ADVANCES IN ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER IN SHEEP AND GOATS

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Reproductive technologies of AI and ET for sheep and goats are becoming more commonplace but still not widely practiced in North America. Costs are high relative to animal values, most procedures are invasive and results are generally inconsistent at best. But, over the last decade, new information and new ideas, much of it suggested by our improved understanding of ovarian activity through the use of transrectal imaging, is being tested under research and field conditions .

Artificial Insemination

Laparoscopic AI

Frozen semen AI in sheep is still most effectively delivered using the laparoscopic approach. It is faster, less labour intensive and, depending on the cost of semen, generally less costly pre lamb born than alternatives. In the past few years little has changed to improve results. But information on variation in timing of ovulation between individuals, breeds and seasons made available through the use of of transrectal imaging helps to explain the variation in results and encourages the use of GnRH administration and/or teaser males to enhance LH release (see B. Buckrell, advances in the control of reproduction in sheep and goat, in these proceedings).

Transcervical AI

Transcervical AI (TAI) has received considerable attention over the past 15 years. A system that is practical, humane and consistently successful would revolutionize artificial breeding of sheep and improve the systems presently used in goats.

The Guelph System[1], first developed and tested since 1988, showed considerable early promise under research and farm conditions. In the following decade over 400 units were sold and others copied and sold similar products. Its widespread use and testing did show that most sheep (about 70%) could be inseminated transcervically in a reasonable time but success of penetration and pregnancy results were variable and unpredictable. Few operators were patient enough to become competent and in many cases cervical injury resulted or was suspected. Still, in capable hands it was shown to be a low cost, non-invasive alternative to laparoscopic AI and is a system that can be used to pass the cervix in about 70 percent of ewes presented producing pregnancy rates in the range of 40 to 50% (or about 35% overall) when using good quality frozen semen in adequate numbers (generally $100x10^6$).

The Gourley scope (Dennis Gourley, Elite Genetics, Iowa) was built on the initial promise of the Guelph system by adding low cost industrial endoscopy to view the passage of the insemination equipment into the cervix. The Gourley system was expensive, suffered damage from rough handling and generally did not live up to its initial promise and is no longer available.

Lewis and co workers [2] have spent many years examining the effect of exogenous hormone administration on improving the ease of passage of transcervical insemination equipment. Oxytocin administration facilitated passage of insemination equipment but affected pregnancy rates negatively. They recommend its use only in training programs. Recently the team developed a new system for TAI. Similar to the Guelph System, animals were restrained in a foot trimming cradle, a speculum, assisted by a bovine AI rod, was used to visualize the cervical os and a 17.5 cm semi flexible stainless steel tube with a 4 mm brass bulb on the end was introduced through the cervical rings. The tube has a proximal attachment for retro loading the AI gun. Their research shows promise in that penetration did occur in a small group of ewes in less than 2 minutes each, embryo fertilization resulted and no damage to sperm could be detected from use of the system. Much testing remains to be done. In the early years of working with the Guelph System our group learned that operator, breed, period since last lambing, age and fecundity all affected the success of cervical penetration and the overall pregnancy rates and need to be tested on any emerging systems.

Cervical AI

Cervical insemination is still the goal for practical on-farm AI programs. It is widely used in Europe but with mixed success. In an attempt to answer many of the questions that continue to face the AI industry, Irish workers [3] undertook a large project which examined factors affecting the success of cervical AI programs using frozen semen in sheep. Using 5 breeds and large ewe numbers in each trial, they determined that:

- there was no difference in pregnancy rate between ewes inseminated to a natural or synchronised oestrus using vaginal progestagen and eCG
- the affect of operator (inseminator) was significant ($p<0.05$)
- there was an interaction between semen type (fresh or frozen) and oestrus type for litter size reflecting the fact that the adverse effect of frozen-thawed semen on litter size was greater in synchronised ewes
- a highly significant effect of ewe breed was shown on pregnancy rate with Finish landrace ewes having the highest pregnancy rate and purebred Suffolk ewes having the lowest pregnancy rate
- there was no effect of breed on either the interval from sponge removal to the LH surge or on the interval from LH surge to ovulation.
- there was much less variation among ewes in the timing of ovulation in the case of Finnish landrace breed than in any of the other breeds, possibly a factor in the breed effect on pregnancy rate
- no breed effects were detected with respect to depth of penetration of the cervix at AI or in the amount of mucus secretion
- cervical measurements were taken by breed but the differences in anatomical measurements were not associated with corresponding differences in pregnancy rate
- neither anatomical differences or the state of the cervix (mucus, penetrability) at the time of AI were implicated in the breed differences in pregnancy rate

- ewes were bred at varying intervals following pessary removal but it was found that timing of AI is not a likely explanation for the effect of ewe breed on pregnancy rate following AI
- some ewes were double inseminated 6 h apart but the double insemination did not greatly influence pregnancy rate.

