Proceedings of the Society for Theriogenology 2013 Annual Conference

Aug. 7-10, 2013 – Louisville, KY, USA

www.therio.org/

Next SFT Meeting:

Aug. 6-9, 2014 – Portland, OR, USA

Reprinted in the IVIS website with the permission of the Society for Theriogenology
Assisted reproductive technologies in small ruminants
J.E. Romano
Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX

Assisted reproductive technologies or techniques (ARTs) refer to achieving a pregnancy by artificial means or partial artificial means.1,2 However, there is not complete consensus about this definition, because ART for others includes all the fertility treatments in which both female and male gametes are handled.3 The first ART was artificial insemination (AI). Then the following ARTs were developed: embryo transfer (ET), in vitro production of embryos (IVP), assisted fertilization techniques (AFTs), gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), and cloning.1,2 Most recent ARTs are the use of sexed semen, cytoplasmic transfer to zygote or oocytes, and others. Most of the ARTs are inter-related to each other; for example, the production of an embryo by in vitro fertilization will require embryo culture and further embryo transfer procedure to obtain an offspring.

Keywords: Artificial insemination, embryo transfer, intracytoplasmic sperm injection, cloning, assisted reproduction

Artificial insemination
Artificial insemination consists of the introduction of semen into the female genital tract with the help of some instrument.4 It could be regarded as the oldest and most widely applied ART in livestock. Artificial insemination has changed the small ruminant industry and has fostered genetic improvement by using proven sires, storage of male genetic material, and use of this genetic material independent of time and space; it has also provided control of reproduction, allowed breeding of females out-of-season, and restricted potential diseases that could be transmitted through the semen.5-7 In general, AI is a simple, economical, and successful ART.

The equation of fertility for AI (also known as Bartlett’s equation) is based on four components: estrus detection, estrus fertility, semen, and technician. The percentage of pregnancies resulting from AI is the product of the four factors and not the average of the four factors. The final results are multiplicative rather than additive.8

In small ruminants, estrus synchronization programs are used to concentrate estrus for either estrus detection or for AI at a fixed time. On the other hand, estrus synchronization protocols are not free of potential negative effects. In ewes, a substantial reduction in the total number of spermatozoa in the different organs (cervix, uterine horns, oviduct) of the female genital tract and times (samples collected at 1, 12, 24, 36, and 48 hours after AI with fresh semen) after estrus synchronization with intravaginal sponges compared with natural estrus was noticed.9 The transport and survival of spermatozoa were affected by the progestogen-treated ewe.9,10 The minimum number of fresh spermatozoa for natural or synchronized estrus is around 100 to 300 to 500 million, respectively.10-12 In general, AI at a fixed time requires that the insemination be performed between 45 and 60 hours after pessary removal. However, the interval between intravaginal pessary removal and estrus, is affected by different factors and modifies the time to ovulation.14,15 In small ruminants, a large variation in the time of ovulation after standard progestogen pessary treatment that has been shown to exist between some flocks may have resulted in less appropriate timing of insemination in some of the flocks/herds.16-19 The factors could be responsible for the variation found among and within experiments using the same estrus synchronization protocols, the same batch of the same drugs, and the same personnel.16-19 Among the factors that influenced this interval are: drugs used for estrus synchronization,20-22 presence of a male at the time of pessary removal,23,24 presence of females around estrus at the time of pessary removal,25 climatic conditions,26 stress,27 level of nutrition,28 use of equine chorionic gonadotropin (eCG),29 time of administration of eCG,30 and dose of eCG.31 In addition to these external factors, the potential influence of the stage of follicular ovarian activity could also be an internal factor that affect this interval.
The semen used for AI in small ruminants can be fresh, diluted and fresh, diluted and refrigerated, or frozen-thawed semen.4

In small ruminant AI, there are three basic possibilities for where the semen is placed: vaginal (VA-AI), cervical (CE-AI), and intrauterine through the cervix (transcervical AI, TR-AI) or through the abdomen (laparoscopic AI, LA-AI). The site of placement of semen has influence on pregnancy rates. There is a marked effect from vaginal, cervical, to uterine deposition on pregnancy rate.32 The deeper the placement of the semen, the better the pregnancy rate.33,34

Sire effects on pregnancy rate have been described with the use of fresh semen,11 with frozen semen after cervical insemination,35-37 and after laparoscopic insemination.17,38,39 In sheep, from several parameters studied on frozen-thawed semen, only the total and motile numbers of spermatozoa per inseminated ewe were associated with fertility (r = 0.25 and 0.26, respectively).17

In general, the fertility of refrigerated or frozen semen is lower than the fresh semen for the same quantity of spermatozoa per inseminating dose.7,40 The storage of semen, either fresh, refrigerated, or frozen, causes ultrastructural, biochemical, and functional damage to the spermatozoa that results in reduction of motility, viability, and fertility compared with non-stored spermatozoa.7 Different studies have revealed that preservation techniques induce sublethal damage that affects the functional capacity of spermatozoa; as a result, the spermatozoa behave differently from fresh spermatozoa even if they are fertile.41,42 Irrespective of the type of extender/diluent or the technique of storage used, many spermatozoa show reduced motility and viability (longevity) and decreased fertility when maintained in refrigeration or frozen storage.12,35,42-44 The low fertility rate of the frozen semen can be reverted by depositing the spermatozoa directly into the uterus.

