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Advanced assisted reproduction technologies (ART) in goats

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Abstract

Assisted reproduction technologies (ART) are reviewed with special emphasis on goat genetic improvement programs. Estrous synchronization and artificial insemination are the most commonly used ART worldwide because of their simplicity and excellent cost/benefit, especially when proven sires are used. Multiple ovulation and embryo transfer (MOET) has not become widely used due to its unpredictability. In vitro embryo production using oocytes collected by laparoscopy from valuable donors has the potential to improve the results obtained from MOET and expand its applications (for example, using prepubertal donors). However, the costs and inefficiencies of the system might restrict its use to special situations. Finally, transgenesis and cloning are expected to have a significant impact on the future genetic improvement of livestock. However, because of low efficiencies and high costs, their present use is restricted to applications with high returns such as the production of recombinant proteins of pharmaceutical and biomedical interest.

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1. Introduction

The application of assisted reproduction technologies (ART) enables the rate of genetic progress to be increased (Nicholas, 1996; Vivanco-Mackie, 2001). Some of these techniques increase the selection differential (artificial insemination: AI; embryo transfer: ET) while others accelerate progress by shortening the generation interval (juvenile in vitro embryo technology or JIVET). ART allow animals of high genetic merit to produce more offspring than would be possible by natural breeding. Moreover, in combination with hormonal synchronization of estrus and ovulation, some of these techniques allow the production of offspring and milk in times of the year that are not the natural breeding period of seasonally reproductive species such as the goat (Corteel et al., 1988; Chemineau and Cognié, 1991).

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Artificial insemination is the most widely used ART 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). Combined with an appropriate system for sire evaluation (progeny testing), AI offers a relatively simple and low cost method for dissemination of valuable genes. The use of frozen semen facilitates international exchange of genetic material, allows the use of semen in both the reproductive and non-reproductive seasons, and extends the effective reproductive life of a valuable male beyond its own life. AI and heat synchronization are a 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.

Multiple ovulation and embryo transfer (MOET) is often referred as the ART that is “to the female, what 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. While the statement is theoretically correct, MOET has not yet become a widespread tool for genetic improvement for a variety of reasons that will be discussed later in this review including its costs, technical demands, and variable and unpredictable efficiency (Baril et al., 1993; Cognié, 1999; Cognié et al., 2003).

Laparoscopic ovum pick-up (LOPU) in combination with in vitro production (IVP) of embryos has the potential to improve the number of offspring produced by genetically valuable does (Tervit, 1996). The procedure is less traumatic than surgical embryo recovery, so it can be repeated more times during the reproductive life of a female and, as will be discussed, it is more predictable than multiple ovulation (superovulation).

Transgenesis and somatic cell nuclear transfer (SCNT) have the potential to play an important role in accelerating/facilitating genetic improvement, assuming that they become more efficient and less costly (Wall, 1996; Wheeler et al., 2003). Currently, the main use of these technologies in goats is for the purpose of generating and propagating transgenic founder animals that produce valuable recombinant proteins of pharmaceutical or biomedical value in their milk (Ebert et al., 1991; Baguisi et al., 1999; Baldassarre et al., 2002, 2004b).

2. Hormonal synchronization of estrus and ovulation

Synchronization of estrus and ovulation (SEO) is a key component of all the ART-protocols and has a major influence in the overall efficiencies of these programs. Since goats are seasonal breeders, SEO plays a major role in allowing AI, MOET and LOPU to be performed during the non-breeding season.

The most widespread method of heat synchronization used in goats involves progesterone/progestagen treatment for 9–11 days followed by a luteolytic dose of prostaglandin (or an analogue) administered in the period 48 h prior to sponge removal. Depending on the local availability of licensed hormones, the progesterone/progestagen treatment may be delivered by means of an intravaginal sponge, a CIDR or a subcutaneous implant (Evans and Maxwell, 1987; Ritar et al., 1989; Freitas et al., 1997).