Semen Processing

The Irish researchers {Boland MP, Byrne GP, et al. 2002 #14321}showed considerable variation among rams in conception rate following insemination of frozen-thawed semen. They used in vitro fertilization to evaluate the potential fertility of semen frozen from the different rams. They concluded that the IVF technique has great and practical potential as an effective means of assessing the fertilisation capacity of frozen-thawed semen and that it may enable the identification of individual rams whose semen is better able to survive the freeze-thaw process and yield high pregnancy rates.

Their results suggest that an IVF assay system maybe the only effective tool presently available short of conducting an actual AI trial to evaluate rams brought into a semen collection center. They calculated that if rams could have been effectively screened using IVF in advance and the best 25% identified it would have raised pregnancy rate in their project by 15% regardless of ewe breed. Results in Guelph on the ram effect on success of in vitro fertilization support their findings [4].

It has been clearly shown that pregnancy rates increase the further frozen semen is introduced through the cervical rings and into the uterus [5]. Because of the difficulty in developing a practical system for TAI attention has been paid to the effect of freeze-thawing on sperm hoping that changes to processing and handling might improve the success from vaginal and cervical insemination. It is know that a large proportion of the frozen-thawed cells are killed by processing, others cells have reduced motility possibly affecting cervical transit and undergo membrane changes similar to capacitation reducing fertilizing life. In 1999, Maxwell[6] described the beneficial effects they achieved from adding antioxidants and seminal plasma to the thawed semen. The frozen thawed semen treated with seminal plasma acted like fresh semen resulting in pregnancy rates from cervical AI comparable to those normally achieved with laparoscopic AI. I had expected that their initial findings with such exciting results might be supported by more work, however, no further progress has been reported.

The Irish group {Boland MP, Byrne GP, et al. 2002 #14321}also examined the addition of seminal plasma to frozen semen finding that including 10 or 20% seminal plasma in the diluents increased the viability of spermatozoa by 10 to 15 percent over the standard diluent. However, in a second study, in which 20% seminal plasma was incorporated in the diluent, there was no significant effect on sperm viability or structural integrity of spermatozoa compared with the standard diluent. Furthermore there was no evidence for any beneficial effect on fertilization rate in the IVF assay. The jury is still out on the value of adding seminal plasma. As with semen quality, seminal plasma may be an individual ram factor, both the source of the plasma and the ram to which it is applied.

Chilled fresh-extended (liquid) semen continues to be an alternative to frozen semen for sheep and goats. Properly processed, extended and managed, the semen remains viable with acceptable fertility for up to a week – particularly if used laparoscopically or transcervically. The addition of antioxidants was shown to extend the life of liquid semen for up to 14 days if deposited intrauterine in both sheep and goats[7]. Systems used for shipping chilled equine and canine semen should work quite well for sheep and goats. Recent reviews covering fresh and frozen semen processing are available for each species. [8] [9]. In Ontario, a small project using ram semen extended with a commercial tris based extender (Triladyl – Minitub®) and the semen shipped in an Equitainer® at 5^0C within 48 hrs to producers previously trained using disposable equipment to deposit the semen vaginally ($400x10^6$ total sperm per dose) in synchronized ewes. The program resulted in pregnancy rates ranging from 20 to 42 % in 6 trials. The program was simple and at an acceptable cost per lamb born and there was considerable opportunity to improve success through improved producer training.

In my opinion, regional sheep and goat organizations could benefit from developing practical liquid semen insemination programs with respectable cost: benefit.

Advances in Embryo Transfer

The same problems, shared with cattle embryo programs, continue to limit results in sheep and goat ET programs. Response to superovulation is highly variable and unpredictable. A large proportion of embryos fail to be fertilized. Goat donors experience premature failure of the corpora lutea with loss of transferable quality embryos. And most procedures still require surgical intervention for best results.

Improvements in Superovulation

It is known that the ovulatory response to commercial FSH preparations used in superovulation programs is related to the number of follicles (2–3 mm) present in the ovaries, and that the final number of transferable embryos is negatively affected by the presence of large follicle(s) (>6 mm) at the start of the FSH treatment [10] . Treatment in sheep with FSH begun soon after ovulation (Day 0), has been shown to increase follicular recruitment, ovulation rate, embryo quality and the number of transferable embryos recovered over treatment begun when larger follicles were present [10]. Day 0 protocols in goats also showed improved production of follicles and transferable embryos.

