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**A STUDY OF TRANSCERVICAL ARTIFICIAL
INSEMINATION IN SHEEP**

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ABSTRACT

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The study describes two trials on artificial insemination in Romney ewes. Trial 1 was conducted to examine the anatomical site in the reproductive tract of the inseminating needle after transcervical insemination, and to evaluate the effect of the needle in causing cervical tissue damage based on the microscopic assessment of cervical slides. Trial 2 was undertaken to compare the effect of intrauterine, cervical or transcervical methods of insemination with fresh semen on fertility.

Two hundred and five mixed-aged ewes (3-8 years old) were hormonally treated with CIDR-G for 12 days and these were removed after this period. Forty-eight hours later, to monitor the onset of oestrus, they were joined with 10 harnessed vasectomised rams. Oestrous detection was undertaken twice daily, at 1.00 am and 1.00 pm. Most ewes were synchronised in oestrus over 2 days after CIDR withdrawal but there was an extended period of 'second' oestrus when the inseminations were conducted. In Trial 1, transcervical insemination with Indian ink was performed in 29 ewes at the second oestrus, and then they were euthanased with Sodium Pentobarbitone. Position of the inseminating needle *in situ* was determined at

dissection. The genital tract was removed and the uterus opened to determine whether Indian ink had penetrated. The cervix was then split into three similar sized portions, fixed in Bouins solution, and sections histologically processed and stained for slides. The slides were microscopically examined by two evaluators to determine damage scores.

In trial 2, semen samples (concentration at least 3×10^9 spermatozoa ml^{-1} , motility minimal 4) were collected per artificial vagina from 5 Romney rams, pooled and freshly diluted with UHT-milk to 8×10^8 spermatozoa ml^{-1} . One hundred and seventy-five cyclic ewes were randomly assigned to either of three AI techniques (intrauterine, cervical and transcervical), and were inseminated with freshly diluted semen at a mean interval of 6.1 ± 0.26 h after second oestrus was detected.

In slaughtered ewes, penetration of the modified needle through the lumen of the cervix and even into the uterus occurred more than with the unmodified needle (90% vs 68%; $0.05 < P < 0.1$). Both types of needle used caused damage in the genital tract, and even caused rupture through the wall of the cervix. This was less frequent with the modified needle and therefore it was used for transcervical insemination in Trial 2. The predicted location of the needle in the tract and its actual location were highly correlated.

In Trial 2, the overall mean for the conception rate to AI was 82%, but no differences were noted between methods of inseminations. With lambing rate,

transcervical inseminations gave the best results, but there were no differences in the litter size between methods.

For transcervical insemination depth of penetration of the needle had an effect on conception rate and lambing rate ($P < 0.05$) and thus the method should aim to place the semen well into and even through the cervix. The results for transcervical insemination were not affected by the interval from oestrous detection to insemination, or the age of the ewes or whether the inseminations were conducted in the morning or afternoon. The technique was more difficult to accomplish in maiden than older ewes.

It was concluded that while transcervical insemination with freshly diluted semen gave a satisfactory result, it is not always suitable for maiden ewes and others, where difficulty is encountered in penetrating through the cervix. Further work to evaluate the technique with larger numbers of ewes is required.

DEDICATION

To THE BLESSED VIRGIN MARY

for her unfathomably maternal love, guidance and care.

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A proverb, 'No man is an island', is greatly applicable to this thesis. This small work is a resultant of the constructive involvement of several animal scientists at Department of Animal Science, which were dedicative to share their knowledge and skills, and therefore they are deserved to be named personally.

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TABLE OF CONTENTS

ABSTRACT	ii
DEDICATION.....	vi
ACKNOWLEDGEMENTS.....	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF PLATESxiii
CHAPTER I: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	4
2.1. A High Reproductive Rate, Its determinants and importance	4
2.2. Oestrus and ovulation	5
2.2.1. Endocrinology of oestrus and ovulation	5
2.2.2. Synchronisation of oestrus and ovulation	7
2.2.3. Time of ovulation	13
2.2.3.1. Breed	13
2.2.3.2. Season	14
2.2.3.3. Ram effect	14
2.2.3.4. Age	15
2.2.3.5. Hormonal treatment	15
2.3. Methods of Artificial Insemination (AI)	16
2.3.1. Vaginal insemination	16
2.3.2. Cervical insemination	17
2.3.3. Intrauterine insemination	18
2.3.4. Transcervical insemination	19
2.4. Factors influencing the success of AI	20
2.4.1. The fertility and dose of the sperm	21
2.4.2. Proper handling of the semen	22
2.4.3. Time of insemination	24
2.4.4. Proper site of semen deposition and sperm transport.	27
2.4.5. Technician	29
2.5. Purpose and scope of the study:	30

CHAPTER 3: MATERIALS AND METHODS	32
3.1. Synchronisation	32
3.2. Training of rams and handling of collected semen	33
3.3. Trial 1: Development of transcervical insemination instruments	35
3.3.1. Instruments	35
3.3.2. Insemination and slaughter	37
3.3.3. Histological examination	38
3.4. Trial 2: Comparison of insemination techniques	40
3.5. Analysis of data	41
3.5.1 Data analysis of development of TCAI instruments and damage of the cervix	41
3.5.2. Data analysis of comparison of AI techniques.	41
 CHAPTER 4. RESULTS	 42
4.1. Trial 1: Development of Transcervical Instruments	42
4.1.1. Placement of inseminating needle	42
4.1.2. Effect of needle penetration on histological damage	47
4.2. Trial 2: Oestrous control and comparison of insemination techniques	53
4.2.1. Incidence of oestrus in ewes.	53
4.2.2. Artificial insemination and fertility	55
4.2.3. Effect of age on fertility	56
4.2.4. Fertility in ewes after transcervical insemination, relative to depth of penetration of the inseminating needle.	58
4.2.5. The interval from oestrous detection to insemination on fertility.	60
4.2.6. Fertility relative to technicians and AI technique	62
 CHAPTER 5: DISCUSSION	 63
5.1. The incidence of oestrus.	63
5.2. Evaluation of the needle penetration	64
5.3. Effect of AI technique on fertility	68
5.4. Effect of age on fertility	72
5.5. Effect of depth of penetration with transcervical insemination on fertility	73
5.6. Effect of interval oestrous detection to the time of AI on fertility.	75
5.7. Effect of the technician on fertility	76

CHAPTER 6: CONCLUSION 77

APPENDIX 79

REFERENCES 83

LIST OF TABLES

TABLE 1: A summary of synchronisation of oestrus and fertility in the ewes.	11
TABLE 2. Fertility after AI technique with different doses	23
TABLE 3: Time interval to AI on fertility	25
TABLE 4: Distribution of ewes relative to position of the inseminating needle in the genital tract, determined at slaughter	45
TABLE 5: Comparison of predicted depth of penetration (Mean \pm SEM) and actual depth (Mean \pm SEM) as determined at dissection using scale values.	46
TABLE 6: Scores for histological damage caused by inseminating needle at different sites within the cervix.	52
TABLE 7: Effect of AI technique on fertility	55
TABLE 8. Effect of age of the ewes on fertility after insemination	57
TABLE 9: Fertility relative to depth of penetration of inseminating needle	58
TABLE 10: Fertility after insemination relative to the interval from onset of oestrus to AI.	60
TABLE 11: Fertility of ewes inseminated at morning or afternoon	61
TABLE 12: Fertility after insemination by individual technician	62

LIST OF FIGURES

FIGURE 1: Schematic diagram of the patterns of change in peripheral plasma hormone concentration throughout the oestrus cycle in the ewe... ..	6
FIGURE 2: Oestrous incidence after CIDR removal	54
FIGURE 3: Depth of penetration after morning or afternoon insemination.	59

LIST OF PLATES

PLATE 1: Collecting semen using the artificial vagina.	34
PLATE 2: Transcervical insemination instruments.	36
PLATE 3: Needles used in the AI trial.	37
PLATE 4: Reproductive tract of a ewe, showing penetration of the inseminating needle through the wall of the cervix.	42
PLATE 5: The genital tracts of 19 ewes removed after slaughter.	43
PLATE 6: Damage in cervical tissue at score 1 and 2.	49
PLATE 7: Damage in cervical tissue at score 3 and 4.	50
PLATE 8: Damage in cervical tissue at score 5.	51

CHAPTER 1: INTRODUCTION

CHAPTER 1: INTRODUCTION

The role of reproduction as a factor affecting the efficiency of animal production in farm animals is important in maintaining herd and flock numbers as well as being a parameter of performance. High levels of reproduction improve biological and economic efficiencies (Dickerson, 1976). In terms of the genetic view point, high rates of fertility and prolificacy result in a large number of offspring being available for selection and can lead to accelerated genetic gain in other productive traits (Rae, 1980). This improvement in the genetic merit of livestock has been greatly aided by artificial insemination using progeny tested semen. Thus, high merit sires, because of AI, can generate large numbers of offspring and so raise the level of production in the flock or herd. This is well shown in the New Zealand dairy industry, where the merit of cattle has increased consistently because of the artificial breeding programme which has been adopted over 40 years. Because of improvements in semen dilution technique the number of insemination from a proven bull in a breeding season has provided in excess of 150,000 calves per year. In the sheep industry, it has been suggested that frozen-thawed semen collected over a period of 8 months at 9 collections per week can be used to inseminate 100,000 ewes and leave 50,000-80,000 lambs (Maxwell & Garnett, 1991).

The use of AI to generate many offspring from a valuable sire is widely accepted and pedigree breeders of livestock frequently sell semen for this purpose. AI also has an

important role as a genetic tool in co-operative breeding schemes where breeders want to make use of reference sires to aid in the evaluation of untested rams. Such schemes have been developed in the former Soviet Union, France, Australia and New Zealand. These schemes frequently make use of freshly collected semen. This material must be inseminated within a few hours after collection for satisfactory fertility to be achieved. There also would be practical advantages if semen from rams could be obtained over a longer period and placed in frozen storage in the liquid nitrogen for later use or for transportation to distant sheep flocks. Frozen-thawed semen when inseminated cervically in sheep gives poor fertility. This drawback is largely due to fertilisation failure resulting from faulty transport of spermatozoa through the complicated cervix which is very folded, constricted, tortuous and narrow. To overcome the problem, methods have been investigated whereby frozen-thawed sperm are injected directly into the lumen of the reproductive tract (usually the uterus) either at laparotomy or by intrauterine insemination with the aid of a laparoscope. This method avoids the cervical barrier. Fertility rate after use of this method was frequently in an excess of 50%, and is almost comparable to cervical insemination with fresh semen. However, there are disadvantages associated with laparoscopic insemination because it is expensive, demanding of a competent technician, normally done under local or general anaesthesia and involves penetration of the laparoscope into the abdominal cavity. In addition, there is increasing concern that the procedure is not welfare-friendly and that it does not apply very well to a modern sheep industry.

The development of transcervical artificial insemination has been a long term goal of sheep breeders who wish to use frozen semen. Recently workers in Canada have reported a procedure to effectively restrain the ewe and pass an inseminating pipette or needle through the cervix; the results have been promising with a high penetration through the cervix and entry to the uterus but conception rate is often low with frozen-thawed semen. If the technique can be further developed for using frozen-thawed semen and result in acceptable fertility rates then the increased application of AI in sheep would be feasible. Research needs to be conducted to evaluate the application of transcervical artificial insemination under New Zealand sheep industry conditions and to determine factors affecting fertility and to test possible improvements in the procedure.

CHAPTER 2: LITERATURE REVIEW

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2.1. A High Reproductive Rate, Its determinants and importance

Reproductive rate is sometimes defined as the number of lambs weaned per ewes joined. It is a complex trait and involves fertility, fecundity and survival rates. Firstly, fertility includes the ability to breed, and it indicates whether or not a ewe will produce a lamb. Secondly, fecundity is reflected in litter size. In sheep, this refers to the number of lambs produced per ewe lambing. A reduction in fecundity or prolificacy results in a smaller litter size and even an increase in ewe barrenness. Thirdly, survival rates can be described as the ability of offspring to survive to weaning (Rae, 1980), or the proportion of offspring weaned of those born (Piper & Bindon, 1979). However, as litter size increases, survival of the offspring tends to decrease (McGuirk, 1976).

Despite such a limitation, it is generally accepted that a high reproductive rate is the main goal of livestock producers regardless what the specific aims of each are. Benefits can be viewed from economic and from biological aspects. Maintaining the breeding female is one of the major costs associated with most production systems (McGuirk, 1976). By increasing the number of offspring each year per female, the overhead costs can be spread over a large number of offspring available for sale (Piper & Bindon, 1979). Thus, having increased this number, there should be

improvement in the return on capital that has been invested. Further, a high reproductive rate brings about a large number of offspring available for selection and this contributes to accelerated genetic gain in other productive traits (Van Vleck, 1981; Rae, 1980). Furthermore, having improved genetic potential may result in decreased cost to the consumers, because, as each livestock unit produces more, the maintenance cost is a smaller portion of the product (Van Vleck, 1981).

2.2. Oestrus and ovulation

2.2.1. Endocrinology of oestrus and ovulation

Oestrus is governed principally by the hypothalamic-pituitary axis, and, in turn, modified by the hormonal feedback mechanism, involving ovarian steroid hormones. The concentrations of progesterone remain low throughout the follicular phase and begin to increase 2-3 days after ovulation, the time where a new corpus luteum (CL) becomes active. A small increase occurs through Day 5-9, followed by an abrupt rise on Day 10 and maintained at that level through Day 14-16 (Thornburn *et al.*, 1969; McNatty *et al.*, 1973; Pant *et al.*, 1977; Scaramuzzi *et al.*, 1993). During the mid-luteal phase, a high concentration of progesterone and low concentration of oestradiol have a synergistic effect on tonic luteinizing hormone (LH). This prevents any positive feedback of LH, thus they prevent the preovulatory gonadotrophin surge. Progesterone concentrations then decline sharply on Day 17-just before reoccurrence of the next oestrus. This decline is associated with the regression of corpus luteum caused by the release of prostaglandins from the uterus (Smith, 1982). It allows the removal of a synergistic effect of progesterone and oestradiol and permits tonic LH

concentration increases. This phase drives the final stages of follicle maturation and its associated rise in oestradiol secretion until the threshold concentration of oestradiol required to provide the positive feedback trigger for the preovulatory surge of LH and follicle stimulating hormone (FSH) to be attained (Haresign, 1985). This dramatic increase (LH) results in structural changes within the follicle, which eventually culminates in the rupture of the follicle and the expulsion of the ovum and formation of the CL. When there is no fertilisation, the CL will undergo a regression period by the end of the cycle, 2-3 days before the next oestrus takes place (Haresign, 1983; Baird & McNeilly, 1981). This mechanism is illustrated in Figure 1.

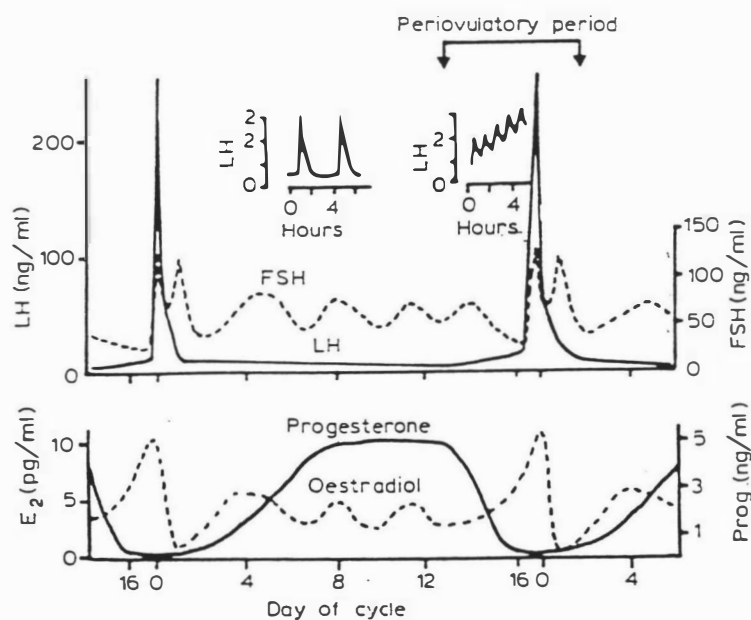


Figure 1. Schematic diagram of the patterns of change in peripheral plasma hormone concentrations throughout the oestrous cycle in the ewe (cited from Haresign, 1985).

The length of the oestrous cycle is 17 days (Maxwell & Evans, 1987), with approximately 24 h (4 to 72 h of variation) for the duration of oestrus (Smith, 1982); at least 30 h in the adult ewes with some variation with breed (Robertson, 1977; Thimonier, 1979), age, degree of prolificacy (Doney & Gunn, 1981) and degree of contact with the ram (Smith, 1982).

2.2.2. Synchronisation of oestrus and ovulation

In both the breeding season and the non-breeding season, the oestrous cycle requires the integration of the hypothalamic-pituitary-ovarian axis and involves both positive and negative feedback systems (Haresign *et al.*, 1983). During the breeding season, synchronisation of oestrous cycles is achieved either by inducing luteolysis (using prostaglandins), or by blocking progression of the oestrous cycle in the luteal phase (using progestagen). The first method requires the ewe to have an active corpus luteum which will undergo luteolysis soon after ovulation. Prostaglandins should be given on two occasions between Days 4 and 14 of the cycle with an interval of 8-9 days between injections; a good synchronisation of oestrus should be achieved 2 days after injection (Hunter, 1982). Beck *et al.* (1987) reported that two doses of 20 mg PGF_{2α} administered with a space of 11 days, resulted in levels of synchronisation and fertility comparable to that obtained with progestagen pessaries. They also found that acceptable levels of oestrous synchronisation can be achieved with a single dose of

20 mg PGF_{2α}. However, compared to progestagen, the use of PGF_{2α} is limited, for it is only applied to cycling ewes (Cognie & Mauleon, 1983), and is less effective as a mean of synchronising oestrus (Henderson *et al.*, 1984).

The second method for synchronisation involves the administration of progestagen by injection, feeding, implant or intravaginal application using polyurethane sponges or controlled internal drug release device (CIDR). These techniques, in principle, have a similar action, namely to lengthen the period of progesterone dominance. In other words, it prolongs the luteal phase by depressing follicular development. This can be achieved by means of intravaginal administration of a sponge containing 30 mg fluorogestone acetate (FGA), or 60 mg medroxyprogesterone acetate (MAP) for between 12 and 14 days. In anoestrous ewes to stimulate ovulation PMSG is usually given either up to 48 h before or at the removal of the sponge (Haresign, 1978).

The development of the CIDR in addition to causing good levels of fertility (Welch *et al.*, 1984; Maxwell *et al.*, 1986; McMillan, 1986) has other advantages. In cycling ewes the CIDR in comparison to the sponge (i) does not absorb nor impede drainage of vaginal secretion, (ii) is more pleasant to handle, (iii) its release characteristics can be monitored because it contains native progesterone, and (iv) it results in high joining and lambing rates (Carlson *et al.*, 1989) and most are retained in the vagina for the duration of the treatment period (Hamra *et al.*, 1986).