For the purpose of performing AI, as well as for the synchronization of embryo recipients, the synchronization treatment is supplemented with a dose of 250–500 IU of eCG which

is administered with the luteolytic agent, i.e. in the period 48 h prior sponge removal. The eCG promotes follicular development and synchronizes ovulation, allowing AI/ET to be performed at fixed times. The dose of eCG should be adjusted for each animal based on season, breed, weight, age, desire for multiple kidding and, where available, previous response.

Repeated use of eCG has been reported to result in poor fertility in fixed-time AI programs. These results have been attributed to the presence of anti-eCG antibodies developed as an immune response to previous treatments (Roy et al., 1999; Drion et al., 2001). The presence of such antibodies has been clearly linked to a delay in the occurrence of estrus, LH peak and ovulation in the synchronized animals, which may explain the lower fertility if the fixed insemination time is not modified accordingly.

The use of progestagen sponges in “long protocol” treatments (18–21 days) does not require the use of a luteolytic agent, but has been shown to result in lower fertility rates, possibly due to poor semen transport (Corteel et al., 1988).

Rubianes et al. (2003) proposed the use of a “short priming protocol” in which goats are treated with progestagen for 5 days and 200–300 IU of eCG is administered at sponge/CIDR removal. They reasoned that progestagen treatment for longer than 5 days results in sub-luteal concentrations of progesterone that promote excessive growth and persistence of the largest dominant follicle, leading to lower fertility following AI. Using their proposed “short priming protocol” and eCG, a 68% pregnancy rate was achieved following fixed-time AI (54 h from sponge removal) with fresh semen.

Ovulation can be synchronized more precisely by administering GnRH around the time of estrus (Pierson et al., 2003). This improves the success of fixed-time insemination, and the collection of ova/embryos at a controlled stage of development for specific applications such as oocytes for SCNT and zygotes for pronuclear microinjection.

3. Artificial insemination

AI may be regarded as a first generation ART and it is the one that has made the greatest contribution to genetic improvement programs, mainly due to well-established methods for identifying males with the highest genetic merit. Three methods of semen preservation (fresh, refrigerated and frozen) and three techniques of insemination (vaginal, cervical and intrauterine) are used worldwide in goats (Evans and Maxwell, 1987; Chemineau and Cognié, 1991; Leboeuf et al., 2000).

Fresh semen is the preferred method of preservation when the male is present in the herd, especially during the breeding season when semen production and quality are at their peak. The use of refrigerated semen is a common strategy in circumstances where a particular male is shared by a group of farmers located within a relatively small area. In such cases, the semen is stored at $\sim 4^{\circ}\text{C}$ and can be used for up to 24 h from collection. Finally, semen is frozen for long-term preservation allowing it to be marketed over a wide area, used throughout the year, and conserving the genetic material in case the animal dies.

Freezing of goat semen is technically challenging due to the presence of bulbourethral gland secretions (lipases) which interact with egg yolk and milk to create substances that are toxic to sperm (reviewed by Leboeuf et al., 2000). This 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. More recently, commercial extenders with no biological components have been developed to improve sanitary safety in semen processing (Hinsch et al., 1997; Gil et al., 2003). Although these extenders were originally developed for cattle, preliminary data from our laboratory indicate that Bioexcell[®] (IMV, L'Aigle, France) can be used for goat semen cryopreservation, without the need to centrifuge the semen prior to addition of the extender (unpublished).

In general, the method of semen preservation dictates the preferred method of insemination. A good practical guide is “the more damaged the sperm is, the deeper semen needs to be deposited in order to achieve good fertilization rates”. Vaginal insemination is successful for fresh semen, whereas intra-cervical insemination is used for refrigerated and frozen semen. However, in order to achieve high pregnancy rates (>70%) with frozen semen, intrauterine deposition of semen is required. While in many does it is possible to by-pass the cervix and deposit the semen intrauterine, in certain categories of animals (e.g. doelings), breeds (e.g. Nigerian dwarf) and individuals this will only be possible using a laparoscopic technique, making the procedure more technically challenging (Evans and Maxwell, 1987; Chemineau and Cognié, 1991).

