ESTRADIOL-17β-OXYTOCIN INDUCED CERVICAL DILATION IN SHEEP: APPLICATION TO TRANSCERVICAL EMBRYO TRANSFER

By

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Abstract

Experiments were initiated to determine whether exogenous estradiol-17β (E2) and oxytocin (OT) can be used to dilate the cervix and improve transcervical embryo transfer (ET) procedures for sheep. However, there was concern that the E2-OT treatment may alter luteal function and that embryo quality would decrease as the superovulatory response to FSH increased. In Exp. 1, 32 ewes were assigned to a 2 x 2 factorial array of treatments. On d 7, ewes received an i.v. injection of either 100 μg of E2 in 5 mL of 1:1 ethanol:saline or 5 mL of 1:1 ethanol:saline; 12 h later, ewes received i.v. injection of either 400 USP units of OT or saline. Jugular blood was collected on d 7, 8, 9, 10, 12, 14, 16, and 18. Progesterone concentrations were unaffected by the treatments. Experiment 2 was conducted to determine the dose of pFSH needed to induce approximately six corpora lutea (CL). Ten-day Norgestomet implants inserted between d 8-12 of the estrous cycle were used to synchronize estrus in Hampshire and Hampshire x Dorset ewes (n = 23). Ewes received a total of either 0, 18, 27, or 36 mg of pFSH, which was injected i.m. at -24, -12, 0, 12, 24, and 36 h relative to implant removal. The dose at each respective time was 19.4, 19.4, 16.7, 16.7, 13.9, and 13.9% of the total. Ewes received 400 IU of PMSG i.m. at -24 h. The CL were counted laparoscopically on d 6 (d

0 = estrus). Number of CL increased linearly (P < .01) with dose of pFSH; there were 1.8, 3.6, 6.3, and 11.2 CL/ewe, respectively. Experiment 3 was conducted to determine the effect of the E2-OT treatment, mode of transfer or the interaction of E2-OT treatment x mode of transfer on embryo survival and development. Experiment 3 was conducted over two breeding seasons and across two trials. In the first trial ewes were assigned to one of three randomized treatments. Procedural limitations that were later overcome prevented a true 2 x 2 factorial design; therefore, transcervical transfer without hormonal treatment was excluded in the first trial. In the second trial, ewes were assigned to a 2 x 2 factorial array of treatments. On d 6 of pregnancy, embryos rating a fair or better were transferred into recipients either transcervically or laparoscopically. Recipients were administered either an E2 (d 6) - OT (d 7) treatment or an ethanol:saline-saline treatment following the same protocol as in Exp. 1. Embryos were recovered on d 12 in Trial 1 and d 14 in Trial 2. Embryos were evaluated morphologically for development and ranked on a scale of one to four; one represented no development and four represented development to the morphological stages associated with the day of collection. The treatments did not affect the percentage of embryos recovered after transfer or the percentage of embryos that showed some developed. However, there was an effect of mode of transfer on mean rank of embryo development; embryos transferred laporscopically developed further than embryos transferred transcervically (P < .01). This may have been an artifact of a technician effect between trials. There was an effect of E2-OT treatment on transcervical transfer (P < .01), indicating that it may be detrimental to transfer embryos transcervically without dilating the cervix. In conclusion, the E2-OT treatment did not affect luteal

function, and the E2-OT treatment can be used to dilate the cervix and enhance success of transcervical transfer of embryos. A 400 IU priming dose of PMSG and a total dose of 27 mg of pFSH can be used to induce the target number of six CL.

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Chapter I

INTRODUCTION

Producers believe that embryo transfer (ET) is the key to achieving rapid genetic gain and improving breeding stock in the sheep industry. In the last 20 yr, ET has produced rapid genetic progress in segments of the dairy and beef cattle industries. However, milk and carcass yields do not justify the expense of ET. The justification for ET in both industries is the rapid production of improved breeding stock. In the cattle industry, artificial insemination (AI) is the most cost effective method of transferring superior genetics into a herd; however, depending on the starting point the initial genetic gains achieved from ET are greater. In all species, ET in conjunction with AI and appropriate selection techniques provide good mechanisms for improving a flock or herd.

A second more promising application of ET in sheep is as a research tool. A transcervical ET technique could become a less expensive and more effective method for studying the effects of in vitro embryo treatments in vivo, maternal-embryo relationships, embryo biochemistry, uterine biochemistry, and cervical function.

The procedures for AI and ET in cattle are well established and relatively easy, but two physical barriers prevent the ET and AI techniques used in cattle from being used in sheep: 1) the occlusive nature of the cervix; and 2) the inability to manipulate the cervix rectally. For the sheep industry to match the success of the cattle industries, ET and AI technologies must become available for use on farms.

The anatomy of the sheep cervix prevents routine transcervical passage of ET or AI instruments. Currently, ET is a surgical procedure that requires a reasonable amount of

training and a significant initial investment. Several methods for transcervical AI have been modified for transcervical ET; however, these methods are difficult to master and have shown only limited success. A marketable method of transcervical ET will still involve a considerable amount of training, but it will include a method that makes the cervix less of a barrier. Taking advantage of the known mechanisms at parturition, there are several methods for dilating the cervix that may serve as a useful adjunct to ET procedures for sheep.

Until ET and other reproductive technologies become routine in the sheep, the rate of embryo research and genetic progress will remain slow. Therefore, the purpose of this research was to determine the usefulness of estradiol-17ß-oxytocin-induced cervical dilation to ET success.

Chapter II

REVIEW OF LITERATURE

Embryo Transfer in Sheep

The exploitation of embryo technology in sheep research and industry has been impaired by a number of inadequacies with the existing technologies. However, with improving technology, increasing success rates, and subsequent reduction in cost, ET has the potential to be a useful tool for researchers and sheep producers. Transcervical ET is important from the perspective of research and industry. A method for transcervical ET will increase our ability to study embryonic development in vivo and in vitro. At the same time, any efficacious technique developed for research will provide the sheep industry with a reproductive technology that could allow the rapid transmission of superior genetics. More important to industry, a usable transcervical ET technique could lead to the development of a practicable transcervical AI technique.

In the cattle industry, AI is the most cost effective method of transferring superior genetics into a herd, but initial genetic gains are greater with ET (Robinson et al., 1989). Indeed, embryo transfer allows producers to change breed or breed type in a single season. Despite that, consecutive embryo transfers within a flock or herd will not yield genetic gains consistent with the cost. After the initial changes are made with ET, intense selection and AI are the most effective means of maintaining and increasing these gains.

Under the correct financial management, ET is an effective tool for creating and marketing breeding stock. Several factors affect the value of ET: 1) economic superiority of the embryos transferred; 2) the percentage of pregnancies maintained after ET; 3) the

number of live lambs produced; and 4) the minimum return on the investment. For the sheep industry to remain competitive with the cattle or hog industries, it is imperative that these types of reproductive technologies become available. However, these technologies must be implemented in a profitable manner. Returns on a single animal must exceed the cost of producing that animal.

Genetic Improvement. Genetic improvement associated with ET can be defined in two terms: 1) the improvement of the entire breed of sheep; or 2) the improvement of a specific population of sheep. In either case, genetic improvement will only occur if ET causes a significant improvement in at least one of four criteria in an average population including 1) selection accuracy, 2) genetic variation, 3) selection intensity, and 4) generation interval. These four traits interact to affect overall genetic progress. Genetic progress can be calculated with this simple formula:

$$\frac{\textbf{Genetic Progress}}{\textbf{Year}} = \frac{\textbf{Selection Accuracy x Genetic Variation x Selection Intensity}}{\textbf{Generation Interval}}$$

For ET to have a significant effect on genetic progress in a breed or flock, one or more of these elements must be affected to a large degree (Aitchison, 1982).

In an average flock, a single round of embryo transfers from superior donors gives the maximum genetic progress. Subsequent transfers have little effect. If the offspring from the first transfers are incorporated into the flock, subsequent transfers into these offspring yield minimal genetic progress.

Multiple Ovulation and ET (MOET). Current ideas suggest that the future of ET in the sheep and cattle industries is dependent on the implementation of MOET schemes.

The idea of MOET is based on the idea that very few breeding herds provide the seedstock for genetic improvement in entire populations. Therefore, ET should be used as a tool to assemble the very best animals available to in a nucleus herd. This herd via ET then provides either stock for multiplier herds or sires for AI use by commercial breeders. In Australia, MOET is mostly used to proliferate new breeds or superior stock of an established breed (Evans, 1991). It is estimated that a grading-up program can be shortened from 10 to 6 yr using MOET (James, 1982).

In dairy (Nicholas and Smith, 1983) and beef cattle (Land and Hill, 1975), the rate of genetic gain can be doubled by MOET schemes. However, estimates of improvement apply to recurrent selection in closed nucleus herds and require that traditional progeny testing is abandoned in favor of sibling testing for male and female replacements. Accuracy of selection is abandoned for speed of replacement of breeding stock. In practice, any scheme advocating selection for improvement must be wary of selection techniques. Selection cannot be limited to a single trait, but will include several traits that affect performance. Depending on selection accuracy, MOET schemes can just as easily increase the propagation of negative genetic traits.

Systems involving nucleus herds are specifically designed to decrease the genetic variation among animals and make it difficult to change negative traits within a herd. The goal of a nucleus flock is to decrease the number of parents producing progeny, while maximizing genetic gain. Decreasing the total number of parents decreases the genetic pool and increases inbreeding. In contradiction to the concept of a nucleus herd, it is necessary to maintain large herds of parents to achieve a large genetic gain while

maintaining moderate degrees of inbreeding (Smith, 1986). Estimates suggest that to maintain low inbreeding levels in a nucleus herd, it is necessary to maintain 200 ewes and 1,000 progeny (Smith, 1986). Although maintaining a nucleus flock leads to a more uniform population of animals, the loss of genetic variance may be devastating.

Disregarding the genetic disadvantages associated with MOET schemes, there are several other problems that may prevent application of these theories in the sheep industry. Implementing multiple ovulation schemes involves superovulation, an extremely variable process that has yet to produce consistent results. Although the majority of breeds of sheep are multiple ovulators, to financially justify ET a donor should produce an average of six embryos, which cannot be accomplished without some type of superovulation procedure. The genetic impact of MOET is inversely proportional to the natural litter size (Smith, 1986). The impact of MOET in cattle is large because cattle typically only produce a single offspring, but it is drastically reduced in animals such as sheep that produce multiple offspring.

Profitability of ET. In cattle, the initial interest in ET procedures was generated by a small number of producers that derived a large proportion of their income from the sale of purebred cattle (Coffey and Tigges, 1983). However, ET has not become a useful tool for generating herd replacements on commercial dairy farms (Cassell, 1994). Typically, the expense involved cannot be recovered by increased milk yield from the ET replacements (Cassell, 1994). Another problem with ET in dairy cattle is that the ET costs must be paid when incurred, but the extra income starts about 3 yr after the ET procedure is performed (Cassell, 1994). Although, these problems are relevant to sheep producers, they may be

less of a financial burden. A shorter generation interval shortens the interval between the initial investment and the returns. Ewes and rams can be sold as replacements or introduced into flocks at 8 mo. In the sheep industry, returns on the initial investment will be noticeable as early as 13 mo after the investment. Embryo transfer also offers a more convenient way to export breeding stock. For owners of superior highly regarded ewes, exporting embryos offers a means of increasing the sales of offspring, and therefore, income almost immediately.

For ET to be profitable in the sheep industry, producers must ensure that each lamb produced from ET is worth more than the transfer. The cost of ET is well established in the cattle industries, but this is not true in the sheep industry. Cost will be based on the cost of production of an embryo (Table 1). Embryo transfer will never be a profitable procedure to produce market lambs (Table 2). However, it may be a profitable procedure to produce replacement stock (Table 3).

Table 1. Embryo transfer cost analysis for sheep:
Cost/embryo based on a net yield of six embryos/donor

Description of Fees	Fees in Dollars
Professional Fees	
a. Superovulation Drugs	35.00/donor
b. Surgical Recovery	90.00/donor
c. Improvement Fee	50.00/embryo
Donors	
d. Delayed Reproduction 20 d @ .50/d	10.00/donor
Cost/embryo = (a+b+d/6) + 50.00 =	72.50/embryo

Table 2. Embryo transfer cost analysis in sheep: Cost/market lamb based on five embryos transferred to each recipient and an average of three live lambs

Description of Fees	Fees in Dollars
Recipient cost	
e. Delayed Reproduction	
40 days open @ .50/day	20.00
f. Cost of Synchronization	16.00
Embryo Cost	
g. 5 Embryos/Recipient	362.50
Cost/3 Lambs	398.50
Income	
h. Market Price for Lambs	
3 lambs @ 56.25 kg @ 1.05/.45 kg	393.75
(Income/Lamb)-(Cost/Lamb)=	-1.58

Table 3. Embryo transfer cost analysis in sheep: Cost/breeding ewe based on five embryos transferred to each recipient and an average of three live lambs

Description of Fees	Fees in Dollars
Recipient cost	
e. Delayed Reproduction	
40 days open @ .50/day	20.00
f. Cost of Synchronization	16.00
Embryo Cost	
g. 5 Embryos/Recipient	362.50
Cost/3 Lambs	398.50
Income	
i. Price for Replacement Ewes	
3 ewes @ 150.00	450.00
(Income/Ewe)-(Cost/Ewe)=	17.51/ewe

*The current typical price of a breeding ram is $300.00. Including only the production of replacement ewes in this scheme is conservative. For every ram lamb produced from the ET, the profit increases.

Tables 1, 2, and 3 present an optimistic estimate of the cost to produce lambs from ET, because the tables only include the estimated costs of putting lambs on the ground, not raising them to market weight.

Although there may be ways to implement profitable ET programs in the sheep industry, it is difficult to assess the advantages of ET for a flock of sheep. At this time, it is difficult to determine whether the genetic gain from ET will be greater than the genetic gain achieved from normal phenotypic selection. Unlike the cattle industry, the sheep industry does not benefit from nationalized selection data.

Embryo Transfer Technology in Sheep

Historical Background. Embryo transfer in cattle was not popularized until transcervical ET was perfected. Development of ET technology in sheep began in the 1930's. Unfortunately, there have not been significant improvements in this technology in the last 65 yr. Early attempts at ET used embryos recovered from reproductive tracts of ewes after slaughter. Following surgical transfer, these attempts yielded an encouraging 20% lamb crop (Warwick et al., 1934; Warwick and Berry, 1949). In the early to mid-1950's, surgical recovery and transfer of sheep embryos became somewhat popular in research. As surgical procedures for ET were perfected, the success increased. Hunter et al. (1955) transferred 19, 2- to 16-cell embryos to 18 synchronous recipients and recovered eight lambs. Averill (1958) transferred 30 embryos and recovered 24 lambs. Few modern ET protocols produce the 80% lamb crop achieved by Averill (1958).

Methods of Embryo Transfer. Currently, there are two viable methods for ET in sheep. Embryos can be collected and transferred via midventral laparotomy or by using a laparoscope. Because the occlusive nature of the cervix makes transcervical embryo collection and transfer extremely difficult, the most common procedures bypass the cervix. Transcervical methods of intrauterine ET are currently being developed by modifying AI methods. To develop a viable ET method for sheep, two problems must be addressed: 1) the collection of embryos from donors; and 2) the transfer of embryos into recipients.

Today, surgical embryo collection in sheep is almost identical to the methods outlined by Hunter et al. (1955). Under general anesthesia, the ovaries, oviducts, and uterus are exposed through a midventral incision. The oviducts are catheterized with a

glass or plastic tube through the infundibulum. A portion or all of the uterine horns and oviducts are flushed by introducing and expressing media along the horns and the oviducts. Flushing the uterine horns and oviducts, rather than just the oviducts, depends on the day of collection. In ewes, embryos enter the uterus between the third and fourth day after estrus.

Surgical ET in ewes is performed under general or local anesthesia. A midventral incision is made, the uterus is exposed, and the embryos are injected directly into the oviduct or the uterus depending on the day of transfer. Embryos collected until 3.5 d after estrus are transferred into the oviducts (Moore and Shelton, 1962). Older embryos, d 4 to 7, are transferred into the uterine horns with a 70 to 75% survival rate (Rowson and Moore, 1966).

Recovery and transfer of embryos through a midventral laparotomy is highly invasive, resulting in surgical trauma and adhesions surrounding the reproductive tract. It is difficult to use a donor or a recipient more than once, and repeated performance of this procedure may reduce subsequent reproductive performance. Surgery is also time consuming and costly. Recently, laparoscopy has allowed more routine practice of ET in sheep. Although costly and training intensive, laparoscopy is much less invasive, causing less damage than laparotomy, and allows the donors and the recipients to be used several times.

Laparoscopic embryo recovery is more difficult than laparoscopic ET. The recovery rates with the most successful laparoscopic embryo collection techniques are similar to those with laparotomy recovery procedures (McKelvy et al., 1986). For the

procedure, ewes are anesthetized and suspended at 45° in a laparoscopy cradle. Three stab incisions are made in front of the udder to accommodate a straight laparoscope, a pair of grasping forceps, and a latex Foley two- or three-way pediatric bladder catheter. Before inserting the Foley catheter, a needle is used to puncture the uterus. Using the forceps to manipulate the uterus, the Foley catheter is passed through the hole in the uterus and into the uterine lumen. With the forceps clamping the uterus at the tip of the uterine horn, fluid is flushed through the uterus via the catheter and back out of the catheter into collection dishes. Embryos can be collected repeatedly from ewes with this technique. Laparoscopic embryo recovery dramatically reduces the formation of adhesions, allowing valuable ewes to be used for repeated embryo donations with only a slight risk of reducing their future fertility (McKelvey et al., 1986).

Embryo transfer in sheep using a laparoscope was first investigated by Moore (1977), but it was not until the mid-1980's that a successful technique was developed (Killeen and Caffrey, 1982; Mutiga and Baker, 1984; Walker et al., 1985; McKelvey et al., 1986). Ewes are anesthetized or sedated and usually suspended at 45° in a laparoscopy cradle. Two stab incisions are made anterior to the teats. The laparoscope is inserted through one incision and a needle designed to puncture the uterus is inserted into the second. The needle is pushed into the uterine lumen and the embryos are injected into the uterine lumen through the needle. As with the laparoscopic embryo recovery method, this method of transfer reduces the formation of adhesions. It is particularly important to avoid damaging the reproductive tract of recipients. Excessive trauma and formation of

adhesions in recipients are linked to decreased reproductive performance and embryo survival (McKelvey et al., 1986).

Although laparoscopic embryo recovery and transfer have overcome many of the problems associated with multiple surgeries, they are still invasive, labor intensive, and expensive procedures. Embryo transfer under these circumstances is a viable research tool; however, it is not a viable production tool.

Transcervical embryo recovery protocols have been proposed for sheep, but most require a laparoscope (Mylne et al., 1991). Unfortunately, these methods have the same limitations associated with laparoscopic recovery. Mylne et al. (1991) dilated the cervix by continually applying PGE_2 to the external surface of the cervix, which enabled a Foley catheter to be passed through the cervix with a minimal amount of damage. A laparoscope and probe were used to manipulate the uterus to force fluid back out through the Foley catheter. Dilating the cervix with PGE_2 manages the occlusive nature of cervix, making it easier and less intrusive to pass an embryo recovery instrument (Coonrod et al., 1986; Mylne et al., 1991). Using PGE_2 to dilate the cervix presents another problem; the cost of the amount of PGE_2 required to dilate the cervix is prohibitive.

Transcervical AI and ET are deterred by the same problems. Recently, interest in developing a transcervical AI method for use in sheep has been mounting. Transcervical AI methods are being designed to minimize the influence of the tortuous nature of the cervix in two ways: 1) decrease the diameter of the AI rod; and(or) 2) dilate the cervix. The methods using a small diameter AI rod are easily adapted to ET by extending the length of the rod (B. Buckrell, personal communication; D. Marsh, personal

communication). Methods for dilating the cervix require oxytocin and must be adapted to the luteal phase for ET versus at estrus for AI (Khalifa et al., 1992).

Early Embryonic Development in Sheep

Fertilization is considered the beginning of embryonic development. There are several critical aspects of ovum and sperm development that contribute to proper embryonic development. In ewes, embryonic development occurs in the oviducts and uterus. Embryos migrate from the oviducts to the uterus at around d 4. Development to the 16-cell stage is called cleavage. Cleavage involves an increase in cell numbers but not in cell mass, resulting in a sphere of cells of indistinguishable number called a morula. Rapid fluid accumulation and a series of contractions transforms a morula into a blastocyst. Continued contractions and expansions in conjunction with the production of proteolytic enzymes cause the embryo to hatch from the zona pellucida. Following hatching, the embryo undergoes a period of rapid growth, preparing for attachment to the endometrium on approximately d 15.

Before fertilization, there are several aspects of ovum development that are important for proper embryonic development. The first meiotic divisions begin during prenatal development. Near the time of birth, the first meiotic divisions arrest at the diplotene stage of prophase I. Meiotic divisions are resumed following an ovulatory surge of gonadotropin. The chromatin condenses, the germinal vesicle breaks down, and the first polar body is protruded. Meiosis II is halted at metaphase. In sheep, the oocyte is ovulated at metaphase II. The second meiotic division is not completed until fertilization occurs (Davies and Hessaldahl, 1971; Anderson, 1977). Meiotic divisions are the greatest source of genetic abnormalities. The delay from the initiation of the second meiotic division to completion at fertilization can increase developmental complications (Hawk, 1979).