Differences among semen sires in surviving the freezing and thawing process were detected. The reasons are unknown. Thus, knowledge of the ability of each sire is essential to determine the correct number of spermatozoa per each inseminating unit, as in the cattle, is required.45 In general, wide variation in total number and motility after thawing among inseminating units has been found.35,46 A difference from cattle, small ruminant’s fertility is affected by multiple factors that interact with each other, making recommendations difficult. The number of spermatozoa per inseminating dose depends, among other factors, on: type of insemination procedure (vaginal, cervical, intrauterine transcervical, or laparoscopic), type of semen (fresh, fresh diluted, refrigerated or frozen-thawed), time of the year (in-season or out-of-season), type of estrus (induced or natural), category of animal (pluriparous vs. nulliparous), and physiological state (lactating or dry).

Goat semen presents important differences compared with ram semen regarding the type of extender used for freezing semen. Goat semen has an inherent difficulty due to seminal plasma exposed to egg-yolk or milk-based extender that affects the viability of spermatozoa. Goat semen has an enzyme from the Cowper’s glands (bulbourethral glands) which acts on lecithin of egg yolk producing lysolecithin that is toxic for the spermatozoa (egg yolk coagulating enzyme, EYCE.47,48 In addition, seminal plasma contains a second enzyme that acts with some components of milk-based extender, depressing the survival of spermatozoa in vitro.49 Different approaches have been used to deal with this situation. First, use extenders that do not contain any egg yolk.50 Second, wash the semen to remove seminal plasma51,52 and then use extender containing a high percentage of egg yolk. Third, use extenders containing egg yolk at no more than 2% at the final extender semen dilution.53,54

Removal of seminal plasma by washing the spermatozoa (once or twice) immediately after semen collection increases the percentage of live cells, percentage of motile cells, motility rate, and percentage of normal acrosomes after thawing when extenders containing egg yolk, milk-based extender or TRIS are used.51-54 The effect of washing was better for spermatozoa collected during the breeding season than for out-of-season collections.30 On the other hand, elimination of the washing protocol is an important component to decrease spermatozoa loss and the mechanical damage to spermatozoa.7,55 Different solutions have been used for washing the semen such as Krebs-Ringer phosphate buffer (with or without glucose), extenders without glycerol, and others.51,52 The semen and washing solution ratios used were from 1:5 to 1:10, and centrifugation was for 10 to 15 minutes at 600 to 1000 g.51,52
The use of components of animal origin for extender preparation such as egg yolk, seminal plasma, bovine serum albumin, and milk proteins, increases concerns about the potential risk of microbial contamination by these products. Soybean lecithin (from 1 to 2%, weight/volume) is a real alternative as a chemically defined pathogen-free component to be part of the extenders instead of products of animal origin. Different studies have shown that these new extenders preserve the semen adequately after freezing and thawing compared with diluents containing products of animal origin. In small ruminants the method of dilution of semen for further freezing is based either on a constant rate (volume/volume) or constant spermatozoa concentration. When the semen is diluted to a constant concentration, the quantity of glycerol/cell remains constant, compared with the constant rate (volume/volume ratio; 1:2 to 1:5), where the quantity of glycerol/cell is variable. Both approaches have produced successful pregnancies.

In rams, length of storage in liquid nitrogen (LN) does not affect the fertilizing ability of frozen spermatozoa. However, in bucks some studies found a decrease in percentage of motile spermatozoa during storage when skim milk-glucose extender or tris-glucose-citric acid-yolk extender was used. In small ruminants, different reproductive parameters are used to evaluate the performance of a program. Estrus response is the number of females that showed estrus during a certain time from all the females that were submitted to treatment. The fertility rate is measured either as nonreturn rate, conception rate, pregnancy rate, or lambing/kidding rate. The nonreturn rate is based in the number of females bred that do not return to estrus after exposure to a male; for example, nonreturn rate at 25 days. The conception rate is the number of pregnant females compared to the total number of females that were detected in estrus and bred. The pregnancy rate is the relation between the number of pregnant females and the total number of females in the breeding program. In the case of AI at a fixed time, the conception rate is identical to the pregnancy rate. The lambing/kidding rate is the relation between the number of females that lambed/kidded and the total number of females at the beginning of the breeding program. Fecundity is defined as the number of offspring produced and the total number of females that lambed/kidded. Prolificacy is defined as the relation between the total number of kids/lambs produced and the total number of females at initial at the breeding program.

Vaginal insemination
Vaginal insemination is based on the deposition of an inseminating dose of semen in the anterior vagina without localization of the external cervical os. This type of insemination requires the use of fresh or diluted semen containing a high number of spermatozoa for optimal pregnancy rate. In this insemination, there is a lot of semen loss due to backflow of spermatozoa. It is not recommended with the use of frozen-thawed semen because the low fertility due to impaired transport of sperm through the ewe cervix when deposited in the vagina and/or entrance of cervix. The use of frozen-thawed semen by this route requires a high number of spermatozoa to obtain a satisfactory fertility rate.

Cervical insemination
Cervical insemination is accomplished when semen is deposited into the cervical canal. There are different degrees of deposition, from shallow to deep. In this technique careful observation of the external cervical os is required. If large amounts of mucus are present in the vaginal fornix drainage is mandatory. It has been reported in sheep and goats that deep deposition of semen into the cervix results in the highest pregnancy rate or lambing or kidding rate. Cervical insemination could utilize fresh non-diluted semen, fresh diluted semen, or refrigerated diluted semen. The use of frozen-thawed semen containing high numbers of spermatozoa is only recommended for deep cervical insemination. In case of natural estrus or estrus synchronized with progestagen the minimum quantity of fresh spermatozoa per inseminating dose is not less than 100 and from 400 to 500 million, respectively. The minimum number of frozen-thawed spermatozoa is between 600 to 700 million per female in estrus or more. Two cervical insenminations with frozen semen (1600 million of spermatozoa/dose) 12 hours apart during natural estrus resulted in a lambing rate of 50%, however, this number of spermatozoa is more than 30 times higher than the dose used for intrauterine insemination by laparoscopy.
Intrauterine transcervical insemination