Maxwell et al. (1999) demonstrated that re-suspension of frozen-thawed semen in seminal plasma, resulted in a significant increase in pregnancy rate after cervical insemination in sheep. It would be interesting to see if similar results can be achieved in goats, but such experiments are pending.

The use of sex-sorted sperm for AI 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. Sex-sorted semen has been used successfully in several species including cattle (Seidel et al., 1999), horses (Buchanan et al., 2000), pigs (Rath et al., 2003), and sheep (Hollinshead et al., 2002) but not in goats. However, it is expected that this technology can be applied to goat semen (David Cran, personal communication).

4. Multiple ovulation and embryo transfer (MOET)

Many practitioners consider MOET to be the most frustrating of all ART, since the results can vary from complete failure to total success without any variation in the standard operating procedure. The main factors contributing to the unpredictability of this technique are the variability of the superovulatory response, the poor fertilization associated with high ovulatory responses, and early regression of corpora lutea (reviewed by Cognié, 1999; Cognié et al., 2003). These unpredictable results, combined with high costs and the use of surgical procedures for collecting and transferring embryos, have prevented large-scale use of MOET in goat improvement programs.

An average of six to eight transferable embryos per donor can be produced in a successful goat MOET program (Baril et al., 1993; Cognié, 1999; Cognié et al., 2003). These results, however, depend on many factors (including breed, age and nutrition) that contribute to the high variability. It is common for the number of transferable embryos to range from 0 to

30 per donor with 25–50% of the donors failing to produce any transferable embryos due to fertilization failure and early regression of corpora lutea (ERCL).

Variation in superovulatory response is believed to reflect the follicular population present at the initiation of gonadotropin treatment (Gonzalez-Bulnes et al., 2003), which is not controlled by standard superovulation protocols. Several strategies have been suggested for increasing the number of small recruitable ovarian follicles at the time of FSH treatment, while avoiding the presence of large (dominant) follicles. Some of these strategies include the use of GnRH agonist/antagonists and the administration of FSH shortly after an induced estrus/ovulation. Pre-treatment with a Buserelin implant (PepTech Animal Health, NSW, Australia) one week prior to superovulatory treatment did not improve the response of superovulated oocyte and embryo donors (Baldassarre et al., 2001). It is possible that GnRH pre-treatment should be administered for more than 1 week to deplete the pituitary of gonadotropins and allow the ovary to build-up a large number of small follicles. Pre-treatment with Antarelix (GnRH antagonist) for 10 days prior to superovulation resulted in an increased number of small follicles at the time of FSH administration and an increased number of ovulations (Cognié et al., 2003). However, this improvement in superovulatory response did not yield a larger number of transferable embryos because of poor fertilization (>30%). A so-called “day 0 protocol” has been proposed recently to avoid the deleterious effects of large dominant follicles and improve results from superovulation (Menchaca et al., 2002). This protocol is based on initiating FSH administration immediately after ovulation and resulted in a 30% increase in the number of CL following superovulation, but only a small number of does was treated. However, improvements in terms of number and quality of embryos recovered have not been reported.

Poor fertilization may, partly, be due to poor sperm transport following heat synchronization (Evans and Armstrong, 1984) as well as to poor synchrony between time of insemination and ovulation. The first of these problems may be avoided by the use of intrauterine (laparoscopic) insemination. The second may be improved by synchronizing ovulation by GnRH administration around the time of heat detection and AI, as discussed previously. Alternatively, Baril et al. (1996) improved the synchrony of ovulation in superovulated goats by administering a GnRH antagonist 12 h after sponge removal, followed by LH administration 24 h later.