Proper transport of ova and sperm through the female reproductive tract is imperative for fertilization. Ovulation results in the release of one or several oocytes. These newly ovulated oocytes are picked up by the fimbria of the infundibulum and directed into the oviduct. In sheep, sperm cells are ejaculated into the vagina. For fertilization to occur, transport of oocytes down the reproductive tract to the site of fertilization must be coincident with transport of sperm cells from the site of deposition. Sperm transport through the female tract is fairly rapid with relatively few sperm cells ultimately reaching the site of fertilization. Freshly ejaculated spermatozoa are incapable of fertilizing an oocyte (Austin, 1961). The spermatozoa must spend a period of time in the female reproductive tract to undergo capacitation (Austin, 1961b). Penetration of the oocyte by a sperm cell results in a series of phenomena leading to normal development. Penetration initiates a block preventing polyspermy and stimulating the resumption of meiosis II. The resumption and completion of meiosis II is visualized by extrusion of the second polar body. The male and female pronuclei are formed and unite during syngamy establishing a diploid one cell zygote. Fertilization also activates cleavage.

Development to the 16-cell stage is called cleavage. Following fertilization, the zygote undergoes continual mitosis resulting in the formation of a two-cell embryo, a four-cell embryo, an eight-cell embryo, and a 16-cell embryo. These cell divisions are not completely synchronous and uneven numbers of blastomeres are often seen. During cleavage, an embryo does not increase in mass, but it increases in cell number (Anderson, 1977; Bazer et al., 1987), and the cytoplasm of the zygote is continually partitioned into smaller and smaller units. At the eight-cell stage, tight junctions begin to form between the

blastomeres (Ducibella, 1977). These tight junctions may seal the blastocoele allowing active transport of ions into the blastocoele (Bazer et al., 1987). Metabolic rate remains constant throughout cleavage; however, it increases rapidly between the morula and blastocyst stage (Bazer et al., 1987).

In sheep, embryos develop through the first stages in the oviducts, migrating to the uterus around d 4. Embryos of the 2-cell stage were found in the oviducts of ewes 39 h after fertilization (Green and Winters, 1945). In this same study, four- to eight-cell embryos were flushed from the uterus 42 h after fertilization; however, the flushing procedures used were undefined, and these embryos were probably flushed from the oviducts (Green and Winters, 1945). In sheep, 16-cell embryos are found 77 h after fertilization. After the 16-cell stage, the number of cells becomes indistinguishable, and a morula forms (Anderson, 1977).

A cavity, or the blastocoele, begins to form in the morula creating a blastocyst (Anderson, 1977). The blastocyst consists of two cell types, which are divided into two layers. The outer layer is the trophoblast, and the inner layer makes up the inner cell mass. The inner cell mass develops into three primary germ layers: ectoderm, mesoderm, and endoderm (Bazer et al., 1987). During the blastocyst stage, there is an increase in fluid accumulation in the blastocoele and a series of contractions and expansions begins (Anderson, 1977). The expansions and contractions are involved in the shedding of the zona pellucida called hatching (Biggers et al., 1978). Embryos accumulate fluid and increase in size with each expansion. Embryos also produce proteolytic enzymes including hyluaronidase, which weaken the zona. The zona tears creating a channel for blastocyst

expulsion (Mintz, 1962; Cole, 1967; Bergstrom, 1972; Biggers et al., 1978). Continual contractions and expansions push the blastocyst out of the zona pellucida. Hatching occurs around d 8 in sheep (Winters and Green, 1945). After hatching, embryos continue rapid fluid accumulation and growth (Anderson, 1977).

In livestock, "implantation" is a superficial noninvasive process (King et al., 1982); therefore, it is more appropriate to use the word attachment to describe this process in ruminants. In sheep, much of the embryonal loss occurs at the time of attachment (Guillomot et al., 1981). Attachment begins around d 15 (Eckstein and Kelly, 1977); although, changes occur in caruncular zones on d 14 (Gaviria and Hernandez, 1994). Attachment begins with a loose apposition of trophoblastic microvilli and uterine glands. The trophoblast temporarily loses microvilli in specific areas, allowing a close apposition of uterine epithelium to the trophectoderm and redevelopment of trophoblastic microvilli (Bazer et al., 1987). The net result of advancing pregnancy is a definite increase in the area of attachment to the uterus (Gaviria and Hernandez, 1994). By d 24, approximately 85% of the trophoblastic tissue is attached to the uterus; before d 20, that value is less than 50% (Gaviria and Hernandez, 1994). This attachment becomes vital for embryonic development and survival.

Embryo-Uterine Synchrony, Progesterone Supplementation, and the Number of Embryos Affect Embryo Transfer Protocols

Much of the information about early embryonic development was developed in connection with ET in livestock. In particular, the relationship between the embryo and the uterine environment are of interest. An appropriate affinity must exist between the developing conceptus and uterine environment during the preattachment period to overcome luteolysis and to achieve immunological tolerance (Ashworth and Bazer, 1989). Understanding the relationship between the embryo and the uterine environment increases the chances of successful ET. Maximizing physiological knowledge of these interactions provides simple ways of augmenting ET protocols in sheep: 1) synchronize the stage of embryo development with the proper uterine environment; 2) supplement with progesterone; and 3) transfer an appropriate number of embryos.

Uterine secretions change throughout embryonic development. These changes must occur at the appropriate developmental time to ensure development and survival of the embryo; therefore, it is imperative that the embryo and the uterus are in synchrony. Even though the way to attain chronological synchrony in ET protocols is to transfer embryos to a recipient that exhibited estrus on the same day as the donor, this may not attain biochemical/developmental synchrony (Rexroad and Powell, 1991). A more accurate method of attaining synchrony between the embryo and the new uterine environment is to base it on the developmental stage of the embryo rather than on the onset of estrus of the donor (Rexroad and Powell, 1991).

Rexroad and Powell (1991) increased the success of ET by transferring cocultured embryos to recipients that were in estrus 1 d later than the donors; the success rate was increased by 33% compared with transfer into synchronous donors. After 2 d of coculture, embryo development was no longer synchronized with the uterine environment of the donors. Culture had slowed down and required recipients with an earlier uterine environment than the donors. Earlier data indicate that immediate transfer of early stage embryos to recipients synchronous with donors (± 12 h) resulted in the maximum lambing rate (Moore and Shelton, 1964). However, these studies involved immediate transfer versus embryos that were cultured or preserved for any period of time. Removing embryos from the uterine environment for as little as 1 h slows down development enough to warrant transfer into a recipient that came into heat 24 h after the donor (C. E. Rexroad, Jr., personal communication).

There is evidence that the effect of asynchrony varies with the stage of embryo development. Older embryos (> d 8) survive better in an asynchronous environment than younger embryos (< d 8; Rowson and Moore, 1966). Embryos transferred during the first week of pregnancy seem to be especially vulnerable to transfer into advanced recipients (Ashworth et al., 1989). Embryo transfer in ewes is usually done on or before d 6. Embryos transferred between d 6 and 8 can survive in asynchronous environments; however, survival rates are greater if embryos are transferred to a synchronous environment (Vincent et al., 1986). It seems particularly important that these young embryos are placed into biochemically compatible uterine environments. In an advanced

uterine environment, embryos lose the ability to establish pregnancy (Ashworth et al., 1989).

In summary, researchers have defined a synchronous uterine environment on the basis of when the donor and the recipient are detected in estrus. A more appropriate definition would be based on the developmental stage of the embryo, particularly in protocols that do not immediately transfer the embryos. Embryos should be transferred into biochemical environments that best represent their developmental stage, rather than their chronological age.

In ewes maintained under good husbandry and nutritional conditions, 20 to 48% of fertilized oocytes are not represented by lambs (Edey, 1969; Bolet, 1986). Progesterone insufficiency during early pregnancy is often cited as a possible cause of embryonal mortality. Changes in the progesterone profiles of ewes after mating may be critical for the establishment of pregnancy (Wilmut et al., 1986). Wilmut et al. (1986) defined three times in which progesterone concentrations are critical: 1) d 1 after mating (.1 to .2 ng/mL); 2) d 3 and 7 (3 to 6 ng/mL); and 3) throughout the remainder of pregnancy. However, the effect of progesterone during early pregnancy on embryonal survival is equivocal (Peterson et al., 1984; Smith et al., 1985; McMillan et al., 1987; Parr et al., 1987; Murray et al., 1989; Scaramuzzi et al., 1988). Low progesterone concentrations before d 4 have been associated with embryonal mortality (Ashworth et al., 1989).

Kleeman et al. (1991) used Boorola-Merino ewes to examine the effects of progesterone supplementation at different times during early embryonic development on the incidence of multiple births. All treatments beginning on d 4 after ovulation improved

litter size when compared with the controls; however, pregnancy rates did not improve. They were unable to determine when during early embryonic development that progesterone supplementation had an effect. In a similar study, Scaramuzzi et al. (1988) reported an improvement in litter size in ewes treated with progesterone between d 10 and 25 after ovulation. Several contradictory studies indicate that supplementation with progesterone does not improve litter size (Smith et al., 1985; Murray et al., 1989). Opposing reports also exist concerning a change in pregnancy rates with progesterone supplementation. In studies when pregnancy rates were low to medium in the controls, there seemed to be a positive effect of progesterone (Peterson et al., 1984; McMillan et al., 1987; Parr et al., 1987). In studies when pregnancy rates were high (>85%), there was no significant influence of progesterone supplementation (Smith et al., 1985; Murray et al., 1989; Disken and Niswender, 1989; Kleeman et al., 1991).

Currently, there are three proposed mechanisms involved in increased litter size associated with progesterone supplementation. First, progesterone helps regulate uterine protein secretion (Murphy et al., 1977; Miller and Moore, 1976; 1983). Inclusion of progesterone in the coculture of preimplantation mouse embryos increased the percentage of embryos developing to later stages (Lavranos and Seamark, 1989). In multiple births, the availability of proteins secreted from the uterus may limit embryo development; thus, supplementing with progesterone may increase the availability of uterine proteins allowing a greater number of embryos to develop (Kleeman et al., 1991). Second, sheep embryos develop to a critical size before attaining the ability to produce estrogen in amounts adequate to influence intrauterine migration (Nephew et al., 1989). Exogenous

progesterone may act on conceptuses by improving critical mass before migration. Third, exogenous progesterone may improve embryo viability by reducing uterine asynchrony (Pope, 1988; Wilmut and Sales, 1981; Kleeman et al., 1994).

Determining the optimal number of embryos to transfer into a single recipient has also provided information concerning embryo-maternal interactions. Specifically, embryo survival rates and pregnancy rates in ET must be evaluated separately. Typically, embryo survival rate is depressed when five or more embryos are transferred, but litter size from recipients receiving a large number of embryos is still larger (Moore et al., 1960). Cumming and McDonald (1970) transferred either 1, 2, or 4 embryos to one of three groups of 36 ewes. Pregnancy rates in all three groups remained constant at approximately 50%; however, litter size was larger in the ewes that received four embryos. This is consistent with other studies. Decreases in pregnancy rates in ewes following ET are not generally seen until five or more embryos are transferred into an individual ewe (Bradford et al., 1974). Initially, this phenomenon was attributed to an insufficient number of CL; however, analyses of embryo survival in relation to the number of CL have failed to show a significant effect of CL number (Moore et al., 1960; Cummings and McDonald, 1970; Bradford et al., 1974).

Cervical Anatomy

The cervical canal in ewes anatomically restricts the passage of AI and ET instruments into the uterus. Several research groups, with the goal of improving transcervical AI and ET techniques, have described the cervical anatomy of ewes (Dun, 1955; Fukui and Roberts, 1978; Bunch and Ellsworth, 1981; Halbert et al., 1990a). Three aspects of the reproductive tract in ewes present physical barriers for transcervical AI and ET: 1) the length of the vaginal canal; 2) the shape and size of the external cervical os; and 3) the length, size, and occlusiveness of the cervix. Locating the external portion of the cervix is difficult, because the length of the vaginal canal is highly variable in ewes.(Dun 1955; Halbert et al., 1990a). Folds of vaginal tissue surrounding the cervix create blind spots and obstruct the cervical lumen; hence, the second major barrier to AI or ET instruments (Figure 1). The number and shape of vaginal papillae can be used to classify the tissue folds surrounding the external cervical os into four groups: 1) flap, which has a single fold of vaginal tissue that stretches over the opening of the cervix; 2) duckbill, which has two opposing folds of vaginal tissue; 3) rosette, which has a cluster of vaginal folds that places the external opening of the cervix in the center of the folds; and 4) spiral, which consists of spiral shaped vaginal tissue that is not differentiated into folds (Dun, 1955; Halbert et al., 1990; Figure 1). Halbert et al. (1990) found that the rosette and flap types were the most common in Suffolk ewes; each represented 35% of the ewes.

According to Halbert et al. (1990a), the average length of the cervical canal in ewes is 6.7 cm with approximately five rings. Fukui et al. (1978) reported similar values. In the study by Fukui et al. (1978), the average length of the cervical canal was 6.5 cm

with approximately six folds. Halbert et al. (1990a) using a silicone mold also classified the narrowest (2.7 mm) and widest (6.6 mm) portions of the cervical lumen. The largest rings were located closest to the vaginal canal. The diameter of the cervical rings decreased after the second ring, with the smallest rings located proximal to the uterine body. Contrast imaging revealed funnel shaped cervical rings with the openings pointing caudally (Halbert et al., 1990). Visual assessment and the silicone molds also confirmed earlier reports that the cervical rings in ewes are arranged in an eccentric manner (Bunch and Ellsworth, 1981; Halbert et al., 1990). Halbert et al. (1990a) reported that the third cervical ring was the most eccentric, but Bunch and Ellsworth (1981) reported that the second ring was the most eccentric. The external cervical os was 9.8 mm from the most eccentric ring, which most often correlated to the third ring (Halbert et al., 1990a).

The occlusive nature of the cervix in ewes has made it difficult to develop methods for passing instruments into the uterus transcervically (Andersen et al., 1973; Fukui and Roberts, 1976; Coonrod et al., 1986). Salamon and Lightfoot (1970) reported that it was only possible to pass a standard AI rod more than 2 cm into the cervix of 13% of the ewes. The small openings of funnel shaped cervical rings, eccentrically positioned, restricted passage (Halbert et al., 1990a). Passage may be difficult for a number of reasons. The vaginal tissue surrounding the cervix creates blind spots and makes it difficult to identify the opening of the cervix. The instruments must enter the narrow end of the funnel shaped rings first. The rings are eccentrically aligned. The size and spacing of the rings is different in each ewe. Fukui and Roberts (1978) determined that the structure of the external cervical os, not the length or number of rings, prevented transcervical passage

of an insemination or ET device through the cervix. However, Halbert et al. (1990a) could not correlate differences between the types of cervical openings and ability to pass an insemination rod.

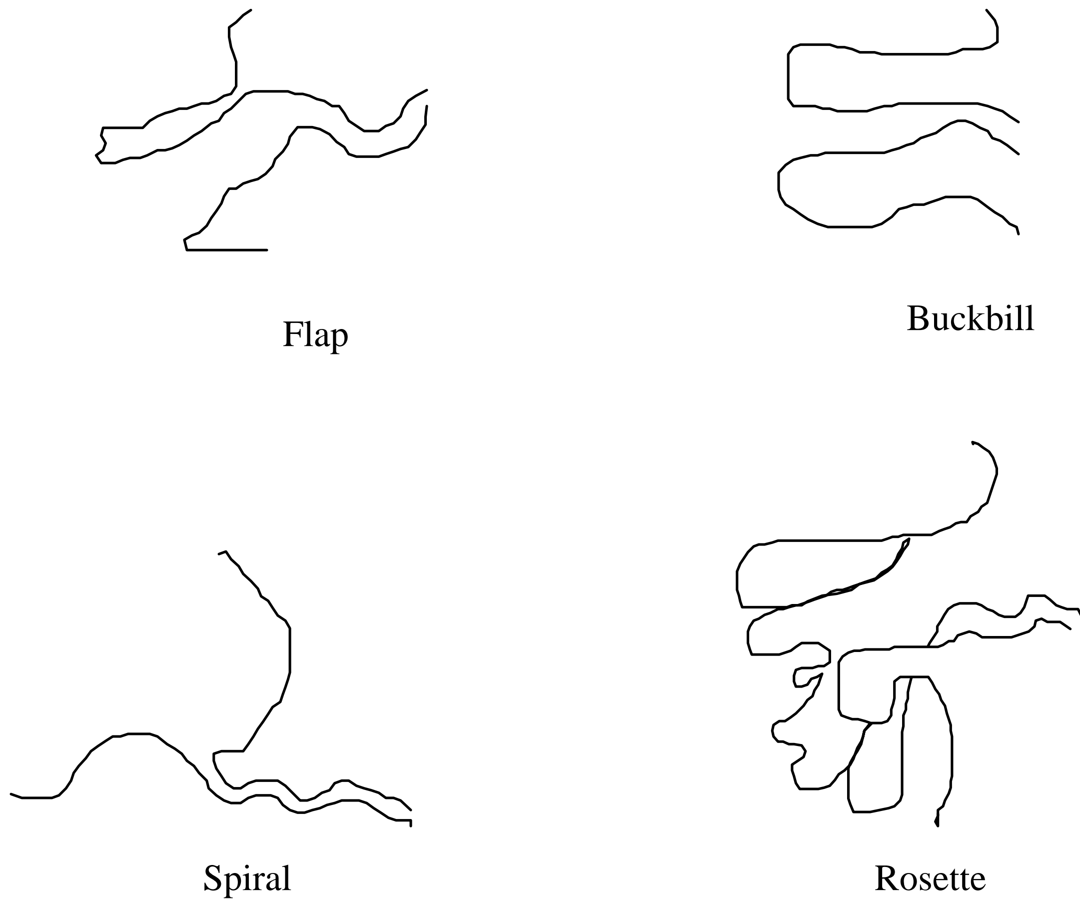

Figure 1. Diagram of various anatomical types of sheep cervix.

Parturition and Cervical Physiology

Hormonal changes mediate the physiological changes associated with parturition. The hormonal trends and environment at parturition are similar to those before the onset of estrus; although, the temporal relationships are somewhat different. During, the periparturient period, progesterone decreases, estrogen increases, and the estrogen:progesterone ratio increases. These hormonal changes cause the primary events of parturition, including induction of uterine contractions and cervical dilation. Oxytocin seems to be involved in the induction of labor in goats, ewes, and cows (Catchpole, 1977). Oxytocin can stimulate uterine contractions and induce cervical dilation (Arulkumaran et al., 1989). However, the exact mechanisms causing cervical dilation at parturition are not fully understood.

In sheep, progesterone peaks between d 125 to 135 of pregnancy, and it decreases rapidly near parturition (Basset et al., 1969; Fylling, 1970; Stabenfeldt et al., 1972; Thompson and Wagner, 1974). Decreases in progesterone cause increases in myometrial contractions. In ewes, estrogen is nearly constant throughout most of pregnancy (50 pg/mL). On the day of parturition, estradiol increases rapidly to approximately 400 pg/mL (Challis, 1971; Thompson and Wagner, 1974). Increased estrogen causes the hypertrophy of smooth muscle cells, synthesis of contractile proteins, synthesis of RNA and DNA, and the deposition of glycogen in myometrial cells (Catchpole, 1977). Hormonal profiles of progesterone and estrogen during pregnancy are similar in cattle (Short, 1968; Pope et al., 1969) and goats (Challis and Linzel, 1971). The timing of the changes in hormonal concentrations is specific for each species.

The cervix and uterus are derived from the Mullerian ducts. The cervix and the uterus can be divided into three distinct layers: 1) endometrium; 2) myometrium; and 3) perimetrium. Unlike the myometrium, the endometrium and perimetrium are similar and continuous throughout the cervix and uterus. In the uterus, the myometrium consists of a large volume of smooth muscle interspersed with connective tissue. In the cervix, the myometrium is composed of connective tissue with irregular bundles of smooth muscle (Rorie and Newton, 1967). Differences in the composition of uterine horn and cervical myometrium indicate that the cervix can respond independently of the uterine horns.