Management of follicular wave emergence by exogenous gonadotrophin administration has been explored as a method to reduce the variation in response to superovulation. French workers [11] have spent a decade testing GnRH agonist (Busereline, 40 mg/day, Receptal –Intervet USA) or antagonist (Antarelix, 0.5 mg/day, Teverelix -Europeptides Argenteuil, France) combined with a progestagen treatment to suppress endogenous gonadotropins and follicular development beyond 1–2 mm followed by exogenous gonadotropins administered over 4 days to produce waves coordinated with the timing of superovulation programs. The pre-treatment over a 2 week period suppresses large follicles, doubles the number of small ones, and improves the response to timed FSH by 50%. As a uniform group of follicles is recruited by the administration of FSH, synchronization of estrus occurs between 20 and 24 h after removal of the progestagen

sponge. An exogenous LH surge (3 mg of pLH) is administered i.v. 32–36 h after sponge removal, allowing synchronization of ovulations 20–28 h later. This is followed by a timed insemination 48–50 h after the end of progestagen treatment. The program has produced more than 10 transferable embryos and seven lambs born per treated donor and overcomes the problem of non-responding females (<5 ovulations). In the goat the beneficial effect of this treatment on the ovulatory response was not realized as there was an increase in the proportion of unfertilized ova and degenerated embryos.

Fertilization failure after superovulation

In sheep and goats, as in cattle ET programs, up to one third of potential embryos are typically recovered unfertilized and the number increases as the response to superovulation increases. In donors with more than 30 ovulations, a drop in both the fertilization and transferable embryo rates is routinely observed [11] and has been associated with sperm transport in through the cervix. Poor synchrony of ovulations after sponge withdrawal is another possible cause after artificial breedings in ET programs. Cognie reported good synchrony in LH surges and in the time at which ewes began to superovulate (58 ± 6 h from progestagen removal) with a median time from first to last ovulation of 6 h. However, in superovulated goats, the spread in ovulations was greater (63 ± 9 hr) and they found that LH surges were observed between 24 and 64 h after sponge removal and, the median time from first to last ovulation was 12 h. .

As stated in the first paper and reviewed by Cognie [6], to synchronize the time of ovulation when frozen-thawed ram semen is used, an injection of GnRH 30–36 h after sponge removal is recommended. In goats, the benefit from an injection of GnRH to synchronize ovulation and to increase the production of transferable embryos remains controversial. In a small-scale experiment, an increase in ovulation rate and in the number of transferable embryos was obtained in goats treated with GnRH 24 and 48 h after progestagen removal. In another trial, authors reported an increase in ovulation rate in GnRH-treated goats, but with a low fertilization rate. The lower fertilization rate observed in high-responding donors following vaginal or cervical insemination may be attributed both to a disturbance in sperm transport and suboptimal ovum quality. However, it continues to be shown that high fertilization rates can be achieved in sheep by intrauterine insemination performed laparoscopically 48 h after sponge removal.

Premature Luteal Failure

Premature luteal regression in superovulated goats continues to be a problem. It is reported to vary among breeds affecting from 10% (Alpine and Saanen breeds) to 32% (Murciana breed) of treated females. Anyone offering ET services in goats will encounter the problem. The reason is believed to be a premature release of uterine prostaglandins which could be induced by the persistence of large oestrogenic follicles 3–4 days after superovulation and results in poor embryo recovery or the recovery of poor-quality embryos [12]. A number of treatments continue to be used [11]. The inhibition of synthesis of prostaglandin with flunixine meglumine between the day of ovulation and the day of embryo recovery has been shown to recude the occurrence and an increase of the number of transferable embryos per treated goat . The induction of new ovulations 3.5 days after estrus with hCG prevents premature luteal regression in superovulated and the

replacement of the source of vaginal progestagen can be used to carry the embryos to the day of recovery.

Transcervical Embryo Transfer

Work at Guelph, using the Guelph transcervical AI equipment [13] showed the potential in transcervical passage during mid diestrus using simple equipment. Embryo survival was poor (16% of embryos transferred) but cervical passage was accomplished during early diestrus and was shown to be easier the second of two attempts only minutes apart. Lewis and coworkers [14] showed that the administration of estradiol the day before and oxytoxin twenty minutes in advance allowed easier passage of instrumentation and did not negatively affect luteal function or embryo development as compared to routine laparoscopic procedures. This could prove to be very useful information and should encourage more work on transcervical transfer of embryos in sheep and could improve successes in goats.

