidelines for efficient breeding, AI, oocyte/embryo recovery and ET programs.

Superovulation: Superovulation is the hormonal treatment for increasing a large number of ova released by the ovary, which ultimately accelerate genetic improvement in any species. It is a means to induce maturation, ovulation and increase the number of follicles available for oocyte recovery. Principles of inducing superovulation in goat are the same as in cattle and sheep. An exogenous follicle-stimulating gonadotrophin is administered that mimics the effect of FSH near the end of the luteal phase of the cycle (days 9-11) or around 48 h before the end of the synchronizing treatments. There are a number of ways to superovulate goat, each of which has its advantages and disadvantages. The major commercial products applied are equine chorionic gonadotrophin (eCG or PMSG) and FSH. Commercial preparations are partly purified from mare's serum and porcine pituitary gland, respectively. Like cattle and sheep, a number of experiments have been performed to compare the superovulatory response between FSH and PMSG, the evidence favors the use

of FSH than PMSG (Tsunada and Sugie, 1989; Pendelton *et al*., 1992). In their study, Tsunada and Sugie (1989) reported that average number of oocytes recovered was significantly higher in FSH-treated goats (9.4) than PMSG-treated (5.7). In another study, embryo recovery was higher in FSH-treated than PMSG (6.8 vs. 3.0, respectively) (Rosnina *et al*., 1992). Our personal experience with superovulation in goats supports both of the works.

Most superovulatory treatments are cumbersome and expensive and over and above, accompanied by endocrine repercussions that take one or more subsequent cycles to subside (Holtz, 2005). Therefore, several attempts have been made to devise less labor-intensive treatment regimes without compromising oocyte/embryo yield. By applying a one shot-treatment regimen consisting of a single dose of FSH combined with a moderate dose of eCG (e.g., 80 μg FSH and 300 IU eCG), Batt *et al*. (1993), Baldassarre *et al*. (2002) and Baldassarre *et al*. (2003b) almost equaled the embryo yield obtained with the traditional multiple injection regimen. The simplicity of this treatment is appealing. Anyway, of all the superovulation protocols in use to date, not a single one fulfills all expectations concerning predictability and reliability of the response. The variability in number of ovulations and yield of viable oocytes/embryos remains the main drawback (Holtz, 2005). Both intrinsic and extrinsic factors are responsible for the variability. Among the intrinsic factors, genetic (Nuti *et al*., 1987), age (Mahmood *et al*., 1991), stage of the cycle at which the treatment applied (Wani *et al*., 1990) are important. A host of environmental factors such as season, nutrition, health state, AI (Holtz, 2005) and type of gonadotrophin administered (Gordon, 1997) are known to contribute to the variability. Therefore, vigorous research efforts are directed for the establishment of suitable superovulation regimes to augment the deployment of LOPU, ET and associated technologies based on them.

Multiple ovulation embryo transfer: The term multiple ovulation embryo transfer (MOET) describes a closed system in which these techniques are used to increase average rates of genetic gain (Nicholas and Smith, 1983). This techniques is often referred as the ART to the female, whereas AI is to the male i.e., a method of producing more offspring from a genetically valuable female than would be possible by natural breeding. MOET has been introduced to overcome reproductive inefficiencies in goats and accelerate genetic gain. Most studies on MOET are focused on variability of the ovulation rate and yield of transferable embryos in response to exogenous FSH treatment (Cognié *et al*., 2003). Recent improvements in administration of gonadotrophin preparations and programed insemination protocols cannot avoid this variability found between treated donors (Cognié, 1999). However, this technique became the most frustrating ART because of its unpredictable outcome from complete failure to total success without any variation in the standard operating procedure (Baldassarre and Karatzas, 2004) which is due to the variability of the superovulatory response, the poor fertilization associated with high ovulatory responses and early regression of corpora lutea (Cognié, 1999; Cognié *et al*., 2003). These unpredictable results combined with high cost, surgical procedure and other associated problems make the techniques less widespread tool for genetic improvement. Although MOET, due to its high cost and other associated problem cannot replace AI as a routine reproductive technology, it can be applied to correctly choose dams to allow extra genetic gain through the production by embryo transfer of males with positive indexes used for AI (Nicholas, 1996).