The cervix dilates at parturition. Cervical dilation is accompanied by uterine contractions. Two theories may account for the mechanisms associated with cervical dilation at parturition. One theory suggests that the contracting uterus may physically dilate the cervix. Oxytocin-induced uterine contractions spread through the continuous uterine muscularis to the cervical muscularis causing the cervix to contract (Valentine, 1977; Garcia-Villar et al., 1984; Arulkumaran et al., 1989; Granstrom et al., 1989). A combination of uterine and cervical contractions may force the cervix to dilate; therefore, the theory suggests that uterine contractions cause cervical dilation (Karim and Prasad, 1979). However, Sayre and Lewis (1996) indicate that cervical dilation induced by exogenous oxytocin occurred while the uterus was in tetany. A second theory suggests that an increase in collagenase activity causes a breakdown in collagen structure leading to a softening of the cervix. The cervix is composed mainly of fibrous connective tissue with large amounts of collagen (Danforth, 1947; Uldberg et al., 1983; Granstrom et al., 1989). Cervical collagen is made of myofibrils of type one collagen bound together as

tropocollagen (Kleissl et al., 1978). Tropocollagen fibers are bound in triplicate to form collagen fibers, which are further bound to each other by ground substance crosslinking. Ground substance crosslinking increases the stability of the matrix, causing the rigidity normally associated with the cervix before parturition. In sheep, cervical connective tissue is altered during late pregnancy and facilitates dilation. The composition of cervical collagen changes with stage of pregnancy, with the greatest changes occurring between d 140 and 145 (Fosang et al., 1984). In late pregnancy, the arrangement of cervical collagen becomes highly disordered with a variable fiber diameter (Fosang et al., 1984). Weight and biochemical composition of the cervix also change near parturition (William et al., 1982) In women near term, it is easier to extract cervical collagen, indicating a change in organization of the cervical collagen matrix (Granstrom et al., 1989). Collagen degradation is related to collagenase activity within the tissue. Collagenase activity increases during cervical softening at parturition (Martin et al., 1983; Rath et al., 1987; Rajabi et al., 1988; Granstrom et al., 1989; Osmers et al., 1992). Oxytocin also induces endometrial release of prostaglandins (Silvia et al., 1991). Prostaglandins specifically, PGE_2, may have a role in cervical softening (Ekman et al., 1986). Prostaglandin E_2 simulates cells to release collagenase. Speculatively, exogenous oxytocin stimulates the uterus and the cervix to release prostaglandins, causing a local effect of PGE_2 on the cervix, enhancing collagenase activity, allowing the cervix to dilate.

Based on the speed of cervical dilation following the administration of exogenous oxytocin, it is unlikely that cervical dilation is due entirely to collagenase activity. Sayre

(1995) proposed that oxytocin-induced cervical dilation is the result of the combined effects of changes in cervical collagen and uterine contractions (Figure 2).

The response to oxytocin varies throughout the cycle. Uterine and cervical responses to oxytocin are mediated by specific membrane bound receptors. Oxytocin receptor concentrations in the cervix vary with day of the estrous cycle (Matthews and Ayad, 1994). This was confirmed by Sayre (1995). Uterine and cervical oxytocin receptor concentrations are lowest during the luteal phase (Matthews and Ayad, 1994); therefore, the administration of exogenous oxytocin alone does not cause cervical dilation during the luteal phase. Khalifa et al. (1992) demonstrated that administration of estradiol-17ß 12 h before administration of oxytocin resulted in cervical dilation (Figure 3).

An increased understanding of the mechanisms involved in cervical dilation at parturition will allow further refinements of dilatory treatments for ET and AI in sheep. Nethertheless, mimicking the hormonal environments at parturition allows dilation of the cervix and easy passage of ET and AI equipment.

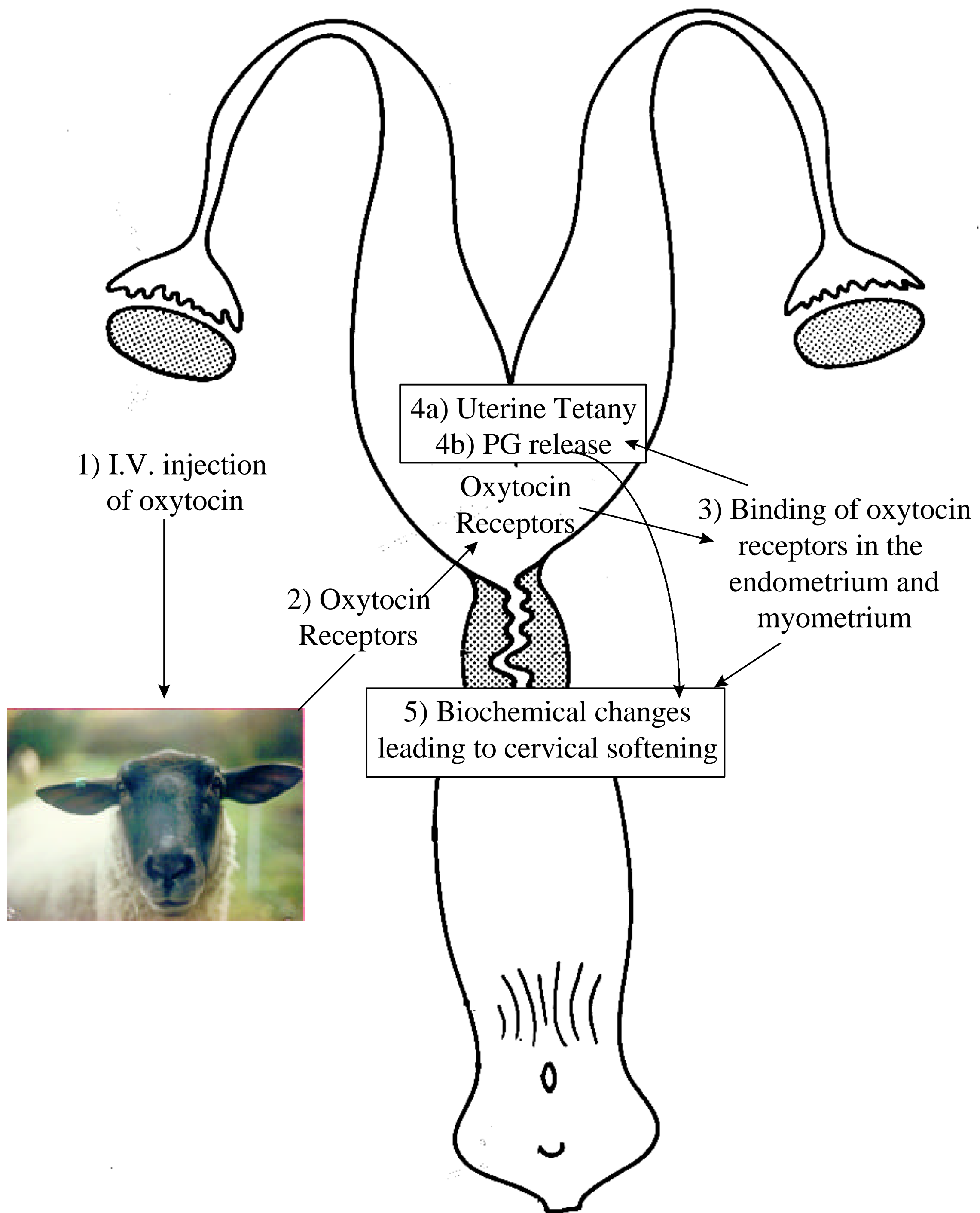

Figure 2. Possible mechanism of induced cervical dilation at estrus.

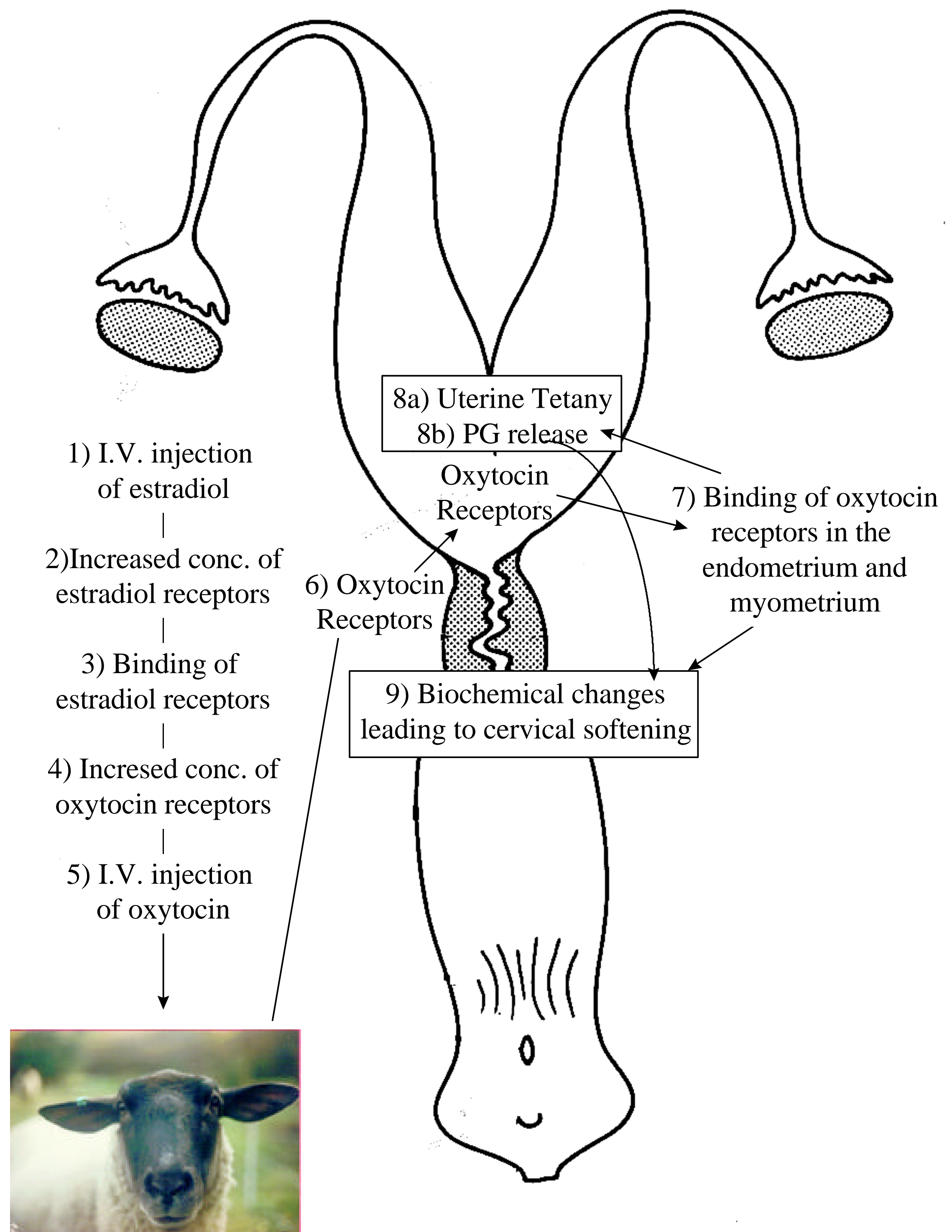

Figure 3. Possible mechanism of induced cervical dilation during the luteal phase

Superovulation in Sheep

Superovulation increases the number of oocytes ovulated by an individual in one episode (Seidel, 1991). However, the results associated with superovulation are highly variable; the numbers of fertilizable oocytes and transferable embryos produced are not predictable (Boland et al., 1991). Factors affecting this response the ewes include 1) type, dose, and method of administration of superovulatory drugs, 2) breed and, 3) season (Maxwell et al., 1990). Understanding the mechanism by which superovulatory protocols cause a greater number of ovulations will allow modifications of the protocols to decrease the variability, creating a more economically and biologically sound process.

The precise pattern and sequence of gonadotropins required to stimulate the growth of a predictable number of follicles in ewes are not understood. Luteinizing hormone and FSH are involved in ovulation rate. However, there are many other factors including estradiol - 17β and inhibin. In sheep, superovulation protocols are designed to do two things with respect to folliculogenesis: 1) recruit more follicles; and 2) prevent atresia of the cohort of subordinate follicles causing a larger number of follicles to grow and ovulate (Draincourt, 1991).

Superovulation in sheep has been induced by the administration of PMSG (Averill, 1958), horse anterior pituitary extract (Moore and Shelton, 1964; Boland and Gordon, 1982), purified forms of FSH (Wright et al., 1981; Bondioli et al., 1982; Evans et al., 1984), and human menopausal gonadotropin (Schiewe et al., 1985). Current research implicates other endocrine and paracrine factors that have important secondary roles in

follicular growth, development, and ovulation and may be used later to refine superovulation protocols (Hammond et al., 1991; Giudice, 1992).

There is considerable variation in the FSH and LH biological activity in the available compounds. Commercially available gonadotropins vary among companies and among batches produced by the same manufacturer. Small amounts of LH in FSH preparations are thought to be beneficial in superovulation of sheep and cattle (Chupin et al., 1984). In sheep, preparations of highly purified FSH (i.e., preparations without any LH activity) do not induce superovulation, demonstrating the necessity of LH contamination. However, preparations with too much LH are detrimental to fertilization rates and quality of embryos produced (Monniaux et al., 1983).

Superovulatory protocols usually involve some type of estrus synchronization program (Betteridge, 1977). Administration of the superovulatory regimen occurs late in the estrous cycle in ewes synchronized with progestogen or relative to injections of $PGF_{2\alpha}$. A link between poor embryo quality and the type, amount, and mode administration of progestogens has been proposed; however, the results vary among studies with the most recent data indicating that there is no link (Scudamore et al., 1991). Administration of superovulatory gonadotropins is based on their half-lives. Typically, PMSG, which has a relatively long half-life in sheep (approximately 21 h), is administered in a single dose (McIntosh et al., 1975), whereas FSH, which has a relatively short half-life in sheep (approximately 2 h), is administered in multiple decreasing doses over several days (Akbar et al., 1974). The long half-life of PMSG is associated with several problems with PMSG-based superovulation protocols. Ewes administered PMSG often have a high incidence of

unovulated follicles leading to a sustained increase in circulating estrogens (Booth et al., 1975). According to Whyman and Moore (1980), the increased concentrations of estrogens increase the rate of transport of ova through the oviduct and decrease the rate of recovery. Stimulation with PMSG also changes the pattern of steroid secretion from the follicles (Moor et al., 1985). This effect is not observed in FSH treated ewes. Also, PMSG has been implicated in premature activation of oocytes resulting in aged or abnormal oocytes at the time of ovulation (Moor et al., 1985). Disadvantages associated with superovulation with FSH include the fact that it is more labor intensive than administering a single injection of PMSG, and some ewes, regardless of the dose, do not superovulate (Ryan et al., 1984). A popular protocol for superovulating sheep involves giving PMSG and FSH. The administration of moderate doses of PMSG in combination with superovulatory doses of FSH seem to overcome the disadvantages of using either alone (Ryan et al., 1984). According to studies, with sheep, there is little difference in ovarian response between multiple and single injection regimens of FSH when given in combination with PMSG (Maxwell et al., 1990).

There is inherent individual variation in spontaneous ovulation rates in sheep. This variation carries over into superovulation responses. Ewes selected for multiple birth, following the administration of various gonadotropins, have a greater ovarian response than ewes not selected for, or ewes selected against, multiple births (Bindon et al., 1971; Butler and Maxwell, 1988). Season is also a source of variation, but this seems to depend on the superovulatory protocol used. Gherardi and Lindsay (1980) reported that the ovulation rate in white faced ewes treated with low doses of PMSG was greater in the fall

than in the spring. However, Ryan et al., (1984) gave greater doses of PMSG and did not find a difference in ovarian response between seasons. Maxwell et al. (1990) reported no differences between seasons in ewes superovulated with a combination of PMSG and FSH.

There is considerable variation in ovulation times associated with different superovulatory protocols. To maximize the number of viable embryos recovered, this variation must be taken into account in breeding schemes. Ewes treated with pFSH seem to ovulate 60 h after removal of progestogenated pessaries; whereas, ewes in the same study treated with PMSG alone ovulated at 54 h after pessary removal (Walker et al. 1986). Ewes treated with a combination of PMSG and FSH ovulate even earlier; the majority of ovulations were detected 42 to 54 h after pessary removal (W.M.C. Maxwell, personal communication). The occurrence of premature ovulations complicates actual determination of the time of ovulation. Premature ovulations are defined as ovulations occurring less than 24 h after pessary removal (Walker et al., 1986).

Based on the increased number of oocytes and the differences in ovulation times in superovulated ewes, it is necessary to amend standard breeding practices to ensure correct timing and adequate sperm numbers to optimize fertilization rates. Intrauterine insemination by laparoscopy is currently the most cost effective method of ensuring insemination at the correct time with adequate numbers of spermatozoa. However, the time of insemination with this method can affect fertilization and recovery rates of ova. Walker et al. (1989) confirmed that the closer insemination was to the time of ovulation, the poorer the embryo recovery rates. In these same studies, fertilization rates were

greater at the times closer to ovulation. This creates a unique problem, suggesting that it may be impossible to optimize fertilization and recovery rates using the same superovulatory protocol.

Although reduced embryo quality is one of the greatest hurdles associated with use of superovulatory protocols in sheep, there is very little literature on the subject. The majority of studies to assess the effects of superovulation on embryo quality are in cattle. In cattle, superovulation is used extensively in an almost standardized fashion, but it is not a well-controlled treatment. Approximately 15 to 20% of donor cattle receiving a superovulatory treatment do not produce transferable embryos (Donaldson, 1985). Typically, this is attributed to the gonadotropin treatment, because unstimulated cows normally yield viable embryos (Screenan and Diskin, 1986). Exogenous gonadotropins affect the development of viable embryos at a variety of stages: oocyte maturation (Callesen et al., 1986; Loos et al., 1991); follicular steroidogenesis (Moor and Kruip, 1984); sperm transport (Hawk, 1988); fertilization; and development of the embryos in the oviduct and the uterus until they are recovered (Hunter, 1988). There is also evidence that even transferred morphologically viable embryos may have subtle injuries inflicted on them during development to the time of transfer that prevents normal development after transfer (Betteridge and Luskutoff, 1993). After removing degenerating and poor embryos, it is almost impossible to distinguish morphologically which embryos will develop. In several studies, embryos recovered after superovulation that were morphologically classified as good or excellent did not develop after transfer. The decreased rates of development have been attributed to the superovulatory protocol (Betteridge and Luskutoff, 1993).

Specifically, superovulatory treatments have been shown to increase circulating estradiol-17β concentrations, suppress LH secretion, cause a premature LH surge, and disrupt normal vacuolization of oocytes (Greve et al., 1995). Although subtle, these changes seem to have profound effects on early embryonic development. Poor fertilization rates or complete lack of fertilization in superovulated animals is attributed to abnormalities in oocyte maturation (Moor et al., 1985) and asynchrony between maturational events in the oocyte and the follicle. Compounding these factors is reduced sperm transport in the oviducts of superovulated animals at the time of fertilization (Hawk, 1988). In sheep, exogenous gonadotropins, administered at high concentrations, disturb biochemical function of the oocyte (Moor et al., 1985). Oocytes are activated prematurely causing abnormalities in the oocytes at ovulation. The abnormalities arise because oocytes are probably in an unsuitable follicular environment for maturation and because the oocytes are aged at the time of ovulation. Aged oocytes are a potential source of embryonal loss. In the study by Moor et al. (1985), PMSG was more liable than FSH to disrupt the normal functions of oocytes. Dattena et al. (1994) demonstrated that fewer high quality embryos were harvested from ewes superovulated with a combination of PMSG and FSH than from animals superovulated with FSH alone.

Poor embryo recovery rates in superovulated ewes may be attributed to a reduction in fertilization rate and to several post fertilization factors that contribute to embryo losses before and during embryo flushing. Endocrine abnormalities may also change the uterine tone making embryo recovery in sheep especially difficult. In addition

to affecting viable embryo recovery from donors, the hormonal abnormalities associated with superovulation may also affect the viability of embryos in recipients after transfer.

Immunological influences on follicular development are currently being studied, and these studies are providing new methods to superovulate sheep. In sheep, passive immunization against inhibitors of gonadotropin secretion may also be a means of increasing the ovulation rate by increasing the levels of endogenous FSH (Maplecroft, et al., 1994). Potentially, ewes with circulating antibodies against endocrine regulators of gonadotropin secretion would have a higher ovulation rate and a larger population of follicles capable of responding to exogenous gonadotropins (Maplecroft et al., 1994).

Interactions Between Estrogen, Progesterone, Oxytocin and $PGF_{2\alpha}$

Estrogen and Progesterone Receptors. Oscillating ovarian production of estrogen and progesterone coordinates uterine functions throughout the estrous cycle and early pregnancy. In sheep, changes in the ovarian production of estrogen and progesterone are accompanied by changes in the endometrial steroid hormone receptor populations (Findlay et al., 1982; Cherny et al., 1991; Ott et al., 1993; Wathes and Hamon, 1993). Progesterone and estrogen receptors are greatest at the time of estrus in response to the increased concentrations of estrogen (Wathes and Hamon, 1993). Progesterone and estrogen receptor concentrations are least during the later portion of the luteal phase in response to prolonged periods of progesterone. Estrogen up regulates estrogen and progesterone receptor expression (Miller et al., 1977; Zelinski et al., 1982; Spencer et al., 1995; Spencer et al., 1995b), whereas progesterone inhibits estrogen receptor expression (Spencer et al., 1995; Spencer et al., 1995b). Specifically, estrogen regulates gene expression of the estrogen and progesterone receptor (Webb et al., 1992) by modulating gene expression through transcriptional and post-translational regulation (Shapiro et al., 1989).

As progesterone in circulation increases, endometrial estrogen receptor mRNA and protein decrease (between d 1 to 11) (Spencer and Bazer, 1995). Estrogen receptor expression increases between d 13 and 15 and is maximal on d 1 when plasma concentrations of estrogens are rising and progesterone levels are low (Baird and Scaramuzzi, 1976). This suggests that progesterone blocks the effects of estrogen during the luteal phase and early pregnancy.