Finally, ERCL may affect up to 30% of the donors in a given program (Pintado et al., 1998). If laparoscopy is used to evaluate the superovulatory response prior to surgery, uterine flushing should not be performed if ERCL is evident since, in most cases, this will be associated with poor recovery of mostly non-fertilized and/or degenerated ova. The causes of ERCL are not fully understood, but its occurrence has been associated with inadequate nutrition (Jabbour et al., 1991), the use of eCG in superovulatory regimes (Pintado et al., 1998), and stress (Baldassarre et al., unpublished observations). Battye et al. (1988) demonstrated the involvement of prostaglandin in premature luteolysis, and suggested the use of flunixin meglumine between ovulation and embryo recovery to increase the recovery of transferable embryos. Other strategies include avoiding the use of eCG in superovulatory regimes (Armstrong and Evans, 1983), and the use of ovulatory treatments (hCG/GnRH) on days 3–4 to neutralize the negative effects of large growing follicles (Saharrea et al., 1998).

5. Laparoscopic ovum pick-up (LOPU) followed by in vitro embryo production

In vitro production of embryos using immature oocytes recovered by laparoscopy has the potential to overcome some of the problems associated with standard MOET techniques (Tervit, 1996; Baldassarre et al., 2002; Cognié et al., 2003). The procedure can be repeated more times since it is less traumatic than standard surgical methods used to recover uterine stage embryos. Moreover, this approach obviates several causes of the poor results observed with superovulation, such as poor ovulation rate, early regression of corpora lutea and poor fertilization. Reliability and reproducibility also are significantly better: while individual variation in the response to gonadotropin treatment remains, LOPU almost always results in >5 oocytes aspirated per donor (Baldassarre et al., unpublished observations). Additionally, the system allows the production of offspring from animals that would not be able to reproduce using AI or MOET, such as prepubertal animals. Finally, as discussed later in this paper, LOPU provides a good yield of oocytes for the production of zygotes for DNA microinjection (transgenic founder generation) or for recipient cytoplasts in nuclear transfer programs.

Snyder and Dukelow first described LOPU in 1974. They aspirated 21 follicles and recovered 6 oocytes from a sheep by laparoscopy. However, the potential of the technique was not fully realized until in vitro embryo production technologies were developed (Baldassarre et al., 1994, 2002; Graff et al., 1999).

The LOPU procedure is described in detail elsewhere (Baldassarre et al., 1994, 2003a). Briefly, donor goats are restrained on a standard laparoscopy table under 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. In the hands of an experienced operator, the procedure takes between 10 and 20 min per goat, depending on the number of follicles to be aspirated, which allows the recovery of over 100 oocytes in a 2–3 h session.

In order to recover high numbers of oocytes per LOPU session, the donor goats are heat synchronized and stimulated with gonadotrophins. Estrous synchronization is most often carried out using intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix[®], Upjohn, Canada) inserted 10 days prior to LOPU and administration of 125 µg Estrumate[®] (Malinkrodt, Canada) on the morning of the 8th day. Sponges are removed at the time of LOPU. Different hormonal regimes have been tested in both sheep and goats (Baldassarre et al., 1996, 2002). These include multi-injection FSH regimes as well as so-called “Oneshot” regimes in which a combination of FSH and eCG is given as a single treatment administered ~36 h prior to LOPU. Since results in terms of follicles aspirated and oocytes recovered were not different between treatments, our group has adopted the “Oneshot” regime as a standard procedure. In the “Oneshot” regime, goats are administered 80 NIH-FSH-P1 of Folltropin[®]-V and 300 IU of eCG in a single application 36 h prior to LOPU. Using this protocol, over the last four years we have conducted 1580 LOPU procedures and have recovered 21,219 oocytes (13.4 oocytes per goat) representing an average recovery rate of around 80% (unpublished).