Laparoscopic ovum pick-up: Laparoscopic ovum pick-up (LOPU) is one of the best techniques for oocyte recovery from live animals. This is the most effective and non-invasive technique, which cause less adhesion-related problems compared to laparotomy or surgical oocyte collection. Thus LOPU offers the possibility of repeated ovum pick-up and allows for repeated production of oocytes/embryos from a single donor. Moreover, LOPU technique obviates several causes of the poor results observed with superovulation, such as poor ovulation rate, early regression of corpora lutea and poor fertilization (Baldassarre and Karatzas, 2004). Additionally, the procedure allows the production of offspring from prepubertal goats that would not be able to reproduce using AI or MOET. Snyder and Dukelow (1974) first conducted LOPU in sheep where 6 oocytes were retrieved from 21 aspirated follicles. However, the potential of the technique was not fully realized until IVP technologies were developed (Baldassarre *et al*., 2002). In LOPU procedure, donor goats are restrained on a standard laparoscopy table using general anesthesia and follicles are aspirated under laparoscopic observation using a 20 g needle mounted in a plastic pipette connected to a collection tube and a vacuum line. The procedure takes less than 30 min for each goat by an experienced surgeon depending on the number of follicles to be aspirated. The LOPU almost always results in >5 oocytes aspirated per donor (Baldassarre and Karatzas, 2004). In agreement with this, in a recent study 6.8 oocytes per goat were recovered by LOPU technique (Rahman *et al*., 2007a). Estrus

synchronization and superstimulation of donor goats are routinely done with gonadotrophins in order to recover high numbers of oocytes per LOPU session. However, oocyte recovery after repeated LOPU in unstimulated goats also provided a high number of oocytes (4-6 per female/session) (Aguilar *et al*., 2002). If the quality of these oocytes for IVP is confirmed, this method could provide a way to produce offspring from genetically valuable females without using hormones (Cognié *et al*., 2003).

***In vitro* production of embryos:** *In vitro* maturation, fertilization and culture (IVMFC) of embryos, which are collectively known as *in vitro* production (IVP), have the potential to improve the number of offspring produced by genetically valuable female goat. Like cattle and sheep, this technique is used in goats to produce offspring from sub-fertile 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. The first kid born after complete IVMFC was reported by Keskintepe *et al*. (1994). In this technique, immature oocytes are collected from slaughterhouse ovaries by aspiration of punctured follicles or slicing, or from live animals by way of laparotomy, ultrasound-guided transvaginal oocyte retrieval (TVOR) or LOPU procedure. The various steps involved in IVMFC or IVP of goat oocytes are quite similar to those employed in the cattle or sheep, where IVP is an established procedure. Three main steps involve in IVP: maturation of the immature oocytes, fertilization of the matured metaphase II (MII) oocytes with *in vitro* capacitated fresh or frozen-thawed semen and culture of the putative embryos for up to 7-8 days until formation of blastocysts that can be transferred to recipients or cryopreserved for future use (Cognié *et al*., 2003; Holtz, 2005). The IVP technique in goats have been detailed by different groups (Crozet *et al*., 1995; Ongeri *et al*., 2001; Baldassarre *et al*., 2003a; Cognié *et al*., 2003; Koeman *et al*., 2003).

***In vitro* maturation:** Embryo development is influenced by events occurring during oocyte maturation (Rajikin *et al*., 1994; Teotia *et al*., 2001). Oocytes must undergo nuclear and cytoplasmic maturation *in vitro* for successful *in vitro* maturation (IVM). A number of IVM media have been developed in different laboratory for IVM of goat oocytes. However, generally goat oocytes are matured in buffered TCM-199 supplemented with pyruvate, heat-inactivated serum and hormones (FSH, LH, estradiol) (Ongeri *et al*., 2001; Wang *et al*., 2002; Izquierdo *et al*., 2002). Low level of efficiency have been achieved after IVM of goat oocytes compared with those of *in vivo* studies, which is almost certainly related to the quality of the oocytes at the outset of IVM (Cognié *et al*., 2003). However, due to advanced research in goat IVP higher maturation rates (70-90%) were achieved with pre-selected oocytes under specific conditions (Samake *et al*., 2000; Bormann *et al*., 2003). Besides the medium, incubation time and temperature in CO_2 incubator have a great influence on oocyte maturation. Therefore, the required incubation temperature and time for goat oocytes in a humidified atmosphere (5% CO_2) of the CO_2 incubator should be 38-39°C and 24-27 h, respectively (Agrawal *et al*., 1995; Samake *et al*., 2000). Incubation time necessary for maturation of goat oocytes seemed to be longer (Rajikin *et al*., 1996) than that needed for sheep/cattle oocytes. A higher proportion of goat oocytes reach metaphase II after 27 h than after 24 h of culture (Rho *et al*., 2001). In our study, more than 80% maturation rates were achieved when cultured at 38.5°C for 27 h (Rahman *et al*., 2006b, 2007b).