In general, the goat cervix can be penetrated and the inseminating dose deposited into the uterus in around 25% to 60% of multiparous females. Transcervical insemination is possible in does in either the standing or the over-the-rail position. In ewes, the cervix presents a series of anatomical characteristics that make penetration of AI pipette difficult due to its length, presence of multiple rings, caudally-oriented blunt annular folds, and rings positioned in an eccentric pattern. In general, deposition of semen more than 10 to 15 mm into the cervical canal is not possible. In ewes, the intrauterine insemination by the transcervical approach requires a special restraint system for the female and the cervix as well as special inseminating equipment. Several factors influence the possibility of passing through the cervix in ewes; these include season, category of animal, parity, interval from lambing to insemination, breed, estrus, stage of estrous cycle, and skill and experience of operator. Among other factors. The factors that increased the percentage of success of TR-AI were AI during the breeding season, AI in pluriparous ewes, AI within three to four months postpartum, AI while the animal was in estrus and when performed by experienced operators. New catheters for TR-AI are in development with promising results; however, more research is required.

In sheep, the number of spermatozoa used by TR-AI were reported to be 80 million, 150 million, 200 million, and 400 million. In general, the numbers of spermatozoa used for TR-AI are higher than the recommended numbers used for LA-AI.

The pregnancy rate from TR-AI using frozen-thawed semen was reported to be 19%, 26%, and 50%. These outcomes of TR-AI on pregnancy rate are similar that of LA-AI. However, the overall fertility rate for LA-AI was always superior to TR-AI due to the difficulties of placing the catheter inside the uterus in all females.

Laparoscopic insemination

Semen can be placed into the uterus by laparotomy or laparoscopy. The low fertility rate from frozen semen can be reversed by depositing spermatozoa directly into the uterus by laparotomy. However, a high incidence of pregnancy loss following this approach was detected. However, the fertility was not affected when the same semen was deposited by laparoscopy. The latter method is quicker and less invasive and stressful for the inseminated females compared with laparotomy. The procedure needs to be performed in a clean, closed, and dust-free area. Laparoscopic artificial insemination allows semen to be placed directly into the lumen of the uterine horns. The first report of this technique performed in ewes was by Killen and Caffery. It is recommended that feed and water be withheld from the females at least 12 hours before the AI procedure. In sheep, this procedure could be performed with no sedation or with tranquilization or general anesthesia depending on clinician preferences as well as country, state, or other regulations. Due to inherent characteristics of goats, females require deep tranquilization to general anesthesia. The female is placed in a laparoscopic cradle in horizontal position; the areas of puncture are sheared, and finally antisepsis is performed. Local anesthesia is applied to the selected area of trocar puncture. Later, the laparoscopic cradle is tilted head down to an angle between 40 and 60°. Pneumoperitoneum is carefully induced by introduction air or CO₂ through a Verres needle or intramammary catheter. Only a small volume of gas sufficient to separate the organs for easier observation and manipulation of the genital tract is required. Use CO₂ is preferred due to its high diffusion rate compared with air. Overinflation causes discomfort to the animal. The first trocar and cannula for the laparoscope (5 mm, 7 mm, or 10 mm diameter) is inserted into the abdominal cavity approximately 5 to 10 mm cranial to the udder and 5 mm to the left of the midline. The second
A trocar and insemination cannula is inserted into the right side of the abdomen. The inseminating tools are either a special modified glass pipette or a special insemination gun designed for that purpose. The recommended site of puncture for semen deposition is the major curvature of the uterine horn. The needle of the inseminating gun or pipette is stabbed into the perimetrium, passing through the uterine wall to the uterine lumen. The same operation is repeated in the other uterine horn. One indication of the correct placement is the lack of resistance to expulsion of the semen and absence swelling at the uterine stab site. Then the instruments are removed, and excess CO₂ is expressed from the abdomen; only the skin is sutured or stapled and sprayed with an antiseptic. The animal is put in ventral recumbence and observed until complete recovery. Then, the female is allowed to return to covered yards or pens for several hours prior to being let out to pasture. Reduce any unnecessary stress for the next four weeks.

In sheep, a linear increase in pregnancy rate from 14%, 38%, to 73% was detected when the numbers of frozen-thawed motile spermatozoa per insemination were augmented from 1, 4, 16, to 64 million, respectively. The same trend was reported by others. A minimum of 20 million normal motile spermatozoa is necessary for LA-AI in fine-wooled breeds; however, the more seasonal breeds need approximately 40 to 50 million normal motile sperm to achieve acceptable fertility rates. Lambing rates greater than 50% were only obtained when more than 20 million motile spermatozoa were deposited.

In goats, 100 million total spermatozoa produce a kidding rate of 61% in animals synchronized with intravaginal pessaries and eCG. In another study, the same number of spermatozoa produced only a 24% rate of kidding. In other reports, 200 million spermatozoa resulted in a pregnancy rate from 49% to 52%. In Alpine goats 100 million spermatozoa are recommended and for Saanen goats 60 million spermatozoa are recommended for LA-AI. In goats, LA-AI executed after estrus detection needs to be implemented between 12 and 24 after estrus detection when two estrus detections per day are performed (Romano, unpublished observations).