Responses following CIDR treatment vary with the physiological status of the ewes. Carlson *et al.*(1989) using ovariectomised ewes found that plasma progesterone levels reached 2.8 ng/ml within 3 days after insertion and declined gradually to 0.3 ng/ml by Day 3, but in cycling ewes, plasma progesterone levels reached 4.5 ng/ml within 3 days after insertion. This occurs as a result of the contribution of luteal progesterone. Exogenous progesterones are able to block oestrus and ovulation for 27 to 31 days after insertion of the CIDR, after which ovulation occurs without oestrus. Oestrus with ovulation takes place by Day 38 to Day 45 from when the CIDR was inserted. Carlson *et al.*(1989) also found that after a 12 day treatment, 91% of ewes were bred and 67% lambed in response to matings within 5 days. Welch *et al.*(1984) found that the higher body weight ewes have high feed intakes and show oestrus earlier than those with low body weight and feed intakes.

Fertility following CIDR and other progestagen analogues varies during the breeding season. Ewes that had received CIDR-S (CIDR-S, CIDR-G are slightly different products but all contain progesterone) exhibited oestrus earlier and with tighter synchrony than animals treated with MAP sponges (Rhodes & Nathanielz, 1988). Shackell (1991) found that CIDR-treated ewes began oestrus sooner than MAP and FGA-treated ewes (30 h vs 42 h and 40 h). Similarly the onset of the Lh surge (31 h vs 46 h and 46 h) and ovulation (59 h vs 69 h and 71 h) occurred sooner in the CIDR ewes compared to those given MAP and FGA sponges. In contrast, Maxwell & Barnes (1986) found no difference in intervals to oestrus between 9% progesterone

CIDR-S and 30 mg Cronolone sponges. Further, most CIDR-treated ewes (80%) succeeded to lamb, which was 10% higher than those treated with progesterone, FGA, or MAP sponges (Hamra *et al.*, 1986). Table 1 provides a summary of results from progestagens used in the control of the oestrous cycle.

Low fertility after insemination in synchronised ewes is associated with transport of sperm (Quinlivan & Robinson, 1969; Hawk & Conley, 1972; Robinson 1973). This problem can be avoided or alleviated either by mating at the second oestrus, or by increasing the number of spermatozoa the ewe receives (Allison & Robinson, 1971), or by a single intramuscular dose of gonadotrophin given at the time of withdrawal of progesterone treatment (Evans & Robinson, 1980).

TABEL 1: A summary of synchronisation of oestrus and fertility in the ewes.

Type of progestagen	Route of administration (no. of papers)	Dose (mg)	Oestrous percentage	Mating method	Fertility
Progesterone:	injection (15)	2.5-65	51-100	natural	60-100 ¹
	feeding (1)	58	81-97		
	vaginal sponge (5)	410	38-98	AI (f.t.s.)	-
	implant	375	66-100		13 ²
MAP	feeding (18)	70	43-100	Natural	14-88 ¹
	vaginal (20)	20-80	51-100	AI (f.t.s.)	16-75 ²
	sponge				

FGA	injection (2)	0.25	67-70	Natural	0-96 ¹
	vaginal (6)	5-80	43-100	AI (f.t.s.)	17-88 ¹
	sponge implant (1)	50	75	AI	15-90 ₂
CAP	injection (2)	2.8	38-100	Natural	0-88 ¹
	Feeding (3)	2.3	88-100	AI (f.s.)	60 ¹
	vaginal (3)	17.5	63-81		60 ¹
	sponge				
MGA	injection (4)	54	6-98	Natural	36-41 ²
	feeding (5)	3.2	0-56	AI (f.t.s.)	40-75 ²
	vaginal (1)	35	88-93		
	sponge				
	implant (1)	74			
CIDR	vaginal sponge	9 & 12	91	AI	62-69 ²

Adapted from Fukui (1976)

^{*}Harvey *et al.*(1987); Carlson *et al.*(1989)

¹=conception rate; ²= lambing rate; f.t.s.=frozen-thawed semen.

Low fertility after insemination in synchronised ewes is associated with transport of sperm (Quinlivan & Robinson, 1969; Hawk & Conley, 1972; Robinson 1973). This problem can be avoided or alleviated either by mating at the second oestrus, or by increasing the number of spermatozoa the ewe receives (Allison & Robinson, 1971), or by a single intramuscular dose of gonadotrophin given at the time of withdrawal of progesterone treatment (Evans & Robinson, 1980).

2.2.3. Time of ovulation

Knowledge of the time of ovulation is crucial to the success of AI and is related to the onset of oestrus. Asynchrony of AI and ovulation is a common cause of the fertility failure after AI (Evans, 1988). As an example, in Merino ewes, the time of ovulation normally occurs 25-30 h after the onset of oestrus (Evans & Maxwell, 1987) or 24 h after the onset of the preovulatory surge of LH (Cumming *et al.*, 1973). Even though Robinson *et al.*(1987) and Evans & Robinson (1980) reported oestrus and ovulation are highly predictable within a homogenous group of oestrous synchronised ewes, Smith *et al.*(1987) found that the onset of oestrus can be highly variable.

2.2.3.1. Breed

Comparisons of breeds and genotypes of low and high prolificacy have consistently shown differences in the timing of the preovulatory LH discharge relative to the

onset of oestrus, ewes with high ovulation rates having a significantly longer interval (Quirke *et al.*, 1979; Cahill *et al.*, 1981) than in single ovulation ewes.

2.2.3.2. Season

Evans & Robinson (1980) reported no difference in progesterone concentration between animals treated at the different time of the year but the peak of oestradiol 17- β was significantly less in spring than in autumn. Similarly in the spring-treated ewes the oestrogen concentration was associated with a longer interval to onset of oestrus than in autumn-treated sheep. They observed a mean time to onset of oestrus of 43.2 h (range: 30 to 46 h) after sponge removal and injection of 400 i.u. PMSG. Boscholt *et al.*(1973) found that the length of oestrus of the ewes treated in the breeding season was longer than in those treated in non-breeding season (35 h vs 29 h).

2.2.3.3. Ram effect

The influence of the ram on occurrence of the commencement of the breeding season suggests that exteroceptive factors may be important. Signoret & Cognie (1975) reported the presence of the male advanced the time of the LH surge, and ovulation occurred 6-8 h earlier and there was a reduction in the duration of sexual

receptivity. With respect to withdrawal of a sponge in ewes that joined a vasectomised ram, Maxwell (1986) found that the mean times of ovulation for teased and unteased ewes were 55.8 h and 59.7 h, respectively.

2.2.3.4. Age

Quirke *et al.*(1981) used young and adult ewes in their experiment and found the interval between sponge removal and the onset of oestrus was shorter in adult ewes than that for young ewes, but the adults had a longer interval between the onset of oestrus and the occurrence of the LH discharge. Quirke *et al.*(1981) noted that the interval from the beginning of LH discharge and ovulation takes 12 to 16 h and ovulation normally occurs at the end of oestrus (Cumming *et al.*1973).

2.2.3.5. Hormonal treatment

The time of ovulation varies with the kind of treatment imposed on the animals. Ryan *et al.* (1992) reported that a combination of 400 i.u. pregnant mare serum gonadotrophin (PMSG) and 12 mg FSH-P treatment in ewes caused ovulation between 42 h and 52 h after sponges were removed. Another report by Walker *et al.*(1986) found that time of ovulation was 60 h after FSH-P or 54 to 60 h after 1200 i.u. PMSG. Similar to the last workers, Evans *et al.*(1984) reported that 22.5 mg FSH-P given in twice daily injection caused ovulation 60 h after sponge removal.

Modification of the time of ovulation using gonadotrophin releasing hormone (GnRH) has been attempted. However Ryan *et al.*(1992) found no apparent effect on the time of ovulation in spring but did so in autumn. Walker *et al.*(1989) found that with the lowest dose of 6.25 µg GnRH for each ewe, the synchrony effect was apparent.

In cyclic ewes, the control of the timing of the LH discharge and ovulation can be achieved either by luteolysis with prostaglandins or its synthetic analogues after Day 4-5 of the cycle, or by artificial lengthening the luteal phase with exogenous progesterone or progestagens (Thimonier, 1979).

2.3. Methods of Artificial Insemination (AI)

Several methods for AI in sheep have been developed and involve deposition of semen into the vagina, cervix or into the uterus. Each technique has both advantages and disadvantages.

2.3.1. Vaginal insemination

This technique (sometimes termed 'shot in the dark') has been reviewed by Evans & Maxwell (1987). This technique is simple and practicable because it simply means depositing fresh semen into the anterior vagina without a speculum or attempting to

locate the cervix and therefore, this technique is more applicable to maiden ewes. With this technique, some workers have demonstrated acceptable results (Maxwell & Hewitt, 1986; Tervit *et al.*, 1984) but others suggest that conception rates are low (Kerton *et al.*, 1984; Rival *et al.*, 1984), especially with frozen semen (Lightfoot & Salamon, 1970a, b). This is caused by reduction in a high proportion of the spermatozoa in inseminate or semen (Mattner *et al.*, 1969).

2.3.2. Cervical insemination

This technique is commonly used in sheep and involves placement of semen into the first fold of the cervix (reviewed by Maxwell & Evans, 1987). It is a cheap and relatively easy method to perform (Wallace, 1992). With fresh semen, cervical insemination has resulted in acceptable lambing rates, which ranged between 55% and 84% (Tervit *et al.*, 1984). The semen should be used within 10 h of collection and preferably at much shorter intervals (Salamon *et al.*, 1977) or 10-15 h (Clarke *et al.*, 1984). However, with frozen-thawed semen, low conception rates occur (Dziuk *et al.*, 1972; Maxwell, 1980; Maxwell & Hewitt, 1986) and part of the problem is impaired transport through the cervix (Mattner *et al.*, 1969; Lightfoot & Salamon, 1967) and reduced viability of frozen-thawed spermatozoa (Evans & Maxwell, 1987). Salamon & Maxwell (1995a) have reviewed causes of unacceptable fertility following cervical insemination and methods to overcome them. Some of the problems may be reduced by an increased concentration of spermatozoa in the inseminate, treatment

of ewes with hormones to relax the cervix, modification of the diluents for semen storage, double and deep cervical insemination, increased depth of deposition of frozen-thawed semen or by transcervical insemination. At present satisfactory and reliable lambing rates have only been reported when frozen-thawed semen has been with inseminated intrauterine.

2.3.3. Intrauterine insemination

Semen can be deposited into the reproductive tract via mid-ventral laparotomy or by use of a laparoscope Killeen & Caffrey (1982). This allows semen in a needle to be inserted across the abdominal cavity to penetrate into the lumen of the tract. The semen can be deposited directly into the infundibulum of the Fallopian tube or more commonly through the wall of the uterus. Thus, the problem of the cervical barrier for sperm is avoided.

Intrauterine insemination has shown promising results with both fresh and frozen-thawed semen in sheep and goats (Wallace, 1992). The following advantages can be attained.

- It increases fertilisation rates (ranging between 50 % and 80%) when using frozen-thawed semen (Maxwell, 1986a, b; Maxwell & Hewitt, 1986; Haresign, 1990).
- Economical semen usage is also achieved. McKelvey & Robinson (1986) estimated that if 5 ejaculates were collected daily from one ram for 6 weeks during the breeding season, the ram could inseminate 10,000 ewes intrauterine. Thus it increases

the mating potential of superior sires (Maxwell, 1984).

- It can increase conception rates in out-of-season breeding, in contrast to cervical insemination which resulted in a low conception rate of 39% (Gordon *et al.*, 1969). This is caused by fertilisation failure and by reduced quantity and quality of semen (Colas, 1983).
- Further, it could potentially facilitate the use of post-partum ewes as embryo donors, as early as 22 days postpartum since it is able to by-pass the hostile environment of the involuting uterus (Wallace *et al.*, 1989; Wallace, 1992).

The timing of intrauterine insemination for satisfactory results will vary with the type of semen used to inseminate ewes. In non-superovulated ewes, the general trend is to inseminate at or just prior to the predicted time of ovulation when using fresh-diluted semen (Davis *et al.*, 1984; McKelvey *et al.*, 1985) and close to the time of ovulation when using frozen-thawed semen (Maxwell, 1986a; Haresign, 1990). In superovulated ewes, insemination is conducted 48-52 hours after progesterone treatment was removed (Robinson *et al.*, 1989).

2.3.4. Transcervical insemination

This technique has been evaluated by Halbert *et al.*(1990a, b). It involves grasping the cervix with forceps and retracting it partly into the vagina, in order to introduce the insemination probe. Buckrell *et al.*(1994) reported that penetration of the cervix ranged between 76% to 98% and resulted in a lambing rate of 51%. Windsor (1994)

reported that pregnancy rate following transcervical AI was lower than that following intrauterine AI with laparoscopy.

The success of the technique is influenced by several factors including the skill to locate, retract and stabilise the cervix prior to depositing the semen (Halbert *et al.*,1990b). Buckrell *et al.*(1994) noted that fertility after transcervical insemination was influenced by reproductive status (maiden or mature ewes), season, and year of breeding.

The technique also brings about disadvantages to the animals. Halbert *et al.*(1990b) reported that retraction and manipulation of the cervix could damage the tissue surrounding the cervical os, and cause trauma and haemorrhage. Similarly, Salamon & Lightfoot (1970) reported that cervical retraction, in cervical AI, potentially led to a reduction in fertility of the ewes. The technique, however, is promising since a high penetration through the cervix of 82% and pregnancy rate of 58% with frozen-thawed semen has been recorded (Halbert *et al.*, 1990b).

2.4. Factors influencing the success of AI

Factors affecting the success of AI in sheep can be grouped into five groups: The fertility and dose of the sperm, proper handling of the semen, an appropriate method of AI, proper site of deposition in the reproductive tract and technician skill.

2.4.1. The fertility and dose of the sperm

Motility of the sperm is an essential pre-requisite for the quantitative distribution of spermatozoa throughout the genital tract. This motility is required for spermatozoa to penetrate between the deeply divided mucosal folds of the cervix (Hawk, 1983) and to pass along the remainder of the reproductive tract.

In practice, visual assessment is widely used to determine the quality of semen. Normally, semen has a motility of 4 to 5 with the concentration of 3 billion spermatozoa is used in the insemination programme. A semen obtained from a normal fertile ram has the number of abnormal spermatozoa varies from 5% to 15%. When this number exceeds 20%, the reduction of fertility will be noticed (Foote, 1974). Fertility after fresh semen is better than that after frozen-thawed semen (e.g. Maxwell, 1986a; Evans *et al.* 1986).

Eppeleston *et al.*(1986) reported that this method of assessment did not correlate with fertility in subsequent insemination trial. Recently, Eppeleston & Maxwell (1995) demonstrated that a significant correlation between motility of the inseminate and reproductive performance. Regression analysis revealed that sperm traits including motility were useful for predicting fertility.

The number of sperm in the dose of semen can be varied according to AI technique that is going to be performed. In general, vaginal, cervical or transcervical

insemination requires more spermatozoa in an inseminate than is required for equivalent fertility following intrauterine insemination. Some examples of fertility rates after AI with different doses of semen are presented in Table 2.

Diluted ram semen has a very limited survival, semen less than 5 h old giving conception rates approximately 10% higher than diluted semen stores for 14 h (Colas *et al.*, 1973). In N.Z. condition, Clarke *et al.*(1984) recommends freshly diluted semen is to be used within 10-15 h since its preparation to avoid marked decreases in lambing rate.

2.4.2. Proper handling of the semen

An appropriate environment is required by ejaculated sperm to survive for a long period. Several diluents for ram semen are available (Evans & Maxwell, 1987; Salamon & Maxwell, 1995a, b). Ram semen has been successfully stored for short periods of time, without serious loss of its fertilising ability, using diluents based on either skimmed milk or egg yolk. The choice between the two types of diluents depends on the temperature selected for storage. Barlow *et al.*(1974) found that at 15°C those based on skimmed milk preserve the fertilising capacity of spermatozoa better than those based on egg yolk.

TABLE 2: Fertility after AI technique with different doses

Dose (x10 ⁶)	Type of semen	AI technique	<u>Fertility</u> CR(%)	LR(%)	References
350	fresh	cervical	81	64)	Fukui & Roberts (1979)
300	frozen	deep cervical	45	20)	
12.5	frozen	intrauterine	70)	-	Davis <i>et al.</i> (1984)
25	frozen	intrauterine	63)	-	
50	frozen	intrauterine	76)	-	
100	frozen	intrauterine	71)	-	
100	fresh	blind	20)	-	Harvey <i>et al.</i> (1986).
200	fresh	blind	38)	-	
100	fresh	cervical	45)	-	
200	fresh	cervical	61)	-	
10	frozen	intrauterine	38)	-	Maxwell (1986a)
20	frozen	intrauterine	53)	-	
50	frozen	intrauterine	62)	-	
100	fresh	cervical	60)	-	Maxwell & Hewitt (1986)
100	frozen	cervical	18.8)	-	
600	frozen	cervical	42.2)	-	
100	frozen	vaginal	17)	-	
600	frozen	vaginal	17.4)	-	
100	frozen	intrauterine	55.6)	-	
600	frozen	intrauterine	64.6)	-	
1	frozen	cervical	14)	-	Eppleston <i>et al</i> (1995)
4	frozen	cervical	38)	-	
16	frozen	cervical	40)	-	
64	frozen	cervical	73)	-	

However, Colas *et al.*(1980) found that a milk diluent alone is not a suitable storage medium at 4°C. When storage time at 15°C was extended to 6 h, the egg yolk proved to be better diluent. The deleterious effect of milk diluent at 5°C is related to rapid accumulation of lactic acid, resulting in a significant drop in pH and leading to a high mortality of spermatozoa.

As an addition, fresh milk should be avoided unless heated at 85°C for 5 minutes (Blackshaw, 1960b). This technique is used to reduce or alleviate the detrimental effect of free sulphhydryl groups in the protein fraction of fresh milk (Johnson *et al.*,1955). Recently, Upreti *et al.*(1995), and Smith *et al.*(1995b) confirmed that a conventional milk based diluent was still superior to a new diluent RSD-1 in maintaining the motility of sperm for 6 h at 38 °C.

2.4.3. Time of insemination

The time of AI is critical since it influences the efficiency of sperm transport in the female genital tract and it will become more critical when frozen-thawed semen is used. This transport depends on the stage of heat at which ewes are inseminated. Salamon (1971) showed that insemination at mid-oestrus will result in the greatest possibility of successful fertilisation. Visser and Salamon (1974) suggest two inseminations at 12-14 h and 23-25 h after the onset of oestrus. The time of AI also depends on the technique of AI and oestrous synchronisation.

Fertility following insemination at different interval between time of vaginal pessary withdrawal and AI time is presented in Table 3.

TABLE 3: Time interval to AI on fertility

AI type	Interval (h)	Fertility (%)		Authors
		Conception	Lambing	
Cervical	50-56 ^a	50	-	Smith <i>et al.</i> (1995)
Intrauterine	50-56 ^a	63	-	
Transcervical	50-56 ^a	40	-	
Intrauterine	3-18 ^a	70)	-	Harvey & McDonald (1995)
Transcervical	3-18 ^a	59) (frozen)	-	
	3-18 ^a	54) (fresh)	-	
Intrauterine	48 ^a	-	62)	Paton <i>et al.</i> (1993)
	60 ^a	-	63)	
	72 ^a	-	48)	
Cervical	51-53 ^a	52.8)	-	Harvey <i>et al.</i> (1986)
Blind	51-53 ^a	29)	-	
Transcervical	54 ^a	-	24.4-50.7	Halbert <i>et al.</i> (1990c)
	54 ^a	-	34.7-56.7	Buckrell <i>et al.</i> (1992)
Intrauterine	12 ^b	58	-	Nephew <i>et al.</i> (1990)
Cervical	55 ^a	-	47.5	Maxwell (1986a)
Intrauterine	48 ^a	-	45.9)	
	60 ^a	-	55.1)	
	72 ^a	-	57.5)	
	78 ^a	-	39.3)	

AI type	Interval (h)	<u>Fertility (%)</u> Conception	Lambing	Authors
Cervical	56 ^a	94.7	94.7)	Haresign <i>et al.</i> (1986)
	50 or 60 ^a	48.3 (frozen	48.3)	
Intrauterine	60 ^a	50 (frozen)	36)	
Cervical	48 ^a	83)	-	McKelvey <i>et al.</i> (1985)
	60 ^a	66.7)	-	
Intrauterine	52 ^a	70.5)	-	
Intrauterine	24 ^a	60)	-	Evans <i>et al.</i> (1986)
	44 ^a	93.8)	-	
	64 ^a	87.7)	-	
	24 ^a	48.7)	-	
	44 ^a	98.2)	-	
	64 ^a	26.1)	-	

^{a)}= after intravaginal pessary removal.