***In vitro* fertilization:** Following IVM, *in vitro* fertilization (IVF) is done with fresh (Izquierdo *et al*., 2002; Wang *et al*., 2002) or frozen-thawed (Rho *et al*., 2001; Bormann *et al*., 2003) buck semen. Several types of IVF media are used which includes, defined medium (Brackett and Oliphant, 1975; Younis *et al*., 1991), TALP medium (Mogas *et al*., 1997) and synthetic oviductal fluid (SOF) medium (Tervit *et al*., 1972; Takahashi and First, 1992). Some laboratories use heparin (Teotia *et al*., 2001), heparin and calcium ionophore (Jimenez-Macedo *et al*., 2006), caffeine or PHE (penicillamine, hypotaurine and epinephrine) (Izquierdo *et al*., 1998) in capacitation medium for better fertilization and cleavage.

***In vitro* culture:** After IVF the presumptive zygotes are cultured in *in vitro* culture (IVC) medium. Presently, a commonly used IVC medium for goat embryos is SOF (Tervit *et al*., 1972; Takahashi and First, 1992) with amino acids and BSA in the absence of serum and somatic cells at 38.5°C in a humidified atmosphere of CO_2 (5%) (Gardner *et al*., 1994). However, some laboratories routinely supplement SOF medium with 5-10% FCS (Jiménez-Macedo *et al*., 2005, 2006, 2007) or estrus goat serum (EGS) (Rahman *et al*., 2007a, b) at 2-3 days post-insemination to promote a higher viability after transfer of such IVP embryos (Cognié, 1999). Recent progress in understanding of the requirements of the developing embryo resulted in the development of sequential media where components change according to the needs of the embryo (Thompson, 2000). These two-step culture media is still not popularized in goat and used mainly in humans and cattle.

Although, IVP has a big potential to propagate useful genetics and improve livestock production, but lower embryo production and survival rate hindering the technique to be popularized. However, progress achieved in recent years in developing new oocyte harvesting techniques and improving early embryo culture has shown promise to increase pregnancy and parturition rates. The development of IVP of embryos has led to the next generation ARTs including ICSI, production of transgenic animals and cloning. With ICSI, 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.

Intracytoplasmic sperm injection: Intracytoplasmic sperm injection is the mechanical insertion of a single sperm directly into the ooplasm of a matured, metaphase II oocyte using a microscopic needle. It is a special type of IVF and one of the advanced ART that is now widely used to overcome male factor infertility in human. The ICSI proved it efficacy in producing thousands of human babies since its first success in 1992 (Palermo *et al*., 1992). In animals including livestock, by applying this technique many live births have been achieved in a number of species. Although the technique has potential to improve livestock production, however, till now it has not been commercially used in goat production. Compare to cattle ICSI, few studies have been done in goat (Keskintepe *et al*., 1997; Wang *et al*., 2003; Zhou *et al*., 2004; Jiménez-Macedo *et al*., 2005, 2006; Rahman *et al*., 2006a, b, 2007a; Jimenez-Macedo *et al*., 2007) with only one live birth (Wang *et al*., 2003). The ICSI can also be applied for the production of transgenic goats for the production of biopharmaceuticals in their milk (Wheeler and Walters, 2001; Wheeler, 2003; Wheeler *et al*., 2003). Compare to human and cattle, ICSI in goat is not much successful yet. Blastocyst production after ICSI remains very low. In a study in sheep, 80% of the embryos produced by ICSI underwent development arrest on day 4 (16-cell to morula stage) (Gomez *et al*., 1997). Like in sheep, developmental arrest also observed in goat at the same stages of embryo development (Rahman *et al*., 2007a). This could be due to a number of factors such as oocyte maturation, culture system, oocyte activation and abnormal fertilization (Gomez *et al*., 1997, 1998). ICSI resulted in a higher rate of abnormal fertilization and lower total activation rate compared to IVF (Catt *et al*., 1996). As with our experience, inadequate oocyte activation after ICSI could be responsible for lower sperm decondensation and pronuclear formation. Activation and fertilization rates were higher when treated the sperm with calcium ionophore before ICSI (Jimenez-Macedo *et al*., 2006) and oocytes after ICSI with calcium ionophore alone (Keskintepe *et al*., 1997) or with DMAP (Jiménez-Macedo *et al*., 2005). Better fertilization and cleavage rates also can be obtained when treating the oocytes with calcium ionophore before and after ICSI or twice after ICSI (Rahman *et al*., unpublished observation). Recently, ICSI with goat oocytes having cytoplsmic dysmorphism also reported by Rahman *et al*. (2007b). Although till now, goat ICSI is not much successful like in human; however, reproductive biotechnologists are engaging themselves to develop suitable and cost-effective ICSI techniques to boost goat production. In future, when it will be possible to utilize sperm as carriers for altered chromosomal material, ICSI could also become a useful method of generating transgenic animals (Perry *et al*., 2001).