The mechanism by which estrogen enhances progesterone receptor transcription is well documented (Kastner et al., 1990; Kraus et al., 1994). However, estrogen enhancement of estrogen receptor transcription or translation is not well understood. According to Ing et al (1996), estrogen regulates estrogen receptor expression at the mRNA and protein level, not at the level of the gene. The short half lives (2 to 3 h) of the mRNA and protein products of the estrogen receptor gene make estrogen receptor levels susceptible to transcriptional and(or) post-transcriptional regulation (Nardulli and Katzellenhogen, 1986; Borras et al., 1994). This accounts for the rapid turnover of estradiol receptors following a low dose of exogenous estradiol-17β. Following the administration of the exogenous estradiol-17β, the mRNA products are modified, and the protein product is produced and remains for approximately 2 to 3 h unless there is continued stimulation with estrogens (Ing et al., 1996).

Oxytocin. In ewes, the uterus is not responsive to oxytocin during portions of the luteal phase (Roberts et al., 1976). The absence of oxytocin receptors during the luteal phase prevents oxytocin from and causing an effect. The uterus is least responsive to oxytocin during periods of progesterone dominance. The amount of uterine activity seems to correspond to uterine sensitivity to oxytocin. In sheep, the uterus is most active at estrus or just before estrus (Croker and Shelton, 1973; Naaktgeboren et al., 1973).

Actions that mimic contractions stimulate oxytocin release in many species. Physical manipulation of the vagina in cows (DeBackere and Peeters, 1960) and ewes (DeBackere et al., 1961), and the vulva in cows (Hays and Van Demaark, 1953) can induce a moderate oxytocin release (40 to 100 mU). In ewes, cows, and goats, stretching

the cervix or the vagina causes milk release (Andersson, 1951; Hays and VanDemark, 1953; Debackere and Peeters, 1960; Debackere et al., 1961). Therefore, it is important to take into account the effects of oxytocin during transcervical ET and AI procedures when the vulva, vagina, and cervix may be inadvertently stimulated.

Estrogen, Progesterone, and Oxytocin Receptors. Estrogen increases oxytocin receptor expression (Soloff, 1975; Hixon and Flint, 1987; Beard and Lamming; 1994), and progesterone decreases oxytocin receptor expression (Burgess et al., 1990; Beard et al., 1994). During the luteal phase, the endometrium is under the influence of progesterone. It has been suggested that progesterone blocks the action of estrogens, probably at the level of the estrogen response element on the oxytocin receptor gene (E.E. Custer, personal communication). In ewes, increased progesterone concentrations on d 12 down regulate the progesterone receptor. The decrease in response to progesterone correlates with an increase in the response to estrogen. Increased estrogen up regulates the production of oxytocin receptors. In rats and cows, there is clear evidence that estrogen binds to an estrogen response element on the oxytocin receptor gene (Adan et al., 1991; Adan et al., 1993; Zingg et al., 1995; Bale and Dorsa, 1997). It is reasonable to conclude that similar events occur in other animals. After estrogen binds to the response element on the oxytocin receptor gene, synthesis of oxytocin receptors begins. Exogenous estrogen increases oxytocin receptors approximately 10 to 12 h after the treatment (E.E. Custer, personal communication).

Progesterone may block the effects of estrogen; however, the block is limited. Initially, in ovariectomized ewes, progesterone suppresses the effects of estrogen on

oxytocin induced secretion of $PGF_{2\alpha}$, but after 10 d of progesterone treatments it enhances the effects of estrogen (McCracken et al., 1981). Homanics and Silvia (1988) reported similar findings after 15 d of progesterone treatment. These findings indicate a short-term suppressive effect of progesterone on oxytocin-induced secretion of $PGF_{2\alpha}$ that disappears after longer periods of treatment. After longer periods of treatment, it also suggests an interaction between estrogen and progesterone that results in an increased responsiveness of the uterus to oxytocin. Endometrial concentrations of oxytocin receptors are greater after 12 d than after 5 d of progesterone treatment (Zhang et al., 1992). The interaction between progesterone and estradiol to enhance $PGF_{2\alpha}$ secretion may not be because of changes in oxytocin receptor concentrations.

Because $PGF_{2\alpha}$ is secreted by the endometrium in response to oxytocin (Sharma and Fitzpatrick, 1974; Mitchell et al., 1975; Roberts et al., 1976), a role has been postulated for oxytocin in luteolysis (Flint et al., 1990). However, reports of the effects of exogenous oxytocin on corpora lutea (CL) have been equivocal. Hatjiminaoglo et al. (1979) is the only study that reports that exogenous oxytocin given between d 1 and 7 of the estrous cycle causes premature luteolysis in some ewes. However, the statistical validity of this experiment is questionable. Premature luteal regression was observed in a significant number of the ewes treated with oxytocin; however, the significance was inflated by the small number of ewes on the study. Milvae et al. (1991) administered exogenous oxytocin over a series of 4 d periods throughout the estrous cycle, without observing premature luteolysis or a decrease in cycle length. Flint and Sheldrick (1985)

reported that continuous infusions of oxytocin on d 13 to 21 in cyclic ewes prolonged luteal lifespan for up to 7 d.

Summary

Development of transcervical ET procedures depends on decreasing the influence of the cervix. Combining the mechanisms of cervical dilation associated with parturition and the current understanding of the influence of the hormones involved in dilation may lead to a viable transcervical ET method.

Chapter III

QUESTIONS AND OBJECTIVES

Earlier research conducted by our group indicated that a better understanding of cervical physiology was needed before an effective transcervical AI technique could be developed. Thus, our previous research increased our understanding of cervical physiology and the mechanisms by which the cervix can be dilated. Many of the problems encountered with perfecting a transcervical AI technique indicated that it may be physiologically more feasible to develop an efficacous transcervical ET technique first. The research initiated for this thesis was designed to test a transcervical ET technique. Many complications involving intrauterine ET were encountered and many new questions emerged. The ET procedure proved to be more difficult to develop than originally anticipated, and it was necessary to retreat from the original approach of developing a transcervical ET technique and first answer some basic questions.

Initially, there were several physical limitations associated with transcervical ET procedures. Other groups attempting transcervical AI and transcervical ET have used techniques very similar to those used in the cattle industry (B. Buckrell, personal communication; D. Marsh, personal communication). These techniques are not effective on sheep for two key reasons: 1) the occlusive nature of the cervix in ewes; and 2) the inability to rectally manipulate the cervix in ewes. It was necessary to develop new ET instruments that would overcome these barriers to perfect a transcervical ET procedure. Several experiments conducted by other members of our group were performed to test the safety and effectiveness of the new equipment.

It was also key to confirm that the new ET instrument was passing through the cervix and into the uterus. Thus, experiments were designed to test the following questions:

Question 1: Is it possible to pass the modified ET instrument through the cervix of a ewe?

Question 2: Does the modified ET rod physically damage tissue as it is manipulated through the cervix?

Question 3: Will embryos flow through the modified ET rod?

Previous research indicated that dilating the cervix in ewes at estrus and during the luteal phase could be accomplished using oxytocin. Dilating the cervix presents a unique opportunity to use fundamental physiology to produce a technique that could be applied in industry. During the luteal phase, estradiol is administered in conjunction with oxytocin to dilate the cervix; therefore, it was this combined treatment that was of particular interest. Although, it is possible to dilate the cervix, it is imperative for us to know that the method of dilation does not interfere with normal reproductive function. Aspects of each experiment addressed questions concerning this issue:

Question 4: Does the estradiol-17β-oxytocin treatment associated with transcervical ET affect luteal function? Or more specifically:

> **Question 4a:** Does estradiol-17β treatment on d 6 of the estrous cycle affect luteal function?
>
> **Question 4b:** Does oxytocin treatment on d 7 of the estrous cycle affect luteal function?
>
> **Question 4c**: Does estradiol-17β-oxytocin treatment on d 6 and 7, respectively, affect luteal function?

Question 5: Does the estradiol-17β-oxytocin treatment associated with transcervical ET make ET more practical?

Question 6: Does the estradiol-17β-oxytocin treatment associated with transcervical ET affect embryonic development?

Question 7: Does the estradiol-17β-oxytocin treatment associated with transcervical ET affect embryonal survival?

There are several inherent financial problems associated with ET protocols in all species. For ET to be financially successful, a maximal number of healthy embryos must be retrieved from a signal donor. Generally, producers and researchers apply superovulatory techniques to achieve this goal. However, the results of superovulatory techniques are highly variable. Therefore, to minimize cost and maximize the number of embryos collected, it was essential to begin by evaluating our superovulatory protocols.

Question 8: Is it possible to decrease the variability of the superovulatory response?

Before performing a large transcervical ET field study, it was necessary to establish that the techniques designed for this thesis were effective in a research environment. Criteria designed to determine whether this was a worthwhile technique included answers to the following questions.

Question 9: Is it possible to deposit embryos in the uterus via the cervix using the modified ET rod and the hormonal treatments to dilate the cervix?

Question 10: Do the embryos remain in the uterus after they have been deposited through a dilated cervix?

Question 11: After the embryos are transferred transcervically, do they develop?

Question 12: After transcervical ET, is embryonic development consistent with the age of the embryo?

It was possible to address of all of these questions within the scope of the research projects described in this thesis. Experiments testing the effect of estradiol-17β-oxytocin treatments on luteal function and superovulation are addressed in chapter IV. Experiments testing the ET technique and the success of transcervical ET using estradiol-17β-oxytocin to dilate the cervix are described in chapter V.

Chapter IV

ELEMENTS OF A TRANSCERVICAL ET PROCEDURE IN SHEEP

Introduction

A previous study (Khalifa et al., 1992) indicated that estradiol-17β-oxytocin induced cervical dilation during the luteal phase may be a useful adjunct to transcervical ET techniques. Experiments were initiated to determine whether exogenous estradiol-17β and oxytocin could be used to improve transcervical ET. However, the effects of estradiol-17β-oxytocin treatment on luteal function were unknown, and therefore, controversial. Superovulatory protocols were used to increase the number of transferable embryos obtained from each donor. However, embryo quality seemed to decrease as the superovulatory response to pFSH increased. It appears that optimal embryo quality is achieved when six ovulations occur. Therefore, two experiments were conducted to determine 1) the effect of exogenous estradiol-17β-oxytocin treatment associated with transcervical ET on luteal function (Exp. 1) and 2) the optimal dose of pFSH needed to induce approximately six CL (Exp. 2).

Materials and Methods

General

Mature Hampshire, Dorset, Finnsheep, and crossbred ewes were penned twice daily during the breeding season with vasectomized rams. Ewes standing firmly to be mounted were considered to be in estrus. Ewes were observed in estrus for two

consecutive cycles, to ensure normal estrous cycles, before they were assigned to an experiment.

Ewes received one half of a Syncro-Mate-B (Sanofi, Overland Park, KS) implant between d 8 and 12 of an estrous cycle. Lutalyse® (Pharmacia and Upjohn, Kalamazoo, MI) was administered in two 5 mg (1 mL) injections 4 to 6 h apart 6 d after the ewes received implants to ensure that the CL regressed. Implants were removed 10 d after they were inserted. Ewes were penned twice daily with vasectomized rams to check for estrus after the synchronization treatment. Ewes that were not detected in estrus within 60 h after implant removal were not assigned to an experiment.

Experiment 1: Does Estradiol-17b-Oxytocin Treatment Affect Luteal Function In Ewes?

Experimental Design. The aim of Exp. 1 was to evaluate luteal function in response to an estradiol-17β-oxytocin treatment designed to dilate the cervix and aid in transcervical ET in ewes. This experiment was designed in a 2 x 2 factorial array. Ewes were assigned to one of four randomized treatment groups: 1) EtOH:saline, saline; 2) estradiol-17β, saline; 3) EtOH:saline, oxytocin; and 4) estradiol-17β, oxytocin. Eight ewes were assigned to each treatment group. On d 6 of the estrous cycle, the estradiol-17β treated ewes received injections in the jugular vein of 100 μg (5 mL) of estradiol-17β diluted in 50% ethanol:50% saline, and control ewes received 5 mL of the EtOH:saline solution. On d 7 of the estrous cycle, the oxytocin-treated ewes received injections in the jugular vein of 400 USP units (20 mL) of oxytocin, 12 h after the estradiol-17β, treatment and control ewes received 20 mL of sterile saline at the same time. Ewes were penned

with vasectomized rams twice daily throughout this experiment and cycle length was recorded. This experiment was conducted from October until November.

Blood Sampling. Blood samples were taken on d 7, 8, 9, 10, 12, 14, 16, and 18. Approximately 12 mL of blood were collected from the jugular vein into heparinized Vacutainers® (Becton Dickinson, Rutherford, NJ) at each collection time. The heparinized Vacutainers® were inverted several times to mix the heparin solution with the blood. Blood was stored in an ice water bath until it was processed approximately 45 min after collection. The samples were centrifuged at 1,700 x g at 10° C for 30 min. Plasma samples were decanted into separate polypropylene tubes and stored at -20° C.

Changes in progesterone concentrations were evaluated in response to each treatment. Progesterone concentrations were used to evaluate luteal function.

Assay Procedures. A [^{3}H] progesterone RIA (Gengenbach et al., 1977) was used to quantify progesterone concentrations in all blood samples. All samples were assayed in duplicate.

Statistical Analysis. The GLM procedures of the Statistical Analysis System (SAS, 1985) were used to determine the effect of treatment on progesterone over time. When the F-tests were significant, least squares means were compared. Least squares means were used to account for missing samples.

To determine whether estradiol-17β, oxytocin, or their interaction affected the mean concentrations and whether the mean concentrations of progesterone changed over time the following GLM statements:

```
PROC GLM; CLASSES EWE E2 OT DAY;
MODEL PROGESTERONE = E2 OT E2*OT EWE(E2*OT) DAY
E2*DAY OT*DAY E2*OT*DAY;
LSMEANS E2 OT E2*OT E2*DAY OT*DAY E2*OT*DAY;
```

where E2 was defined estradiol-17β, and OT was oxytocin.

Experiment 2: Can Refinements in Superovulatory Techniques Improve Ovulation Rates In Ewes Prepared for ET?

Experimental Design. The aim of Exp. 2 was to determine the dose of pFSH (Sioux Biochemical, Sioux City, IA) needed to induce approximately six CL. Estrus in Hampshire and Hampshire x Dorset ewes (n = 23) was synchronized as described in the General methods section. Ewes received a total of either 0 (n = 5), 18 (n = 6), 27 (n = 6), or 36 (n = 6) mg of pFSH, which was injected i.m. at -24, -12, 0, 12, 24, and 36 h relative to implant removal. The dose at each respective time was 19.4, 19.4, 16.7, 16.7, 13.9, and 13.9% of the total. As a portion of the superovulatory protocol, ewes received a priming dose of 400 IU of PMSG i.m. at -24 h.

Counting Corpora Lutea. The CL were counted using a modification of the laparoscopic procedure described in Appendix B. After insertion of the laparoscope and the manipulation probe, the uterus was maneuvered into view. The uterine bifurcation was exposed. Following the uterine horns, the oviduct was located. Using the probe, the mesosalphinx and mesovarian tissue were elevated, the ovary was exposed, and CL were counted. This procedure was repeated for both ovaries.

Statistical Analysis. The GLM procedures of SAS (1985) were used to determine the effect of dose of pFSH on the number of CL. Orthogonal contrasts were used to determine whether the number of CL increased linearly or quadratically with dose of

pFSH. To determine whether the number of CL increased linearly or quadratically with dose of pFSH the following GLM statements:

```
PROC GLM; CLASSES TRT;
MODEL CL = TRT;
CONTRAST 'LIN' TRT -3 -1 1 3;
CONTRAST 'QUAD' TRT 1 -1 -1 1;
```

where TRT was defined as the dose of pFSH, CL was the number of CL per ewe, LIN was linear and QUAD was quadratic. The mathematical commend to test for a linear fit was -3 -1 1 3, and the mathematical commend to test for a quadratic fit was 1 -1 -1 1.

Results

Experiment 1.

Progesterone Response. The changes in progesterone concentrations over time are shown in Figure 1. The main effects of estradiol-17β and oxytocin did not affect progesterone concentrations (Figure 1), and the estradiol-17β x oxytocin interaction was not significant. Progesterone concentrations changed ($P < .01$) with day after estrus (Figure 1). There was a significant oxytocin x day interaction ($P < .001$); luteal function was enhanced in the oxytocin treated ewes. Mean progesterone concentrations in the oxytocin treated ewes did not begin to decrease until d 14 of the estrous cycle, but, they started to decrease on d 12 in control ewes (Figure 1).

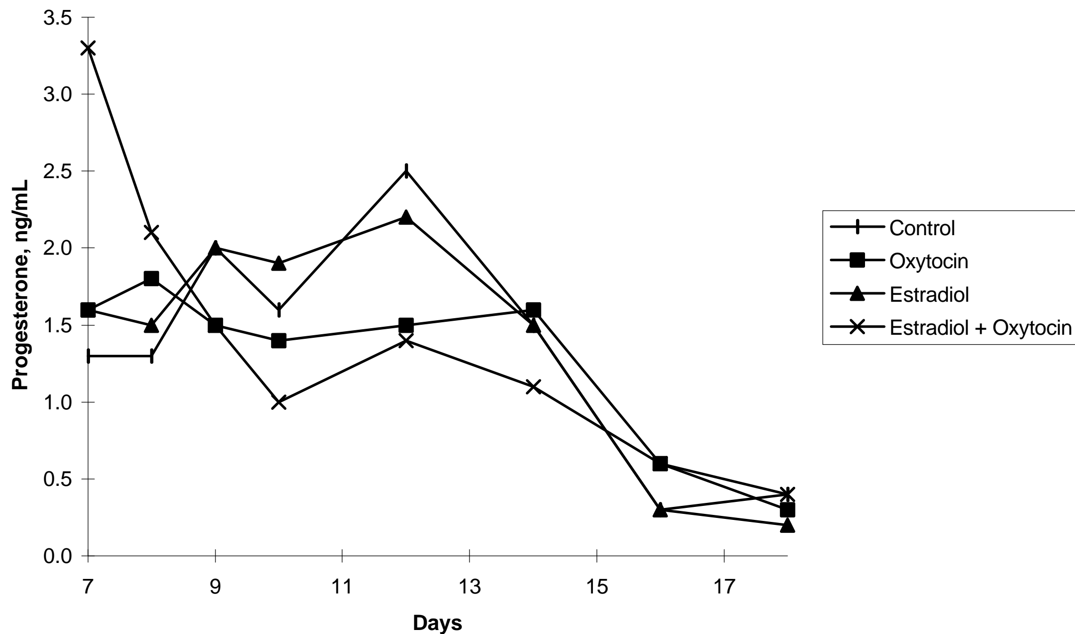

Figure 1. Effect of E_2-OT treatment on progesterone concentrations in Exp. 1. The E_2 (200 µg) or saline was injected on d 6 followed by injections of OT (400 USP units) or saline 12 h later on d 7. The E_2 and OT did not affect progesterone concentrations or the duration of the estrous cycle, but the OT x day interaction was significant ($P < .001$). The mean square for error = .9685, and n = 8 ewes per group and n = 8 samples per mean.

Experiment 2.

Dose response Curve. The effect of dose of pFSH on the number of CL for Exp. 2 is shown in Figure 3. The dose of pFSH affected the number of CL ($P < .001$; Figure 3). The number of CL increased linearly with dose of pFSH ($P < .0001$; Figure 3). Ewes receiving 27 mg of pFSH responded with a mean of 6.2 CL, which was closest to the target of six CL per ewe.

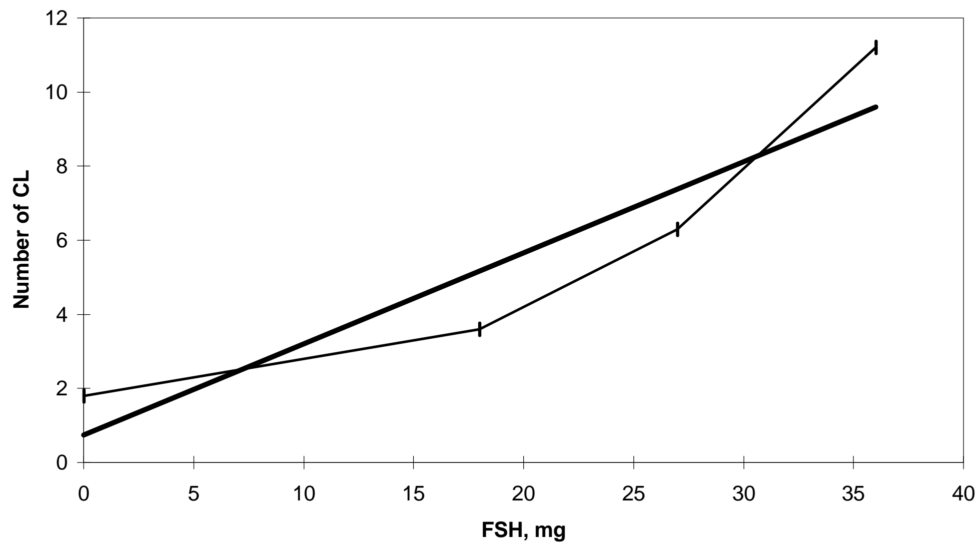

Figure 2. The pFSH dose response curve in Exp. 2. The number of CL observed increased linearly as the dose of pFSH was increased ($P < .001$). The mean square for error = 2.18, and n = 6 ewes per group except for control group for which n = 5 ewes. The adj. $r^2 = .789$ and the equation of the best fit line is $y = .2457x + .7743$.