Evans *et al.*(1986) suggest that the optimum time for intrauterine insemination of superovulated ewes is 44 h after pessary removal, but the time of insemination was less critical using fresh than frozen semen. Regarding intrauterine insemination, Maxwelll1 (1986); Walker *et al.*(1986) reported that low fertility obtained when this technique was performed close to or coincident with the time of ovulation.

2.4.4. Proper site of semen deposition and sperm transport.

When conducting cervical and transcervical insemination it is clear that it is difficult to pass the inseminating needle through the cervix, even in the cervix of old and oestrous ewes (Dun, 1955; Bunch & Ellsworth, 1981; Halbert *et al.*, 1990a,b).

The cervix, anatomically, consists of connective tissue, musculature and secretory glands. It also has ridges and crypts which fit together so as to make the cervix directly impassable (Evans & Maxwell, 1987). It is 4-7 cm long and has 4-5 folds (Dun, 1955). Halbert *et al.* (1990a) found that failure to identify the cervical opening and the nature of one fold to the next which was not concentrically aligned, were the reasons for poor penetration of inseminating instruments through the cervix.

Four sites have been used for insemination in sheep:

- (1) Vaginal insemination involves depositing semen into the vagina without attempting to locate the cervix.
- (2) Cervical insemination. The target site of semen deposition is the first fold of the os cervix (Wallace, 1992), or up to 3 cm deep into the cervix (Evans & Maxwell, 1987).
- (3) Intra-uterine insemination. This technique can be done in conjunction either with laparotomy or laparoscopy. Even though these techniques are different, they are similar in the target site of insemination, namely the lumen of the uterus.

Nephew *et al.*(1990) reported a higher conception rate resulted when semen was deposited at the utero-tubal junction than at the external uterine bifurcation (conception rate: 71.2% vs 16.7%). Regarding laparoscopic intrauterine insemination, semen is generally deposited into the mid-section of the uterine horn rather than into the tip or bottom of the uterine horn. (Maxwell, 1986).

(4) Transcervical insemination. The target site of this technique is the uterine lumen. In deep transcervical insemination the AI pipette reaches a depth of at least 2.5 cm into the cervix and little semen back flow occurs, whereas in shallow transcervical insemination it reaches a depth of less than 2.5 cm and with a substantial semen back flow (Windsor *et al.*,1994).

It is increasingly evident that transport of sperm from the site of semen deposition to the site of fertilisation after mating or insemination represents a critical phase in the reproductive process of farm animals (Mattner, 1963; Mattner & Braden, 1963; Mattner *et al.*,1969; Lightfoot & Salamon, 1970a). In cattle and sheep, major barriers of sperm transport to the site of fertilisation include the cervix/mucus complex in natural service (Krzanowska, 1974), and for both natural and AI, the uterus (Mitcell *et al.*,1985), and the utero-tubal junction/oviductal isthmus regions (Wilmot & Hunter, 1984). Semen is transported at different speeds passing through each segment of the genital tract including the cervix with

its folds and crypts, the uterus, the tubal-uterine junction and the isthmus of the oviduct. Contributing factors to the success of sperm transport have been reviewed elsewhere (Lehrer *et al.*, 1978; Bedford, 1972) and this matter is out of the scope of this thesis.

2.4.5. Technician

There is an ample evidence that technician variation influences level of fertility. For example, cervical penetration is likely to be influenced by technician skill (Buckrell *et al.*, 1994; Windsor *et al.*, 1994). In general, fertility following inseminations which are performed by an experienced technician is higher than that by unexperienced technician. However, it is difficult to make judgements of the performance of a technician at different localities, and at present, no standard criteria for this. In cattle, Graham (15) suggested that it may be possible to follow up the type of mistakes of the technician by analysing the length of return cycles and by the decrease in the non-return from 30-60 to 60-90 d. If technicians make technical errors there will be a low 30-60 d non-return rate but the deviation in cycle length of the return cows will be normal. If technicians make mistakes in detecting oestrus then there will be less cows returning at normal intervals and more returning after abnormal cycles. (Kalay, 1980).

2.5. Purpose and scope of the study:

In the ewe the complex nature of the cervix due to its anatomy, makes deposition of semen into the uterus difficult. To overcome this problem laparoscopic AI has been developed as an alternative method to cervical insemination. This technique is able to by-pass the cervix and to deposit semen directly into the uterus. It is well accepted that this technique results in a consistent and high fertility. However, it requires expensive instruments, use of an anaesthetic drug and a well trained technician to perform it, and it has been the subject of growing concerns of animal welfare.

The development of a procedure for passing an inseminating needle well into and even through the cervix to enable semen to be deposited into the uterus was the main objective of the present work. Work in a previous year (Harvey & McDonald, 1995) showed that the method described by Halbert *et al.*(1994b) could be applied but that the fertility following the use of frozen-thawed semen was still considerably less than what might have been hoped for. In that work the passage of the inseminating needle through the cervix was sometimes thought to be complete yet few sperm were recovered from within the upper portion of the reproductive tract. Post mortem observation of the tract suggested that in some ewes the inseminating needle probably penetrated through the cervical wall and semen might thus have been deposited in the abdominal cavity instead of the genital tract.

Two trials were therefore conducted to examine the placement of the inseminating needle when transcervical insemination was performed and also to assess the significance of some factors affecting the fertility in the ewes when freshly collected semen was used. The specific objectives were

- 1) to determine the anatomical site of the inseminating needle.
- 2) to examine effect on the histological appearance of the cervical tissue following insemination.
- 3) to determine the accuracy of prediction of placement of the inseminating needle in the tract.
- 4) the effect of several factors on fertility following use of inseminating needle.

CHAPTER 3: MATERIALS AND METHODS

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The work was conducted in February, March and April 1994, on a Massey University flock in Manawatu district, New Zealand. In Trial 1, a total of 29 ewes were chosen, and used to study placement of the inseminating needle in the reproductive tract, and these animals were then slaughtered with the needle *in situ* immediately after insemination was completed. In Trial 2, 176 ewes were used in a comparison of AI techniques. All the ewes were chosen without selection on age, size, bodyweight, performance, and health. All the ewes were identified by serially numbered brass and large ear tags.

The animals were grazed on ryegrass and white clover pasture without supplementation, for 2 weeks prior to the time of insemination. They were weighed twice both before insertion and after removal of CIDR-G for programming of the oestrous cycle. The mean weights were 50.9 ± 5.8 kg and did not differ between weights.

3.1. Synchronisation

The ewes were hormonally synchronised with CIDR-G (EAZI-breed, Carter Holt Harvey, Hamilton, New Zealand) for 12 days (the day of insertion was counted as Day -12) without considering the current ovarian status of the animals at the time of insertion of the devices and removal of CIDR-G on Day 0.

Forty-eight hours later they were joined with ten vasectomised Romney rams - which were equipped with mating harness and colour crayon.

Daily oestrous detection was undertaken twice, starting at 1.00 am and at 1.00 pm. The first oestrous detection was begun in Day 3 and the next day, and was repeated on Day 12 and the next eight consecutive days for the second oestrus. At this last period, between Day 12 to Day 20, the ewes found in oestrus were drafted out from the flock and then were randomly assigned to the treatments (AI methods).

3.2. Training of rams and handling of collected semen

Six mixed-aged Romney rams were chosen from a Massey University flock to be trained for service into an artificial vagina (AV). Each ram was led close to an entire ewe, which was restrained in a service crate. After two days of training, each ram, in turn, was allowed to serve the ewe once per day, in the presence of the operator. By Day 7 five of the rams could serve into the artificial vagina (Plate 1). One ram was excluded because of lack of performance due to grass staggers.

The AV was prepared with warm water (40-45°C) filled inside its liner and at a pressure of 40-60 mm Hg. One end of the liner up to 3 cm deep was lightly lubricated with petroleum jelly, and at the edge of the end fitted with a scaled test tube for collection of semen. Immediately after collection, semen in the test tube was placed in a water bath at 32°C. A small amount of the semen was taken and microscopically examined to determine its motility (graded 1 to 5) and the

concentration (density) of semen was determined with haemocytometer, both as described by Evans & Maxwell (1987). The volume of semen was recorded. However, consistency of semen, colour and morphology of spermatozoa were not examined.

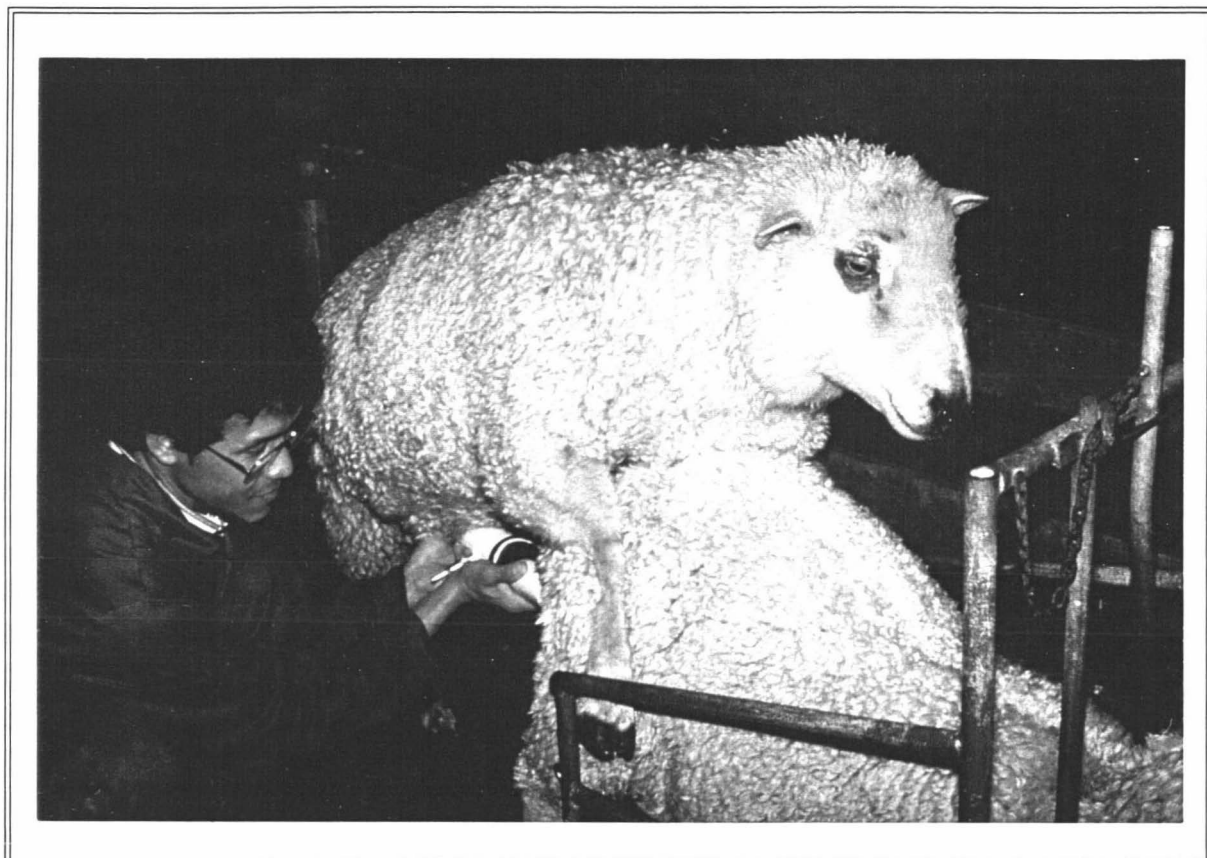


PLATE 1. Collecting semen using the artificial vagina

Dilution of the semen was undertaken 30 minutes before insemination. Collection of fresh semen was made from each ram and if of satisfactory quality samples were pooled together (motility graded 4 and 5, concentration of spermatozoa $3-5 \times 10^9$ spermatozoa/ml semen).

The material was diluted with skim milk until the concentration of freshly diluted semen was 8×10^8 spermatozoa/ml. The diluted semen was removed from the water bath at 32°C and was allowed to cool to 15°C over a 30 minute period using a 500 ml beaker of water as a buffer.

3.3. Trial 1: Development of transcervical insemination instruments

3.3.1. Instruments

Instruments used for performing transcervical artificial insemination (TCAI) are described and shown in Plate 2. The spinal needle was 8.9 cm long (Luer-lok hub). The end of the needle was soldered and its tip inclined 45° . Just anterior to the inclination there was an opened orifice, which was useful to allow exit of the diluted semen through the needle.

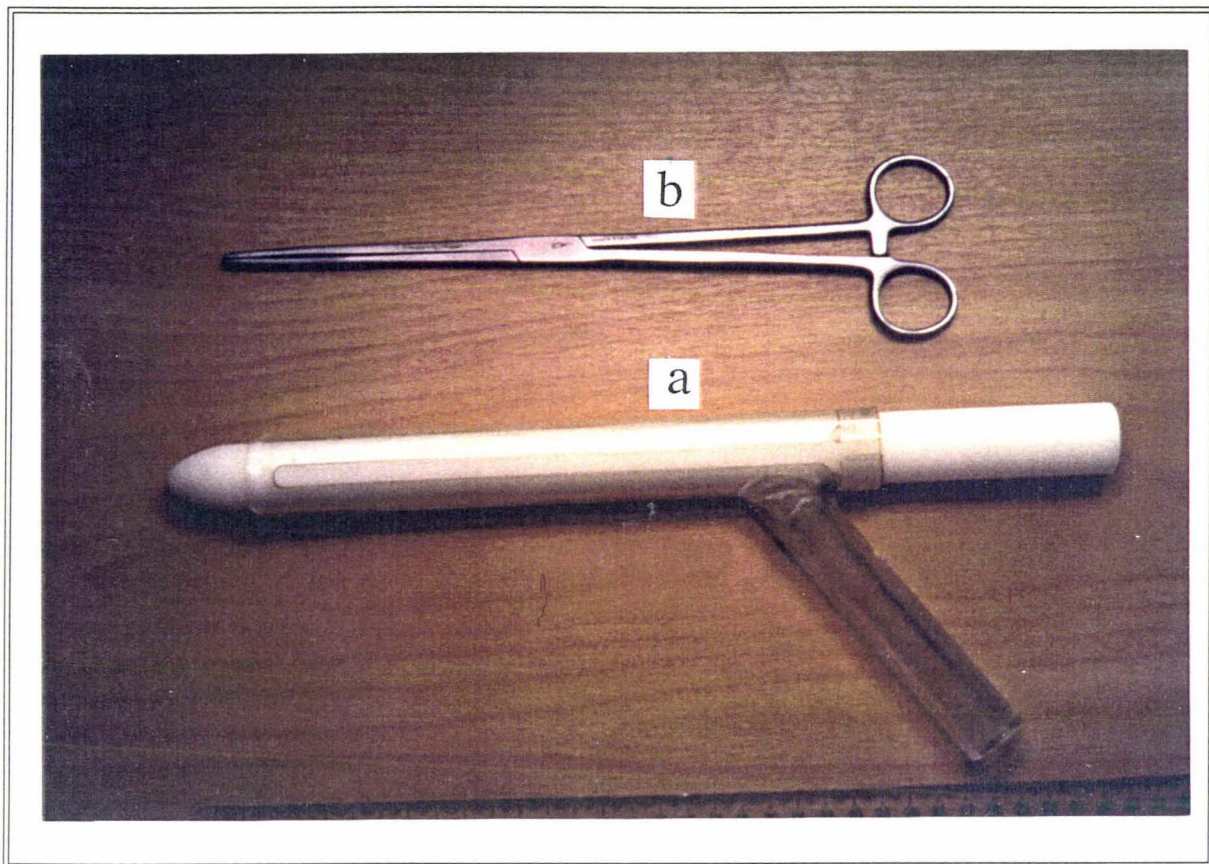


PLATE 2. Transcervical insemination instruments.

From bottom to top:

a) The speculum containing a solid insert was fitted into the vagina and with the handle.

b) A Bozeman forceps (267mm) was used to grasp the surrounding tissue in the cervix and hence aid in passing the needle to aid in passing through the distal opening.

Other instruments used in this present trial were needles (Plate 3).

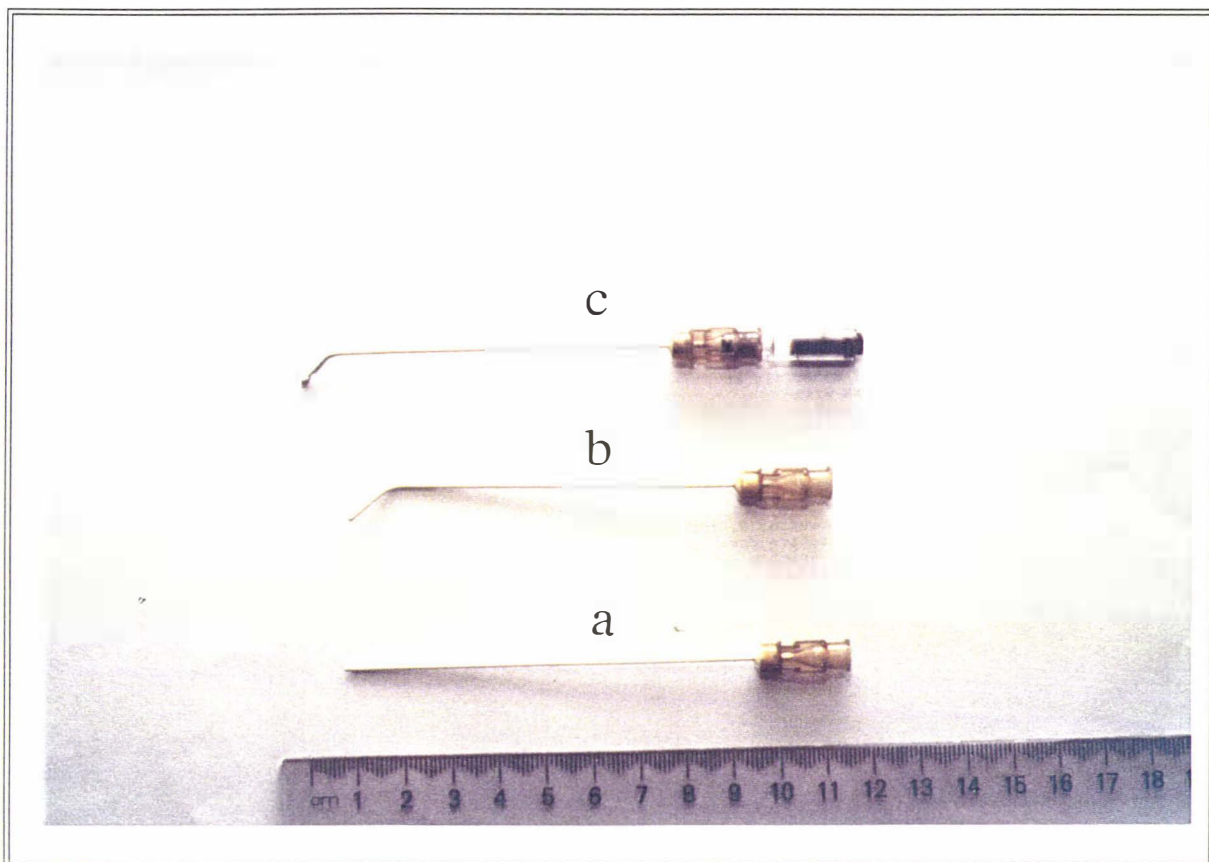


PLATE 3. Needles used in the AI trial

From bottom to top:

- a) 19 gauge (8.9 cm) spinal needle, unaltered.
- b) Unmodified needle.
- c) Modified needle showing bulb end.