Cryopreservation of sperm: Cryopreserved sperm is an important integral component for the advancement of goat production. By virtue of this technique sperm could be stored indefinitely, used widely and can be exported easily. The frozen sperm facilitates international exchange of genetic material, allows AI in both the reproductive and non-reproductive seasons and extends the effective reproductive life of a valuable male beyond its own life. Development of goat sperm cryopreservation, however, has progressed at a slower rate than the cattle and sheep (Abdullah *et al*., 1997). The ability to cryopreserve sperm from all of the domestic animals is challenging. It is a complex process, which involves balancing many factors for obtaining satisfactory results. Although, all of the sperm cells must endure similar physical stresses associated with the cryopreservation processes, sperm of different animals are not similar in size, shape and lipid composition, all of which affect cryosurvival (Purdy, 2006). Thus, when a cryopreservation protocol has been optimized for sperm of one species, it may not be ideal for another.

Cryopreservation of goat semen is technically challenging due to the presence of seminal plasma (bulbourethral gland secretions a lipases), which interact with egg yolk to create substances that are toxic to sperm (Iritani *et al*., 1964). This situation is not observed with cattle seminal plasma and egg yolk that has led to the development of alternative methods of freezing in which either the sperm is centrifuged to eliminate the seminal plasma prior to dilution in standard extenders (e.g., 20% egg yolk), or low-egg yolk concentrations (2%) are used which may result in insufficient protection to sperm membranes (Baldassarre and Karatzas, 2004). Until now, the use of the Tris-egg yolk cryopreservation diluents,

such as the one described by Salamon and Ritar (1982) is recommended, as it is easy to use. The sperm freezing protocol with Tris-egg yolk developed in our lab also working very well (Abdullah *et al*., 1997, 2002). However, commercial extenders with no biological components (e.g., egg yolk) have been developed to improve sanitary safety in semen processing (Hinsch *et al*., 1997; Gil *et al*., 2003). One of the these commercial expender, Bioexcell® (IMV, L'Aigle, France) that was originally developed for cattle, could be used for goat semen cryopreservation, without the need to centrifuge the semen prior to addition of the extender (Baldassarre and Karatzas, 2004).

Although, nowadays, frozen-thawed goat sperm are utilized successfully for AI, IVF, ICSI and embryo production, but the results presented in literature are quite variable. To overcome this variability the scientific community should endeavor to reach a consensus that addresses generally accepted practices for freezing goat sperm (medium, cooling/freezing rate, sperm concentration, etc.) and its subsequent uses across breeds (Purdy, 2006).

Cryopreservation of oocytes and embryos: Cryopreservation or freezing of oocytes and embryos is an established commercial practice, especially in cattle. Oocytes or embryos can be stored for a long period of time in liquid nitrogen tanks by using a programmable freezer, quick-freezing, direct plunging and vitrification methods. Various cryoprotectants (substances used to protect cells or tissues from damage during freezing) such as glycerol, DMSO, ethylene glycol, polyethylene glycol and sucrose can be used to preserve oocytes and embryos. Mixtures of cryoprotectants have less toxicity and are more effective than single-agent cryoprotectants. A mixture of formamide with DMSO, propylene glycol and a colloid was for many years the most effective of all artificially created cryoprotectants. The cryopreservation technique is useful as a conservation strategy for endangered species or breeds and every effort should be made to select embryos representing the maximum range of current diversity. Preservation of embryonic stem cells could represent an important method of genome conservation.