Chapter V

LAPAROSCOPIC VERSUS TRANSCERVICAL EMBRYO TRANSFER AND THE EFFECTS OF EXOGENOUS ESTRADIOL-17β-OXYTOCIN TREATMENT ON EMBRYO RECOVERY AND SURVIVAL IN EWES

Introduction

Currently, ET in sheep is performed laparoscopically, which is an expensive technique that requires extensive training. For ET to be widely used in the in research or the sheep industry, a feasible transcervical method must be developed. It is necessary to ensure that any new technique can be used with an acceptable degree of success in a research setting before it can be tested in the field.

Data from Exp. 1 indicated that the estradiol-17ß-oxytocin treatment did not affect luteal function in ewes. Therefore, Exp. 3 was designed to determine whether the transcervical ET method developed as part of the research for this thesis could be used effectively in a research setting.

Materials and Methods

Experiment 3.

Experimental Design. In Exp. 3, the effects of exogenous estradiol-17β and oxytocin (estradiol-17β-oxytocin vs EtOH:saline-saline) and type of ET (laparoscopic or transcervical) on embryo recovery rate and survival were evaluated.

Ewes were assigned to a donor or a recipient flock. In the first trial of the experiment, recipient ewes were assigned to one of three randomized treatment groups: 1) EtOH:saline-saline, laparoscopic; 2) estradiol-17β–oxytocin, laparoscopic; and 3)

estradiol-17β-oxytocin, transcervical. The recipient ewes in the second trial were randomly assigned to one of four randomized treatment groups: 1) EtOH:saline-saline, transcervical; 2) EtOH:saline-saline, laparoscopic; 3) estradiol-17ß-oxytocin, transcervical; and 4) estradiol-17β-oxytocin, laparoscopic. The experimental unit in this experiment was considered the embryo not the recipient. Treatment 1 was excluded from the first trial because of technical limitations (i.e., the inability to pass an ET instrument through the cervix in EtOH:saline-saline ewes), which were overcome by the beginning of the second trial. Treatment groups will be referred to by the designations assigned in the second trial. The initial design called for 30 embryos per group, but because of technological and time constraints this was not achieved. The uneven distribution of embryos across the four treatments can be attributed to changes in the randomization scheme with the addition of treatment 1 during the second trial.

On d 5 of the estrous cycle, the estradiol-17β-oxytocin treated ewes received injections in the jugular vein of 100 μg (5 mL) of estradiol-17β diluted in 50% ethanol:50% saline, and control ewes received 5 mL of the EtOH:saline solution on d 5 of the estrous cycle. Oxytocin (400 USP units; 20 mL) was administered in the jugular vein 10 h after the estradiol-17β, or ewes received 20 mL of sterile saline. Embryos were transferred to ewes transcervically or laparoscopically 20 min after they received oxytocin or saline.

The first trial of this experiment was conducted between January and April. The second trial was conducted between November and March of the following breeding season.

Donor Preparation. Donor ewes were synchronized as described in Chapter IV. During the first trial, ewes were superovulated with 20 mg of pFSH (Folltropin; Vetrepharm, London, Ontario, Canada) administered as a series of five injections: 5, 5, 5, 2.5, and 2.5 mg at -36, -24, -12 , 0 and 12 h, respectively, relative to Synchro Mate-B implant removal (Rexroad and Powell, 1991). In the second trial, ewes were superovulated with a combination of 400 IU PMSG (Sioux Biochemical, Sioux City, IA) and 24 mg of pFSH (Sioux Biochemical). The pFSH was administered as a series of six injections: 5.25, 5.25, 4.5, 4.5, 3.75, and 3.75 mg at -24, -12, 0, 12, 24, and 36 h relative to implant removal. The dose for the second trial was based on the results from Exp. 2. The preparation of FSH was changed based on availability and product consistency.

In trial one, two rams known to produce offspring were trained to an artificial vagina for semen collection. A ram was introduced to a ewe in estrus. The ram was allowed to mount, and his penis was directed into the artificial vagina. This process was continued until the ram ejaculated. The semen was collected into a glass graduated conical tube and was stored in a water bath at 37°. All semen was used within 1 h after collection. Sperm motility was estimated in each ejaculate by examination at 40X. Only ejaculates with greater than 60% motility were used.

In the first trial, ewes were artificially inseminated using the laparoscopic procedures described by Evans and Maxwell (1987; for procedural details see Appendix

B). Ewes were inseminated with .25 mL of fresh neat semen in each uterine horn 48 h after implant removal.

In the second trial, ewes were bred naturally. Following implant removal, ewes were placed in a small pasture with rams at a stocking density of at least one ram per ewe. The rams were rotated every 12 h, and each ewe was exposed to a total of 24 fertile rams. The ewes were left with the rams for a total of 5 d after the removal of the implants. The high stocking density was designed to avoid the low fertility associated with natural mating of superovulated ewes (J. Stellflug, personal communication).

Recipient Preparation. Estrus was synchronized using the procedures described in Chapter IV. In the first trial, implants were removed from the recipients on the same day as from the donors. Recipients and donors were synchronized based on the day of implant removal. During the second trial, implants were removed from the recipients 1 d after they were removed from the donors in an attempt to synchronize the embryo with the uterine environment.

Embryo Collection. Embryos (morulas or blastocysts) for transfer were collected surgically 6 d after insemination (for general procedural details see Appendix A). After the uterus was exposed, the oviduct was located, and the infundibulum was carefully fanned out over one finger. The infundibulum was folded back to expose the lumen of the oviduct. The lumen of the oviduct was catheterized with a sterile polyvinyl tube. The catheter was placed a third of the way into the oviduct. Using a sponge, the catheter was grasped, holding it in the oviduct, and the tip was placed over a Petri dish. The uterine horn was clasped near the cervix with the thumb and forefinger. Using a 12 cc disposable

syringe with an 18 ga blunt needle, 12 mL of medium were flushed through the uterine horn, out the catheter, and into the Petri dish. This procedure was repeated for the opposite uterine horn. If there were no CL on the ovary, the adjacent horn was not flushed. The flushings were immediately transferred to a slide warmer and maintained at 36° until embryos were found and removed.

After the uterine horns were flushed, the uterus was washed with sterile saline and allowed to return to its normal position. The body wall and skin incisions were closed with polyamide suture.

Embryo Processing. Embryos were held in a modified Dulbecco's media (Appendix B) that had been filter sterilized (.2 µm; Tuffryn Membrane Filter, Gelman Sciences, Ann Arbor, MI) and was maintained at a neutral pH (7.1). The morulas were graded based on morphology. Embryos were considered excellent, good, fair, or poor, based on the shape and compaction of the cells (Moore, 1977). Embryos that were classified as poor were removed from the experiment. Depending on the number of embryos collected on a given day, one to three embryos were loaded into a .5 mL semen straw. The first quarter of the straw was filled with media, and then a small bubble of air was loaded followed by media plus an embryo. Small air bubbles were loaded between each of the embryos. The last quarter of the straw was loaded with media. The straws were examined under the microscope to verify that the embryos were loaded correctly. The straws were stored on the slide warmer until the embryos were transferred within a time space of 5 to 25 min.

Transcervical ET Rod. Previous transcervical ET techniques developed in our laboratory used a typical cattle AI rod. For sheep, modified AI rods have been developed for transcervical AI (Halbert et al., 1990b). At least two research groups have used these modified AI rods for ET (B. Buckrell, personal communication; D.L. Marsh, personal communication); however, we found them too short and flimsy to be useful (Figure 1).

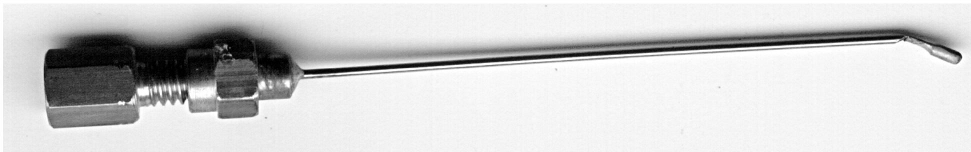

Figure 1. Modified AI rod for ET.

In an attempt to accommodate ET procedures, a new rod was developed (Figure 2). The transcervical ET rod used in these experiments was a 17.5 cm long, 17 ga semiflexible, stainless steele tube with a 4 mm brass bulb attached at the tip. This rod was long enough to allow deposition of the embryos into a uterine horn rather than at the base of the uterine body. The flexibility allowed passage through the cervix without kinking the rod and blocking the passage of embryos through it. The internal diameter (1.07 mm) was large enough for embryos to pass through undamaged. The external diameter of the tube was 1.47 mm. The brass bulb at the end of the tip served two purposes. First, it helped when manipulating the rod through the cervical rings. Second, the brass bulb is echogenic and easily picked up on ultrasound allowing confirmation of the position of the tip of the rod inside the reproductive tract. The end of the tip was brass and was machined to couple securely to a retroload AI gun. Each tip was checked to ensure that it fit snugly with the

retroload gun to prevent embryos from getting caught in any gaps between the rod and the gun.

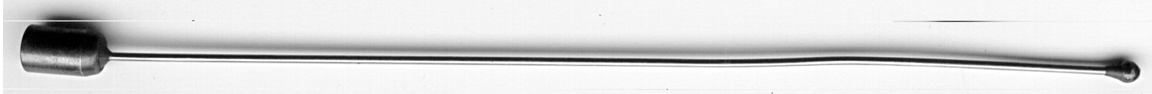

Figure 2. Transcervical ET rod used in Exp. 3.

Transcervical ET. Recipient ewes were restrained in a dorsal recumbent position in a Poldenvale Commodore chute. Wool was removed from the area surrounding the vagina and the anus. This area was scrubbed with an antiseptic soap and rinsed with water three times. Dry sponges were used to remove excess water and antiseptic. A coating of KY jelly was applied to a vaginoscope (tubular speculum), and the vaginoscope was inserted into the vagina and pushed against the tissue surrounding the cervix to help center the cervix. A sponge was used to dry the inside of the vaginoscope and remove any fluid near the cervix. A cattle AI rod was placed into the folds of tissue surrounding the external os to position the cervix in the vaginoscope. The cattle AI rod was removed. The ET rod was placed at the external os and manipulated through the cervix (Figure 3). The rod was believed to have passed through the cervix when it was no longer obstructed by cervical rings, when there was a change in tissue tone between the cervix and the uterus, or when the internal bifurcation was found (Figure 3). After passing the rod through the cervix, the bifurcation was located with the tip of the rod, and the rod was angled into a uterine horn. A .5 cc straw containing the embryos was then placed in a retroload gun, and the embryos were expelled through the modified ET rod and into the uterine horn. The contents of a second straw containing media without embryos were injected through the

ET rod into the uterine horn in an attempt to prevent the embryos from sticking to the inside of the embryo transfer rod. The rod was slowly removed, the vaginoscope was removed, and the ewe was left in the dorsal recumbent position for 10 min after transfer.

The ET rod and the straw were flushed with saline. The flushings and straws were checked under a dissecting microscope to determine whether embryos were left in the straw. If an embryo was found in the flushings or in the straws, it was removed from the experiment.

The ET rod and the retroload gun were autoclaved before each use. A separate ET rod was used for each sheep. The vaginoscope was washed with antiseptic and dried thoroughly between uses.

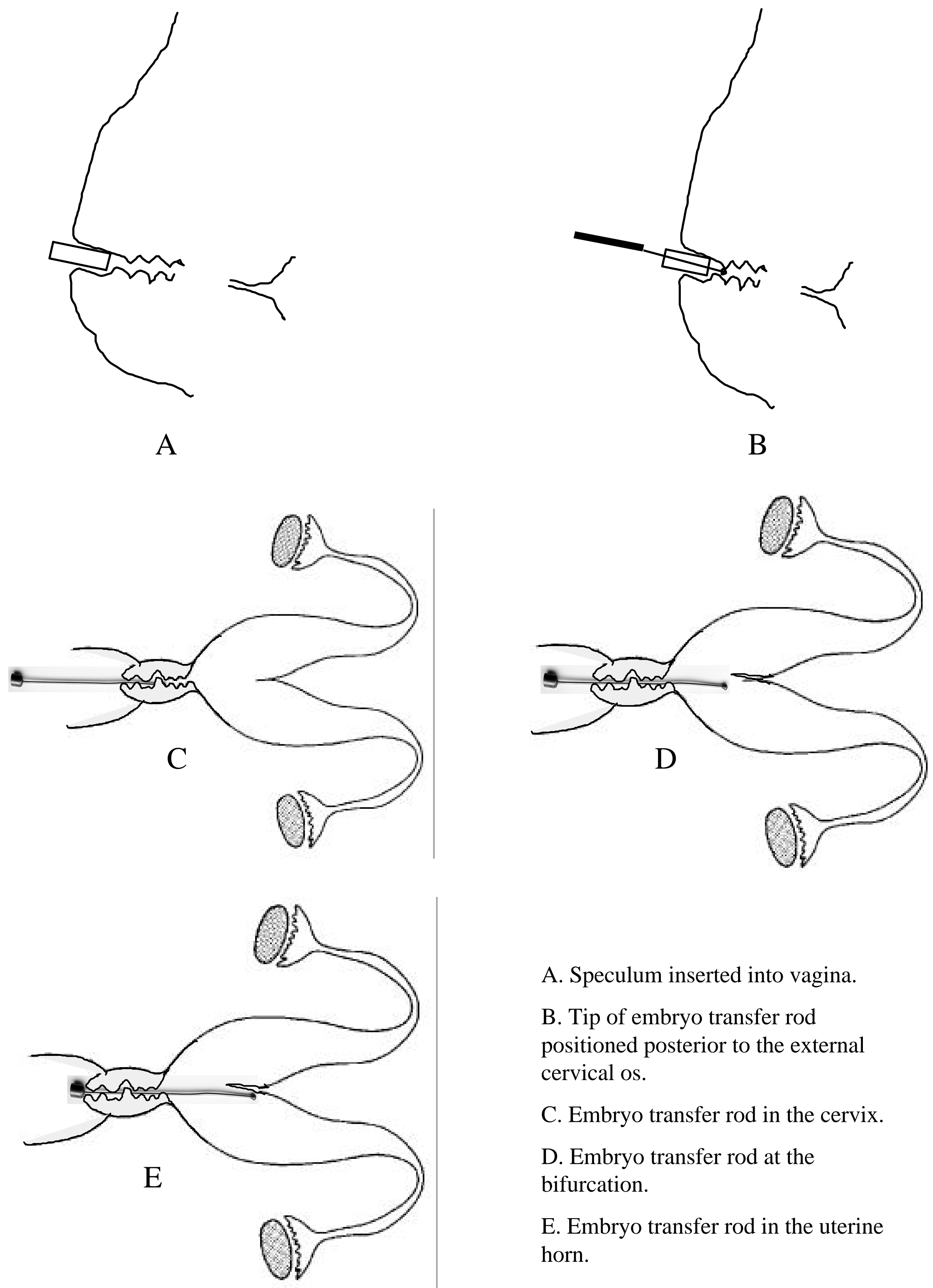

Figure 3. Transcervical embryo transfer: Exp. 3.

Laparoscopic ET. General laparoscopic procedures are described in Appendix B. Ewes without CL were removed from the experiment. The uterus was positioned with both uterine horns in view. The probe was removed and a cattle ET rod, containing a straw with embryos, in a sheath with a 16 ga needle at the end was placed in the 5 mm cannula. Embryos were assigned to randomized sides of the uterus (right or left uterine horn). The needle was plunged into the uterine horn. A second person slowly depressed the plunger of the ET rod. If liquid leaked out at the injection site it was assumed that the needle was not positioned in the uterine lumen. The needle was adjusted until it was through the endometrium and in the uterine lumen. The straws contained adequate liquid before the embryos to allow injection of a small amount of fluid to confirm position of the needle. After the needle was in the uterine lumen, the second person slowly expelled the embryos by depressing the plunger.

The straws that contained the embryos were flushed with 10 mL of media. The flushings and straws were checked under a dissecting microscope to ensure that the embryos were not left in the straw. If an embryo was found in the flushings or in the straws it was removed from the experiment.

Maintenance of Recipients. Recipients were housed indoors in pens with ad libitum access to feed and water until they were killed on d 12 or 14 of pregnancy. During the second trial, 5 mg of supplemental progesterone (2 mg/mL of sesame oil) were administered. Progesterone injections were given immediately after the transfer and administered every 12 h until the ewes were killed. Ewes were given 650 mg of sodium

pentobarbital (Sigma Chemical Co., St. Louis, MO) and anesthetized after which they were exsanguinated.

Embryo Recovery from Recipients. The uterus and a portion of the cervix were removed post mortem and placed in a surgical pan, which was labeled with the ewe number. The CL were counted, and the reproductive tracts were processed within 20 min after removal. A renal vascular clamp was used to occlude the cervix. The ovaries and the majority of the oviduct were removed. Tissue surrounding the uterine horns was carefully removed in an attempt to straighten the uterine horns. The tip of one uterine horn and the remainder of the oviduct were enlarged by inserting the tip of scissors and making a longitudinal cut approximately 5 mm in length. The uterine tip was grasped with a hemostat and placed over a Petri dish (Fisher Scientific Co., Pittsburgh, PA). The opposite uterine horn was clamped with a hemostat. Using a 12 cc disposable syringe with a 20 ga needle, 12 mL of sterile saline were injected into the uterine lumen of the clamped uterine horn. The fluid was injected at a slow steady rate to allow complete flushing of the uterine contents into the Petri dish. A second 12 mL of sterile saline were injected. The fluid remaining in the uterine horns was removed by massaging with the thumb and forefinger beginning with the clamped uterine horn and following the tract to the end of the opened uterine horn. The tract was then cut longitudinally from the initial 5 mm cut to expose the lumen of both uterine horns. The lumens were gently scraped with the blade of scissors to remove remaining embryos. The flushings were examined under a dissecting microscope for the presence of embryos. Embryos were classified developmentally according to their morphology (Killeen, 1969). Embryos were placed into one of four morphological

categories: 1) embryos that did not develop from the time of transfer; 2)embryos that developed to the blastocyst stage; 3) embryos that hatched; and 4) embryos that developed to as tage indicative of d 12 in Trial 1 or d 14 in Trial 2.

Embryos recovered in trial one of this experiment were recovered on d 12 of pregnancy. Embryos recovered in trial two of this experiment were recovered on d 14 of pregnancy. After the first trial, we believed that it would be easier to identify the embryos on d 14. The range of morphological stages varies less on d 14.

Conformation of Intrauterine Transcervical ET Technique. To verify the passage of the transcervical ET rod through the cervix, through the uterine body, and into the uterine horn, eight ewes were used at various times of the year over a 2 yr period of time. The ET rod and tip were echogenic and were easily discerned with ultrasound. Initially, the modified ET rod was evaluated ultrasounically in water to confirm that the tip and the rod were echogenic and to produce sonograms of the ET rod and tip to serve as guides.

The ultrasound evaluation was conducted with an Aloka 500V instrument equipped with a 5.0 MHz transducer (linear array; Corometrics Medical Systems, Inc., Wallingford, CT). During ultrasonography, ewes were restrained in a Poldenvale Commodore chute and placed in dorsal recumbency. A coating of KY jelly was applied to a vaginoscope, and the vaginoscope was inserted into the vagina, until it reached the cervix. A cattle AI rod with a sheath over it was used to place the cervix in the center of the vaginoscope. Pressure was applied to the vaginoscope to maintain the position of the cervix. The ET rod was inserted into the vagina and passed through the cervix, into uterine body, and into the uterine horn. Each position was confirmed ultrasonically.

A liberal coating of KY jelly was placed on the transducer as a lubricant and coupling medium. The transducer was inserted into the sheep's rectum until an image of the vagina, cervix, uterine body, and uterine horn were obtained. The transducer was rotated clockwise and counterclockwise across the reproductive tract until the rod was detected. The proposed position of the ET rod was confirmed by the actual position depicted by ultrasound.

Statistical Analysis. The FREQ and GLM procedures of SAS (1985) were used to determine the effect of hormonal treatment and mode of transfer on number of embryos recovered, embryo development, and the stage of embryo development. Chi-square was used to determine whether the rate of embryo recovery was affected by the fixed variables estradiol-17β-oxytocin treatment, the mode of transfer, or an interaction between the treatments. Chi-square was then used to determine whether embryo development was affected by these variables. The GLM procedures were used to determine whether the stage of embryo development was affected by treatment. Embryos were assigned discrete rankings. The discrete rankings were treated as continuous data that were normally distributed, because rankings assigned over the biological spectrum can be treated as normally distributed data (R. Pearson, personal communication). Even though there is some loss of statistical power, the analysis presents data in the most biologically meaningful terms (R. Pearson, personal communication). The traditional method for analyzing this type of data is by chi-square. However, chi-square analysis places data in cells, which are then placed in rows and columns. Comparisons are then made between the rows and the columns, not between the individual embryo transfer groups, making it

possible to compare only the rows and the columns versus the individual cells. This decreases the biological meaning.

Data were analyzed including both trials, and then the trials were partioned. Partitioning the treatments out of the trials was difficult because of the uneven design in trial 1. Data for trial 2 were analyzed in the same manner as the combined data set. Comparisons in trial 1 were limited to comparisons between treatments during laparoscopic transfer and a comparison of laparoscopic transfer and transcervical transfer when ewes were treated with estradiol-17β-oxytocin treatments.

Results

Conformation of the Position of the Modified ET Rod in the Reproductive Tract.