Embryo transfer

Embryo transfer is an ART by which at least one embryo is collected from a female (donor) and transferred to another female (recipient) that serves as a surrogate mother for the remainder of gestation. The first successful embryo transfers in livestock species were performed on sheep and goats in Texas. The indications of embryo transfer are multiple, and include an increased number of offspring from a donor with valuable genotype for high quality productive traits, introduction of new breeds, importation and exportation of embryos, control of transmission of certain diseases, preservation of endangered species, breeding during out-of-season, required for other ART (i.e., in vitro production of embryos), for interspecific embryo transfer, as potential diagnosis for repeat breeders and research purposes. Embryo transfer requires strict selection of the donor female and recipients, an adequate superovulatory treatment, breeding, embryo collection and evaluation, potential embryo conservation, and finally transfer of the embryo. Each of these steps is fundamental to obtain satisfactory results.

Multiple ovulation and embryo transfer (MOET) is one of the most frustrating ARTs in small ruminants, because the results vary from complete success to complete failure even when careful standard operating procedures are followed. The main factors that are involved in the process are: variability in the superstimulation response, poor fertilization rate or failure, especially in females with high ovarian responses, surgical approaches for embryo collection, and early luteal regression. These unpredictable results combined with high costs, difficulty of assessing the ovarian response, and use of surgical procedures for collecting and transferring the embryos, have prevented the use of MOET on a large scale.

The selection of the donor is the first step. The donor needs to be clinically healthy, free of internal and external parasites, have sound feet and legs, have good mothering ability, have adequate milk production, have previously lambed or kidded normally, and have a regular estrous cycle. Prior to
beginning the superovulation treatment, it is recommended that a reproductive examination including vaginoscopy and transrectal ultrasonography be performed. Also, collection of samples for testing for potential diseases is recommended, especially for export/import requirements. For the recipients, most of the same criteria listed for donors are required.

Superovulation could be performed during the breeding season as well as the nonbreeding season. Better response is obtained during the breeding season. Several gonadotropins have been used for superovulation, including eCG, follicle stimulating hormone (FSH; porcine, ovine), equine pituitary extract (ePE), horse anterior pituitary hormone (HAPH), and human menopausal gonadotropin (hMG). The two most frequently used products for superovulation in small ruminants are FSH and eCG (also called pregnant mare serum gonadotropin, PMSG). In general, the administration of FSH or eCG will start at the end of the luteal phase (day 12 in ewes and day 17 in does) or at the end of a progestagen treatment. Between 1000 and 2000 IU eCG is given at the time of pessary removal or one or two days prior to pessary removal as a single intramuscular injection due to its long half-life in vivo. If FSH is used, several protocols can be used for superovulation; most commonly the injection of multiple doses of FSH from two to four days, or a single injection of FSH mixed with a long release carrier or a single injection of a combination of FSH and eCG. In the multiple injection protocol, FSH was administered every 12 hours at a decreasing or constant dose. In general, FSH was superior to eCG in number of corpora lutea, embryos recovered, and transferable embryos. In addition, the proportion of nonovulatory follicles and short estrous cycle were significantly reduced with FSH compared to eCG. To produce luteolysis, a single or second dose of prostaglandin F2α is used at the fifth and sixth FSH injection in the three-day protocol or at the time of eCG administration or 24 hours later. In general, donors will show standing estrus at approximately 24 hours after progestagen removal and earlier than synchronized recipients; therefore, recipients need to have their intravaginal pessaries removed 12 to 24 hours earlier than the donors. The benefits of eCG are a single dose and low cost; however, the long half-life produces persistent ovarian stimulation that could produce variable results and affect the number and quality of embryos collected. In addition, in goats, repeated eCG treatments are followed by decreased fertility in females inseminated at a fixed time after treatment due to the production of antibodies against eCG. However, this response was variable depending on the goat population. The major histocompatibility complex (MHC) was involved in these immune responses to eCG. To reduce this deleterious effect of eCG, antibodies against it were used in donor goats and ewes and the results showed this to be an efficacious treatment that increases the number of viable embryos collected compared with eCG alone. A new alternative for superovulatory treatment in goats is the active immunization against inhibin which enhances ovarian follicular development and ovulation rate by promoting an increase in pituitary FSH secretion. The superovulatory response to FSH is positively correlated to the number of small follicles of 1 to 2 mm and negatively correlated to the number of large follicles determined by laparoscopic observation prior to gonadotropin administration.

The variable response to superovulation is the bottleneck of ET as it is in cattle. Multiple factors influence the response to superovulation, such as individual and group sensitivity to gonadotropins (there are breed differences in response), breeding season, age of donor, level of stress, nutritional status, drug used for superovulation, and batch number, among others. Donor females are bred by natural service, AI or a combination. In case of natural service, several controlled matings need to be performed per estrus. It is mandatory that donor males have a previous satisfactory breeding soundness examination. Hand mating is recommended every 6 to 12 hours throughout estrus. In donor ewes fertilization failure due to the effect of either the pessary or gonadotrophin treatment or both causes impaired spermatozoa migration. Therefore, to reduce the chances of this potential problem, fresh semen deposited by the intrauterine route (laparotomy or laparoscopy) is recommended. In donor does however, high fertilization rates occur after natural mating. The frequency of fertilization failure was high in does treated with eCG compared with FSH. Fertilization failure also took place when a high superovulatory response occurred.

Use of fresh or refrigerated semen requires cervical or transcervical depositions with high numbers of motile spermatozoa per insemination every 6 to 12 hours, with at least two inseminations per
estrus. Frozen-thawed semen is not recommended by the vaginal or cervical route. In sheep, frozen-thawed semen requires intrauterine deposition by laparoscopic or laparotomy insemination due to reduced viability and fertilizing capacity. However, intrauterine deposition of semen by laparotomy has been associated with a high level of embryo loss. In goats, it is possible to use intrauterine insemination by the transcervical approach; however, as mentioned previously, this is not achievable in all females. In does, fertilization failure is not a problem as in ewes after natural mating. Therefore, service every 12 hours is recommended. It is mandatory with the use of frozen-thawed semen that information about the percentage of normal spermatozoa and total and motile number of spermatozoa per dose be available.