Cryopreservation of oocytes and ovarian tissues: Cryopreservation of ovarian tissue may be a potential alternative for the conservation of genetically superior animals, including high milk and meat-producing goat breeds. Both the ovarian tissue pieces (Rodrigues *et al*., 2004a, b) and isolated prenatal follicles (Rodrigues *et al*., 2006) from goat ovary can be cryopreserved successfully. The ovarian cortex contains a large supply of immature oocytes enclosed in preantral follicles and oocytes can be successfully recovered from ovarian tissue pieces or isolated prenatal follicles. Cryopreserved mature oocytes had premature release of cortical granules contents, hardening of the zona pellucida and increased chromosomal abnormalities. These problems were not observed with cryopreserved, less-differentiated immature oocytes (Rodrigues *et al*., 2004a). However, a high rate of follicular degeneration was observed in goat ovarian tissue due to cryprotectant. Recently, vitrification (i.e., solidification without any ice formation whatsoever) of goat preantral follicles enclosed in ovarian tissue has been reported using conventional and solid-surface vitrification techniques (Santos *et al*., 2007).

Cryopreservation of goat embryos: For the cryopreservation of goat embryos, similar methods those are successful in cattle can be used (Holtz, 2005). In the conventional cryopreservation method, the embryos are incubated in an appropriate concentration of permeating cryoprotectants, cooled 3-7°C below freezing point, then ice nucleation is induced in the medium by touching the vial with a pair of precooled forceps. The cryoprotectant is removed step-wise after thawing and the embryo is ready for transfer. Ethylene glycol is considered the most suitable cryoprotectant (Le Gal *et al*., 1993; Fieni *et al*., 1995), although excellent results were reported with DMSO (Li *et al*., 1990) or glycerol (Nowshari and Holtz, 1995). Further research allowed the development of cryopreservation protocols where embryos are cooled very rapidly in a high concentration of cryoprotectants, which form a glass structure without the formation of ice crystals. This procedure, called vitrification, has been adapted for many species including goat. Vitrification has important application in preserving embryos, biological tissues and organs for transplant. Vitrification is also used in cryonics in an effort to eliminate freezing damage. The first successful transfer of vitrified goat embryos was reported in 1990 (Yuswaiti and Holtz, 1990). Since then, a number of cryopreservation protocols have been proposed, but few results are available concerning pregnancy rates after the transfer of vitrified goat embryos (Traldi *et al*., 1999; Traldi, 2000; Branca *et al*., 2000). In a comparative study between the sheep and goat, a big difference was observed in *in vitro* (41% versus 60%, $p<0.01$) and *in vivo* (9% versus 30%, $p<0.01$) embryonic survival after vitrification and thawing for sheep and goat, respectively (Barillet, 1997).

Sexing of sperm and embryos

Sperm sexing: Sex predetermination in livestock offspring is in great demand and is of critical importance to provide

for the most efficient production of the world's food supply. The application of sex pre-selection to livestock production systems becomes increasingly necessary with the changes in animal agriculture over the past generation (Johnson, 2000; Maxwell *et al*., 2004). The current technology is based on the well-known difference in X- and Y- sperm in the amount of DNA present (Johnson, 2000). Sex-sorted sperm has been used successfully in a number of species of livestock with live birth in pigs (Johnson, 1991), cattle (Cran *et al*., 1993), sheep (Catt *et al*., 1996) and horses (Buchanan *et al*., 2000). Until now no report of sex-sorted sperm has been published in goats, however, it is expected that this technology can be applied to goat sperm (Baldassarre and Karatzas, 2004). The use of sex-sorted sperm for AI, IVP or ICSI has been promoted as a means of increasing the efficiency of reproduction in goats, especially in the dairy business where males have little commercial value. Although, the process of commercialization of sexed sperm has accelerated recently, however, this technology is characterized by high costs, complexity of implementation and lower pregnancy rates than with control sperm (Seidel, 2007).