Protocols for in vitro maturation and fertilization of LOPU-recovered oocytes have been described in detail elsewhere (Wang et al., 2002). Briefly, IVM is carried out in 50 µl drops of TCM 199 supplemented with hormones and 10% heat-inactivated estrous goat serum, at

Table 1

Follicles aspirated and oocytes recovered by laparoscopic ovum pick-up (LOPU; mean \pm S.D.) from adult vs. prepubertal (3–5 months of age) donor goats of standard breeds (from Koeman et al., 2003)

Age	N	Follicles	Oocytes	Recovery rate (%)
Prepubertal	23	39 \pm 4.5 a	28.4 \pm 3.5 a	73
Adult	21	19 \pm 1.4 b	15.9 \pm 1.5 b	84

Values in the same column with different superscript are significantly different (ANOVA, $P < 0.01$).

39 °C in a humidified incubator with 5% CO₂ in air for 24–27 h. IVF is performed in 50 μ l drops of TALP medium supplemented with 20% heat-inactivated estrous goat serum at 39 °C in a humidified incubator with 5% CO₂ in air. The fertilization drops are inseminated with pre-capacitated fresh semen at a final concentration of 1×10^6 sperm per ml. After co-culture of the gametes for 15–20 h, the resulting zygotes can be either transferred to the oviduct of synchronized recipient goats or cultured further in vitro until they reach uterine stages of development. Several media have been used successfully for the IVC of goat embryos to the blastocyst stage, including SOF (Keskinetepe et al., 1998; Cognié et al., 2003) and Gardner's sequential media G1–G2 (Koeman et al., 2003). We routinely transfer embryos to recipients within 24 h of IVF, as we believe this method avoids poor/abnormal development associated with in vitro culture of embryos (McEvoy et al., 2000). However, both systems (short and long culture) yield an average kids to oocytes ratio of approximately 10–20%.

An application of LOPU-IVP technology with great commercial interest is the propagation of genetically valuable goats at prepubertal ages, often referred to as JIVET. This interest is based on the fact that one can shorten the generation interval, in combination with the outstanding follicular responses that are obtained in prepubertal animals following gonadotropin stimulation. Using the same "Oneshot" regime previously described, but without the need to synchronize their estrous cycle with an intravaginal sponge, prepubertal goats can produce nearly twice as many oocytes per LOPU session, on average, as adult goats (Table 1). Studies performed by our group have shown that the follicular response is maximized in prepubertal goats >3 months of age (Table 2). In agreement with previous work in lambs (Earl et al., 1995; Ledda et al., 1999; Ptak et al., 1999), acceptable development to term was obtained following the transfer to recipients of in vitro produced embryos from oocytes collected from prepubertal goats (Table 3).

It is noteworthy that the application of this technology to kids >100 days of age results in the birth of their progeny at about the same time that the donor animals reach the age and

Table 2

Follicles aspirated and oocytes recovered by laparoscopic ovum pick-up (LOPU; mean \pm S.D.) from prepubertal goats of standard breeds at two different age ranges at the time of first collection (from Baldassarre et al., 2002)

Age range (days)	N	Average age (days)	Follicles	Oocytes	Recovery rate (%)
60–90	20	74 \pm 7	59.3 \pm 28 a	49.7 \pm 24 a	84
90–150	36	116 \pm 15	34.4 \pm 20 b	27.4 \pm 14 b	80

Values in the same column with different superscript are significantly different (ANOVA, $P < 0.001$).

Table 3

Early propagation of goats with valuable genetics: effect of donor age at the time laparoscopic ovum pick-up (LOPU) on the efficiency of LOPU/IVF (from Baldassarre et al., 2004a)

Variate/age at LOPU	<100 days age	>180 days age	P-value
Number of LOPU donors	5	5	n/a
Follicles aspirated (mean \pm S.D.)	57.0 \pm 16	28.0 \pm 5	<0.05
Oocytes recovered (mean \pm S.D.)	41.0 \pm 9	25.8 \pm 6	<0.05
Embryos transferred (mean \pm S.D.)	139	105	n/a
Embryos transferred/oocytes recovered (%)	67.8%	81.4%	<0.01
Recipients transferred	23	15	n/a
Pregnant (%)	9 (40%)	12 (80%)	<0.05
Kids born (mean/recipient)	15 (1.9)	27 (2.2)	<0.05

stage of development for normal breeding. This highlights the reduction in the generation interval that can be achieved. In summary, JIVET might be the most efficient application of LOPU-IVP technology since it has the potential to accelerate genetic progress by allowing the production of a large number of offspring from valuable animals, as well as shortening the generation interval.