One of the difficulties in small ruminants compared with large ruminants is the impossibility to assess the ovulatory response through palpation per rectum prior to embryo collection because most of the procedures for embryo collection are invasive. The use of laparoscopy or transrectal ultrasonography could be used to assess the number of corpora lutea to decide whether to proceed to embryo collection. These approaches are less invasive than laparotomy. The measurement of progesterone prior to the embryo collection at days six to eight is another possibility; however, this test requires time to determine the progesterone levels, and it is not precise enough to determine the total number of corpora lutea.

The collection of embryos can be performed by laparotomy, laparoscopy, or transcervical approach. The embryo collection from the oviduct can be performed at early stages or from the uterine horns after day four. Oviductal collection is performed on days three or four (estrus = day zero) and is basically performed by laparotomy. These embryos contain eight to 16 blastomeres and are easily differentiated from unfertilized oocytes. Oviductal collection is used in valuable donors that could otherwise undergo premature luteal regression. However, this recovery requires oviductal transfer if no culture (in vitro or in vivo) is obtained. The side effect of this collection is that the high level of ovarian and oviductal adhesions will reduce the reproductive life of the donor.

In ewes, optimum outcomes measured as number of corpora lutea and numbers of embryos collected were obtained by laparotomy and laparoscopy. The best results of uterine collection are between days six to eight after estrus (estrus = day zero). In general, an embryo with an intact zona pellucida is a requirement to reduce the potential of transmission of certain diseases through these embryos by washing multiple times, especially for import or export conditions.

Drawbacks of the surgical approach are stress to the animal, the requirement of general anesthesia and the formation of adhesions. Repeated laparotomies and handling of the reproductive tract can traumatize tissues and increase the incidence of abdominal adhesions, thereby reducing the potential of reproductive life of the donor female. After three procedures, there is a significant reduction of collection of embryos.  

Laparoscopy is less invasive; the incision wounds are smaller, and the incidence of infections is reduced. Additionally, there is less dehydration of tissue, less adhesion formation, and faster recovery than laparotomy. However, laparoscopy requires special equipment and highly skilled and trained personnel. In ewes, repeated embryo collection was possible up to three times a month with an efficiency of approximately 60% to 80%. The first nonsurgical embryo collection from small ruminants was reported in goats.

Nonsurgical uterine collection seems a viable alternative to conventional surgical and laparoscopic procedures. In goats, pretreatment with prostaglandin E₂ and estradiol 24 hours prior to embryo collection was effective to permit passage through the cervix; however, no information about embryo recovery rate was presented. In later research, laparoscopic collection had a significantly higher embryo recovery rate than transcervical collection (79% vs. 37%). Tranescervical embryo collection was successful (90% recovery) when prostaglandin F₂α was administered 16 or eight hours previor collection with oxytocin administered prior to flushing performed at day six. In sheep, nonsurgical collection by the transcervical route was performed; however, the percentage of success was only 42%, and only 74% of the ova were recovered. In ewes, transcervical passage was 100%, with a recovery of 65% in ewes pretreated with prostaglandin E₂ and estradiol cypionate 24 hours prior to collection. In a further study, the transcervical technique of embryo collection was successful in around 46% of the multiparous ewes and in about 5% of nulliparous ewes submitted for hormonal treatment of prostaglandin
E₂ and estradiol cypionate the day before collection. Transcervical embryo collection was 60% and similar to laparoscopic collection.¹²¹

Several flushing media for embryo collection have been used. Basically, all contain a balanced salt solution with a source of protein and antibiotics. The source of protein can be either homologous serum or bovine serum albumin. If serum is used, it should be heat treated at 56°C for 30 minutes to inactivate the complement and it should be filtered through a 0.45 µm Millipore filter before use. The most used medium for embryo collection is Dulbecco’s phosphate-buffered saline. Other media that can be used for flushing are Tissue Culture Medium-199 (TCM-199), Ham F-10 medium, and Brinster Mouse Ova Culture Medium-3. All these media are commercially available. TCM-199 with Hanks’ salts do not depend on CO₂ for buffering, but this medium with Earle’s salts, Ham-F10, and Brinster Mouse Ova Culture Medium-3 must be maintained under an atmosphere of 5% of CO₂ in air.⁹⁴,⁹⁷,¹¹⁰

The criteria of evaluation of small ruminant embryos are the same as those for cattle. The two criteria are stage of embryo development and cytological characteristics at embryo collection day. The embryo development needs to be in concordance with the day of embryo collection. Embryo flushing at day six is expected to collect compact late morula and early blastocyst. The collection of cleavage embryos at those days containing two to four cells suggests nonviable embryos, and presence of 16 to 32 cells means retarded development. Blastomeres should be reasonably symmetrical, uniform, and spherical, with a minimum of extruded cytoplasm. Considerable experience is required to classify embryos. The embryo can be graded using International Embryo Transfer Society criteria.⁹⁸ Good quality embryos can be used for freezing and/or immediate transfer. Low-grade embryos should be used only for immediate transfer.