Several sonograms were made of the modified ET rod and the reproductive tract as a means of orientation and to determine whether the rod and tip were echogenic. The rod and the tip were echogenic. Evaluating the rod and tip in water provided a sagital (Figure 4) and a longitudinal (Figure 5) view. The rounded tip is evident in the sagital view, but the size and the shape of the tip are distorted in the sonogram. The tip appears as a flattened oval. The diameter of the rod in relation to the surroundings is visible in the longitudinal section. Sonograms of the cervix are more difficult to obtain in ewes than in cows. In ewes, the occlusive nature of the cervix and the smaller diameter make it more difficult to distinguish the cervix from other areas of the reproductive tract. Cartilage in the cervical rings produces an echogenic marker. Generally, longitudinal sonograms of the cervix show a pattern of alternating black and white parallel lines (Figure 6). The white lines are the cervical rings. The black lines are the lumenal space between each ring. In

sheep, this pattern is less distinct than in cows because of the eccentric pattern of the rings, although, it is still observable.

The following sonograms were made as the modified ET rod was positioned in the reproductive tract in the same manner as if the ET procedure were being performed. Initially, the tip of the rod was placed against the external cervical os. In most ewes, the rod was still visible outside the vaginal canal. Figure 7 shows the rod after it is passed through the vagina. The tip was placed against the external cervical os. Placement of the rod was confirmed by placing a slight amount of pressure on the rod and feeling it lock into the external cervical os.

The next step in the ET protocol was to manipulate the rod through the cervix. As the tip of the rod moves through the cervix, the cervical rings can be counted as a series of bumps. After the rod passes through the last cervical ring, it slides undisturbed into the uterine body. In Figure 8, the tip and the rod are caudal to the anterior end of the cervix. The cervix is distinguished from the uterus by the wavy outline, indicative of the cervical rings and the pattern of black and white parallel lines. The black and white parallel lines are disrupted by the rod passing through the cervix and making the cervix more difficult to distinguish. At the point where the rod enters the uterus, there is a pronounced increase in the diameter of the lumen. The tip is positioned in the uterine body. The position of the cervix can also be determined in relation to the position of the aitchbone. The cervix and the uterus meet immediately before the curve in the aitchbone (Figure 8).

The ET rod was modified specifically for ET; therefore, it is longer than most transcervical instrumentation. Ideally, the embryos should be delivered into the uterine

horns. After entering the uterine body the rod either slips easily into a uterine horn, or presses against the bifurcation. If the rod presses against the bifurcation, changing the angle a slightly allows it to slip into the horn. Unlike moving the rod from the cervix into the uterus, there is very little change in tone between the uterine body and the uterine horns. The length of the rod is used as the major indicator of its position in the uterine horn. In Figure 9, the end of the cervix with the rod passing into the uterine horn is apparent. The rod was positioned in the uterine horn so that it was not possible to scan the tip. The rod was in too far. The uterine horn is defined by the decreasing diameter of the lumen and the length. A second view of the rod entering the uterine body is presented in Figure 10. At this point, the rod had bent slightly, and it was in several planes so the sonogram only shows a small portion of the middle of the rod. The anterior end of the rod is positioned at the beginning of the uterine horn, and the posterior portion of the rod is positioned at the end of the cervix.

The ultrasound evaluation confirmed that the modified ET rod was passed through the cervix, through the uterine body, and into the uterine horns. Ultrasound was also used to confirm my accuracy in assessing the placement of the rod in the reproductive tract. The ultrasound evaluation indicated that the length of the rod in conjunction with tonal differences of the reproductive tract can be used to predict placement of the modified ET rod.

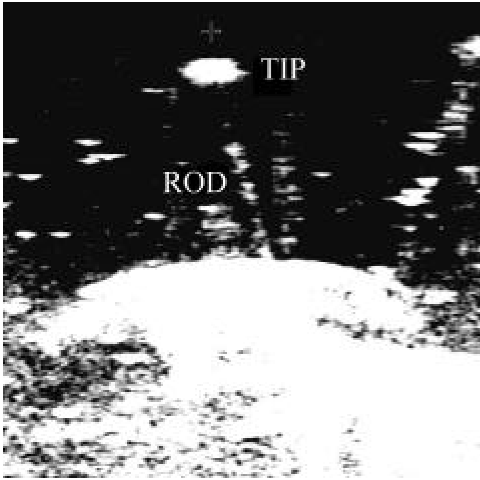

Figure 4. Cross sectional view of embryo transfer rod in water. Exp. 3.

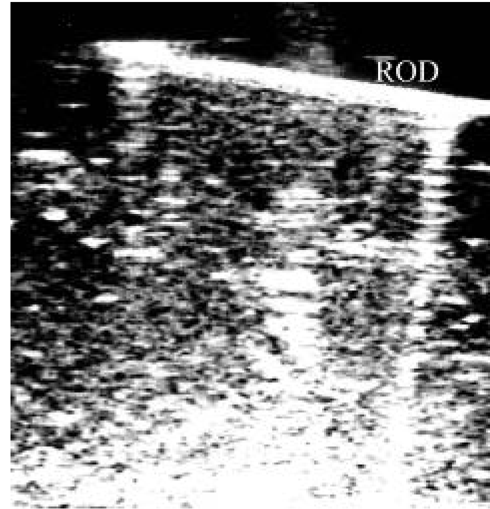

Figure 5. Longitudinal view of embryo transfer rod in water. Exp. 3.

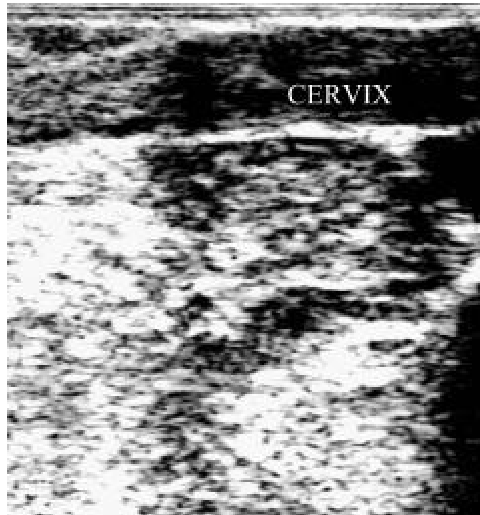

Figure 6. Longitudinal view of dilated ewe cervix. Exp. 3.

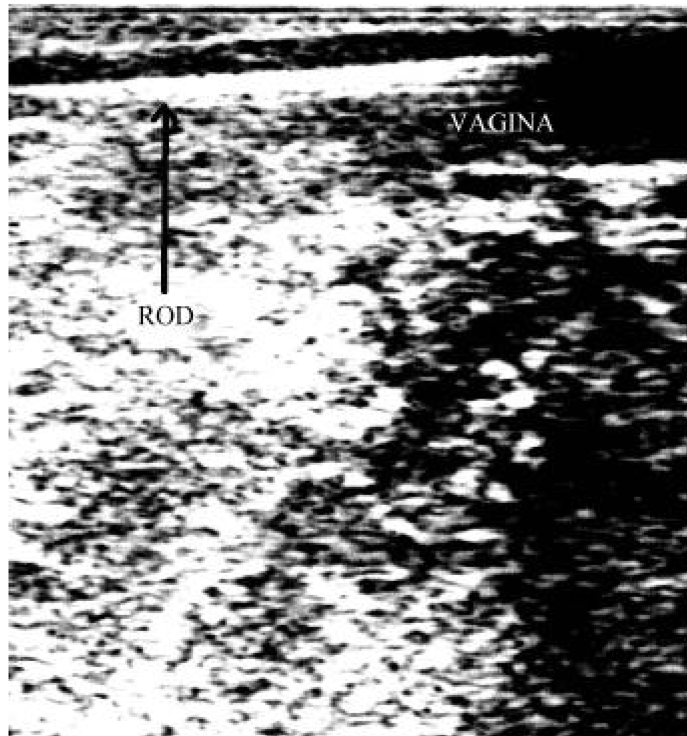

Figure 7. Embryo transfer rod traversing the vagina. Exp. 3.

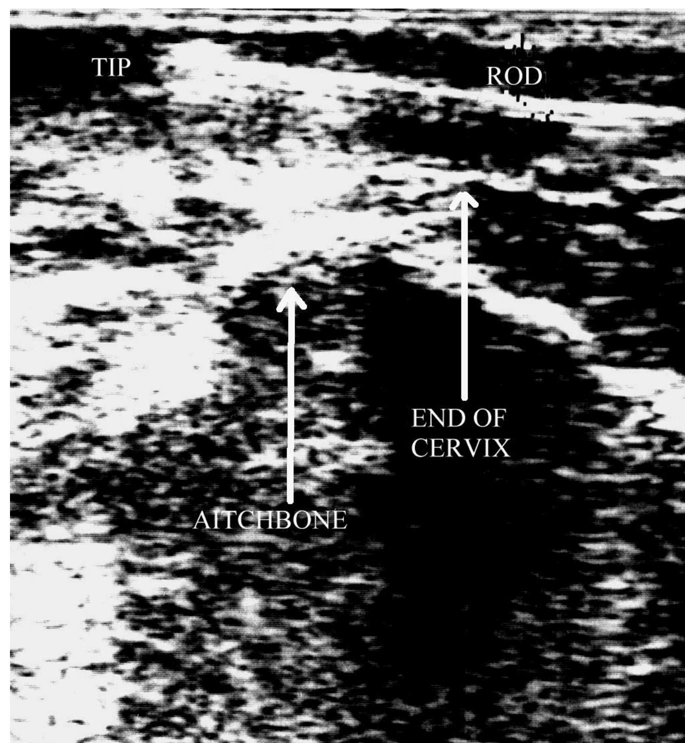

Figure 8. Tip of embryo transfer rod anterior to internal cervical os. Exp. 3.

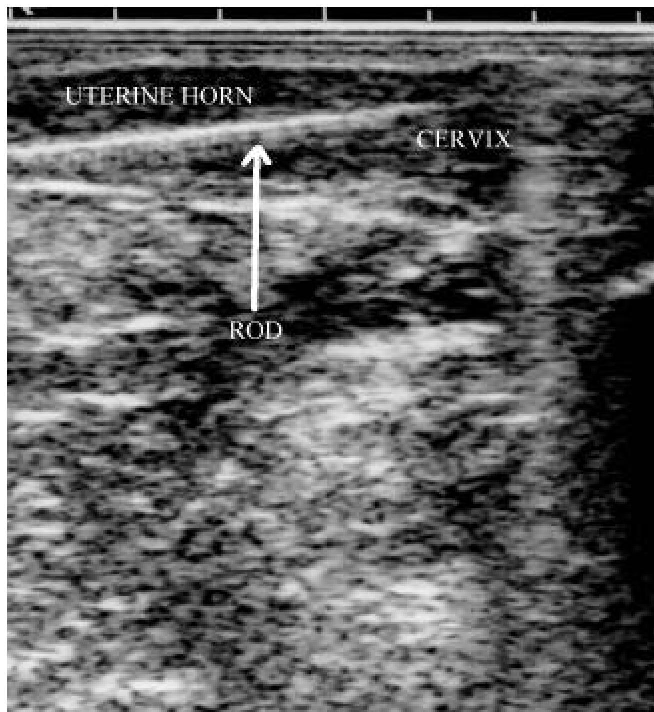

Figure 9. Embryo transfer rod traversing the uterine horn. Exp. 3.

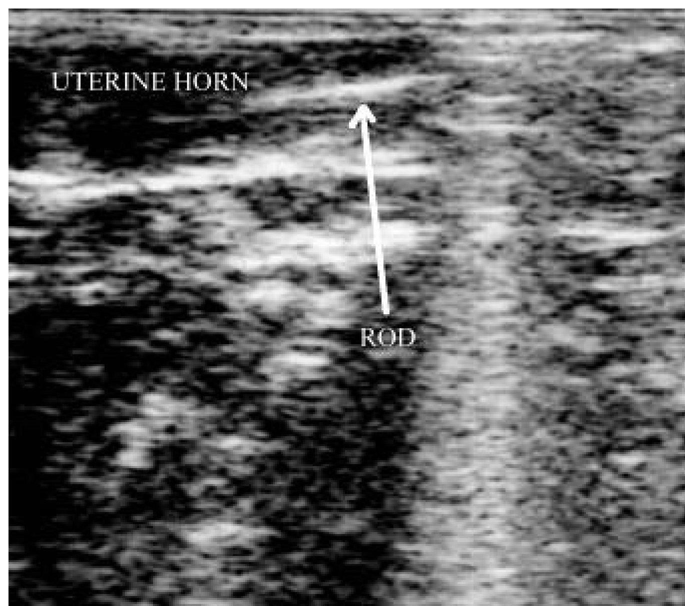

Figure 10. Partial longitudinal view of embryo transfer rod entering the uterine horn. Exp. 3.

Embryo Tranfer Data.

The treatments did not affect the percentage of embryos recovered after transfer (Table 1 and Figure 11). Based on the total number of embryos for all treatments in trials 1 and 2, 66% of the embryos were recovered.

Table 1. Embryo recovery, development, and progression of development in Trials 1 and 2 in Exp. 3

Treatments[A]	Embryos recovered, % (recovered/total)	Embryos developed, % (developed/total)	Mean embryo development score
		Trial 1[B]	
TC	NA	NA	NA
LAP	60.00 (3/5)	100.00 (3/3)[a]	3.25[d]
E2-OT, TC	58.82 (10/17)	60.00 (6/10)[b,U]	2.20[e, W]
E2-OT, LAP	66.66 (4/6)[Y]	50.00 (2/4)[b,U]	2.00[e, W]
		Trial 2 [C]	
TC	68.75 (11/16)[f]	90.91 (10/11)	2.54[i]
LAP	72.73 (8/11)[f]	100.00 (8/8)	4.00[j]
E2-OT, TC	45.45 (5/11)[g]	100.00 (5/5)[V]	3.60[j, X]
E2-OT, LAP	100.00 (2/2)[h, Z]	100.00 (2/2)[V]	4.00[j, X]
		Trials 1 and 2[D]	
TC	68.75 (11/16)	90.91 (10/11)[k]	2.54[m]
LAP	68.75 (11/16)	100.00 (11/11)[k]	3.81[n]
E2-OT, TC	53.57 (15/28)	73.33 (11/15)[l]	2.66[m]
E2-OT, LAP	75.00 (6/8)	66.75 (4/6)[l]	2.83[m]

[A]Transcervical transfers (TC), laparoscopic transfers (LAP), and the estradiol-17β-oxytocin treatment (E2-OT) are abbreviated in the table.
[B]T-tests were used to evaluate the data.
[C]Data in Trial 2 were compared by Chi-square analysis (embryos recovered and embryos developed) and by a comparison of the means (mean embryo development scores).
[D]Data were combined from Trials 1 and 2 and analyzed as in Trial 2.
[a,b,c,d,e,f,g,h,I,j,k,l,m,n]Means with different superscripts in the same column and trial differ (P < .05).
[U,V,W,X,Y,Z]For comparisons between Trials 1 and 2, means with different superscripts in the same column differ (P < .05).

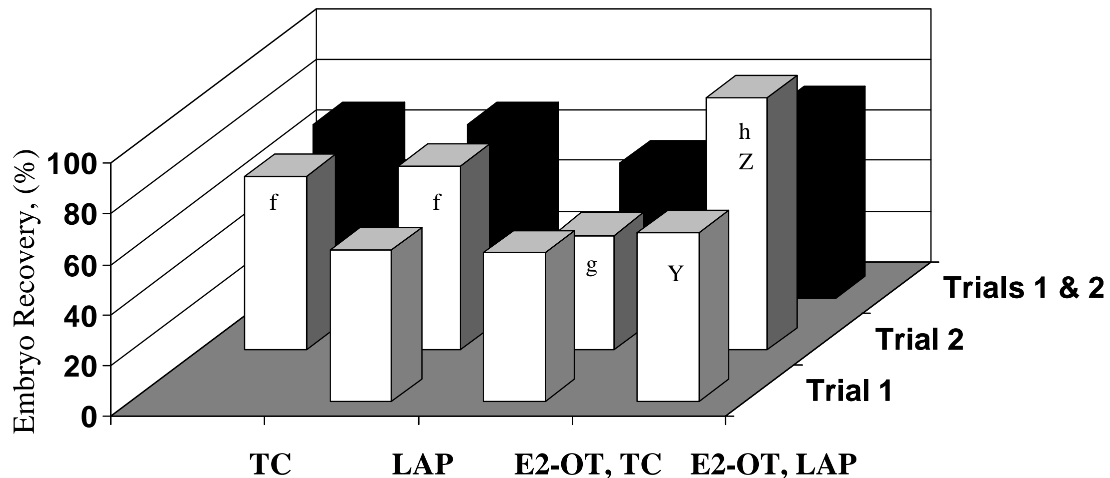

Figure 11. Embryo recovery in Trials 1 and 2 in Exp. 3. Ewes were assigned to randomized treatments, estrus was synchronized, and embryos were transfered transcervically (TC) or laparoscopically (LAP) after EtOH:saline-saline or estradiol-17β-oxytocin (E2-OT). Embryos were collected from recipients 6 d after transfer in Trial 1 and 8 d after transfer in Trial 2. [f,g,h]Different letters indicate that the means in that shade differ ($P < .05$). [Y,Z]Different letters indicate that the means in that treatment group differ between trials ($P < .05$).

The percentage of embryos that developed did not differ between ET techniques (Table 1 and Figure 12). However, the percentage of embryos that developed differed between laparoscopic treatments. A greater percentage of embryos was recovered from the laparoscopic group receiving the control treatment than the group receiving estradiol-17β-oxytocin treatment ($P < .05$; Table 1 and Figure 12). Even though a difference was calculated, it may be an artifact of the low number of embryos in the laparoscopic group receiving estradiol-17β rather than a true difference (Table 1 and Figure 12). The

percentage of embryos that developed differed between the EtOH:saline-saline and the estradiol-17β-oxytocin treated ewes (P < .05; Table 1 and Figure 12).

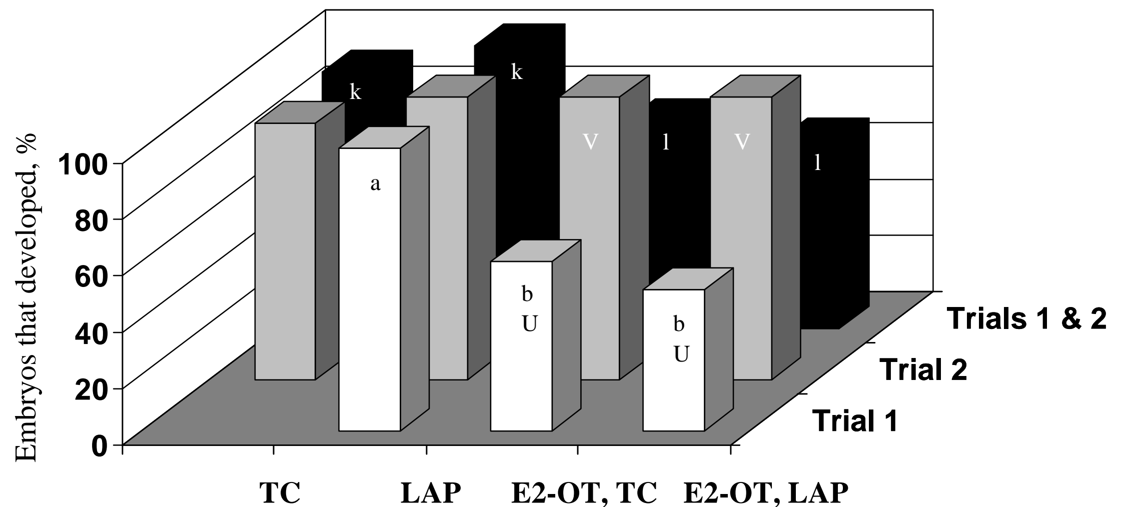

Figure 12. Effect of treatment on embryo development in Exp. 3. Ewes were assigned to randomized treatments, estrus was synchronized, and embryos were transfered transcervically or laparoscopically after EtOH:saline-saline or estradiol-17β-oxytocin. Embryos were collected from recipients 6 d after transfer in Trial 1 and 8 d after transfer in Trial 2.[a,b,k,l]Different letters indicate that the means in that shade differ (P < .05). [U,V]Different letters indicate that the means in that treatment group differ between trials (P < .05).

Development of embryos was assessed morphologically, and embryos were ranked 1 through 4 based on their developmental stage. Embryos that did not develop were ranked 1, embryos that developed to the blastocyst stage were ranked 2, embryos that hatched were ranked 3, and embryos that developed to morphological stages consistant with d 12 in trial 1 and d 14 in trial 2 were ranked 4. There was a significant effect of

transfer on the mean rank of embryo development (P < .05; Table 1 and Figure 13). Embryos transferred laparoscopically developed to more advanced stages than embryos transfered transcervically. There was not a significant difference between transfer techniques when ewes received estradiol-17β-oxytocin (Table 1 and Figure 13). However, there was a significant effect of transfer when the ewes were treated with EtOH:saline-saline (P < .05; Table 1 and Figure 13), indicating that transcervical ET may not be feasible without dilating the cervix.