Embryo sexing: Sexing of embryos prior to transfer has commercial application, especially in dairy goats (Holtz, 2005). The established technique for sex determination of embryos in domestic species is the application of Polymerase Chain Reaction (PCR) to amplify sex-specific gene(s), followed by electrophoresis. An embryo biopsy is performed whereby the biopsied portion is used for the sex determination assay and the remaining portion for further culture. Although in cattle, sexing has become routine, in goats there is still a paucity of available information (Aasen and Medrano, 1990; Rao and Totey, 1992; Leoni *et al*., 1996; Phua *et al*., 2003). In a study, after transferring 12-sexed blastocysts to recipient does 8 (67%) were carried to term (El Gayar and Holtz, 2005). Anyway, the economic factor should be considered in sexing embryos, because half the embryos are usually discarded, as the breeder only wants kids of one sex. This has the effect of doubling the charge that must be made for each embryo transferred.

Embryo splitting: The facility to split embryos generating identical twins was developed two decades ago and embryo splitting was offered as a commercial service. The purpose of embryo splitting is to provide genetically identical twins to be used for experimental purposes or to increase the number of transferable embryos (Holtz, 2005). Compare to cattle a very few studies had been reported on embryo splitting in goat that has been reviewed in McKelvey and Bhattacharyya (1992). In a study of goat demi-embryo transfer, 59% of the embryos were carried to term (Nowshari and Holtz, 1993). However, only 9% kidding rate was found when used cryopreserved demi-embryos (without a protective zona pellucida or agar coating). After transfer of demi-embryos derived from splitting of cryopreserved goat embryos, kids were successfully born (Yong and Wang, 1990). In a very interesting study, split early embryos when transferred to genetically identical females, was developed to term in allogeneic pregnancies, being genetically identical twins to these foster females (Oppenheim *et al*., 2000).

Cloning and gene transfer

Cloning: Tremendous interest in cloning has been generated in the recent years (Loi *et al*., 1999; Colman, 1999, 2000; Paterson *et al*., 2003). It is the asexual production of genetically identical organisms that can be obtained by embryo splitting or by nuclear transfer (NT). Although cloning can be generated by embryo splitting, however, embryo sectioning for more than once will drastically reduce chances of survival (Holtz, 2005). Therefore, NT can be used to generate larger clones. The NT involves the transfer of nuclei from serum-starved fetal or adult cells into enucleated oocytes matured *in vivo* or *in vitro*. Fetal fibroblast cell or a variety of adult cells including mammary epithelial cells, granulosa cells (female), Sertoli cells (males) and skin cells (dermal fibroblasts) (Peura, 2003) can be used. Since the birth of Dolly (Wilmut *et al*., 1997), derived from the transfer of an adult somatic cell to an enucleated oocyte, research on cloning of somatic cells has gained momentum (Holtz, 2005).

In goats, births have been produced from embryos obtained by transfer of either adult or fetal cell lines nuclei into enucleated ova and transfer of reconstituted embryos into recipients at 2-4 -cell stage (Yong, 1998; Baguisi *et al*., 1999; Keefer *et al*., 2001, 2002). Generally, 0.5-2% of reconstructed goat embryos finally becomes live offspring (Baguisi *et al*., 1999; Campbell, 1999). However, there was no indication of increased abortion rate, prolonged gestation or the large offspring syndrome following NT of *in vitro*-derived goat reconstructed embryos those encountered with cloned embryos of other ruminant species (Baguisi *et al*., 1999; Keefer *et al*., 2001). In conclusion, the cloning and NT technology in goats gives rise to an optimistic prognosis that will benefit the propagation of genetically superior individuals. However, vigorous research are needed to gain a better understanding of various intricacies involved at various levels of the process which will definitely increase the efficiency of the cloned goat production.

Gene transfer: Gene transfer or transgenesis has the potential to play an important role in accelerating and facilitating genetic improvement. Although, the definition of transgenic animals is evolving, however, a transgenic animal may be defined as one containing recombinant DNA molecules in its genome that were introduced by intentional human intervention (Wall, 1996). Transgenesis in small ruminants is approximately four times successful than cattle and as a result its application in goats has proven to be more practical (Basrur and King, 2005). Transgenesis of goat is important for developing and propagating founder animals, which will produce valuable recombinant pharmaceutical or biomedical proteins (rc-proteins) in their milk. Goats are particularly an efficient means of producing rc-proteins as they produce considerable amounts of milk and incur lower investment and maintenance costs than cows. Examples of human rc-proteins expressed in the milk of transgenic goats are antithrombin III, blood clotting factor IX, alpha-1-antitrypsin, tissue plasminogen activator, anti-cancer monoclonal antibody, growth hormone and prolactin (Echelard *et al*., 2000). Besides these biopharmaceuticals, a type of transgenic goat was produced that secrete a spider silk protein that is strong and more elastic than silk fibre and is referred to as BioSteel (Keefer *et al*., 2002; Baldassarre and Karatzas, 2004). The BioSteel could be used to produce fine surgical sutures (soluble) and artificial ligaments (Keefer *et al*., 2002).