Ovine and caprine embryos can be cultured for one or two days in bicarbonate- or phosphate-buffered media or be slowly cooled to 4°C for up to two to three days. This last approach may allow asynchronous recipients to be in the adequate day for the transfer with the temporarily arrested embryo. The embryos for transfer could be fresh, refrigerated, or frozen-thawed. Depending of the age of the embryos, oviductal or uterine transfer can be performed. Oviduct transfer requires in most cases a laparotomy approach. In general, more embryo transfers are performed with embryos of morula or blastocyst stages, therefore requiring uterine placement. The transfer of these embryos can be performed through laparotomy, laparoscopy, and the transcervical route. However, optimal pregnancy rates were obtained by laparotomy and laparoscopy. Embryo transfer by laparotomy involves some degree of trauma and often leads to the formation of adhesions that affect the uterus, oviducts and ovaries. This situation could temporarily or permanently affect the future reproductive life of the female. The advantages reported for laparoscopy are quicker transfer and reduction of genital tract adhesions with rapid recovery of recipient females compared with laparotomy. There is also a combined laparoscopy and laparotomy approach in which the endoscope is used to localize the uterine horn that is ipsilateral to the corpus luteum and then raised to the level of the abdominal wall. Then the embryo is transferred via micropipette.¹²² In ewes, laparoscopic transfer was performed through the paralumbar fossa in the standing position¹²³ or with general anesthesia in females in dorsal recumbency with and without exteriorization of the uterine horn.¹²⁴,¹²⁵ The number of embryos transferred varies from one to three embryos per recipient. It is recommended that embryos be transferred to the terminal half of the uterine horn ipsilateral to the ovary containing the most developed corpus luteum or corpora lutea.¹²⁵

Precise synchrony of estrus between donor and recipient is paramount. Asynchrony of plus or minus one day is acceptable; however, the best results are probably obtained when donor and recipient are at the precisely the same point in the estrous cycle.¹²⁶ In does 24 hours of asynchrony is well-tolerated compared with ewes, in which more than 12 hours of asynchrony is not well-tolerated based on pregnancy outcomes. Furthermore, the development stage of the individual embryo, rather than the day of estrous cycle of collection should decide which recipient day is used. For example, a compact morula should preferably be transferred to a recipient on day five or six rather than on day seven even though the donor was collected on that day.¹²⁷ The same principles are used for cultured embryos.

A good pregnancy rate after embryo transfer is between 55% and 65%.¹¹⁰ However, this outcome depends on recipient condition, type of embryo transfer, kind of embryo, number of embryos transferred,
place of transfer, and other factors. In goats, the pregnancy rate was not different between laparoscopic embryo transfer and transcervical embryo transfer (36% vs 39%, respectively).\(^{110,117}\)

The incidence of premature luteal regression in superstimulated goats is considerable and varies from 10% to 30% or higher. That has resulted in low embryo recovery rates with poor quality embryos.\(^{99,100}\)

Short estrous cycles have also been reported at the beginning and end of the breeding season, after estrus synchronization with luteolytics, and after abortion.\(^{95,99,128}\) Short estrous cycles occur between five and nine days after stimulated estrus. Short estrous cycles are due to anovulation and/or early luteal regression. The levels of peripheral progesterone during this period are low compared with does without early luteal regression. The recovery rate from short estrous cycles is reduced and the quality of embryos is low.\(^{95}\) The observation of ovaries by laparoscopy during short estrous cycles around days five to nine is characterized by the presence of small white-pink corpora lutea. Different approaches have been used to deal with this situation: use of progestagens during the initial stage of metestrus and diestrus;\(^{129}\) administration of antiluteolytic drugs during metestrus and early diestrus;\(^{130}\) administration of luteotrophic hormones such as human chorionic gonadotrophin or gonadotripin releasing hormone during metestrus;\(^{131}\) or collection of embryos at day three after breeding. In this last case, an oviduct flushing approach is required. In general, this estrus is estrus.

**In vitro production of embryos**

In general, this technology implies in vitro oocyte maturation (IVM), sperm capacitation, and in vitro fertilization (IVF) and in vitro culture (IVC) of early cleaving embryos until their development to morula or blastocyst stages (transferable embryos). The good quality transferable embryos can be cryopreserved. Some of these steps can be performed in vivo also and then moved to in vitro (oocyte can be from an ovulated follicle) and submitted to in vitro fertilization and vice versa.

In goats the first in vitro fertilization was obtained after capacitation of spermatozoa in a rabbit oviduct.\(^{132}\) The first birth using IVP on ovulated oocytes was achieved by Hanada.\(^{133}\) The first pregnancy from an oocyte matured and fertilized in vitro was reported by Younis in 1991.\(^{134}\) However, the first kid born from an oocyte matured and fertilized in vitro was informed two years later by Crozet.\(^{135}\) Finally, in 1994, the first kid was born after all the steps were performed in vitro (IVM/IVF/IVC).\(^{136}\)

In most studies in goats oocytes were recovered surgically by laparotomy,\(^{137}\) by laparoscopy,\(^{138}\) transvaginal ovum pick-up\(^{139}\) or after the slaughter of animals that had been treated with or without hormones.\(^{137,140}\) The oocytes were obtained from adult goats, prepubertal goats, and pregnant goats.\(^{141}\) In goats the fertilization rate of in vitro matured oocytes was the same as those from recently ovulated oocytes.\(^{137}\) When the entire ovary is available, the collection of oocytes can be performed by aspiration, puncturing, and slicing. The slicing method is simple, rapid and efficient compared with aspiration and puncturing.\(^{134,142}\) In prepubertal goats, the number of ovarian follicles is lower than in adult animals, making it difficult to collect high numbers of oocytes compared with adults.\(^{140}\) It is possible to obtain oocytes from pregnant females after hormonal treatment (eCG) with good results within the first four months of pregnancy.\(^{141}\)

Oocytes with three or more layers of cumulus cells showed better capacity for in vitro maturation than oocytes with fewer layers of cumulus cells or no cumulus cells.\(^{140}\) The development competence of goat oocytes was acquired progressively during follicular growth; oocytes collected from large antral follicles have the capacity to progress to the blastocyst stage, after in vitro maturation, fertilization, and culture.\(^{135}\) Frozen-thawed oocytes were used, but fertilization was low.\(^{143}\) Oocytes from small and medium-sized antral follicles yield a significantly lower proportion of blastocysts than those from large follicles.