3.3.2. Insemination and slaughter

In 19 mixed-age ewes and 48 hours after CIDR-G removal transcervical insemination was conducted. The technician made an attempt to pass the needle through the cervix into the uterus and injected a non-toxic dye (Indian ink). This trial was

intended to assess where the dye was placed in the tract. The depth of penetration was also assessed, and scored on a 1-5 scale (1 = slight penetration of the cervix; 5 = penetration into the lumen of the uterus). Data on condition of the vagina (mucus) and depth of penetration were recorded. Each ewe was killed by intravenous injection of Sodium pentobarbitone. The abdomen was opened by an assistant, whilst the technician firmly held the insemination gun *in situ* (Figure 5) and with care to avoid changing its position. The assistant advised the recorder the exact position of the needle and then removed the whole internal genital tract for histological examination. Because puncture of the wall of the cervix sometimes occurred, this procedure was repeated with 10 additional ewes, but they were inseminated after modification of the inseminating needle. This modification involved placing a rounded tip to the end of the needle. Indian ink was also injected through the modified needle prior to slaughter.

3.3.3. Histological examination

The cervix collected from each of the 20 ewes was split into anterior, middle and posterior parts and each of them placed in Bouin's solution. Twenty-four hours later, the solution was replaced with ethanol (75%) at similar volume. The parts of the cervix were preserved in

ethanol for 24 hours. To obtain a good result each one-third part of the cervix was further cut into tissue blocks of 5 mm. They were then processed for paraffin histology using a Shandon Elliot automatic tissue processor. Each tissue sample was given a code number prior to the embedding stage. At this stage, each sample was embedded in wax in order to make a block sufficiently rigid for uniform cutting. Each section (cut at 6 μ thick) was consecutively stained with Alcian Blue, Haematoxylin and Eosin, or Haematoxylin van Giesen according to Culling *et al.*(1985).

Slides obtained were randomly chosen and were given codes before microscopic examination by two technicians, at magnification of x 400. This was done to assess any damage that had occurred in tissue of the reproductive tract following TCAI.

Damage scores were classified as follows:

- 1 = no damage;
- 2 = minor epithelial damage;
- 3 = major epithelial damage;
- 4 = swelling and bruising in sub-epithelial layer;
- 5 = puncture in sub-epithelial layer.

3.4. Trial 2: Comparison of insemination techniques

Each oestrous ewe was restrained and prepared for insemination using one of three techniques (cervical, transcervical and intrauterine artificial inseminations). The cervical inseminations were performed in an angle-park sheep inseminating crate. The transcervical and intrauterine procedures were done using a laparotomy cradle. The first and the second techniques were performed by Technician 1 (the first undertaken as described by Evans & Maxwell, 1987; and the second was adapted from Halbert *et al.*(1990b). The intrauterine insemination were performed by Technicians 2 and 3, 6.10 ± 0.26 h after drafting on detection of oestrus. The dose of semen used was similar for the three techniques and contained 2×10^8 spermatozoa/ 0.25 ml.

At transcervical insemination, data were recorded for time of AI, duration of AI completed, depth of penetration(a 1-5 scale) and condition of vagina. For the intrauterine insemination with a laparoscope, data were recorded on time of AI and duration of AI. Cervical insemination: Data on time of AI and duration of AI were not recorded.

Aspects of fertility assessed in the trial were conception, lambing rates and litter size. The first variable was determined by non-return to oestrus after insemination. The second was a ratio of number of ewes which produced lambs and number of ewes present at lambing. Thus, those ewes which were dead or missing were excluded and put in a separate group. Litter size was defined as ratio between total number of lambs relative to the number of ewes which gave birth.

3.5. Analysis of data

3.5.1 Data analysis of development of TCAI instruments and damage of the cervix

Analysis of the technician predictions and actual depth of needle location and section of the cervix were made using the general linear model of SAS. The analysis of the needle location being in or out of the reproductive tract, was done using the CATMOD procedures of SAS and the frequency table was generated from the frequency procedures of SAS. Technician's prediction of AI needle location to actual location and the correlation of the two technicians damage scores were analysed using the correlation procedures of SAS.

3.5.2. Data analysis of comparison of AI techniques.

Conception and lambing rate data were treated as binomial traits. Effects of AI techniques on conception rate and on lambing rate were analysed with categorial modelling (CATMOD)(SAS, 1985) with $P < 0.05$ for statistical significance. Differences between AI techniques, age, depth of penetration, time intervals between time of draft to time of insemination were compared using Chi-square. Mean values of variable, and correlation between these independent variables to conception and lambing rate, were calculated (SAS, 1985)

CHAPTER 4: RESULTS

CHAPTER 4: RESULTS

4.1. Trial 1: Development of Transcervical Instruments

4.1.1. Placement of inseminating needle

This trial was to assess whether the inseminating needle was located in the genital tract at the completion of insemination. In several cases the needle had penetrated through the wall of the cervix and was visible in the abdominal cavity at dissection. Plate 5 shows what was found in one ewe after dissection where the needle had penetrated through the wall of the cervix.

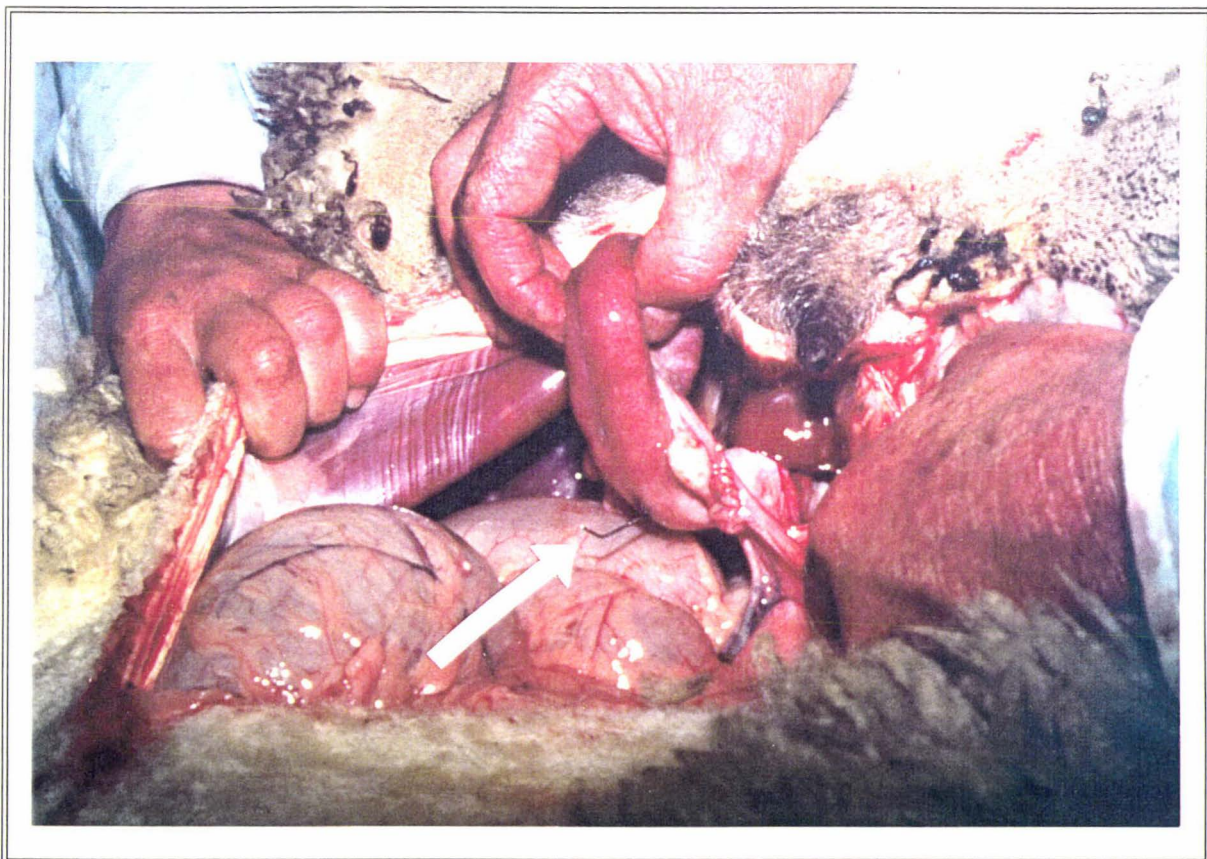


PLATE 4: Reproductive tract of a ewe showing penetration of the inseminating needle through the wall of the cervix.

In this situation, if semen had been expelled through the needle then fertilisation would have been unlikely as the sperm were deposited into the abdominal cavity rather than within the tract. With the unmodified needle in 6 out of 19 ewes the needle penetrated through the wall of the cervix. The observation with the modified needle showed that in only one out of 10 animals had the needle penetrated through the wall.

Following recording of the position of the inseminating needle, the genital tract of each euthanased ewe was removed and placed on a piece of paper.

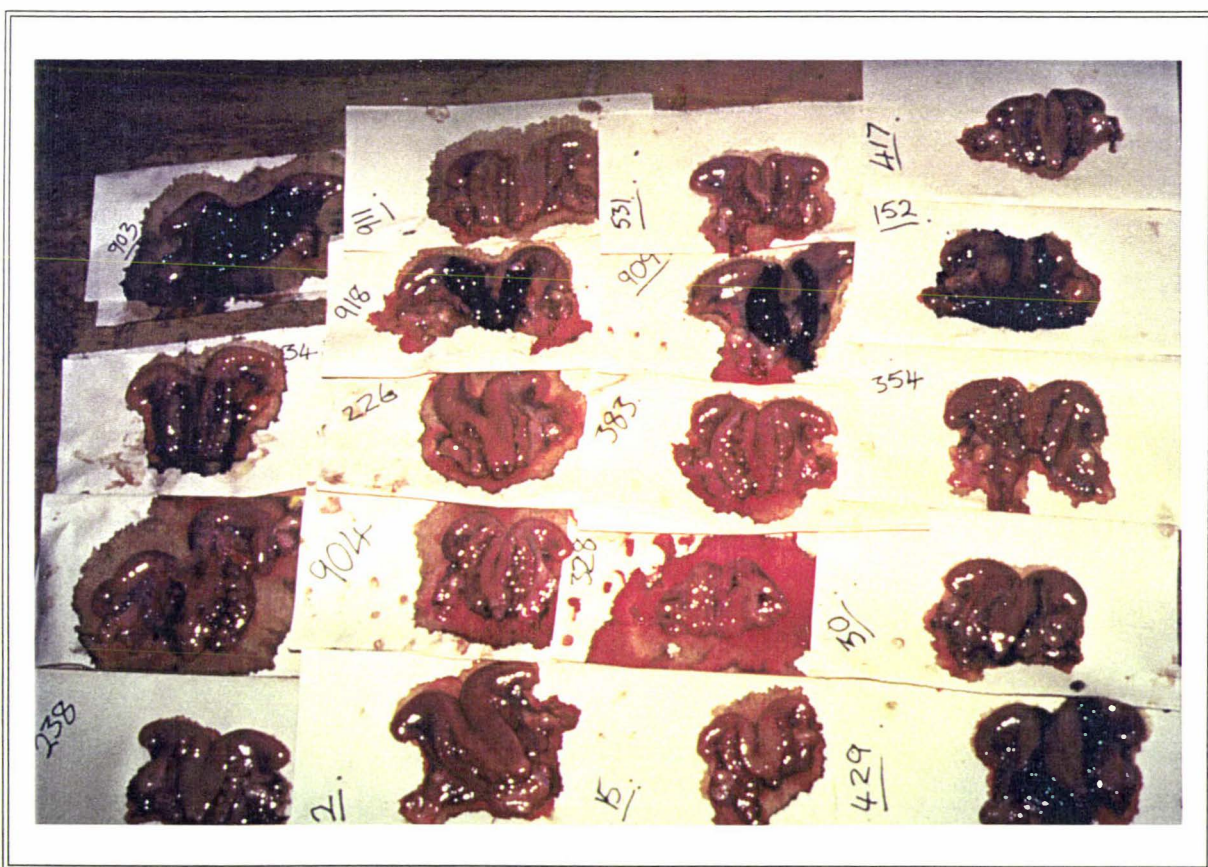


PLATE 5: The genital tracts of 19 ewes removed after slaughter.

The reproductive tract was dissected to open the interior surfaces in the cervix and uterus, and to record the presence of the Indian ink, which had been expelled through the inseminating needle. Plate 5 shows the tracts from 19 animals inseminated with the unmodified needle and reveals a range of positions where Indian ink was found in the lumen of the tract. This information enabled confirmation of the site where the needle had reached at completion of the insemination procedure. In six animals (no.918, 909, 903, 429, 152, and 34) the dye was clearly present within the uterus, but in others the dye was in the cervix or not at all. In the latter case, penetration through the wall of the cervix was confirmed or suspected. Table 4 shows data for the position of the inseminating needle after euthanasia. Data from one ewe in which AI was attempted using the unmodified needle, was excluded as it was a maiden animal and difficulty in locating the cervix was noted. The data show that when the two types of needles were compared, a greater percentage of penetration of the needle occurred through the wall of the cervix with the unmodified needle than when the modified needle was used (32% vs 10%). The modified needle remained in the tract more frequently than with the unmodified needle (90% vs 68%), but this difference was not statistically significant ($P>0.05$). It was concluded that the design of the inseminating needle did not determine the rate of penetration into the cervix.

TABLE 4: Distribution of ewes relative to position of the inseminating needle in the genital tract, determined at slaughter.

Position in the genital tract	Type of needle	
	Unmodified (%)	Modified (%)
In cervix	7 (36.8)	7 (70)
In uterus	6 (31.6)	2 (20)
Out of tract	6 (31.6)	1 (10)
TOTAL	19	10

A chi-square analysis indicated that there was no significant difference between the modified needle and the unmodified needle, in terms of being in the genital tract when inseminations were accomplished ($P>0.05$). The frequency of the modified needle being in the reproductive tract was greater than that for the unmodified needle (90% vs 68%). It was concluded that as the modified needle punctured the wall less frequently than the unmodified needle did, it was preferred for use in Trial 2.

The prediction of depth of penetration by the technician and the actual depth as determined at dissection of the animal is shown in Table 5.

TABLE 5: Comparison of predicted depth of penetration (Mean \pm SEM) and actual depth (Mean \pm SEM) as determined at dissection using scale values.

Item	<u>Type of needle</u>		Predicted value
	Unmodified (n=19)	Modified (n=10)	
Scale:1-5;			
Technician prediction ^a	4.36 \pm 0.33	3.70 \pm 0.33	0.12
Actual	4.68 \pm 0.40	3.90 \pm 0.40	0.12
Scale:1-3:			
Technician prediction ^b	1.63 \pm 0.16	1.30 \pm 0.16	0.09
Actual	1.95 \pm 0.25	1.40 \pm 0.25	0.09

^a Depth score as predicted by technician or actual observation ranked 1 to 5 where 1 is slight penetration of the cervix and 5 is penetration into the lumen of uterus.

^b Depth score as predicted by technician or actual observation ranked 1 to 3, where 1 is in the cervix; 2 is in the uterus and 3 is out of the reproductive tract.

The predicted location of the needle and actual location as determined by dissection were highly correlated (79% and 92% for the unmodified needle and the modified needle respectively; $P < 0.001$). The actual comparisons between the predicted and the observed needle location are shown in Table 5. When given a score of 1 to 5 the trend ($P = 0.12$) was for technician's prediction to be less than the actual depth of penetration in both needle groups (4.36 ± 0.33 and 3.70 ± 0.33 for technician; 4.68 ± 0.40 and 3.90 ± 0.40 for actual depth; the unmodified needle and modified needle, respectively). When the data were analysed by using a depth score 1 to 3, where 1 was in the cervix, 2 was in the uterus, and 3 being out of the reproductive tract, the trend ($P = 0.09$) was similar (1.63 ± 0.16 and 1.30 ± 0.16 for technician prediction; 1.95 ± 0.25 and 1.40 ± 0.25 for actual depth; the unmodified and modified needle, respectively).

4.1.2. Effect of needle penetration on histological damage

When the inseminating needle was located into the cervix and manipulated through the tract, some damage to the tissue could be expected. The observations were made of any bleeding seen down through the speculum at the time of insemination and the appearance of the mucus in the vagina. As well after slaughter and opening of the tract observations were also made for microscopic tissue damage. Histological examination of tissue shown within the cervix was the main means to describe damage.

Three staining techniques were used on the cervical tissues and were Haematoxylin and Eosin (H & E), Haematoxylin/van Gieson (H.vG) or Alcian Blue (AB) as described by Culling *et al.*(1985). Haematoxylin and Eosin and Haematoxylin and van Gieson were mostly used in the study. Having used these techniques, slides obtained showed fair resolution of nuclei of epithelial cells, collagen and muscle, from those parts which built up most the cervical tissues. On the other hand, Alcian blue was used to distinguish whether there was acid polysaccharides in the tissue.

The damage, as seen in the histological slides, was variable from a minor level, which was indicated by minor epithelial damage, and up to a major effect, indicated by puncture by the inseminating needle through the sub-epithelial layer of the cervix. Force was used to retract the surrounding tissue close to the entrance into the cervix and to introduce the inseminating needle as deep as possible. This pressure of the needle was responsible for damaging the cervix. Lifting or removing the forceps and inseminating needle from the cervix was also suspected to increase the level of damage. In the present trial, the force used for insemination was not recorded. However, it was reasonable to expect that the more force used in introducing inseminating needle, the more severe the damage might occur. In addition, it was found that damage was likely to become severe as the inseminating needle was moved deeper into the genital tract and manipulated to find a pathway.

Plates 6, 7, and 8 show different degrees of damage which occurred as a result of introducing the inseminating needle into the cervix.