6. Transgenesis and cloning

The production of valuable recombinant (rc) proteins in the milk of transgenic animals has been the subject of several recent reviews (Wall, 1996; Wheeler et al., 2003) and the production of transgenic goats has been reviewed recently (Baldassarre et al., 2004b). Goats are a particularly efficient means of producing rc-proteins since they produce considerable amounts of milk, and incur lower investment and maintenance costs than cows.

The traditional method for producing transgenic founder goats involves the microinjection of a DNA construct into the pronuclei of in vivo produced zygotes (Ebert et al., 1991). This method is reliable but rather inefficient due to random integration, resulting in unpredictable results in terms of transgenesis rate (usually <10% of kids born) and expression (0–10 g of recombinant protein/l of milk). Advances in the production of transgenic goats by pronuclear microinjection have been reported recently by using in vitro produced zygotes from LOPU-derived oocytes (Baldassarre et al., 2003a). This method increases the number of procedures performed in the life of each donor, is more predictable in terms of the number embryos/ova produced, and enables controlled timing of fertilization and, subsequently, DNA microinjection, which is a critical factor in successful integration.

Following the report of the birth of the first cloned sheep (Wilmut et al., 1997), further improvements in the efficiency of producing transgenic goats have been made possible by the use of somatic cell nuclear transfer (Baguisi et al., 1999; Keefer et al., 2001, 2002; Baldassarre et al., 2003b). The method allows the incorporation of a DNA construct into target cells while in culture using lipid mediated transfection, followed by the selection of donor cells for transfer based on proper integration. Although the reproductive efficiencies from NT-reconstructed embryos are significantly lower than those on PN-microinjection

(due to lower pregnancy and higher perinatal losses), all of the animals born are transgenic and most of them produce the rc-protein of interest in milk when induced to lactate (Baldassarre et al., 2003b, 2004b; Wheeler et al., 2003).

The application of transgenic/SCNT technologies to improve production traits of economic value, such as increased milk, meat and/or mohair production, has not been reported in goats. While the opportunity for such applications is envisioned in the near future, it is unlikely that widespread implementation will occur unless technical efficiencies and costs of producing transgenic and cloned goats improve dramatically.

7. Conclusions

Estrous synchronization and artificial insemination are the most widely utilized ART in genetic improvement programs for goats, due to their simplicity, relatively low cost and proven efficiencies. MOET reproducibility and cost/benefit efficiency need to be improved prior to widespread application. MOET will continue to be utilized in the propagation of elite stud animals and especially for the export of genetically superior livestock from countries where goat breeding is more developed. LOPU-IVP has great potential for more efficient propagation of valuable animals, but its use is restricted by the need for more demanding laboratory conditions than those required for MOET. Of special interest is the efficient use of LOPU for prepubertal propagation of valuable animals (JIVET), as discussed above. A specific application that is potentially very beneficial is the combination of JIVET with the import of frozen embryos of an exotic breed of goats into a particular region or country. In this case, the female kids born from the frozen embryos could be subjected to several LOPU sessions during pre-pubertal life, allowing rapid multiplication, which of particular significance in the early phase of development of newly introduced breeds/strains.

Goat production traits including the modification of milk composition, increase of growth rate or the improvement of mohair fiber production may benefit from transgenic technology. However, for this technology to be widely used to improve these production traits, the genes that influence the specific traits need to be identified, efficiencies need to be improved and, equally important, the costs of achieving and assessing the genetic gain must be reduced.

Similarly, cloning has the potential to improve the efficiency of transgenesis in these applications, as well as a role for the multiplication of animals of proven production. However, issues of high cost, low pregnancy and survival rate, and lack of genetic diversity need to be resolved before it becomes part of livestock improvement strategies.

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