Culture media used for IVM such as Tyrode albumin lactate pyruvic (TALP), modified Dulbecco’s phosphate buffered saline, Tissue Culture Medium-199 (TCM-199) with 20% heat-inactivated goat serum with or without hormones have produced satisfactory results. The duration of in vitro maturation is approximately 24 hours. Most investigators maintained a droplet of medium under paraffin oil and in a humidified environment of 5% CO\(_2\), 5% O\(_2\), and 90% of N\(_2\) at 38.5°C. The use of
FSH was effective at 10 µg/mL in the media.\textsuperscript{144} It was found that the combination of TCM-199 supplemented with hormones (FSH, luteinizing hormone, and estradiol) supplemented with 10% fetal calf serum was the most efficacious medium for IVM and subsequent embryonic development.\textsuperscript{145} The most frequent medium for in vitro maturation of oocytes is a culture medium supplemented with FSH, luteinizing hormone, estradiol, and 10% fetal calf serum. The extrusion of the first polar body at MII occurs in small ruminants between 16 and 24 hours after the beginning of maturation.\textsuperscript{95}

In vitro fertilization was obtained satisfactorily by using fresh semen. The semen was prepared by using heparin in the medium from 2.5 to 10 µg/mL depending on the male.\textsuperscript{146} The process requires an adequate level of calcium in the medium. Caffeine depresses the fertilization rate in goats.\textsuperscript{147} The number of spermatozoa per oocyte used was from 400 to 100,000 to 200,000 spermatozoa.\textsuperscript{137,145} The duration of IVF was also variable, from six to 24 hours.\textsuperscript{136,148}

Studies of IVF have shown that oocytes and sperm can unite in a different oviduct. For example, when oocytes and spermatozoa from goats are placed in the sheep oviduct, fertilization will occur (gamete intrafallopian tube transfer). The inverse also is possible, oocytes and spermatozoa from sheep in a goat oviduct. The fertilized oocyte can be cultured in vivo or in vitro. In vivo culture was performed in oviducts of the same or other species (xenoculture). The in vitro co-culturing of two- or four-cell embryos to obtain morulas or blastocysts was successful using oviduct cells rather than uterine cells or without co-culture.\textsuperscript{149} Fertilized goat oocytes placed into the oviducts of pseudopregnant rabbits developed to the morula or blastocyst stage.\textsuperscript{150,151} Synthetic oviduct medium supplemented with serum proteins and amino acids under specific levels of O₂, CO₂, and N₂ resulted in high levels of transferable embryos.\textsuperscript{152,153}

The cleavage embryo can be cultured in a medium without or with cells (co-cultured medium). Multiple studies have shown that there is an in vitro block at the stage of eight to 16 cells that can be overcome by using co-cultured cells with supplemented serum.\textsuperscript{154} More transferable embryos are obtained in co-culture systems.\textsuperscript{152} Different types of cells were used in the co-culture media such as caprine cumulus cells, bovine cumulus cells, oviduct epithelial cells, bovine oviduct cells and porcine uterine epithelial cells. Caprine cumulus cells produced a high proportion of morula forms; however, the caprine oviduct epithelial cells produce more blastocysts. Therefore, a co-culture that involves a sequence of two co-cultures using first caprine cumulus cells and then caprine oviduct cells seems to be appropriate.\textsuperscript{136}

In small ruminants, transfer of in vitro-produced embryos resulted in lower pregnancy rates compared with in vivo-produced embryos.\textsuperscript{153,155} This is probably because of differences in cell structure, biochemistry and other differences that can make these in vitro-produced embryos more sensitive to the freezing/thawing process.\textsuperscript{156}

**Intracytoplasmic sperm injection**

Intracytoplasmic sperm injection (ICSI) is the ART procedure that consists of the microinjection of single spermatozoa across the plasma membrane and inside of the cytoplasm of a metaphase II oocyte leading to fertilization. This method bypasses the process of spermatozoa selection and interaction with the oocyte; it could also use immotile or dead spermatozoa.\textsuperscript{1,2} Intracytoplasmic sperm injection was reported for the first time in hamsters.\textsuperscript{157} In small ruminants, this technology produced a lamb for the first time in ewes in 1996,\textsuperscript{158} it was later reported in goats in 1997.\textsuperscript{159} However, the first goat born by ICSI was described some years later in 2003.\textsuperscript{160}

The indications for ICSI are: previous failure of IVF, depression of semen parameters (low concentration, low motility, high levels of abnormal spermatozoa, and low total number of spermatozoa), frozen semen (low number of spermatozoa, low motility), few oocytes from in vivo- or in vitro-matured process for fertilization (also cryopreserved).\textsuperscript{1,2} In small ruminants, ICSI has potentially important applications in animal production systems, primarily its use with semen of valued animals, with testicular sperm, with epididymal sperm, oocytes from prepubertal females, cryopreserved oocytes, or other possibilities.\textsuperscript{161} Intracytoplasmic sperm injection was shown to be an effective technique to solve a problem when few straws of frozen semen from a ram with altered genes for hemophilia A were
available. The use of a small injection pipette was effective in producing a number of blastocysts that were transferred and produced numerous live offspring, of which some had the desired genotype.\textsuperscript{162,163} The use of sperm-mediated gene transfer and its combination with ICSI has the potential to be an interesting approach as a transgenic technique for transgenic animals.\textsuperscript{161}