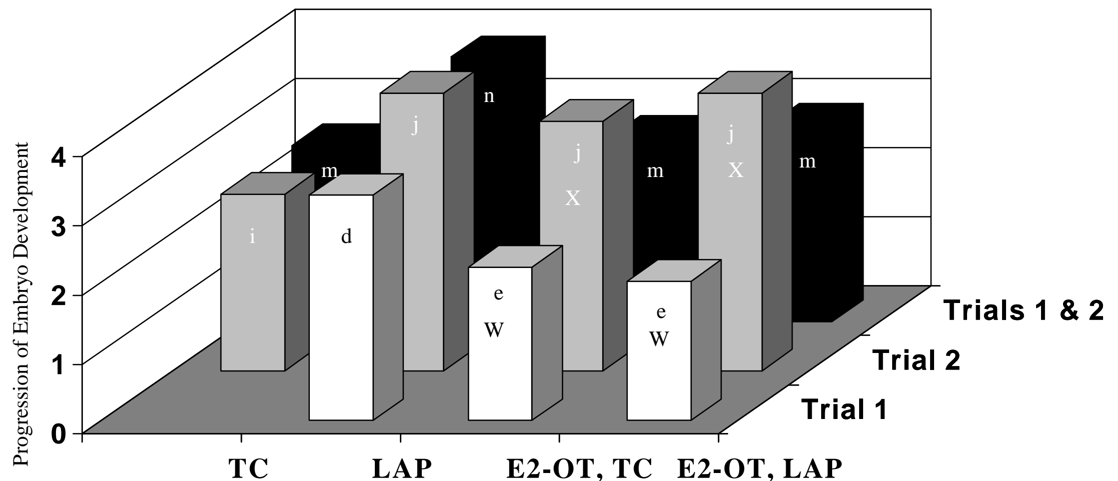

Figure 13. Effect of treatment on the progression of embryo development in Exp. 3. Ewes were assigned to randomized treatments, estrus was synchronized, and embryos were transfered transcervically or laparoscopically after EtOH:saline-saline or estradiol-17β-oxytocin. Embryos were collected from recipients 6 d after transfer in Trial 1 and 8 d after transfer in Trial 2. Embryos were scored based on morphological progression. Embryos were assigned a 1 if they had not developed, a 2 if they had developed to the blastocyst stage, a 3 if they had hatched, and a 4 if they developed to a morphological stage indicative of d 12 in Trial 1 and d 14 in Trial 2. MSE = 1.069. [d,e,i,j,m,n]Different letters indicate that the means in that shade differ (P < .05). [W,X]Different letters indicate that the means in that treatment group differ between trials (P < .05).

There was a significant effect of trial on the development of laparoscopically transferred embryos ($P < .05$; Table 1 and Figure 13). This is probably indicative of a technician effect. Embryo handling, embryo recovery, and general laparoscopic procedures improved for over time.

Because of technical limitations, an EtOH:saline-saline, transcervical group was not included in the first trial. Therefore, it is not possible to determine whether there would have been an effect of trial on this group. However, there was an effect of trial on estradiol-17β-oxytocin, transcervical and the estradiol-17β-oxytocin, laparoscopic groups. Again, this is indicative of a technician effect; in the transcervical technique improved between the two trials.

Because of the improved technique, the second trial provides a better indicator of the effect of the treatments on embryo development. In trial 2, there is an effect of transfer in the EtOH:saline-saline ewes ($P < .01$; Table 1 and Figure 13). In general, development in the transcervical transfer ewes receiving EtOH:saline-saline arrested before hatching; whereas, the laparoscopic embryos developed to the trophoblastic stage. There was not a significant effect of transfer in the estradiol-17β-oxytocin groups, suggesting that the treatment is actually beneficial, particularly for transcervical transfers ($P < .01$; Table 1 and Figure 13).

Comparisons within trial 1 are difficult to make because of the uneven design; however, useful information about the effects of the estradiol-17β-oxytocin treatment

(Table 1 and Figure 13) and the difference between the laparoscopic and transcervical technique receiving this treatment (Table 1 and Figure 13) was obtained. The raw data for each embryo are listed in Appendix D.

Chapter VI

DISCUSSION

Previous studies have indicated that transcervical ET is extremely difficult in ewes; however, this study indicates that dilating the cervix with an estradiol-17β-oxytocin treatment allows transcervical intrauterine deposition of embryos (Chapter V; Figure 8), without affecting development of the embryos (Chapter V; Table 1, Figures 12 and 13) or luteal function (Chapter IV; Figure 1). In fact, the estradiol-17β-oxytocin treatment may enhance the ability of the embryos to develop following transcervical transfer. Even though the ET procedure seems to offer real promise for the sheep industry, there are several production problems associated with ET that are not specifically related to the transfer process (i.e., collecting enough transferable embryos) to make ET in sheep financially sound. Currently, most embryo production schemes incorporate superovulation; however, the response to superovulation is highly variable, and this reduces the economic value of the embryo transfers (Maxwell et al., 1990).

In Exp. 3, embryo development and survival of embryos transferred transcervically was greater in the groups receiving the estradiol-17β-oxytocin treatment (Chapter V; Table 1, Figures 12 and 13). Even though it is not yet possible to attribute this effect to either the estradiol-17β, oxytocin, a combination of the two, or the increased physical ease of the transfer, it is possible to speculate on potentially positive effects of the treatments.

There are several mechanisms by which estradiol-17β might have improved embryo survival and development following transcervical transfer. For example, embryos

in Exp. 3 were collected from superovulated donors. Generally, the oocytes, zygotes, and early embryos from superovulated animals develop in a highly estrogenized environment. Although there is limited evidence in sheep (W.M.C. Maxwell, personal communication), there is an abundance of evidence in cattle that indicates an increased number of luteinized follicles associated with superovulation (Guay and Bedoya, 1981; Monniaux et al., 1983). Following ovulation in normally cycling ewes, estrogen concentrations decrease and progesterone concentrations increase. In superovulated ewes, even if estrogen concentrations decrease there is an early increase in serum progesterone concentrations. Compared with naturally cycling ewes, serum progesterone concentrations increase significantly in pFSH treated ewes 48 h before they do in controls (Schiewe et al., 1991). The progesterone concentrations are on average three times greater in superovulated animals between d 2 and 6 (Schiewe et al., 1991). There is even an increase in the number of small and total luteal cells per CL following superovulation (Hilde-Petito et al., 1987). Dramatic increases in progesterone immediately following ovulation have been associated with changes in uterine secretion of $PGF_{2\alpha}$ and premature luteal regression (Ottobre et al., 1980).

The recipients in this study were not superovulated; therefore, the embryos might have been placed into an asynchronous environment. It is imperative for donor embryos to be placed into a [biochemically] synchronous environment for proper development. The estradiol-17β treatment might have decreased the differences between the uterine environments and decreased the environmental "shock" to the embryos. This could be especially true of embryos transferred from ewes with luteinized follicles. A follicle may

not become fully luteinized until d 4. The cells that remain unluteinized during this period continue to secrete estrogens. Superovulated ewes with luteinized follicles often sustain increases in estrogens through at least d 4 (Booth et al., 1975). By d 6, follicles destined to be luteinized have completed the process. The embryos in this experiment were recovered on d 6 of pregnancy. At this time in the cycle, it is extremely difficult to tell the difference between normal luteal tissue and a luteinized follicle.

The estradiol-17β-oxytocin treatment may be beneficial for the transcervical procedure by reducing the trauma to the cervix. The exogenous oxytocin dilates the cervix. Dilating the cervix allows the rod to be passed into the uterine horn within 30 seconds with a minimum of damage compared with 10 to 20 minutes without dilating the cervix. The ease of transfer may prevent damage to the reproductive tract creating a more stable environment for the embryos.

Even though Exp. 3 was not designed to determine whether cervical manipulation has an unfavorable outcome on ET, it produced some evidence suggesting detrimental effects of manipulation. The estradiol-17β-oxytocin treatment did not improve laparoscopic transfer; therefore, the significant improvement associated with this treatment in the transcervical groups is most easily attributed to something specific in the transcervical procedure. There is evidence that transcervical passage of an AI rod or deep cervical AI may be detrimental to fertility (Salamon and Lightfoot, 1970; Ritar et al., 1990; Sayre and Lewis, 1996); however, there have not been any transcervical ET studies that suggest this outcome. In ewes, dilation of the cervix with estradiol-17β-oxytocin

treatments dramatically reduces the need to manipulate the cervix. The modified ET rod is passed easily through the lumen of the cervix with little resistance after passage through the first cervical os (B.A. Costine, unpublished results). If the cervix is not dilated, it is only occasionally possible to pass an ET rod without exerting a considerable amount of force or slowly manipulating the rod through the cervix. The increased time that the rod is in the cervix or the increased manipulation of the cervix may have a negative effect on ET. It has been postulated that a stressed cervix may produce a spermicidal compound (Hawk, 1983); therefore, it is possible to speculate that a stressed cervix during the luteal phase may produce an embryocidal compound. It is also possible to speculate that manipulating the cervix changes the hormonal environment of the uterus; a change in uterine function, may cause the uterine environment to discontinue supporting embryo development.

The effects of the estradiol-17β-oxytocin treatment may only be beneficial during transcervical transfer. In Exp. 3, the percentage of embryos that developed after laparoscopic transfer into recipients receiving the estradiol-17β-oxytocin treatment was less than that for embryos transferred laparoscopically into ewes receiving the control treatment (Chapter V; Table 1 and Figure 12). This was the most difficult set of data in this study to analyze, because the accuracy of the statistical tests was compromised by the small number of embryos. Only initial development was affected by the estradiol-17β-oxytocin treatment. There was no difference between the degree of development between treatment groups. If the embryos began to develop, the estradiol-17β-oxytocin treatment did not affect continued development.

Neither estradiol-17β, oxytocin, nor the combination of the two hormones negatively affected luteal function as measured with progesterone concentrations (Chapter IV; Figure 1)). This was confirmed by the presence of intact CL in recipients when the embryos were collected on d 12 or 14 (Chapter VI; Appendix A). This indicates that estradiol-17β and oxytocin administered in amounts similar to those used to dilate the cervix to facilitate ET do not cause luteal regression. Because progesterone concentrations were "normal", the CL were deemed fully functional (Chapter IV; Figure 1). This concurs with literature on the luteolytic effects of estradiol-17β and oxytocin at the levels administered.

Exogenous estradiol is presumed to be luteolytic on d 7 of the estrous cycle in sheep (Stormshak et al., 1969; Hixon and Flint, 1987). However, luteolytic doses of estradiol are approximately 3 to 10 times greater than the dose administered in this study. (Exp.1 and 3).

Current "dogma" implies that oxytocin is a luteolysin; however, there is no direct evidence to support this statement. Because $PGF_{2\alpha}$ is secreted by the endometrium in response to oxytocin (Sharma and Fitzpatrick, 1974; Mitchell et al., 1975; Roberts et al., 1976), a role has been postulated for oxytocin in luteolysis. However, this hypothesis is not consistent with the literature. Milvae et al., (1991) administered 50 USP units of oxytocin to ewes twice daily between d 0 to 3, 2 to 5, 4 to 7, 6 to 9, 8 to 11, 10 to 13, 12 to 15, or 14 to 17 of the estrous cycle. Treatment with oxytocin twice a day for any of the consecutive 4-d periods did not affect concentrations of progesterone or the duration of

the estrous cycle. In this same study, continuous intraluteal infusion of oxytocin on d 2 to 9 and d 10 to 15 did not affect luteal weight, cycle length, or plasma progesterone concentrations. The results of Exp. 1 concur with Milvae et al. (1991) that there is no direct inhibition of the CL in sheep by oxytocin (Chapter IV; Figure 1).

The only effect of treatment on luteal activity in Exp. 1 was the extension of luteal function in ewes receiving the oxytocin treatment (Chapter IV; Figure 1). This effect has not been reported in the literature on the effects of oxytocin on midcycle luteal function. Flint and Sheldrick (1985) reported that oxytocin administered from d 13 to 21 prolonged luteal lifespan approximately 7 d.

Superovulation is a highly variable process in ewes. In Exp. 2, the superovulatory response to the pFSH increased linearly with dose (Chapter 4; Figure 2). Measurements of superovulation were made using a moderate dose of pFSH in black-faced ewes in an attempt to limit the variation between ewes. The black-faced ewes were chosen for their decreased natural ovulation rates to decrease the chances of over stimulation. Even though an average of seven CL was achieved in Exp. 3, the mean number of transferable embryos per donor was low (1.25 transferable embryos/ewe) (Chapter VII; Appendix D). The question becomes, is superovulation a worthwhile adjunct to ET protocols in sheep? In breeds that have low ovulation rates, superovulation of the donors is a necessity, but in breeds with higher ovulation rates superovulation is probably not an economically sound practice. In a research setting where the goal is to produce embryos for nongenetic studies, it may be more cost effective to invest in a high fecundity breed that will be used strictly as embryo donors.

Practical Applications. Transcervical ET is important from the perspective of research and industry. A method of transcervical ET will increase our ability to study embryonic development in vivo and in vitro. At the same time, any efficacious technique developed for research will provide the sheep industry with a reproductive technology that could allow the rapid transmission of superior genetics. More important to industry, a usable transcervical ET technique could lead to the development of a practicable transcervical AI technique. The studies in this thesis stemmed from knowledge accrued while attempting to develop a transcervical AI technique. Much of the data collected was qualitative and difficult to measure quantitatively; therefore, the data are not described in the results section. However, these data were instrumental in developing this technique and should be addressed.

Experiment 3 was conducted over two breeding seasons. During that time period, many procedural details were reevaluated. However, major changes were not made between the two trials in an attempt to maintain consistency. Based on observations throughout Exp. 3, it may be possible to improve dilation of the cervix and transcervical ET by amending several procedural details. First, the amount of oxytocin administered to cause dilation of the cervix may be reduced. Second, visual assessment of the cervix provides information about individual ewes; therefore, briefly studying the cervix of each ewe may change the transfer approach. Third, transcervical ET techniques with or without dilation of the cervix require extensive training. Increased training decreases the amount of damage to the cervix, and it may increase the success of the transfer. Fourth, a speedy

procedure often compromises the integrity of the cervix and it is necessary to weigh the advantages and disadvantages caused by conducting the procedure as quickly as possible.

The minimum dose of oxytocin in Exp. 3 was 400 USP units. Previous reports indicate that a dose of 200 USP units is practical (Khalifa et al., 1992; Sayre and Lewis, 1996). A dose of 400 USP units induces rapid enough cervical dilation to allow the passage of an ET rod through the cervix in 12 to 15 min after administration (B.A. Costine et al., unpublished data). As the dose of oxytocin is decreased, the minimum amount of time until a rod can be passed through the cervix increases. It is possible to use as little as 50 USP units of oxytocin and pass a rod through the cervix, but one must wait for approximately 40 min after administration of the oxytocin before attempting the procedure (Flohr et al., unpublished data). In an attempt to minimize the time the embryos were held in vitro and to decrease the time between transfers, the 400 USP unit dose of oxytocin was used in this study. In the future, it will be necessary to weigh the perceived advantages of dilating the cervix with a reduced dose of oxytocin against the inconvenience of increasing the time necessary for the cervix to dilate.

Even though the dose of oxytocin is pharmacological, it may be beneficial. Large doses of oxytocin induce uterine tetany. Sayre and Lewis (1996) reported that after a dose of 400 USP units, the uterus remained in tetany for at least 30 min. In that study, uterine tetany did not affect sperm transport. In fact, Sayre and Lewis (1997) indicate that the exogenous oxytocin seemed to improve fertilization rate. Possibly, uterine tetany is beneficial during ET in ewes with a dilated cervix. According to Khalifa et al. (1992), the cervix remains dilated for upwards of 6 h. The lack of uterine contractions may help

prevent retrograde flow of embryos (i.e., from the uterus to the cervix). Several procedural details may also help prevent retrograde flow, including placing the embryos into the uterine horn rather than the uterine body (Exp. 3) and leaving the ewes in a dorsal recumbent position for 10 to 20 min after transfer (Exp. 3). A significant number of the embryos transferred in all treatment groups (34%) were missing at the time of the final collection; however, the number was not significantly different between treatments indicating that dilating the cervix does not seem to increase embryo lose during ET (Chapter 5; Figure 11).

The anatomy of the cervix was studied in detail by Dun (1955), Fukui and Roberts (1978), and Halbert et al. (1990) with the intent of developing transcervical AI procedures. Even though these studies indicate at least four anatomical classifications of cervices in sheep, they do not suggest the need for different approaches to each type. In my experience, when dilated the rosette cervix is the least difficult to pass an instrument through, followed by the duckbill, the flap, and the spiral type of cervix. However, this ranking may be technician specific, and it changes when the cervix is not dilated. If the cervices are not dilated, the order from least difficult to most difficult is reversed. Dilation causes the flaps of vaginal tissue surrounding the external cervical os to swell, changing the exposure of the lumen. The lumen of the rosette cervix is easiest to find after dilation because the folds swell and push the lumen up; however, when the cervix is undilated it is difficult to manipulate through the folds. When a rosette cervix is undilated, it causes the same type of obstruction caused by the fornix vagina in a cow. At the opposite extreme, it

is easier to find the lumen of the spiral cervix before dilation, but after dilation vaginal tissue surrounding the external os swells and occludes the opening of the lumen.

A second and more extreme cause of problems stemming from the anatomy of the cervix is the use of yearling ewes for transcervical ET. In my opinion, it is not advisable to attempt to pass a transcervical ET instrument through either a dilated or an undilated cervix in yearlings, because the success rate is very poor and it causes substantial damage to the cervix. The cervix in yearling ewes does not seem to respond well to the estradiol-17β-oxytocin treatment; therefore, there is very little dilation. In yearling ewes, the lumen is much smaller and more occluded (Dun, 1955). The ET instrument developed for these studies is too flexible to pass through the cervix in these ewes when their cervices are not dilated. The instrument has a tendency to bend and break. In these ewes, damage to the cervix is indicated by bleeding and bruising. In this study, yearling ewes were excluded from the treatment groups.

Transcervical ET procedures for sheep should be inexpensive, quick, relatively easy, and relatively reliable. However, the current industry idea maintains that a viable ET technique should be easy enough for anyone to perform with a short training period. This is a virtually impossible goal to achieve. In the cattle industry, AI and ET industries suffer because of inconsistencies between technicians. Even in a laboratory setting with a single technician, there was an effect of trial (Exp. 3). In this study, the two trials were conducted over two breeding seasons, with an initial 3-month training period. The best measure of improvement was the ability to add an EtOH:saline-saline transcervical treatment to the second trial, representing considerable technical improvements between

the two trials. A second indication was the decreased time needed to perform each procedure, the decreased external bruising, the lack of cervical trauma observed throughout the second trial, and the increase in embryo survival and development in the second trial (Chapter 5; Table 1, Figures 12 and 13). In my opinion, extensive training and practice are needed to perform this technique proficiently. I would approximate that it is necessary for a person to pass an embryo transfer instrument through the cervix of 70 to 100 ewes before the person could proclaim proficiency at the technique. The most highly publicized transcervical AI and ET technique has been plagued by problems with technician effects (B. Buckrell, personal communication). Even a method of transcervical ET that bypasses the cervix as a major barrier requires extensive training.

The degree of trauma to the cervix varies with technician and between ewes. It is possible to perform transcervical ET when the cervix is not dilated without causing any obvious damage; however, the time required for each transfer averages approximately 12 min (D. Marsh, personal communication; B. Buckrell, personal communication). The two most common methods of transferring embryos transcervically through undilated cervices are modifications of AI procedures (D. Marsh, personal communication; B. Buckrell, personal communication). Even with the cervix dilated, it is possible to cause trauma to the cervix if too much force is exerted on the ET instrumentation. It is possible to transfer embryos transcervically by dilating the cervix and exerting very little force on the ET instrument; however, these transfers take approximately 5.5 min (B.A. Costine et al., unpublished data). Popular "theory" suggests that any force on the ET instrumentation causes trauma to the cervix. However, Costine et al. (unpublished data) found that a fast

slightly more forceful passage through the cervix produces less damage than a slow passage with very little force.

Implications

This study indicates that an estradiol-17β-oxytocin treatment dilates the cervix enough to allow easy passage of the ET equipment described in this thesis and intrauterine deposition of embryos. The extent of embryo development was not different between the laparoscopic transfers and the transcervical transfers receiving the estradiol-17β-oxytocin treatment, suggesting that transcervical ET is possible in sheep when the cervix is dilated with estradiol-17β-oxytocin. Contrary to popular opinion, the estradiol-17β-oxytocin treatment does not affect luteal function, and may actually enhance ET success rates. In conclusion, with an appropriate amount of training, transcervical ET could be a useful research tool with the possibility of becoming a useful industry tool.

Chapter VII

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Appendix A

Surgical Procedures

Laparoscopic Procedures

1. All ewes were housed and denied access to feed and water for 24 h before the laparoscopic surgeries.

2. Ewes were anesthetized with an initial combination of ketamine (150 mg) and zylexene (50 mg). The ketamine and zylzene were administered i.v. until ewes were considered to have reached a surgical plane of anesthisia.

3. Wool was removed from around the udder and from the 12 to 18 cm of the belly proximal to the udder.

4. The surgical area was scrubbed with antiseptic soap.

5. The ewes were restrained in a laparoscopic cradle, and the hind legs were lifted to present the ewes at a 45° angle.

6. Two small incisions were made through the skin to the left and right of the midline 2 cm below the teats.

7. A 7 mm diameter trocar and cannula were inserted into the peritoneal cavity through an incision left of the midline.

8. A 5 mm diameter trocar and cannula were inserted into the peritoneal cavity through an incision right of the midline.

9. The peritoneal cavity was inflated with CO_2.

10. The trocars were removed from each cannula.

11. The laparoscope was placed in the 7 mm cannula, and a probe was placed in the 5 mm cannula. The interior of the abdomen could then be viewed through the laparoscope.