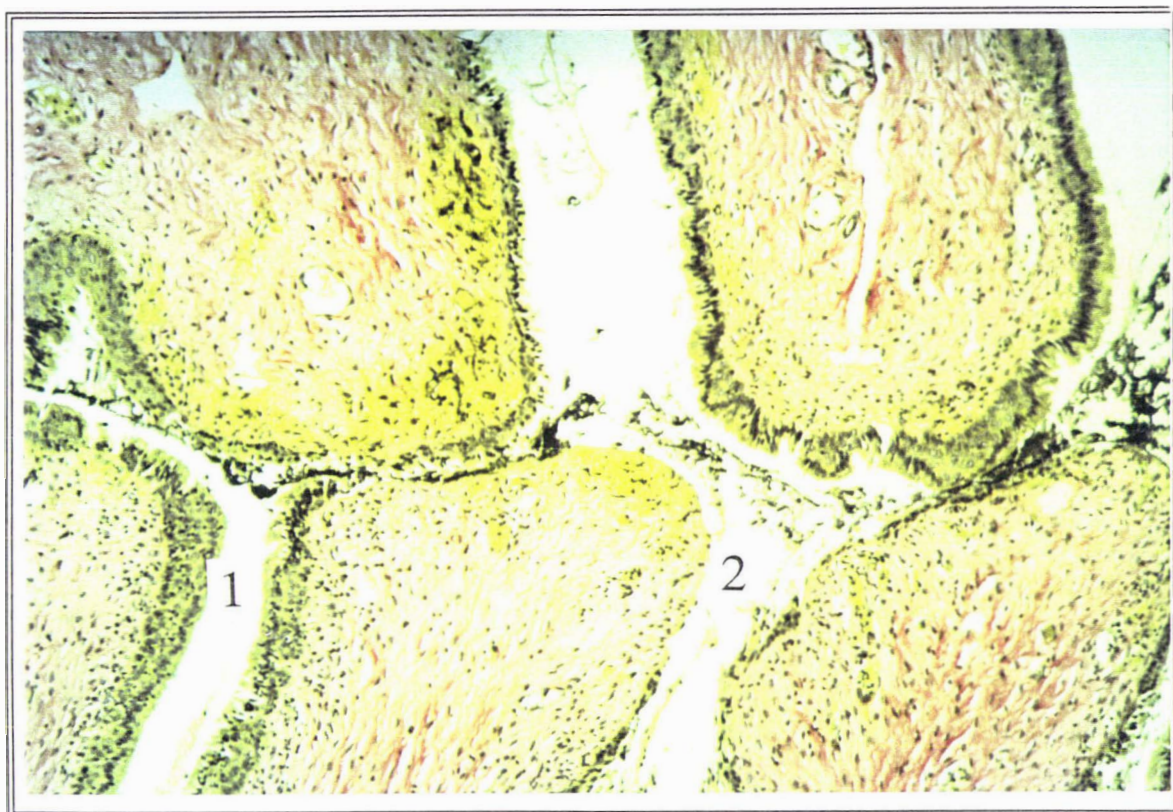


PLATE 6: Damage in cervical tissue at score 1 and 2

In Plate 6, the damage to the epithelium of the cervix was limited in extent and very localised. Abrasion of the lining had occurred and cells were present in the lumen of the tract.

In tissue from the animals as depicted in Plate 7, the damage in the epithelium was more extensive, and clearly showed that tissue had been torn from the tract. The wall of epithelium was frequently ruptured and sub-epithelium layes also showed bruising.

The most extensive type of damage found was that where the inseminating needle had penetrated through the wall of the genital tract. This effect could be seen in consecutive sections. In this part of the tract, anterior to the exit point of the needle from the tract, the cervix appeared undamaged.

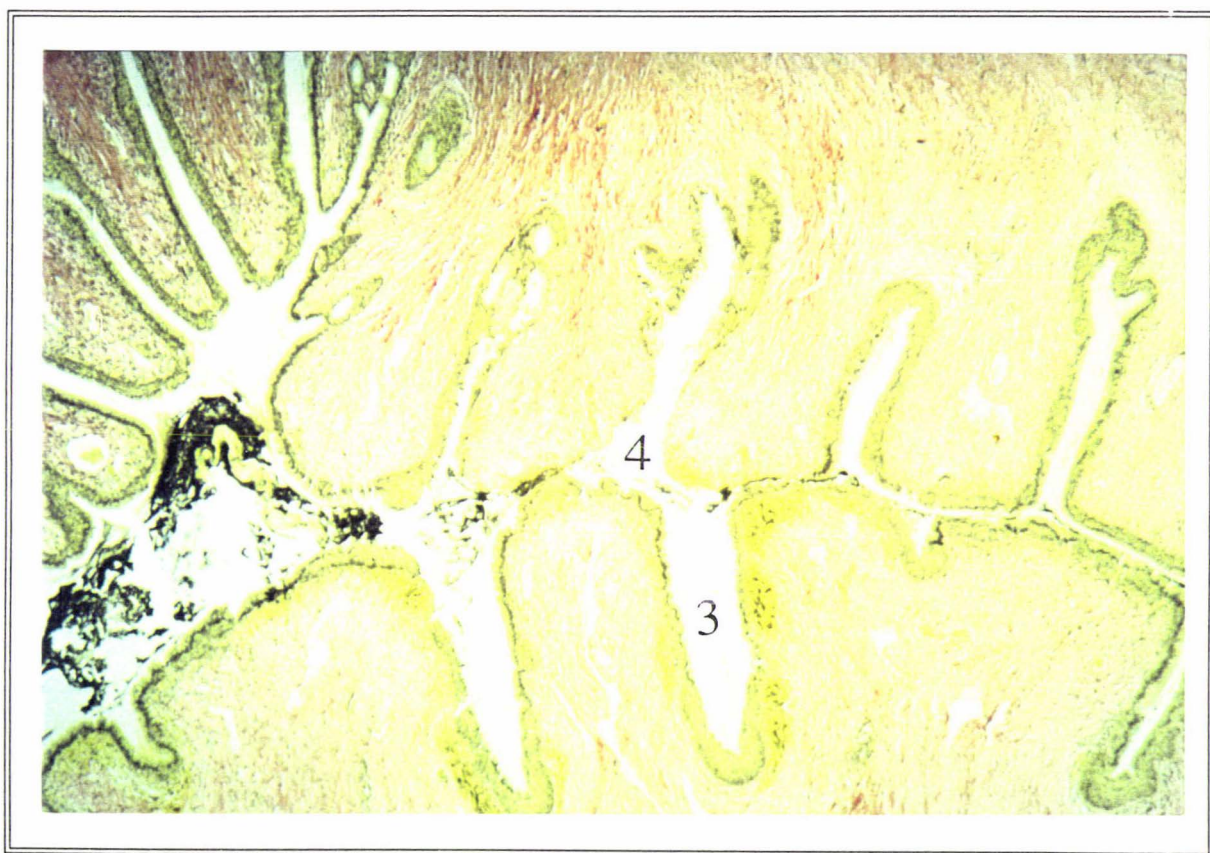


PLATE 7: Damage in cervical tissue at score 3 and 4.



PLATE 8: Damage in cervical tissue at score 5.

Table 6 shows the mean scores for damage in the tract recorded by two evaluators.

TABLE 6: Scores for histological damage caused by inseminating needle at different sites within the cervix.

Item	Section of the cervix		
	Posterior (n=9)	Middle (n=9)	Anterior (n=9)
Damage ^a	2.73 ± 0.20 ^b	2.24 ± 0.20 ^b	1.72 ± 0.20 ^c

^a Damage = Histological evaluation where 1 = no damage; 2 = minor epithelial damage; 3 = major epithelial damage; 4 = sub-epithelial layer damage; 5 = puncture in sub-epithelial layer.

^{b c} Row means with different superscripts (P<0.001).

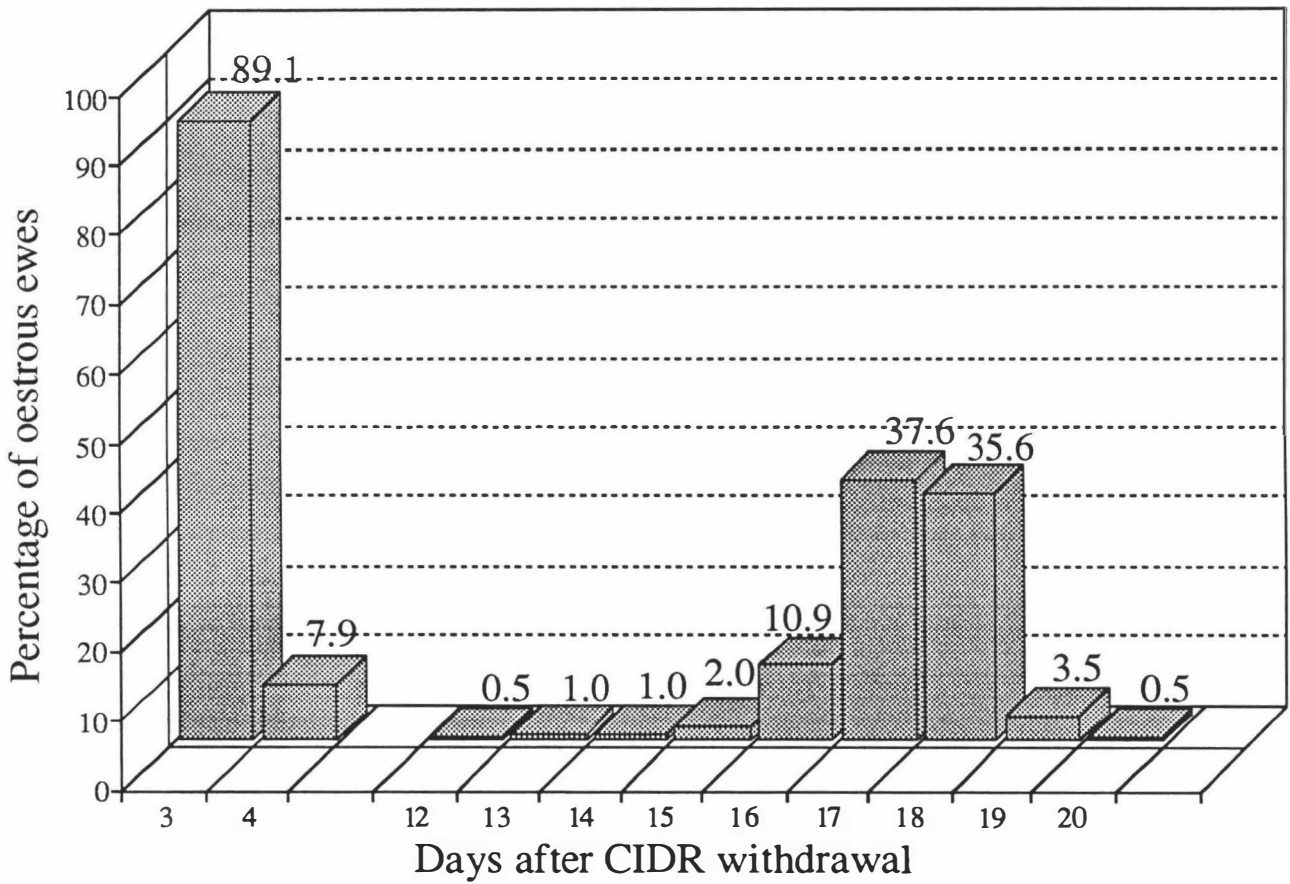
The damage scores given by the two evaluators, were highly correlated ($r = 93.9\%$; $P < 0.001$). The damage scores either at the posterior (2.73 ± 0.20) or the middle of the cervix (2.42 ± 0.20) did not differ significantly but were greater than that found in the anterior part of the cervix (1.72 ± 0.20). This was to be expected as passage of the needle to approximately half-way through the cervix occurred in all animals.

4.2. Trial 2: Oestrous control and comparison of insemination techniques,

4.2.1. Incidence of oestrus in ewes.

Figure 2 (p.54) shows the percentage of ewes in oestrus after withdrawal of CIDR-G. Almost all CIDR-G-treated ewes were synchronised in oestrus during 2 days (mean 1.12 ± 0.04 d). Most of the ewes returned to second oestrus although 15 were not recorded in heat and one was missing. The interval from the first and second oestrus was 15.4 ± 0.2 days. The day of oestrus and hence day of insemination did not significantly affect conception rate or lambing rates ($P > 0.1$).

Figure 2. Oestrous incidence after CIDR removal



4.2.2. Artificial insemination and fertility

Seven ewes assigned for transcervical insemination, were changed to cervical insemination because the technician could not perform the transcervical insemination successfully; they were either maiden or had blind vaginas. In a further 6 ewes, semen was observed to flow back into the vagina immediately after transcervical insemination. The data from these ewes were included in the analysis. Fertility after insemination with three different techniques is presented in Table 7.

TABLE 7: Effect of AI technique on fertility

AI technique	<u>Conception rate</u>		<u>Lambing rate</u>		<u>Litter size</u>
	%	(No.)	%	(No.)	Mean (No.)
Intrauterine	80.4	(37/46)	50.0	(21/42)	1.24 (26/21)
Cervical	75.6	(34/45)	56.4	(22/39)	1.18 (26/22)
Transcervical	86.9	(73/84)	75.9	(60/79)	1.22 (72/59)
Mean \pm SEM	82.3 \pm 3.8		63.7 \pm 3.8		1.21 \pm 0.04

The type of AI technique did not significantly affect conception rate ($P=0.23$). When intrauterine insemination was omitted, and the two remaining procedures (cervical and transcervical techniques) were compared, the difference was not statistically

significant ($P>0.05$). There were significant differences between AI techniques on lambing rate ($P<0.05$). Litter size was not different relative to AI methods. Mean of the litter size was 1.21 ± 0.04 .

Table 7 also shows that a major loss of fertilised embryos, occurred in the group of intrauterinely inseminated ewes (30.4%), followed by cervically inseminated ewes (19.2%) and transcervically inseminated ewes (11%).

4.2.3. Effect of age on fertility

The results from the three insemination treatments, were pooled and classified relative to the ages of the ewes. These are presented in Table 8. Statistical analysis revealed that the age of the ewe was not associated with differences in pregnancy ($P>0.05$) and lambing rates ($P>0.05$) and in litter size ($P>0.05$). When the ewes were split into three groups (3, 4 and ≥ 5 years), it was found that pregnancy rate was the highest in the four-year group among the three aged groups (76.5% vs 60.5% vs 50%; $P<0.05$) for lambing rate, but it was not statistically different ($P>0.05$) for conception rate and litter size.

TABLE 8: Effect of age of the ewes on fertility after insemination

Age (years)	<u>Conception rate</u> % (No)	<u>Lambing rate</u> % (No.)	<u>Litter size</u> Mean (No.)
3	80.9 (72/89)	60.5 (49/81)	1.20 (59/49)
4	87.5 (49/56)	76.5 (39/51)	1.23 (48/39)
5	87.5 (7/8)	62.5 (5/8)	1.20 (6/5)
6	66.7 (10/15)	46.7 (7/15)	1.14 (8/7)
7	75.0 (3/4)	66.7 (2/3)	1.50 (3/2)
8	100 (3/3)	-	-
≥ 5 [§]	76.7 (23/30)	50.0 (14/28)	1.21 (17/11)

[§] Calculation for the last three-aged groups.

4.2.4. Fertility in ewes after transcervical insemination, relative to depth of penetration of the inseminating needle.

The inseminating needle was introduced as deeply as possible (within 5 minutes) before deposition of semen was done. The result obtained is recorded in Table 9.

TABLE 9: Fertility relative to depth of penetration of inseminating needle

Depth of penetration	<u>Conception rate</u>		<u>Lambing rate</u>		<u>Litter size</u>
	%	(No)	%	(No)	Mean (No)
1	100	(4/4)	66.7	(2/3)	1.00 (2/2)
2	62.5	(5/8)	50.0	(4/8)	1.25 (5/4)
3	77.8	(21/27)	56.0	(14/25)	1.14 (16/14)
4	100	(13/13)	91.7	(11/12)	1.36 (15/11)
5	93.9	(31/33)	93.5	(29/31)	1.21 (35/29)

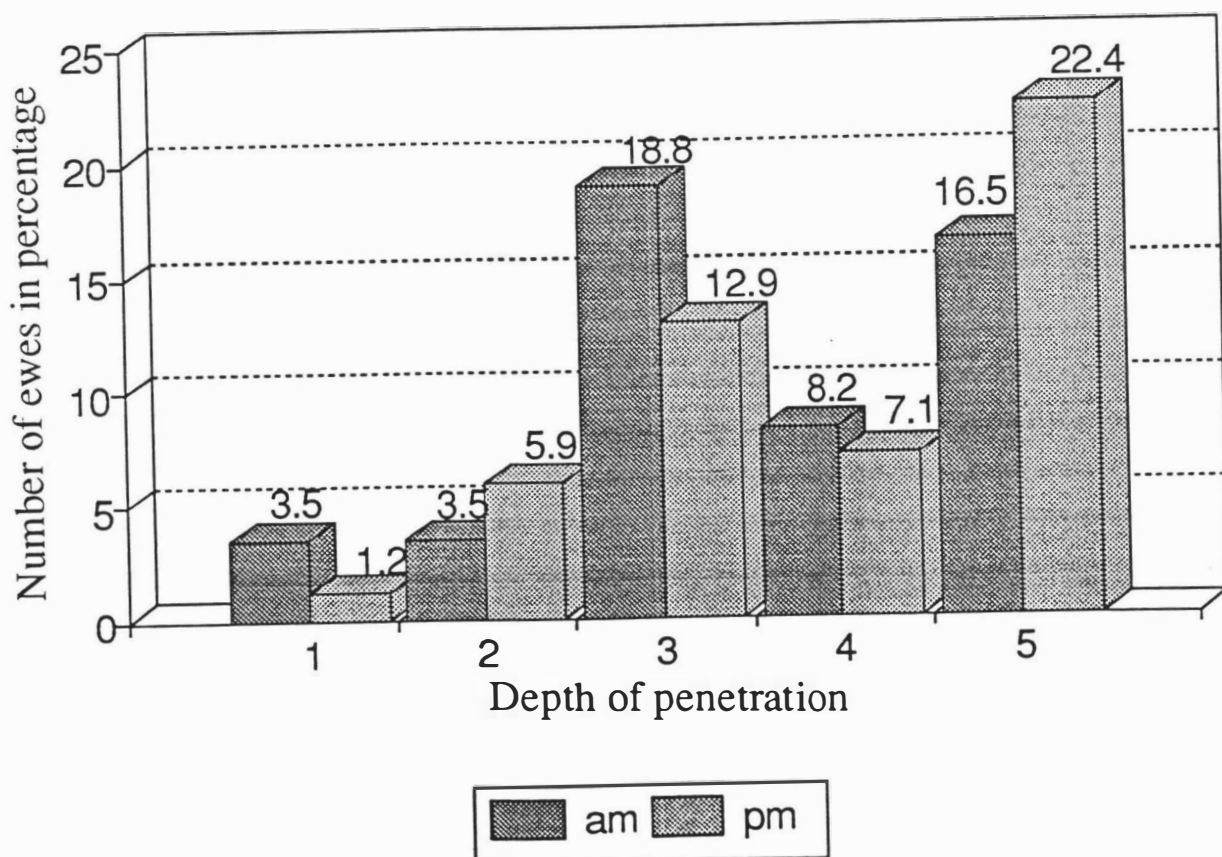
Depth of penetration showed a significant effect ($P < 0.05$) on conception rate and on lambing rate ($P < 0.05$), but not significant on litter size ($P > 0.05$). Further, the depth of penetration and lambing rate was positively correlated ($r = 0.39$, $P < 0.01$).

Classification of the results for conception rate after full penetration (95.7%, 44/46) and partial penetration (77%, 30/39) showed a significant difference ($P < 0.05$).

The depth of penetration achieved with the inseminating needle was not related to the age of the ewe, day of oestrus and interval from detection of oestrus to AI ($P > 0.1$).

Figure 3 shows results for the degree of success in penetrating through the cervix. There was no effect on fertility relative to inseminations done in the morning or afternoon.

Figure 3. Depth of penetration after morning or afternoon insemination



4.2.5. The interval from oestrous detection to insemination on fertility.

The interval from detection of oestrus to the time AI was undertaken was very variable, its minimum and maximum being 1.30 h and 13.50 h and a mean 6.10 0.26 h. Regarding to these intervals, the ewes were classified into two groups with intervals to insemination (a) up to 5 h or (b) 6-13.5 h after the onset of oestrus. Table 10 shows fertility results relative to the interval to insemination.