**Cloning**

The first approach to produce cloned embryos was by embryo splitting.\textsuperscript{164,165} Embryo splitting produces monozygotic twins. A four- to seven-day-old embryo is surgically dissected with a glass knife or steel blade into halves or embryo parts. The demi-embryo is placed into the original or surrogate empty zona pellucida. This demi-embryo can be immediately transferred or can be cultured for one or two days to evaluate the development rate before transfer. Best results were obtained when two demi-embryos were transferred per recipient.\textsuperscript{166}

Another method is separating the totipotent blastomeres from the inner cell mass from early embryos and putting individual cells into a surrogate empty zona pellucida. This possibility will create more identical animals than the preceding procedure. Willadsen\textsuperscript{127} produced identical twins by microsurgical separation of the blastomeres of two-cell embryos, their insertion into a foreign zona pellucida, embedding them in a protective cylinder of agar and cultured in a ligated sheep oviduct. In a further study, blastomeres from four-cell embryos or eight-cell embryos were used, then cultured to develop to blastocyst stage and further transferred to recipient females producing pregnancies.\textsuperscript{167}

The third procedure for cloning consists of enucleation of oocytes in metaphase II (as a source of cytoplasm, named cytoplast), insertion of the donor cells (or nuclei, as a source of the nucleus, named karyoplast), activation of the reconstructed embryo, and in vitro culture. At the beginning of this new technique of cloning, the donor cells used were the totipotent blastomeres originated from the inner cell mass and transferred to an enucleated mature oocyte to produce a new embryo.\textsuperscript{168} These findings indicated that at least some nuclei derived from transcriptionally active embryos are totipotent and able to be reprogrammed to support full-term development when fused to enucleated secondary oocytes.\textsuperscript{169} This new embryo can be used for immediate embryo transfer or a source of more blastomeres. A further improvement of the type of cloning technique is to use embryonic cells rather than adult differentiated cells. This last procedure is named somatic cell nuclear transfer (SCNT). The first mammal cloned by using differentiated mature cells obtained from the mammary gland from a ewe was Dolly.\textsuperscript{170} Then two years later the first goat produced by this type of cloning was reported.\textsuperscript{171} This tactic destroyed an old myth in biology that a differentiation in cells cannot de-differentiate anymore and in addition opened a new concept in mammal reproduction, asexual reproduction.

The benefits of cloning by somatic nuclear transfer can be seen in superior genetic animals to increase their numbers (reproductive cloning) or in animals that cannot reproduce naturally or by using one of the other ARTs (ovarian or uterine pathology, orchiectomized or ovariectomized animals, terminal animals, or recently deceased animals).\textsuperscript{165,172} In addition, this technology is a powerful resource for understanding the cellular and molecular aspects of nuclear re-programming.\textsuperscript{172}

In general, a sterile skin biopsy from the ear containing fibroblasts (as the source of karyoplast) is obtained, then cultured in the laboratory, and finally used immediately for cloning or preserved in liquid nitrogen for further cloning. The source of the cytoplast could be in vitro or in vivo mature enucleated oocytes in metaphase II. Different cloning approaches for inserting the donor cells are used. The fibroblast is included in the perivitelline space of the cytoplast and then activated and electrically fused; only the nucleus of the fibroblast is injected directly into the cytoplast. Subsequently, the cloned embryos are cultured in vitro for a period, and when they reach the optimal stage for embryo transfer, they are transplanted into a surrogate mother animal. In general, the efficiency of this process is very low.\textsuperscript{172,173} There is a lot of loss, not only during the in vitro process, but also during in vivo process (high level of pregnancy loss). Different types of cells have been used as a source of karyoplasts such as fibroblasts, granulosa cells, Leydig cells, and others. A recent investigation opened a new dimension of potential cells such as somatic cells found in frozen-thawed semen, which can be used as nucleus donors to produce cloned blastocyst-stage embryos.\textsuperscript{174}

---

303  Clinical Theriogenology • Volume 5, Number 3 • September 2013

Proceedings of the Society for Theriogenology Annual Conference, Louisville, KY, USA - Aug. 7 - 10, 2013
Production of transgenic animals

Transgenic animals are expected to have high impact in the future genetic improvement of livestock.97 However, the drawbacks of this approach are low efficiency of the technique and high costs involved. There are growing numbers of recombinant proteins that have been expressed in milk. Commercial production of human pharmaceutical proteins in the milk of dairy goats has already been achieved. Transgenic goats carry the genes for a longer-acting form of tissue plasminogen activator (tPA); some goats are capable of producing this factor at level of 2 g/L of milk.175 Transgenic cloned goats were produced using fibroblasts transfected with human coagulation factor IX as a source of karyoplasts.176 Also, transgenic goats for human acid beta-glucosidase have been produced.177 Additionally, removing immunogenic factors that are expressed in milk could be an interesting way to control epidemic diseases through drinking milk. Therefore, this new concept of transgenic animals as bioreactors opens a new dimension for pharmaceuticals products. Efficient transgenic animal production provides several new opportunities for agriculture and medicine.172

Other technologies

It is possible to pre-select the sex of offspring from different species prior to fertilization with an accuracy of 85-95%.178 In small ruminants, sexed semen has been used for AI in estrus synchronized females,179 superovulated females,180 in vitro fertilization,181 and the possibility of used in ICSI.161 In sheep, the results are satisfactory compared with the results in cattle.179,180,182 Other new technology available is the use of ooplasmic (cytoplasm) transfer to an ovulated oocyte or zygote.183 This new technology could be used in certain cases of mitochondrial disorders.

References


78. McKelvey WAC: Recent research on AI and MOET in sheep and their commercial application in the UK. Proc NZ Embryo Transfer Workshop 1994, p. 16-17.