Transgenic animals can be produced by several techniques, including DNA transfer by retroviruses, microinjection of genes into pronuclei of fertilized ova, injection of embryonic stem or germ (ES or EG) cells into the blastocyst cavity previously exposed to foreign DNA, sperm-mediated exogenous DNA transfer during IVF or through ICSI, liposome-mediated DNA transfer into cells and embryos, electroporation of DNA into sperm, ova or embryos, biolistics and NT with somatic cells, ES or EG cells. However, the most common method for the generation of transgenic animals is the microinjection of DNA constructs into the pronuclei of zygotes. While the procedure is somewhat reliable, it is rather inefficient procedure yielding less than 1% of microinjected oocytes as transgenic offspring (Echelard *et al*., 2000; Baldassarre *et al*., 2003b) due to low integration of foreign DNA into the host genome and variable expression due to position effects (Pursel and Rexroad, 1993; Wall, 1996). Therefore, large number of recipients are required to produce a few transgenic animals (Ebert *et al*., 1991; Baldassarre *et al*., 2002, 2003a). Anyway, apart from gene pharming, production traits such as growth rate, quantity and composition of milk, fibre production or disease resistance could benefit from transgenic technology (Wall, 1996). However, this is to be noted that enormous amounts of resources are needed to produce a transgenic livestock and the cost for one expressing transgenic animals is extraordinarily high (Niemann and Kues, 2000). Although the opportunity for such applications is envisioned in the near future, however, the widespread implementation will not occur unless technical efficiencies and costs decrease dramatically (Baldassarre and Karatzas, 2004).

Marker-assisted selection: Various aspect of goat production has been benefited from the use of different ARTs. However, to achieve directed genetic improvement, the genes controlling the desirable and undesirable traits must be characterized (Basrur and King, 2005). From the available literatures it is found that this has not been accomplished in goat yet. Techniques involving the generation of gene marker (DNA marker) based on the molecular data and the creation and use of genetic maps as selection criteria for breeding i.e., marker-assisted selection (MAS) may help to achieve this goal, especially in cases where pedigree data are not available or the targeted traits are of low heritability (Dentine, 1999). The success of MAS in breeding goats and other livestock has been negligible to date, especially with regard to economically important traits in these animals that are expressed as non-discrete phenotypes (Basrur and King, 2005). Markers are important in screening genetic defects and when genes are introduced from other populations, the markers can be used to track their segregation in the populations (Amoah and Gelaye, 1997). The MAS has little to no risk to the animal donating blood, sperm or embryos for an assay and therefore, imposes no risk to the consumers. Therefore, MAS is expected to be most commonly used techniques in association with other goat ARTs in near future.

CONCLUSIONS

The present state of goat ARTs show a great deal of promise. The last two decades have dramatically changed the management of goat breeding, through improved nutrition and veterinary assistance. However, despite these improvements, AI is the only ART applied in selection programs. Among the ARTs described in this current paper, some of the techniques for example superovulation and all *in vitro* technologies, including cloning, still have a large margin for improvement. It is essential to improve the efficiency of these *in vitro* technologies and also to solve problems with fetal development. Anyway, the understanding of the control of early embryo development at the molecular level may lead to the discovery of factors affecting fetal and placental development and causes of fetal and gestational

abnormalities seen with embryos produced *in vitro*. The ARTs, for example cloning or transgenesis, which are expensive and technically complex, are already being adopted by a number of biotechnological companies and the production of rc-proteins from transgenic goats is very close to reaching commercial applications. Nevertheless, the application of these ARTs in goat production would depend on efficiency and acceptance by consumers.

ACKNOWLEDGMENT

The authors wish to thank Islamic Development Bank (IDB) for providing an IDB Merit Scholarship to the first author.

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