There was no significant effect of interval from time of oestrous detection to time of insemination on conception rate or on lambing rate ($P>0.05$). The mean litter size for the two groups were similar. In addition, the decrease in lambing rate following insemination in the morning was nearly 2 times those following insemination in the afternoon (24.4% vs 12.6%).

TABLE 10: Fertility after insemination relative to the interval from onset of oestrus to AI.

Time interval	<u>Conception rate</u>		<u>Lambing rate</u>		<u>Litter size</u>
(h)	%	(No)	%	(No)	Mean (No)
1-5	81.9	(68/83)	57.5	(42/73)	1.24 (52/42)
≥ 6	82.4	(75/91)	69.8	(60/86)	1.16 (70/60)

Fertility following insemination which was performed at morning or noon, is presented in Table 11. There was no significant difference ($P>0.05$) in fertility or in litter size associated with the time when insemination was performed. The fertility results determined at conception, declined at lambing by 25% and 13% for ewes that were inseminated in the afternoon and morning, respectively.

TABLE 11: Fertility of ewes inseminated at morning or afternoon

Time	<u>Conception rate</u>		<u>Lambing rate</u>		<u>Litter size</u>
	%	(No.)	%	(No.)	Mean (No.)
Morning	82	(73/89)	69.4	(59/85)	1.17 (69/59)
Noon	82.6	(71/86)	57.3	(43/75)	1.26 (54/43)

Fertility following insemination which was performed at morning or noon, is presented in Table 11. There was no significant difference in fertility or in litter size associated with the time when insemination was performed. The fertility results determined at conception, declined at lambing by 25% and 13% for ewes that were inseminated in the afternoon and morning, respectively.

4.2.6. Fertility relative to technicians and AI technique

The cervical and transcervical inseminations were conducted by one technician, while intrauterine insemination was done by two people. Table 12 shows a comparison of the results for the intrauterine insemination for technicians and also the results for the third technician and also the results for the third technician but using different method of insemination.

TABLE 12: Fertility after insemination by individual technician.

Technician	AI Methods	<u>Conception rate</u>		<u>Lambing rate</u>		<u>Litter size</u>	
		%	(No)	%	(No)	Mean	(No)
2	Intrauterine	90.5	(19/20)	50	(10/20)	1.40	(14/10)
3	Intrauterine	73.9	(17/23)	55	(11/20)	1.09	(12/11)
1	Cervical & Transcervical	82.4	(108/131)	67.5	(81/120)	1.20	(97/81)

Overall, the results for the two technicians using intrauterine insemination, were not significantly different in conception and lambing rates ($P>0.05$), and in litter size ($P>0.05$). It was not valid to compare the results from the three technicians using different techniques being used. However, all technicians did achieve fertility levels better than 70% conception which would compare favourably with animals being naturally mated.

CHAPTER 5: DISCUSSION

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5.1. The incidence of oestrus

The present study showed that the synchronisation and incidence of oestrus after CIDR-G removal was more concentrated than that which occurred following the second oestrus (2 days vs 9 days). The effectiveness of the CIDR treatment was good since the incidence of oestrus was 97%, and only a very small number of ewes lost the CIDR-G (less than 1%); similar results have been reported by other workers (Maxwell & Barnes, 1986; McMillan, 1986; Welch *et al.*, 1984). At the second oestrus the percentage of ewes which responded was 92%. Compared to published results for Romney ewes, the present result was higher (65% ewes in oestrus after progesterone injection, Cumming & McDonald, 1966; or 77% ewes in oestrus after PMSG injection ceased, Eastwood & McDonald, 1974; McMillan & Hall, 1991). Other factors which might potentially have contributed to the difference, include the time in the season at which treatment was given, age of animals and level of feeding as well as the different methods of synchronisation used.

The period of synchronised oestrus with respect to the onset of the oestrus after CIDR-G removal was less variable than that of the next oestrus (Figure 2), but the latter was more variable than has been reported by Evans & Robinson (1980). Those workers reported a mean time to oestrus of 43.2 h (range: 30-46 h) after sponge

removal and injection of 400 i.u. PMSG. In the present trial, the range was 48 to 96 h after CIDR removal. This difference is understandable since the experiments used different breeds of sheep and PMSG was given in combination with progesterone.

In the present trial, a large number of CIDR-treated ewes (89.1%) exhibited oestrus at Day 3, whilst only few ewes were in oestrus found on Day 4 after CIDR withdrawal. It was likely that the oestrous percentage on Day 3 included those ewes which came into heat earlier than this day, but they were not recorded.

The sheep were left unmated until the next oestrus when there would be the potential for a higher conception rate. This was done in order to avoid the impairment of spermatozoa transport in the reproductive genital tract (Allison & Robinson, 1969; Quinlivan & Robinson, 1969). The next oestrus began on Day 14 after CIDR removal and reached its peak on Day 19 and concluded within 9 days. In animals having normal oestrous cycles, the length of the cycle would be approximately 16-18 days (Fairnie & Wales, 1982). The oestrous cycle of the synchronised ewes was 15.4 0.22 days. Thus, it was shorter than the normal oestrous cycle. It appeared that a "carry-over" effect of CIDR treatment existed at the next oestrus to cause it to occur slightly earlier than might have been predicted.

5.2. Evaluation of the needle penetration

This trial confirms the previous results reported by several workers (Halbert *et al.*, 1990a, 1990b; Buckrell *et al.*, 1992; Buckrell *et al.*, 1994; Windsor *et al.*, 1994;

Windsor *et al.*, 1995) that transcervical passage in oestrous and mature ewes was possible. However, other workers such as Dun (1955) and More (1984) have reported that the cervical canal was impassable to an inseminating pipette. According to Halbert *et al.*(1990) it occurred because (1) the presence of blind spaces from folds of vaginal tissue which make identification of the cervical opening difficult; (2) the narrow end of the funnel- shaped rings are pointed caudally; (3) the rings are eccentrically presented, most commonly the second and third within the canal; and (4) the alignment, number, size and spacing of rings is variable between ewes. In some cases in the present study, penetration of the cervix canal could not be accomplished in several maiden ewes. Thus, it may suggest that transcervical artificial insemination is not very suitable for this group of animal (nulliparous), but is for multiparous ewes.

The results of the first trial showed that the modified needle was more likely to be located in the lumen of the genital tract than was the unmodified needle. Similarly, workers in the former Soviet Union (Zaicev *et al.*, 1989) reported that pipette design increased ease of penetration into the cervix. In contrast, Eppeleston *et al.*(1995) reported that penetrability of the cervix could not be increased after using either standard bent-tipped or helicoid tipped pipettes. The difference might be due to individual variation such as the structure of the cervixes than due to the level of experience of the technician, or in the use of a different pipette (Maxwell & Salamon, 1995b; Windsor, 1995, Windsor *et al.*, 1994).

Correlation analysis showed that there was a good relationship between the technician's prediction of depth of penetration and the actual depth of penetration, which suggest that it is possible to predict the location of the inseminating needle in the genital tract after transcervical insemination. The technician predicted a greater depth score using the unmodified needle as compared to the modified needle as there was a greater number of predicted uterine penetrations when in actuality the penetrations were through the wall of the cervix. Using the modified needle the predictions were more accurate and with the technician having a greater percentage of penetrations into the cervix, the depth score was lower. When the data were analysed using a 1 to 3 scale depth score the same trends were seen.

The number of penetrations into the genital tract of subsequently slaughtered ewes was 68% with the unmodified needle and 90% with the modified needle. This increase was attributable to the improved pipette design and also to the technician gaining experience with the procedure. Penetration into the cervix was 36% with the unmodified needle and 70% with the modified needle. These figures were slightly lower than the results reported by Buckrell *et al.*(1992), namely 87% for uterus penetration, and 70-96% for cervical penetration. In the present experiment, penetration of the uterus occurred in 31.6% (6/19) and 20% (2/10) ewes using the unmodified needle and modified needle, respectively. These penetration rates are comparable to the report of Eppleston (1992), that the success of deeper penetration was low.

He continued to report that insemination beyond 2 cm, was achievable in 6% to 38% of the ewes. With the same pipette as used in the Guelph-System of transcervical insemination, Fukui & Roberts (1978) reported cervix penetration in 35% to 50% of oestrous ewes.

Histological examination of tissue from the ewes after insemination indicated that more severe damage occurred as the needle reached the mid-cervix or the uterus than from which occurred from inseminating only in the vagina or if rupture through the wall of the genital tract took place and the needle was out of the tract. Manipulation, with forceps, of the tissue at the entrance to the cervix, in order to provide a path for introducing the inseminating needle with force, probably caused damage to the cervix. Unfortunately, in this present trial, no attempt was made to measure force used as the inseminating needle was introduced as deep as it permitted. In many of those ewes which underwent uterine penetration, their cervixes showed damage. This was in agreement with the report of McKelvey (1994). From postmortem examination, McKelvey found there was a high incidence of peri-cervical abscessation and pyometra and up to 50% of ewes inseminated had some degree of damage to the reproductive mucosa. Halbert *et al.* (1990a) reported the use of forceps resulted in trauma and haemorrhage, and in some cases, the instrument caused damage to tissue surrounding the cervical os.

The presence of damage did not always influence the reproductive performance as conception rate and lambing rate following transcervical insemination were 86.9% and 63.7% respectively. This result, thus, indicates that while damage might occur, it is not so severe as to cause complete infertility.

5.3. Effect of AI technique on fertility

Overall, in the present experiment the type of AI technique did not significantly alter conception rate ($P>0.05$). But when only the effect of cervical and transcervical artificial inseminations are taken into account on conception and lambing rates, it was found that the effect was close to significant effect ($P=0.09$). These results were higher, but not consistent to the latest published result which was conducted under New Zealand conditions (Smith *et al.*, 1995a). Smith *et al.* claimed that the type of AI technique gave significant differences in conception rate, with transcervical insemination being inferior to either laparoscopic insemination or cervical insemination (40% vs 63% vs 50%). However, the two trials from which this difference emerged, were different in terms of (1) kind of treatment prior to insemination and (2) the type of semen (fresh vs frozen) used in trials. The first reason was related to the treatment for synchronisation prior to insemination. In the present study, insemination was undertaken at the second oestrus after CIDR removal and it was based on oestrous detection, whereas in the work of Smith *et al.* the

inseminations were done 51 h after CIDR removal, and this in turn would be subject to the impairment of sperm transport immediately after hormonal synchronisation (Allison & Robinson, 1970; Quinlivan & Robinson, 1969). The second reason relates to the semen used. Smith *et al.* used frozen-thawed semen whereas in the present trial freshly diluted semen was used. The conception and lambing rates of the present trial after transcervical insemination are also higher than those published abroad (e.g. Buckrell *et al.*, 1992; Windsor *et al.*, 1994).

The conception rate after cervical insemination in the present trial compares favourably to the report of Harvey *et al.* (1984) (75.6% vs 73%). These figures are higher than the conception rate after insemination with frozen-thawed semen, and these agree with the results reported by several workers (Evans *et al.*, 1986; Maxwell & Hewitt, 1986).

The type of AI technique had a significant effect on lambing rate ($P < 0.05$). There is no clear explanation for this result when considering that AI technique did not affect conception rate. However, the most probable explanation for this, is a big prenatal loss occurred prior to the determination of lambing rate. The results also may be partly caused by loss of data for missing and dead ewes during the gestational period (Table 7) and this chance factor biased the result.

It is normal that there is a difference from conception rate to lambing rate. Reasons for this may be reflected either in anoestrus or poor oestrous condition in the ewes

or some overall infective agents (J.F. Smith, personal comm., 1995). But if these causes did take place in the present trial, there should be equal losses in all three AI groups. However, this was not the case in the present result as all the AI groups were different in the percentage of loss.

This result was also complicated as the biggest loss was not found in transcervical insemination group, but found in the intrauterine insemination group (11% vs 30.4%). This is contradictive to the result of Salamon & Lightfoot (1970) which showed that cervical traction - as performed in transcervical insemination - was a potential source of reducing fertility. From this disagreement, one is tempted to conclude from the present results that, by chance might be the cause for this present result.

A major loss of 30.4% occurred following intrauterine insemination, whereas cervical loss was moderate (19.45%), and transcervical was the slightest (11%). These losses were less than the result of Lightfoot & Salamon (1976) that reported that uterine insemination caused embryo loss of 47%, and this indicates the discrepancy in the potential performance between conception and lambing. There are three possibilities for this present loss. Firstly, the growing embryos become increasingly dependent on the uterine environment for survival and growth. A hostile environment which does not favour the maintenance of the embryo will often lead to an increase in embryo mortality (Edey, 1969). Secondly, a number of physiological processes may not function as expected when there is much variation in the embryo stage and the uterus in development. This consequently will lead to an increased level of prenatal mortality (Wilmot & Sales, 1981; Wilmot *et al.*, 1985, 1986). Thirdly, manipulation

of the cervix using AI techniques which cause damage to the reproductive tract may induce embryo mortality. The damage to the tract might be expected to repair quite rapidly after it was caused, but the effects might "carry over" to influence the early development of the embryo coincident with the ewe not returning to oestrus.

This would mean it was included in the calculation of conception rate but subsequently failure of the embryo to develop will result in the lower lambing rate.

Lambing rate following transcervical insemination in the present study is higher than the result reported by Buckrell *et al.*(1994) obtained from inseminating during the normal season. The result is also higher than those others recently published (Windsor *et al.*1993; Smith *et al.*,1995a).

Comparisons between transcervical insemination and other AI techniques on conception rate using frozen semen have shown that AI technique strongly affects conception rate. Several workers report that transcervical insemination is superior to cervical insemination but inferior to intrauterine artificial insemination with the aid of a laparoscope (Smith *et al.*, 1995a; Windsor *et al.*, 1994).

AI technique did not influence litter size ($P>0.05$). Maxwell (1986b) reported that the percentage of lambs born was significantly better after insemination into the middle of the uterine horns than the other sites of the genital tract. One of the possibilities attributed to the difference is the current trial used mixed-aged Romney ewes but Maxwell studied mature Merino ewes.

The mean litter size obtained (1.21 ± 0.04) in the range noted by a number of authors (e.g. McDonald, 1958; Averill, 1959; Quinlivan *et al.*, 1966a; Allison, 1968, 1975; Hight & Jury, 1976) who found that the means varied between 1.00 to 1.88 lambs for Romney ewes not given any superovulation treatment.

5.4. Effect of age on fertility

Like the report of Eppleston *et al.*(1994), the present result did not demonstrate a significant difference among the different-aged ewes in terms of conception and lambing rates and of litter size ($P > 0.05$). When they were split into three groups (3, 4 and ≥ 5 years old), difference was found on lambing rate ($P < 0.05$). No unequivocal explanation can be made to this surprising result.

On the other hand, no statistically significant difference was found either ($P > 0.05$) on conception and on litter size. These results are in parallel to the finding of Shackell *et al.*(1990), that conception rates decreased as age of ewe increased. Further, Harvey *et al.*(1986) demonstrated that conception rates were higher in the two-tooth ewes than in three- or four year old ewes. Conversely, Paton *et al.*(1993) reported that conception rate after cervical insemination in the mixed-aged ewes (3-5 years old) was higher than that in two-tooth ewes (58% vs 43%). The fertility of N.Z. Romney sheep reaches its peak at around 5 years of age, with a decline after that age (Dalton & Rae, 1978). They also stated that the two-tooth ewes are a critical group for reproductive problems, through lower multiple birth rate, greater barrenness

and higher lamb mortality. Although the above results suggest that the difference in fertility between ages of ewes might occur, there does not appear a consistent trend. In the present work, only small numbers of animals were present in the several age classes, thus to demonstrate any true effect of age it would be necessary to consider much large numbers of animals to show a statistically significant result.

Litter size was not statistically influenced by age in the present trial. On the contrary, Goot (1952) found that litter size increased with age until it reached its peak at 5 year old, and a decline occurred after that (1.01 lambs at 2 years, 1.34 at 5 years and 1.32 at 6 years).

5.5. Effect of depth of penetration with transcervical insemination on fertility

The conception rate was influenced by the depth of insemination ($P < 0.05$). This result was different from the results reported by several workers (Windsor *et al.*, 1994; Graham *et al.*, 1978; Fukui & Roberts, 1976; Aamdal, 1974; Anderson *et al.*, 1973; Salamon & Lightfoot, 1976), who reported that fertility did not always increase as the depth of semen deposition increased; but it is in parallel to the result reported by Eppleston *et al.* (1994), that cervical insemination with frozen semen showed an increase in conception rate as the depth of penetration increased. Further, they found that there was a linear increase of 7%-12% in conception rate per cm as depth of insemination increased.

This is in agreement with the result obtained from the present experiment, where there was a relationship between the depth of penetration and conception rate ($r=0.16$; $P=0.03$). Other factors that might influence depth of penetration are structure of the cervix, stage of oestrus, age and parity of ewes, presentation of ewes for insemination and skill of the operator (Salamon & Maxwell, 1995).

The conception rates in the present trial were also different for full and partial penetration at insemination (97% and 80.1%) These results were much higher than the results after insemination with frozen-thawed semen reported by Smith *et al.* (1995).

The time of oestrous detection did not show a significant effect with depth of penetration, the result which was similar to the report of Smith *et al.*(1995a). On the contrary, Eppleston *et al.* (1994) demonstrated that depth of insemination at 12 h was deeper than at 24 h after detection of oestrus. This difference is closely associated with different length of interval from oestrous detection to AI time. In the present trial, interval from the detection of oestrus to AI classified into two groups (up to 5 h and more than 5 h with mean 6.10 ± 0.26 h), while in the work of Eppleston *et al.* ewes were inseminated either 12 h or 24 h after the onset of oestrus.

The effect of depth of insemination on lambing rate showed a significant effect ($P < 0.01$) but there was no significant effect on litter size. This result is in line with the previous findings of several workers (Fukui & Roberts, 1976a, 1976b; Maxwell & Hewitt, 1986) that, a significant difference was associated with depth of penetration. Thus, it could be summarised that as depth of penetration increased lambing rate proportionately increased.

5.6. Effect of interval from oestrous detection to the time of AI or technician on fertility.

Table 10 suggests that the interval between oestrous detection and AI did not affect conception and lambing rates and litter size ($P > 0.05$) in cyclic ewes. Regarding intrauterine insemination, this is in agreement with the result reported by Evans *et al.* (1986), that there was no difference in fertilisation rates between various of times of insemination with fresh semen. In contrast, Paton *et al.* (1993) found that fertility to cervical insemination was associated with the interval from CIDR removal and time of AI. Similarly, Maxwell (1986a) demonstrated that the interval between oestrous detection and intrauterine insemination had a significant influence on the number of ewes lambing and lambs born in PMSG-treated Merino ewes; the optimal interval to produce the highest fertility was 72 h after PMSG withdrawal. In the present trial, the ewes were naturally in oestrus and were inseminated anytime during

the period 1.5-13.5 h after oestrus was noted. It is likely that these sheep would have been in oestrus for a period of several hour longer, so that many inseminations probably in the mid to late oestrous period. These inseminations would have occurred quite close to the time for ovulation and thus satisfactory conception rates should be expected.