Guelph Embryo Program Results

An industry-supported sheep improvement program at the University of Guelph (Ontario Lamb Improvement Breeding Strategy) produced results in Table 1. The table summarizes three years of embryo production, continuous throughout each year, using a mix of maternal and sire breeds conducted in an attempt to increase the numbers of offspring from industry-selected donors, many of which were aged or in poor health. The program used traditional AI and ET, as well as juvenile and mature oocytes aspiration with in vitro embryo production.

The in vivo embryo program used accepted protocols for superovulation and procedures of surgical recovery and laparoscopic transfers. Table 1 compares the results from the University program, using research facilities and staff, with results from a commercial service (Small Ruminant Genetics, Georgetown, Ontario) where producers managed the animals and administration of superovulation protocols etc.

Results are in line with what others routinely report. Of interest was the rejection rate of recipients based on lack of a CL indicating failure of response to standard stimulation protocol (all Rideau Arcott maternal breed, n=360). The rejection had a seasonal pattern that was consistent over the three years of the program. From September through February (traditional breeding season) the rate of rejection ranged, by month, from none to 5%. From March through July, the rate ranged from a low of 11 % to a high of 27% in June for all three years. Interesting, there was no difference between the seasons in the pregnancy rates or embryo survival from recipients selected. The high numbers of rejected recipients during the out-of-season period increased the costs of the program during that period. Practitioners need to account for the increased numbers of recipients required and the added costs to clients programs during the out of season period.

Table 1: Results of in vivo embryo transfer comparing research program with commercial service

	Commercial ET Service	**University Program**
Embryo recovery procedures	132	330
CL count	12.2	11
Embryos recovered	9.3	8
Recovery rate (% of CLs)	77%	73%
Unfertilized oocytes	28%	32%
Donors failing to respond	4%	8%
Recipients rejected	12%	18%
Embryos/Transfer	2.2	2.9
Pregnancies established	76%	67%
Lambs born per flush	3.6	2.8
Embryo Survival	63%	52%
Males	52%	54%

Embryo Cryopreservation

Few advancements in the efficiency of cryopreservation of sheep and goat embryos have been reported. Typical programs today employ programmable freezers using ethylene glycol instead of glycerol and slow freezing rates. Martinez and Matkovic [15] compared the effect of glycerol and ethylene glycol for sheep embryos. They evaluated the effect of both cryoprotectant (glycerol vs ethylene glycol) and the effect of extending the thawing process for ethylene glycol frozen embryos. They confirmed previous studies that showed that ethylene glycol is superior to glycerol for sheep embryos and that this advantage was very significant for embryos at the compact morula stage. They also found there was no difference in the pregnancy rates (48%; 12/25) between thawing processes that removed ethylene glycol in 10 minutes and a longer protocol that required 30 minutes.

Sheep and goat embryos are able to survive vitrification procedures and considerable research has been reported using this system and with Cognie [11] suggesting that it may provide an economical alternative to the current freezing. Vitrification does not require any special equipment and, therefore, may be very well adapted to routine field use.

When fresh or vitrified sheep embryos recovered 7 days after estrus were transferred to synchronized recipients (two embryos/recipient), the pregnancy rates (72% in both cases) and the numbers of lambs born per embryo transferred were not different (60 and 50%), respectively [16]. These results with vitrified embryos were similar to those reported and routinely achieved by more traditional slow freezing. However, using the same vitrification and thawing methods with goat embryos, [11] have produced lower kidding and embryonic survival rates after vitrification (48 and 39%, respectively) than after conventional slow freezing (69 and 55%, respectively). Considerably more works needs to be done testing vitrification under field conditions.

A good review paper[17] covers recent developments in vitrification and compares the success to traditional commercial freezing methods. In spite of the many systems used to vitrify there is a growing improvement in results and in the simplification of

methodologies. And while results are improving, particularly for fresh embryos the impracticality of handling embryos individually (generally about 3-4 minutes per straw) vitrification may never be practical for practitioners where embryos are recovered in large numbers from field ET programs.

A small trial in the University of Guelph program produced the results in Table 2. Comparing the vitrification of in vivo produced embryos with those produced from juvenile oocytes aspirated and produced in vitro.