12. Modifications for counting CL, artificailly inseminating and transferring embryos are discussed in the appropriate materials and methods sections.

13. The laparoscope and the probe were removed.

14. The bellies of the ewes were depressed to remove excess CO_2.

15. The cannulas were removed, and incisions were stapled.

16. The ewes were allowed to remain undisturbed in pens for 2 to 3 h after the procedure.

Laparotomy

1. Ewes were restricted from feed for 36 h and from water for 24 h before surgery.

2. Ewes were anesthetized with sodium pentobarbital (Sigma Chemical Co., St. Louis, MO) in sterile saline (65 mg of sodium pentobarbital/ml). The sodium pentobarbital was administered i.v. until ewes were considered to have reached a surgical plane of anesthisia.

3. Anesthetized ewes were placed on a stainless steel surgical table. The ewes were placed in a dorsal recumbency position, and the surgical field was sheared.

4. The surgical field encompassed approximately 20 cm anterior to the udder and 15 cm in both directions from the midline. The surgical field was scrubbed at least three times with an antiseptic soap. Following the final scrub, a 10% iodine solution was applied to the surgical area.

5. Aseptic surgical techniques were used throughout all procedures. A sterile disposable drape was placed over the surgical field, and a longitudinal opening was cut directly anterior to the udder.

6. A 10 cm skin incision was made with a scalpel to one side of the midline in an attempt to avoid severing the mammary vein.

7. The subcutaneous layer of tissue was blunt dissected from the body wall.

8. At the midline, a scalpel was pushed through the body wall at the most anterior portion of the incision. Forceps were inserted into the hole created by the scalpel and used to lift the body wall as it was cut with the scalpel. The body wall incision was slightly shorter than the skin incision.

9. The uterus was located using fingers and carefully lifted to the surface in an attempt not to damage the uterus or the ovaries.

10. The ovaries were examined and desribed.

11. After the embryo collection procedures, which are desribed in more detail in the appropriate chapter, where performed, the uterus was washed with sterile saline and allowed to return to its normal position.

12. The body wall and skin incisions were closed with polyamide suture.

Appendix B

Modifed Dulbecco's Media

Compound	Amount (g/L)
$CaCl_2$	.050
KCl	.100
KH_2PO_4	.100
$MgCL_2.6H_2O$	.050
NaCL	4.000
$Na_2HPO_4.7H_2O$	1.080
Penicillin	.030
Streptomycin	.025
BSA	2.000 (BSA was only added to the media in the first trial.)

Appendix C

Recipient Data

Table 1. Raw recipient data. Exp. 3.

Ewe #[a]	Rep.[b]	Trt.[c]	Embryos at Transfer Stage	Embryos at Transfer Quality[d,e]	Embryo Stage at Recovery[f,g,h]	# of CL at Recovery	Development[i]	Rating[j]
R2590	2	1	BLASTOCYST		BROKEN ZONA	2	YES	3
R2590	2	1	BLASTOCYST		BROKEN ZONA	2	YES	3
P1065	2	1	MORULA	GOOD	BROKEN ZONA	1	YES	3
L2292	2	1	MORULA	GOOD	MORULA		UK[l]	0
L2292	2	1	MORULA	GOOD	BLASTOCYST	1	YES	2
T4272	2	1	MORULA	GOOD	--------------	0	UK	0
T4272	2	1	MORULA	GOOD	--------------	0	UK	0
T4272	2	1	MORULA	FAIR	--------------	0	UK	0
T2086	2	1	MORULA	GOOD	--------------	2	UK	0
T2086	2	1	MORULA	FAIR	--------------	2	UK	0
L0726	2	1	MORULA	GOOD	MORULA	2	NO	1
L0726	2	1	MORULA	GOOD	BLASTOCYST	2	YES	2
L0726	2	1	MORULA	GOOD	BLASTOCYST	2	YES	2
R2559	2	1	MORULA	GOOD	SPHERICAL	2	YES	4
R2559	2	1	MORULA	GOOD	SPHERICAL	2	YES	4
R2559	2	1	MORULA	GOOD	--------------	2	UK	0
R2758	2	1	MORULA	FAIR	BLASTOCYST		YES	2
R2758	2	1	MORULA	GOOD	BLASTOCYST		YES	2
S0272	1	2	BLASTOCYST		E BLASTOCYST[k]	1	YES	3
S0272	1	2	BLASTOCYST		E BLASTOCYST	1	YES	3
S0272	1	2	BLASTOCYST		--------------	1	UK	0
N2187	1	2	BLATOCYST		TROPHOBLAST	2	YES	4
N2187	1	2	BLASTOCYST		--------------	2	UK	0

Table 1. Continued.

Ewe #[a]	Rep.[b]	Trt.[c]	Embryos at Transfer Stage	Quality[d,e]	Embryo Stage at Recovery[f,g,h]	# of CL at Recovery	Development[i]	Rating[j]
T4168	2	2	MORULA	GOOD	TROPHOBLAST	1	YES	4
T4168	2	2	MORULA	FAIR	--------------	1	UK	0
T4168	2	2	MORULA	FAIR	--------------	1	UK	0
S2376	2	2	MORULA	GOOD	TROPHOBLAST	1	YES	4
S2376	2	2	MORULA	GOOD	TROPHOBLAST	1	YES	4
S2376	2	2	MORULA	GOOD	--------------	1	UK	0
L2038	2	2	MORULA	FAIR	TROPHOBLAST	1	YES	4
R2530	2	2	MORULA	FAIR	TROPHOBLAST	1	YES	4
R2530	2	2	MORULA	FAIR	TROPHOBLAST	1	YES	4
P2255	2	2	MORULA	FAIR	TROPHOBLAST	2	YES	4
P2255	2	2	MORULA	FAIR	SPHERICAL	2	YES	4
O2218	1	3	MORULA	FAIR	MORULA	1	NO	1
O2218	1	3	MORULA	GOOD	MORULA	1	NO	1
O2218	1	3	MORULA	GOOD	MORULA	1	NO	1
O2279	1	3	BLASTOCYST		TROPHOBLAST	1	YES	4
O2279	1	3	BLASTOCYST		TROPHOBLAST	1	YES	4
O2279	1	3	MORULA	GOOD	--------------	1	UK	0
N0392	1	3	BLASTOCYST		TROPHOBLAST	1	YES	4
N0392	1	3	BLASTOCYST		--------------	1	UK	0
R0079	1	3	MORULA	GOOD	--------------	1	UK	0
R0079	1	3	MORULA	GOOD	--------------	1	UK	0
S2005	1	3	MORULA	FAIR	BLASTOCYST	1	YES	2
S2005	1	3	MORULA	GOOD	BLASTOCYST	1	YES	2
S2005	1	3	MORULA	GOOD	--------------	1	UK	0
T4257	1	3	MORULA	GOOD	BLASTOCYST	1	YES	2
T4257	1	3	BLASTOCYST		--------------	1	UK	0
S0160	1	3	MORULA	FAIR	MORULA	0	NO	1

Table 1. Continued.

Ewe #[a]	Rep.[b]	Trt.[c]	Embryos at Transfer Stage	Embryos at Transfer Quality[d,e]	Embryo Stage at Recovery[f,g,h]	# of CL at Recovery	Development[i]	Rating[j]
S0160	1	3	MORULA	GOOD	--------------	0	UK	0
R2591	2	3	BLASTOCYST		SPHERICAL	1	YES	4
R2591	2	3	BLASTOCYST		--------------	1	UK	0
N2220	2	3	MORULA	GOOD	--------------	3	UK	0
N2220	2	3	MORULA	GOOD	--------------	3	UK	0
P2271	2	3	MORULA	GOOD	FOOTBALL	3	YES	3
P2271	2	3	MORULA	GOOD	FOOTBALL	3	YES	3
T4136	2	3	MORULA	GOOD	SPHERICAL	2	YES	4
T4136	2	3	MORULA	GOOD	SPHERICAL	2	YES	4
U2046	2	3	MORULA	GOOD	--------------	3	UK	0
U2046	2	3	MORULA	GOOD	--------------	3	UK	0
U2046	2	3	MORULA	FAIR	--------------	3	UK	0
O0352	1	4	MORULA	GOOD	SPHERICAL	1	YES	4
O0352	1	4	MORULA	FAIR	--------------	1	UK	0
O0352	1	4	MORULA	FAIR	--------------	1	UK	0
P1338	1	4	BLASTOCYST		BLASTOCYST	2	NO	1
P1338	1	4	BLASTOCYST		BLASTOCYST	2	NO	1
P1338	1	4	BLASTOCYST		E BLASTOCYST	2	YES	3
T2226	2	4	MORULA	FAIR	SPHERICAL	2	YES	4
T2226	2	4	MORULA	GOOD	TROPHOBLAST	2	YES	4

[a]Embryos are labeled by the tag of the recipient ewes.
[b]There were two trials. Trial 1 was conducted between January and April and Trial 2 was conducted between November and March of the following breeding season.
[c]Treatments 1, 2, 3, and 4 were EtOH:saline-saline (transcervical), EtOH:saline-saline (laparoscopic), estradiol-17β-oxytocin (transcervical), and estradiol-17β-oxytocin (laparoscopic), respectively.
[d]Only morulas received a quality grade.

[e]A quality grade of poor, fair, good, or excellent was assigned to each of the embryos. Embryos rating a poor were discarded from the experiment.
[f]At recovery broken zonas found in the absence of an embryo were considered representative of hatching.
[g]The description spherical applies to hatched embryos that had not begun to elongate.
[h]The description football applies to trophoblasts prior to elongation.
[i]Embryos that had developed beyond the initial stage at transfer were given a development rating of yes. Embryos that were lost were not rated.
[j]Depending on the amount of morphological development embryos received a rating of 1, 2, 3, or 4. Embryos that did not develop rated a 1. Embryos that developed to the morphological stage associated with the day of pregnancy were rated a 4. Even though the end point in both trials was at the stage of a trophoblast there is a considerable difference between a trophoblast at d 12 and 14 and this was accounted for in the ratings.
[k]Elongated blastocyst.
[l]Embryos missing at recovery.

Appendix D

Donor Data

Table 1. Raw donor data. Exp. 3.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
A0006	1	4	1		
A0006	1	4	1		
L0725	1	4	1		
L2137	1	4	0		
M2095	1	4	0		
N0001	1	4	7	UFO[e]	
N0001	1	4	7	UFO	
N0038	1	4	17	MORULA	GOOD
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0038	1	4	17	BLASTOCYST	
N0068	1	4	0		
N0118	1	4	12	MORULA	GOOD
O0073	1	4	12	UFO	
O0298	1	4	3	UFO	
O0298	1	4	3	UFO	
O0360	1	4	17	MORULA	GOOD
O0360	1	4	17	MORULA	GOOD
O0360	1	4	17	MORULA	GOOD
O0360	1	4	17	MORULA	FAIR
O0360	1	4	17	MORULA	FAIR
O0360	1	4	17	MORULA	FAIR
O0360	1	4	17	MORULA	GOOD
O0360	1	4	17	UFO	
O0360	1	4	17	UFO	
O0360	1	4	17	UFO	
O0360	1	4	17	UFO	
O0360	1	4	17	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	

Table 1. Continued.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2064	1	4	16	UFO	
O2079	1	4	23	UFO	
O2079	1	4	23	UFO	
O2079	1	4	23	UFO	
O2079	1	4	23	UFO	
P0065	1	4	3		
P0106	1	4	12	MORULA	GOOD
P0106	1	4	12	MORULA	GOOD
P0106	1	4	12	MORULA	GOOD
P0106	1	4	12	BLASTOCYST	
P0106	1	4	12	BLASTOCYST	
R0005	1	4	18	UFO	
R0005	1	4	18	UFO	
R0005	1	4	18	UFO	
R0005	1	4	18	UFO	
R0005	1	4	18	MORULA	GOOD
R0005	1	4	18	UFO	
R0005	1	4	18	UFO	
R0005	1	4	18	UFO	
R0005	1	4	18	UFO	
R0042	1	4	1		
R0113	1	4	12	BLASTOCYST	
R0113	1	4	12	MORULA	GOOD
R0113	1	4	12	MORULA	GOOD
R0113	1	4	12	MORULA	GOOD
R0113	1	4	12	MORULA	GOOD
R0113	1	4	12	MORULA	FAIR
R0113	1	4	12	MORULA	POOR
R0113	1	4	12	MORULA	POOR
R2056	1	4	1	MORULA	FAIR
R2140	1	4	4	UFO	
R2140	1	4	4	UFO	
R2140	1	4	4	UFO	
R2230	1	4	0		
R2570	1	4	0		

Table 1. Continued.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
R2584	1	4	29	BLASTOCYST	
R2590	1	4	2		
S 0160	1	4	3	BLAST	
S 0160	1	4	3	MORULA	GOOD
S2020	1	4	14	UFO	
S2020	1	4	14	UFO	
S2020	1	4	14	MORULA	GOOD
S2020	1	4	14	MORULA	GOOD
S2020	1	4	14	MORULA	GOOD
S2020	1	4	14	MORULA	GOOD
S2020	1	4	14	MORULA	GOOD
S2032	1	4	10	BLASTOCYST	
T4010	1	4	1	MORULA	GOOD
T4264	1	4	1		
T4330	1	4	2		
T4368	1	4	7	UFO	
t4426	1	4	0		
A0001	2	2	6		
A0003	2	2	7	MORULA	FAIR
A0003	2	2	7	MORULA	GOOD
A0003	2	2	7	MORULA	GOOD
A0003	2	2	7	MORULA	GOOD
A0003	2	2	0		
A0004	2	2	9	MORULA	FAIR
A0004	2	2	9	BROKEN ZONA	
A0004	2	2	9	BROKEN ZONA	
A6128	2	2	5	MORULA	GOOD
L2137	2	2	1		
N9110	2	2	16	MORULA	GOOD
N9110	2	2	16	MORULA	FAIR
N9147	2	3	11	UFO	
N9147	2	3	11	UFO	
N9147	2	3	11	UFO	
N9154	2	1	11	UFO	
N9154	2	1	11	UFO	
N9154	2	1	11	UFO	
N9154	2	1	11	UFO	
N9154	2	1	11	UFO	
N9219	2	1	3		
O0102	2	1	10		
P0100	2	1	1		

Table 1. Continued.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
P0103	2	1	6		
P0103	2	2	1	UFO	
P0111	2	1	1	BLASTOCYST	
P1110	2	2	4		
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2056	2	2	8	UFO	
R2101	2	1	9	BLASTOCYST	
R2101	2	1	9	BLASTOCYST	
R2101	2	1	9	BLASTOCYST	
R2101	2	1	9	BLASTOCYST	
R2101	2	1	9	BLASTOCYST	
R2107	2	1	7	MORULA	GOOD
R2107	2	1	7	MORULA	GOOD
R2107	2	1	7	MORULA	GOOD
R2107	2	1	7	MORULA	FAIR
R2107	2	1	7	MORULA	FAIR
R2121	2	1	9	UFO	
R2121	2	1	9	UFO	
R2121	2	1	9	UFO	
R2121	2	1	9	UFO	
R2123	2	2	9	UFO	
R2123	2	2	9	UFO	
R2123	2	2	9	UFO	
R2123	2	2	9	UFO	
R2123	2	2	9	UFO	
R2123	2	2	9	UFO	
R2123	2	2	9	UFO	
R2127	2	2	0		
R2155	2	1	9	MORULA	GOOD
R2155	2	1	9	MORULA	GOOD
R2155	2	1	9	MORULA	GOOD
S2011	2	2	12	MORULA	GOOD
S3003	2	3	15	BROKEN ZONA	
S3003	2	3	15	BROKEN ZONA	
S3003	2	3	15	2 CELL	

Table 1. Continued.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
S3003	2	3	15	2CELL	
S3003	2	3	15	UFO	
S3003	2	3	15	UFO	
S3003	2	3	15	4CELL	
S3003	2	3	15	4CELL	
S3121	2	1	12	BLASTOCYST	
S3121	2	1	12	UFO	
S3121	2	1	12	BLASTOCYST	
S3130	2	1	0		
S3135	2	2	13		
T2004	2	2	2	UFO	
T2004	2	2	2	UFO	
T2170	2	1	19	UFO	
T2170	2	1	19	UFO	
T2170	2	1	19	UFO	
T2170	2	1	19	UFO	
T2170	2	1	19	MORULA	FAIR
T2170	2	1	19	MORULA	FAIR
T2170	2	1	19	UFO	
T2170	2	1	19	MORULA	POOR
T2170	2	1	19	MORULA	POOR
T2170	2	1	19	MORULA	POOR
T2170	2	1	19	MORULA	POOR
T2170	2	1	19	MORULA	POOR
T2170	2	1	19	MORULA	POOR
T2249	2	2	0		
T2477	2	1	0		
T2488	2	2	12	UFO	
T2488	2	2	12	UFO	
T2488	2	2	12	MORULA	POOR
T2488	2	2	12	MORULA	GOOD
T2488	2	2	12	MORULA	FAIR
T2488	2	2	12	MORULA	FAIR
T2488	2	2	12	MORULA	GOOD
T2488	2	2	12	MORULA	GOOD
T2488	2	2	12	MORULA	GOOD
T2488	2	2	12	UFO	
T4128	2	2	7	2CELL	
T4128	2	2	7	2CELL	
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	

Table 1. Continued.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	
T4136	2	2	20	UFO	
T4161	2	2	11		
T4168	2	2	4		
T4254	2	1	8	MORULA	POOR
T4254	2	1	8	UFO	
T4254	2	1	8	MORULA	GOOD
T4254	2	1	8	MORULA	GOOD
T4263	2	2	3		
T4264	2	3	0		
T4264	2	1	0		
T4313	2	2	3	MORULA	GOOD
T4313	2	2	3	MORULA	FAIR
T4353	2	2	7	UFO	
T4353	2	2	7	UFO	
T4379	2	2	5	UFO	
T4379	2	2	5	UFO	
T4379	2	2	5	UFO	
T4380	2	2	7		
T4381	2	3	10	UFO	
T4381	2	3	10	UFO	
T4381	2	3	10	UFO	
T4381	2	2	14	MORULA	GOOD
T4381	2	2	14	MORULA	GOOD
T4381	2	2	14	MORULA	GOOD
T4381	2	2	14	MORULA	GOOD
T4381	2	2	14	MORULA	GOOD
T4381	2	2	14	MORULA	FAIR
T4381	2	2	14	MORULA	FAIR
T4381	2	2	14	4CELL	
T4381	2	2	14	4CELL	
T4382	2	1	12		
T4387	2	1	4		
T4405	2	3	5	BROKEN ZONA	
T4405	2	3	5	UFO	
T4405	2	3	5	BROKEN ZONA	

Table 1. Continued.

Ewe #[a]	Rep.[b]	Ram[c]	Total CL	Description	Grade[d]
T4405	2	3	5	BROKEN ZONA	
T4407	2	1	12	MORULA	POOR
T4407	2	1	12	UFO	
T4407	2	1	12	UFO	
T4407	2	1	12	UFO	
T4407	2	1	12	4CELL	
T4407	2	1	12	4CELL	
T4411	2	1	3		
T4413	2	2	1		
T4417	2	2	6	MORULA	GOOD
T4417	2	2	6	MORULA	GOOD
T4417	2	2	6	MORULA	GOOD
T4418	2	2	6	MORULA	GOOD
T4418	2	2	6	UFO	
T4418	2	2	6	UFO	
T4421	2	1	9	4CELL	
T4421	2	1	9	4CELL	
T4421	2	1	9	2CELL	
T4421	2	1	9	2CELL	
T4421	2	1	9	2CELL	
T4421	2	1	9	UFO	
T4423	2	1	1		
T4425	2	1	6	MORULA	GOOD
T4426	2	3	5	UFO	
T4426	2	3	5	UFO	
T4426	2	3	5	UFO	
T4426	2	3	5	UFO	

[a]Embryos are labeled by the tag of the donor ewes.
[b]There were two trials. Trial 1 was conducted between January and April and Trial 2 was conducted between November and March of the following breeding season. Different superovulatory protocols were used in the two trials.
[c]Rams 1, 2, 3, and 4 were breeding group I (6 rams), breeding group II (8 rams), breeding groups I and II rotated at 8 h intervals, and neat semen from Arthur, respectively.
[d]Only morulas received a quality grade. A quality grade of poor, fair, good, or excellent was assigned to each of the embryos. Embryos rating a poor were discarded from the experiment.
[e]Unfertilized oocyte.

VITA

Meghan Carole Wulster, daughter of William and Carole Wulster, was born on August 15, 1973. She graduated from Hamden Hall Country Day School located in Hamden, Connecticut in June of 1991. She received her Bachelor of Science at University of Wisconsin at Madison in May of 1995. She began working on her Master of Science degree in July of 1995 at Virginia Polytechnic Institute and State University. She plans to remain at Virginia Polytechnic Institute and State University and pursue her Ph.D. degree. She is a current member of the American Society of Animal Science.

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Meghan Carole Wulster