5.7. Fertility relative to technicians and AI technique.

Effect of the technicians, which performed intrauterine insemination in the current study, were not different, which is similar to Smith *et al.*(1995a). In contrast, published papers (e.g. Shackell *et al.*, 1990), which claims that technician had a marked effect on fertility following AI. No clear explanation can be offered to reconcile this difference. Difference in location, non-return rates and AI systems ('fixed' or 'oestrous-based' inseminations) may contribute to the difference, and therefore the interpretation of the difference is more difficult. However, in terms of conception rate, the results of the current study were higher than those reported by Shackell *et al.* (76% - 80% vs 51% - 76%).

The effect of technician, which performed transcervical insemination, has not been made possible since only one technician executed this method.

CHAPTER 6: CONCLUSION

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1) CIDR-treated ewes showed a more concentrated distribution of oestrus immediately after CIDR withdrawal than at subsequent oestrus.

2) From slaughtered animals it was determined that the modified needle used for transcervical insemination more frequently was located in the genital tract. With both types of the needles difference was found in penetration through the cervix and insemination into the uterus.

3. Cervical damage occurred after transcervical insemination. Variable damage often occurred as the inseminating needle was located in the genital tract. However, the damage which occurred did not always exert a substantially depressing effect on fertility.

4) The AI (intrauterine, cervical and transcervical) techniques did not significantly influence conception rate and litter size. However, a significant difference was found for lambing rate with transcervical insemination giving the highest percentage. This result was surprising and there was no ready explanation for this.

5) The interval between time of oestrous detection and AI was not critical to increase conception, lambing rates or litter size when AI with freshly diluted semen was performed within a mean of 6.1 ± 0.26 h relative to detection.

6) The age of the ewe was not associated with differences in conception and litter size, but was with lambing rate.

7) In respect of intrauterine insemination, technician differences were not apparent in conception or lambing rates and litter size.

8) For transcervical insemination, depth of penetration of the inseminating needle had an effect on conception and lambing rate but there was no significant effect on litter size. Depth of penetration was similar when inseminations were performed in the morning or afternoon.

9) Transcervical insemination needs further improvement to be an alternative to laparoscopic intrauterine insemination especially as in maiden ewes it is difficult to penetrate deep into the cervix.

10) Further work is required to evaluate whether transcervical insemination with frozen-thawed semen can be expected to consistently produce similar results to other AI techniques using frozen semen and give results approaching those noted in this work using freshly collected semen.

APPENDIX

APPENDIX

ALCIAN BLUE/PERIOD ACID SCHIFF (AB/PAS)*

METHOD:

1. Dewax and bring the water.
2. Stain in 0.3% Alcian blue in 3% acetic acid for 15 minutes.
3. Wash in water.
4. Oxidise in 1% period acid for 10 minutes.
5. wash in running tapwater for 5 minutes.
6. Rinse in three changes of distilled water.
7. Place slides on staining rods and apply Schiff's reagent for 15 minutes.
8. Return slides to staining rack and wash in running tapwater for 10 minutes.
9. Lightly counter stain nuclei in Mayer's Haemalum for 5 minutes.
10. Wash in tapwater.
11. Blue in Scott's tapwater.
12. Rinse in tapwater.
13. Dehydrate, clear and mount.

RESULTS:

Neutral glycoprotein	pink to red.
Acidic glycoproteins	blue.
Nuclei	blue/black.

HAEMOTOXYLIN & EOSIN (H & E)***METHOD:**

1. Dewax to water
 - a) immerse in 2 charges of Xylene, 5 minutes.
 - b) rinse in absolute ethanol (until slides clear).
 - c) rinse in 70% ethanol (until slides clear).
 - d) rinse in tapwater.
2. Stain in Mayer's Haemalum for 10 minutes.
3. Rinse in tapwater.
4. Blue in Scott's tapwater.
5. Rinse in tapwater.
6. Stain in 1 per cent aqueous eosin for 2 minutes.
7. Rinse in tapwater.
8. Differentiate and dehydrate in 70% ethanol and two changes of absolute ethanol.

9. Clear in two changes of Xylene.
10. Coverslip and mount with DPX.

RESULTS:

Nuclei	Blue/black.
Collagen	Pale pink.
Muscle	Bright red.
Erythrocytes	Bright scarlet.

HAEMOTOXYLIN/VAN GIESON (H.vG)***METHOD:**

1. Dewax and bring to water.
2. Stain in Celestin Blue for 10 minutes.
3. Was in tapwater.
4. Stain in Mayer's Haemalum for 10 minutes.
5. Wash in tapwater.
6. Blue in Scott's tapwater for 2 minutes.
7. Rinse in tapwater.
8. Stain in Van Gieson for 7 minutes.
9. Rinse rapidly in tapwater.
10. Dehydrate rapidly, clear and mount.

RESULTS:

Nuclei	Blue/black.
Muscle	Yellow.
Collagen	Pink or red.
Erythrocytes	Bright yellow.
Other tissue components . . .	green/brown, khaki.

*) adapted from Culling *et al.*(1985)

REFERENCES

REFERENCES

- Aamdal, J.**(1974) Insemination with deep frozen ram semen. *Proceedings of the 12th Nord. Veterinary Congress, Reykjavic (Abstr.) DII vol.2*, pp.145-146.
- Allison, A.J.**(1968) The influence of live weight on ovulation rate in the ewe. *Proceedings of the New Zealand Society of Animal Production* 28: 115-128.
- Allison, A.J.**(1975) Effect of nutritionally induced live-weight differences on the ovarian response and fertility in ewes treated with pregnant mare's serum gonadotrophin. *New Zealand Journal of Agricultural Research* 18: 101-107.
- Allison, A.J. & Robinson, T.J.**(1970) The effect of dose level of intravaginal progestagen on sperm transport, fertilization and lambing in the cyclic Merino. *Journal of Reproduction and Fertility* 22: 515-531.
- Allison, A.J. & Robinson, T.J.**(1971) Fertility of progestagen-treated ewes in relation to the numbers and concentration of spermatozoa in the inseminate. *Australian Journal of Biological Sciences* 24: 1001-1008.
- Anderson, V., Aamdal, J. & Fougner, J.**(1973) Intrauterine and deep cervical insemination with frozen semen in sheep. *Zuchthygiene* 8: 113-118.
- Averill, R.L.W.**(1959) Ovulatory activity in mature Romney ewes in New Zealand. *New Zealand Journal of Agricultural Research* 2: 575-583.
- Baird, D.T. & McNeilly, A.S.**(1981) Gonadotrophic control of follicular development and function during the oestrous cycle. *Journal of Reproduction and Fertility (Suppl.)* 30: 119-133.
- Barlow, M., Pryce-Jones, D. & Reed, H.C.B.** (1974) MLC sheep artificial insemination fields: A comparison of milk and egg-yolk diluents. *Veterinary Record* 94: 159-160 (Abstr.).
- Beck, N.F.G., Davis, M.C.G., Davis, B. & Lees, J.L.**(1987) Oestrus synchronization and fertility in ewes: A comparison of three methods. *Animal Production* 44: 251-254.
- Bedford, J.M.**(1972) Sperm transport, capacitation and fertilization. In: *Reproductive Biology* [eds. H. Balin & S. Glasser], Excerpta Medica, Amsterdam, pp.338.
- Blackshaw, A.W.**(1960b) The effect of milk diluents on the viability of ram spermatozoa and their revival after freezing. *Australian Veterinary Journal* 36: 432.

- Boschoff, D.A., van Niekerk, C.M. & Morgenthal, J.C.**(1973) Time of ovulation in the Karakul ewe following synchronisation of oestrus. *South African Journal of Animal Science* 3: 13-17.
- Buckrell, B.C., Buschbeck, C., Gartley, C.J., Kroetsch, T., McCutcheon, W., Martin, J., Penner, W.K. & Walton, J.S.**(1994) Further development of a transcervical technique for artificial insemination in sheep using previously frozen semen. *Theriogenology* 42: 601-611.
- Buckrell, B.C., Buschbeck, C., Gartley, C.J., Kroetsch, T., McCutcheon, W., Martin, J., Penner, W.K. & Walton, J.S.**(1992) A breeding trial using a transcervical technique for artificial insemination in sheep. *Proceedings of the 12th International Congress of Animal Reproduction, The Hague, 3*: 1531-1533.
- Bunch, T.D. & Ellsworth, H.S.**(1981) Gross anatomy of the ovine cervix. *International Goat and Sheep Research* 4: 282-285.
- Cahill, L.P., Saumande, J., Ravault, J.P., Blanc, M., Thimonier, J., Mariana, J. & Mauleon, P.**(1981) Hormonal and follicular relationships in ewes of high and low ovulation rates. *Journal of Reproduction and Fertility* 62: 141-150.
- Clark, J.N., Tervit, H.R., Welch, R.A.S. & Harvey, T.G.** (1984) Artificial insemination in the sheep industry. *Proceedings of the Ruakura Farmers' Conference*, pp 54-58.
- Carlson, K.M., Pohl, H.A., Marcek, J.M., Muser, R.K. & Wheaton, J.E.**(1989) Evaluation of progesterone controlled internal drug release dispensers for synchronisation of estrus in sheep. *Animal Reproduction Science* 18: 205 (Abstr.).
- Cognie, Y. & Mauleon, P.**(1983) Control of reproduction in the ewe. In: *Sheep Production* [ed: W. Haresign], Butterworths, London, pp.381-392.
- Colas, G.**(1983) Factors affecting the quality of ram semen. In: *Sheep Production* [ed. W. Haresign], Butterworths, pp.453-465.
- Colas, G., Thimonier, J., Courot, M. & Ortovant, P.**(1973) Fertility, prolificacy and fecundity during the breeding season of ewes artificially inseminated after treatment with fluorogestone acetate. *Annale de Zootechnie*, 22(4): 441-41.
- Colas, G., Tryer, M., Guerin, Y. & Aguer, D.**(1982) 9th International Congress of Animal Reproduction and Artificial Insemination 5 : 161.

- Culling, C.F.A., Allison, R.T. & Barr, W.T.**(1985) Cellular pathology technique. Butterworths, London, Boston.
- Cumming, I.A., Buckmaster, J.M., Blockey, M. A. de B., Goding, J.R. & Baxter, R.W.** (1973) Constancy of interval between luteinizing hormone release and ovulation in the ewe. *Biology Reproduction* 9: 24.
- Cumming, I.A. & McDonald, M.F.** (1966) A note on oestrous response to progesterone treatment in New Zealand Romney ewes. *The New Zealand Journal of Agricultural Research* 10: 316-318.
- Dalton, D.C. & Rae, A.L.** (1978) The New Zealand Romney Sheep: A Review of Reproductive Performance. *Animal Breeding Abstracts* 46: 60, Commonwealth Bureau of Animal Breeding and Genetics, U.K.
- Davis, I.F., Kerton, D.J., McPhee, S.R., White, M.B., Banfield, J.C., & Cahill, L.P.**(1984) In: *Reproduction in Sheep* [eds: D.R. Lindsay & D.T. Pearce], Cambridge University Press, pp.304-305.
- Dickerson, G.E.**(1976) Efficiency of animal production - Molding the biological components. *Journal of Animal Science* 30: 849-859
- Doney, J.M. & Gunn, R.G.**(1981). Nutritional and other factors in breeding performance of ewes. In: *Environmental Factors in Mammalian Reproduction* [eds: D.P. Gilmore & B. Cook], McMillan, London, pp.169-177
- Dunn, R.B.**(1955) The cervix of the ewe - its importance in artificial insemination of sheep. *Australian Veterinary Journal* 31 : 101-103.
- Dziuk, P.J., Lewis, J.M., Graham, E.F. & Moyer, R.H.**(1972) Comparison between natural service and artificial insemination with fresh or frozen semen at an appointed time in the ewe. *Journal of Animal Science* 35: 572-575
- Eastwood, B.K. & McDonald, M.F.**(1974) Maintenance of oestrus synchronisation in two-year-old ewes at second heat after progestagen sponge treatment. *New Zealand Journal of Agricultural Research* 18: 119-121.
- Edey, T.N.** (1969) Embryo mortality. In: *Sheep Breeding* [eds. G.J. Tome, D.E. Robertson & R.J. Lightfoot], Butterworths, pp. 169-177.
- Eppleston, J. & Maxwell, W.M.C.**(1991) Between ram variation in the reproductive performance of ewes inseminated with frozen semen. *Proceedings of the Australian Society of Animal Reproductive Biology* 23: 130 (Abstr.).
- Eppleston, J.**(1992) Studies on the fertility of frozen-thawed ram semen. Ph.D thesis, The University of New South Wales, in Maxwell & Salamon (1995).

- Eppleston, J. & Maxwell, W.M.C.**(1995) sources of variation in the reproductive performance of ewes inseminated with frozen-thawed ram semen by laparoscopy. *Theriogenology* 43: 777-788.
- Eppleston, J., Maxwell, W.M.C., Battye, K.M. & Roberts, E.M.**(1986) Effect of thawed motility and intra-uterine dose of motile sperm on fertility in ewes. *Proceedings of the 18th Annual Conference of the Australian Society of Reproductive Biology*, p.19.
- Eppleston, J., Salamon, S., Moore, N.W. & Evans. G.**(1994) The depth of cervical insemination and site of intrauterine insemination and their relationship to the fertility of frozen-thawed semen. *Animal Reproduction Science* 36: 211-225
- Evans, G.**(1988) Current topics in artificial insemination of sheep. *Australian Journal of Biological Science* 41: 103 (Abstr.).
- Evans, G., Holland, M.K., Nottle, H.B., Sharpe, P.H. & Amstrong, D.T.**(1984) Production of embryos in sheep using FSH preparations and laparoscopic intra-uterine insemination. In: *Reproduction in Sheep* [eds. D.R. Lindsay & D.T. Pearce], Cambridge University Press, pp.313-315.
- Evans, G., Jabbour H.N. & Moore, N.W.**(1986) Time of intrauterine insemination of superovulated ewes using fresh and frozen semen. *Australian Society of Reproductive Biology. Proceedings of the 18th Annual Conference*, Brisbane, Australia, p.18.
- Evans, G. & Maxwell, W.M.C.**(1987) Salamon's artificial insemination of sheep and goats. Butterworths, Sydney, 194 pp.
- Evans, G. & Robinson, T.J.**(1980) The control of fertility in sheep: endocrine and ovarian responses to prostagen-PMSG treatment in the breeding season and in anoestrus. *Journal of Agricultural Science, Cambridge*, 94: 69-88.
- Fairney, I.J. & Wales, R.G.**(1982) Using genetically superior rams efficiently by artificial insemination. *Proceedings of the World Congress on Sheep and Beef Cattle Breeding*, vol.1 [eds. R. A. Barton & W.C. Smith]. Palmerston North, New Zealand, pp.311-320.
- Findlater, R.C.F., Haresign, W., Curnock, R.M. & Beck, N.G.F.**(1988) Effect of timing of intrauterine insemination with frozen-thawed semen on fertility in ewes. *Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination*, Dublin, Ireland, vol.3, paper 242.
- Foote, R.H.**(1974) Artificial insemination. In: *Reproduction in Farm Animals* [ed. E.S.E. Hafez], Lea and Febiger, Philadelphia, pp. 212-246.

- Fukui, Y.**(1976) Studies in controlled breeding of sheep. Ph.D. Thesis. The University of New South Wales, 476 pp.
- Fukui, Y. & Roberts, E.M.**(1976) Studies on non-surgical intra-uterine insemination of pelleted semen in ewes. *Proceedings of the 18th International Congress of Animal Reproduction and Artificial Insemination 4*: 991-993, Krakow, Polish Academy of Science.
- Fukui, Y. & Roberts, E.M.** (1978) Further studies on non-surgical intrauterine technique for artificial insemination in the ewe. *Theriogenology 10*: 381-393.
- Fukui, Y. & Roberts, E.M.**(1979) Fertility of non-surgical intrauterine insemination with frozen-pelleted semen in ewes treated with prostaglandins $F_{2\alpha}$. In: *Sheep Breeding* [eds: G.J. Tomes, D.E. Robertson, R.J. Lightfoot]. Revised by W. Haresign. Butterworths, London. pp.533-545.
- Goot, H.**(1952) Studies on some New Zealand Romney Marsh stud flocks. Part VII. Lambing percentage. *New Zealand Journal of Science and Technology 34*: 106-116.
- Gordon, I.**(1969) Factors affecting response of anoestrous sheep to progestagen treatment. *Journal of the Department of Agriculture and Fisheries, Republic of Ireland 66*: 232-267.
- Gordon, I., Caffrey, W. & Morrin, P.**(1969) Induction in early breeding in sheep following with progestagen-impregnated pessaries and PMSG. *Journal of the Department of Agriculture and Fisheries, Republic of Ireland 66*: 232-267.
- Graham, E.F.**(1966) The use of dye indicator for testing, training, and evaluating technicians in artificial insemination. In: Kalay, D.(1980) Evaluation of artificial insemination programs. *The Ninth International Congress of Animal Reproduction and Artificial Insemination*, Madrid, pp.147-157.
- Graham, E.F., Crabo, B.G. & Pace, M.M.**(1978) Current status of semen preservation in the ram, boar and stallion. *Journal of Animal Science 47 (Suppl.2)*: 80-120.
- Halbert, G.W., Dobson, H., Walton, J.S. & Buckrell, B.C.**(1990a) The structure of the cervical canal of the ewe. *Theriogenology 33*: 977-992.
- Halbert, G.W., Dobson, H., Walton, J.S. & Buckrell, B.C.**(1990b) A technique for transcervical intrauterine insemination of ewes. *Theriogenology 33*: 993-1010.
- Halbert, G.W., Dobson, H., Walton, J.S. & Buckrell, B.C.**(1990c) Field evaluation of a technique for transcervical intrauterine insemination of ewes. *Theriogenology 33*: 1231-1244.

- Hamra, A.H., Massin, Y.G., Marcek, J.M. & Wheaton, J.E.**(1986) Plasma progesterone levels in ewes treated with progesterone controlled internal drug release dispensers, implants and sponges. *Animal Reproduction Science 11*: 187-194.
- Haresign, W.**(1985) The physiological basis for variation in ovulation rate and litter size in sheep: A review. *Livestock Production Science 13*: 3-20.
- Haresign, W., McLeod, B.J.& Webster, J.M.**(1983) Endocrine control of reproduction in the ewe. In: *Sheep Production* [ed. W. Haresign], Butterworths, London, pp. 353-379.
- Haresign, W.**(1978) Ovulation control in the sheep. In: *Control of ovulation* [eds: D.B. Crighton, N.B. Haynes, G.R. Foxcroft & G.E. Lamming], Butterworths, London, pp.353-379.
- Haresign, W., Curnock, R.M. & Reed, H.C.B.**(1986) A note on the use of laparoscopy for intrauterine insemination of frozen-thawed semen in the ewe. *Animal Production 43*: 553-556.
- Haresign, W.**(1990) Controlling reproduction in sheep. *Occasional Publication no.14 British Society of Animal Production* [eds. C.F.R.Slade & T.L.J. Lawrence], p.23 (Abstr.).
- Harvey, T.G., Baker, R.L. & Johnson, D.L.**(1987) Factors affecting an efficient sheep artificial insemination programme. *The 4th AAA Animal Science Congress*.
- Harvey, T.G. & McDonald, M.F.**(1995) Development and evaluation of transcervical artificial insemination techniques in sheep. A report for Massey Agricultural Research Foundation, 6 pp.
- Harvey, T.G., Johnson, D.L., Baker, R.L., Trust, B.K. & Thomson, B.C.**(1986) Artificial insemination in sheep - Comparison of storage time, dose rate and insemination technique. *Proceedings of the New Zealand Society of Animal Production 46*: 229-232.
- Harvey, T.G., Johnson, D.L., Tervit, H.R. & Welch, R.A.S.** (1984) Synchronisation and artificial insemination of ewes - comparing techniques which have been possible commercial application. *Proceedings of the New Zealand Society of Animal Production 44*.
- Hawk, H.W.** (1973) Estrus, uterine motility and sperm transport after prostaglandins. *Journal of Animal Science 37*: 314.