Table 2. Results of a trail comparing survival of vitrified in vivo and in vitro produced embryos.

	in vitro produced embryos	**in vivo recovered embryos**
Embryos	105	33
Recipients	35	16
Pregnancies (lambing)	14 (35%)	8 (50%)
Lambs Born	15 (14%)	9 (27%)

In vitro Embryo Production

In vitro embryo production continues to hold promise, possibly most particularly in small ruminants, where invasive procedures are routinely used for embryo programs. The mass production of low-cost embryos from abattoir ovaries, production from juvenile donors and the production of offspring from diseased or aged animals at necropsy warrant ET practitioners and providers of bovine IVF services to keep abreast of developments in these species.

In general terms, based on survival as fresh or frozen transfers, it is still safe to say that in vitro produced (IVP) embryos are inferior to those produced in vivo.

Oocytes are recovered from ovaries of slaughterhouse ewes/ does or aspirated from the ovaries of anesthetised mature or juvenile donor females. The method of producing IVP of embryos involves three steps: maturation of oocytes (IVM), fertilization of the matured secondary oocytes with frozen-thawed semen (IVF) and, culture of the putative embryos (zygotes) for up to 1 week until formation of blastocysts that can be transferred to recipients or cryopreserved for future use(IVC).

Cognie's paper reviews the state of oocyte recovery [11]. Abattoir-derived ovaries provide a cheap and abundant source of oocytes, and collection by aspiration provides 1.5–2 cumulus–oocyte complexes of acceptable quality per adult sheep or goat ovary. Oocyte recovery from live animals is accomplished by laparotomy or the laparoscopy-guided "ovum pick up" (LOPU) technique. When LOPU is performed 24 h after the end of a gonadotropin treatment, a mean of six oocytes per ewe are selected for IVF, resulting in about 1.1 blastocysts. Two to three good-quality blastocysts, which resulted in about 1.5 lambs being born per ewe were reported but with a high degree of variation between

donors. Oocyte retrieval after repeated LOPU in unstimulated sheep and goats also provided a high number of oocytes (4–6 per female/session). Cognie suggests that 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. Baldassarre [18] and his group in Montreal are successfully and consistently using laparoscopic ovum pick repeatedly on donor goats in their transgenic program.

Remarkable progress in producing embryos from 5- to 9-week-old lambs has been reported after recovery of oocytes after gonadotropin treatment [19]. However, only 19% of cleaved prepubertal oocytes developed to the blastocyst stage compared to 65% for their adult counterparts. Evidence suggests that prepubertal matured oocytes do not possess the developmental potential of their adult counterparts. The beneficial effect of a single treatment with estrogen and progesterone prior to gonadotropin on the developmental capacity of lamb oocytes has been demonstrated. This could suggest that the acquisition of oocyte competence for embryogenesis in the prepubertal female is progressive with age and could be influenced by the hormonal environment of oocytes before IVM. The fact that the number of oocytes recovered from the prepubertal female declines significantly with increasing age of the donors seems to be balanced by the fact that their development ability is improved when donors are older. Investigations into the control of follicular growth in the juvenile female should allow the production of offspring from juveniles to reduce the generation interval and increase the rate of genetic progress in breeding schemes [16]. Juvenile donors will have an important place in livestock-improvement programs, especially when their embryos or oocytes can be efficiently frozen.

The survival rate of fresh IVP goat embryos is similar to the survival rate obtained with IVP sheep embryos which significantly lower than their in vivo counterparts (47% versus 71%) [16].

Most programs recovering oocytes from mature or juvenile live donors are aspirating targeted follicles on the ovarian surface. In the sheep IVP program at Guelph oocyte recovery was done using a laparoscope-guided procedure, where the ovary was identified using the laparoscope and elevated and exposed through a small midline incision (mini-lap) [20-25]. Aspiration was done systematically just under the surface of the exposed ovary – not by targeting individual follicles. Average oocyte recovery from mature, unstimulated donors was 8.5, from mature stimulated 9 and from stimulated juveniles (between 8 and 12 weeks of age) 18. From mature donors, for every 100 good-quality oocytes recovered 35 blastocysts were produced which yielded an average of 16 lambs from fresh transfers or 13 from frozen-thawed transfers using slow freezing. An interesting observation was that large numbers of oocytes could be recovered from ovaries of diseased animals at necropsy. In one case of copper toxicity 5 donors yielded an average of 27 oocytes producing 11 blastocysts and 3 lambs each. In a trial using 30 juvenile donors for repeat collections, up to three times at monthly intervals beginning at 8 weeks, it was shown that repeated aspirations vial mini-laparotomy had no affect on future fertility or caused ovarian pathology [19].

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