- Hawk, A.W.**(1983) Sperm survival and transport in the female reproductive tract. *Journal of Dairy Science* 66: 2645-2660.
- Hawk, H.W. & Conley, H.H.** (1972) Investigation of sperm transport failures in ewes administered synthetic progestagen. *Journal of Animal Science* 34: 609-613.
- Henderson, D.C., Downing, J.M., Beck, N.F.G. & Lees, J.L.**(1984) Oestrus synchronisation in ewes: A comparison of prostaglandins F_{2α} than salt with a progestagen pessary. *Animal Production* 39: 229-233.
- Hight, G. K. & Jury, K.E.**(1976) Hill country sheep production. VII. Relationship of hogget and two-year old oestrus and ovulation rate to subsequent fertility in Romney and Border Leicester x Romney ewes. *New Zealand Journal of Agricultural Research* 19: 281-288.
- Hunter, R.H.F.**(1982) Reproduction in farm animals. Longman, London.
- Jheltoburch, N.A.**(1979) AI of sheep in the Soviet Union. In: *Sheep Breeding* [eds. W. Haresign], Butterworths, London, pp.
- Johnson, P.E., Flipse, R.J. & Almquist, J.O.** (1955) Effect of diluents on the fertility of sperm in dairy cattle. *Journal of Dairy Science* 38: 53 (Abstr.).
- Kalay, D.**(1980) Evaluation of artificial insemination programs. *The Ninth International Congress of Animal Reproduction and Artificial Insemination*, Madrid, pp.147-157.
- Kerton, D.J., McPhee, S.R., Davis, I.F., White, M.B., Banfield, J.C. & Cahill, L.P.**(1984) A comparison of insemination techniques in Corriedale ewes. *Proceedings of Austalian Society of Animal Production* 15: 701.
- Killeen, J.D. & Cafferey, G.J.** (1982) Uterine insemination of ewes with the aid of laparoscopy. *Australian Veterinary Journal* 59 : 95
- Krznowska, H.**(1974) The passage of abnormal spermatozoa through the uterotubal junction of the mouse. *Journal of Reproduction and Fertility* 38: 81-90.
- Lehrer, A.R., Schindler, H., Brown, M.B. & Fischler, H.**(1978) The effect of mating, artificial insemination, and fright on uterine motility of the oestrous ewe. *Animal Reproduction Science* 1: 297.
- Lightfoot, R.J. & Salamon, S.**(1970a) Fertility of ram spermatozoa frozen by the pellet method. 1. Transport and viability of spermatozoa within the genital tract of the ewe. *Journal of Reproduction and Fertility* 22: 385-398.

- Lightfoot, R.J. & Salamon, S.**(1970b) Fertility of ram spermatozoa frozen by the pellet method. II. The effect of method of insemination on fertilization and embryonic mortality. *Journal of Reproduction and Fertility* 22:399-408.
- Mattner, P.E.**(1963) Spermatozoa in the genital tract of the ewe. III. The role of spermatozoan motility and of uterine contractions in transport of spermatozoa. *Australian Journal of Biological Sciences* 16: 877 (Abstr.).
- Mattner, P.E. & Braden, A.W.H.**(1963) Spermatozoa in the genital tract of the ewe. I. Rapidity of transport. *Australian Journal of Biological Sciences* 16: 473 (Abstr.).
- Mattner, P.E., Entwistle, K.W. & Martin, I.C.A.**(1969) Passage, survival and fertility of deep-frozen ram semen in the genital tract of the ewe. *Australian Journal of Biological Sciences* 22: 181 (Abstr.).
- Maxwell, W.M.C.**(1980) Fertility of ram semen frozen in autumn and spring. *Proceedings of Australian Society of Reproductive Biology*. Abstr, no.8. Annual Conference, Armidale, N.S.W.
- Maxwell, W.M.C.**(1984) Current problems and future potential of artificial insemination programmes. In: *Reproduction in Sheep* [eds.D.R. Lindsay & D.T. Pearce], Australian Academy of Science and Australian Wool Corporation, Canberra, pp.291-298.
- Maxwell, W.M.C.**(1986a) Artificial insemination of ewes with frozen-thawed semen at a synchronised oestrus. 1. Effect of time of onset of oestrus, ovulation and insemination on fertility. *Animal Reproduction Science* 10: 301-308.
- Maxwell, W.M.C.**(1986b). Artificial insemination of ewes with frozen-thawed semen at a synchronised oestrus.2. Effect of dose of spermatozoa and site of insemination on fertility. *Animal Reproduction Science* 38: 309-316.
- Maxwell, W.M.C. & Barnes, D.R.**(1986) Induction of oestrus in ewes using a controlled internal drug release device and PMSG. *Journal of Agricultural Science, Cambridge* 106: 201-203.
- Maxwell, W.M.C. & Evans, G.**(1987) Salamon's artificial insemination of sheep and goats. Butterworth, Sydney, 194 pp.
- Maxwell, W.M.C. & Garnett, N.L.**(1991) The effect of artificial insemination in the Merino stud industry. *Proceedings of the Fourth Conference, Australian Association of Animal Breeding and Genetics*.pp.106-112.

- Maxwell, W.M.C., Hunton, J.R., Ryan, J.P. & Hood, G.**(1986) Effect of GnRH on time of ovulation in superovulated of Merino ewes. *Proceedings of the 18th Annual Conference of Australian Society of Reproduction Biology*, p.20.
- Maxwell, W.M.C. & Hewitt, L.J.**(1986). A comparison of vaginal, cervical and intrauterine insemination of sheep. *Journal of Agricultural Science Cambridge* 106: 191-193.
- Maxwell, W.M.C. & Salamon, S.** (1995a) Frozen storage of ram semen. II. Causes of low fertility after cervical insemination and methods of improvement. *Animal Reproduction Science* 38: 1-36.
- Maxwell, W.A.C. & Salamon, S.**(1995b) Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination: Review. *Animal Reproduction Science* 37: 185-249.
- McDonald, M.F.**(1958) A progress report on ovulation studies in the Romney ewes. *Sheepfarming Annual*, pp.193-200.
- McKelvey, W.A.C., Robinson, J.J. & Aitken, R.P.**(1985) The evaluation of laparoscopic insemination technique in ewes. *Theriogenology* 24: 519-535.
- McMillan, W.H.**(1986) Hogget oestrus synchronization: A comparison of CIDR and sponges. *Proceedings of New Zealand Society of Animal Production* 46: 225-228.
- McMillan, W.H. & Hall, D.R.H.**(1991) Superovulation in ewes: are follicle numbers a useful predictor of ovulation rate? *Proceedings of the New Zealand Society of Animal Production* 51: 133-137.
- McMillan, W.H. & McDonald, M.F.**(1983) Reproduction in ewe lambs and its effect on two-year-old performance. *New Zealand Journal of Agricultural Research* 26: 437-442.
- McMillan, W.H. & McDonald, M.F.**(1985). Survival of fertilized ova from ewe lambs and adult ewes in the uterine of ewe lambs. *Animal Reproduction Science* 8: 235-240.
- McGuirk, B.J.**(1976) Relevance to producers of research into genetic improvement of reproductive performance. *Proceedings of the Australian Society of Animal Production* 11: 93-100.
- McNatty, K.P., Revfeim, K.J.A. & Young, A.**(1973) Peripheral plasma progesterone concentrations in sheep during the oestrous cycle. *Journal of Endocrinology* 58:219-225.

- Mitchell, J.R., Senger, P.L. & Rosenberger, J.L.**(1985) Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. *Journal of Animal Science* 61: 956-963.
- More, J.**(1984) Anatomy and histology of the cervix uteri of the ewe: new insights. *Acta Anatomie* 120: 156-159.
- Moore, N.W.**(1988) The ovariectomized ewe: Its contribution to controlled breeding. *Australian Journal of Biological Sciences* 41: 15-22.
- McKelvey, W.A.C.** (1994). Recent research on A.I. and MOET in sheep and their commercial application in the UK. *Proceedings of the New Zealand Embryo Transfer Workshop*, pp. 16-17
- Murray, J.M., Boland, M.P., Moran, C., Sutton, R., Nancarrow, C.D., Scaramuzzi, R.J. & Hoskinson, R.M.**(1985). Occurrence of haploid and haploid-diploid mosaic embryos in untreated and androstenedione immune Australian Merino sheep. *Journal Reproduction and Fertility*
- Nephew, K.P., Clay, J.C., Thayer, S.L., Baertsche, S.R. Parker, C.F. & Pope, W.F.**(1990) Intrauterine insemination of Merino ewes with frozen semen from Australian Merino rams. *Sheep Research Journal* 6: 5-7.
- Pant, H.C., Hopkinson, C.R.N. & Fitzpatrick, R.J.**(1977) Concentration of oestradiol, progesterone, luteinizing hormone, and follicle stimulating hormone in the jugular venous plasma of ewes during the oestrous cycle. *Journal of Endocrinology* 73: 247-255.
- Paton, A.J., Harvey, T.G. & McCutcheon, S.N.**(1993) Effects of CIDR withdrawal - insemination interval on conception rate in sheep AI. *Proceedings of the New Zealand Society of Animal Production* 53: 291-293.
- Piper, L.R. & Bindon, B.M.**(1979) Selection for increased cow fecundity: A review. *Proceedings of the New Zealand Society of Animal Production* 39: 224-232.
- Quinlivan, T.D., Martin, C.A., Taylor, W.B. & Cairney, I.M.**(1966) Estimates of pre- and prenatal mortality in New Zealand Romney Marsh ewe. II. Pre- and prenatal loss in those ewes that conceived to second service and those that returned to second service and were mated a third time. *Journal of Reproduction and Fertility* 11: 391-398.
- Quinlivan, T.D. & Robinson, T.J.**(1967) The number of spermatozoa in the Fallopian tubes of ewes at intervals after artificial insemination following withdrawal of SC-9880 impregnated intravaginal sponges. In: *The Control of the Ovarian Cycle in the Sheep* [ed. T.J. Robinson], Sydney University Press, Sydney. pp. 177-194.

- Quinlivan, T.D. & Robinson, T.J.**(1969) Numbers of spermatozoa in the genital tract after artificial insemination of progestagen-treated ewes. *Journal of Reproduction and Fertility* 19: 235-239.
- Quirke, J.F., Hanrahan, J.P. & Gosling, J.P.**(1979) Plasma progesterone levels throughout the oestrous cycle and release of LH at oestrus in sheep with different ovulation rates. *Journal of Reproduction and Fertility* 55: 37-44
- Quirke, J.F., Hanrahan, J.P. & Gosling, J.P.**(1981) Duration of oestrus, ovulation rate, time of ovulation and plasma LH, total oestrogen and progesterone in Galway adult ewes and ewe lambs. *Journal of Reproduction and Fertility* 61: 265-272.
- Rae, A.L.**(1980) Selection for reproductive performance and hereditary aspects of sheep reproduction. In: *Current Therapy in Theriogenology* [ed. D. Morrow], W.B. Saunders, p.841-843.
- Rhodes, L. & Nathanielz, P.W.**(1988) Comparison of a controlled interval drug release device containing progesterone with intravaginal medroxy progesterone sponges for estrus synchronisation in ewes. *Theriogenology* 30: 831-836.
- Ritar, A.J., Ball, P.D. & O'Mary, P.J.**(1990) Artificial insemination of Cashmere goats; Effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa, and age of females. *Journal of Reproduction and Fertility and Development* 2: 377-384.
- Rival, M.D., Chenoweth, P.J. & McMicking, L.I.**(1984) Semen deposition and fertility in ovine artificial breeding programmes. In: *Reproduction in Sheep* [ed. D.R. Lindsay & D.T. Pearce]. Cambridge university, pp. 301-303.
- Robertson, H.A.**(1977) Reproduction in the ewe and goat. In: *Reproduction in Domestic Animals* [eds. H.H. Cole & P.T. Cupps], Academic Press, New York, pp. 475-498.
- Robinson, T.J.** (1973) Factors involved in the failure of sperm transport and survival in the female reproductive tract. *Journal of Reproduction and Fertility (Supplement)* 18: 103-109.
- Robinson, J.J., Wallace, J.M. & Aitken, R.P.**(1989) Fertilisation and ovum recovery rates in superovulated ewes following cervical insemination or laparoscopic intrauterine at different times after progestagen withdrawal and in or both uterine horns. *Journal of Reproduction and Fertility* 87: 771-782.

- Robinson, T.J., Scaramuzzi, R.J. & Smith, C.A.** (1987) The time of mating and of LH release and subsequent fertility of anoestrus Border Leicester x Merino ewes treated with progestagen and pregnant mare serum gonadotrophin. *Animal Reproduction Science* 13: 23-26.
- Robinson, T.J., Robinson, T.J., Moore, N.W., Lindsay, D.R., Fletcher, I.C. & Salamon, S.** (1970) Fertility following synchronisation of oestrus in the sheep with intravaginal sponges 1. Effect of vaginal douche, supplementary steroids, time of insemination, and numbers and dilution of spermatozoa (Abstr.0. *Australian Journal of Agricultural Research* 21 : 767.
- Ryan, J.P., Hunton, J.R. & Maxwell, W.M.C.** (1992) Time of ovulation in Merino ewes superovulated with PMSG and FSH-P. *Journal of Reproduction and Fertility* 4:91-97.
- Salamon, S.** (1971) Fertility of ram spermatozoa following pellet freezing on dry ice at -79°C and -140°C . *Australian Journal of Biological Sciences* 24: 183-185.
- Salamon, S. & Lightfoot, R.J.** (1967) Fertilization and embryonic loss in sheep after insemination with deep frozen semen. *Nature* 216: 194-195, London.
- Salamon, S. & Lightfoot, R.J.** (1970) Fertility of ram spermatozoa frozen by the pellet method. III. The effect of insemination technique, oxytocin and relaxin on lambing. *Journal of Reproduction and Fertility* 22: 409-323.
- Salamon, S. & Maxwell, W.M.C.** (1995a) Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. *Animal Reproduction Science* 37: 185-249.
- Salamon, S. & Maxwell, W.M.C.** (1995b) Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Theriogenology* 33: 993-1010.
- Statistical Analysis System** (1985) SAS Institute, Inc., Cary NC, U.S.A.
- Scaramuzzi, R.J., Adams, N.R., Baird, D.T., Campbell, B.K., Downing, J.A., Findlay, J.K., Henderson, K.M., Martin, G.B., McNatty, K.P., McNeilly, A.S. & Tsonis, C.G.** (1993) A model for selection and the determination of ovulation rate in the ewe. *Journal of Reproduction, Fertility and Development* 5: 459-478.
- Shackell, G.H.** (1991) The timing of oestrus, LH surge and ovulation in ewes following synchronisation with MAP, FGA sponges or CIDR-S. *Proceedings of the New Zealand Society of Animal Production* 51: 73.

- Signoret, J.P. & Cognie, Y.**(1975) Determination of the moment of ovulation in the ewe and sow. Influence of environment and hormonal treatment. *Annales de Biologie Animalae, Biochimie, Biophysique* 15: 205-214.
- Smith, J.F.**(1982) Principles of reproduction. In: *Sheep Production*, vol.1 [eds. G.A. Wickham & M.F. McDonald]. New Zealand Institute of Agricultural Science and Richards Publisher, Wellington, pp.211-237.
- Smith, J.F.**(1995) Personal communication.
- Smith, J.F., McGowan, L.T., MacLean, K.S. & Farquhar, P.A.** (1987) The effect of steroid immunisation on the seasonal pattern of oestrus, ovulation and ovulation rate in Coopworth ewes. *Proceedings of the New Zealand Society of Animal Production* 47: 143-146.
- Smith, J.F., Aspinal, J., Oliver, J., Murray, G., Smith, J.K. & Upreti, G.C.**(1995b) Field scale evaluation of semen diluents for cervical AI in sheep: An attempt at technology transfer. *Proceedings of the New Zealand Society of Animal Production* 55: 231-235.
- Smith, J.F., Parr, J., Beaumont, S. Oliver, J.J. & Upreti, G.C.**(1995a) Comparison of cervical, transcervical and laparoscopic insemination of ewes with chilled stored and frozen semen. *Proceedings of the New Zealand Society of Animal Production* 55: 248-250
- Tervit, H.R., Gould, P.G., James, R.W. & Frazer, M.D.**(1984) The insemination of sheep with fresh or frozen semen. *Proceedings of the New Zealand Society of Animal Production* 44 : 11-13.
- Thimonier, J.**(1979) Hormonal control of oestrous cycle in the ewe (A review). *Livestock Production Science* 6: 11-39.
- Thornburn, G.D.; Basset, J.M. & Smith, I.D.**(1969) Progesterone concentrations in the peripheral plasma of sheep during the oestrous cycle. *Journal of Endocrinology* 45: 459-469.
- Upreti, G.C., Oliver, J.E., Dugansich, D.M., Munday, R. & Smith, J.F.**(1995) Development of a chemically defined rams semen diluent (RSD-1). *Animal Reproduction Science* 37: 143-157.
- van Vleck, L.D.**(1981) Potential genetic, impact of artificial insemination, sex selection, embryo transfer, cloning and sexing in dairy cattle. In: *New technologies in Animal Breeding* [eds. B.G. Brackett, G.E. Seidel & S.M. Seidel] Academic Press, N.Y. pp. 221-276.

- Visser, D. & Salamon, S.**(1973) Fertility of ram spermatozoa frozen in a Tris-based diluent. *Australian Journal of Biological Sciences* 16: 513-516.
- Walker, S.K., Smith, D.H., Ancell, P.H. & Seamark, R.F.**(1989) Time of ovulation in the South Australian Merino ewes following synchronisation of oestrus. 2. Efficacy of GnRH treatment and its relevance to insemination programs utilizing frozen-thawed semen. *Theriogenology* 31: 555-564.
- Walker, S.K., Smith, D.H. & Seamark, R.F.**(1986) Timing of multiple ovulations in the ewe after treatment with FSH or PMSG with and without GnRh. *Journal of Reproduction and Fertility* 77: 135-142.
- Wallace, J.M.**(1992) Artificial insemination and embryo transfer. In: *Progress in Sheep and Goat Research* [ed. A.W. Speedy] C.A.B. International, chap.1.
- Wallace, J.M. & McNeilly, A.S.**(1985) Increase in ovulation rate after treatment of ewes with bovine follicular fluid in the luteal phase of the oestrous cycle. *Journal of Reproduction and Fertility* 73: 505-515.
- Wallace, J.M., Robinson, J.J. & Aitken, R.P.**(1989) Successful pregnancies after transfer of embryos from ewes induced to ovulate 24-29 days *post partum*. *Journal of Reproduction and Fertility* 86: 627-635.
- Welch, R.A.S., Andrews, W.D., Barnes, D.R., Bremner, K. & Harvey, T.G.**(1984) CIDR dispensers for oestrus and ovulation control in sheep. *Proceedings of the 10th International Congress on Animal Production and Artificial Insemination*, The University of Illinois, Urbana-Champaign 3: 354.
- Wilmut, I., Ashworth, C.J. & Sales, D.I.**(1986) The influence of progesterone profile on embryo survival in ewes. In: *Embryonic Mortality in Farm Animals*. [eds. J.M. Sreenan & M.G. Diskin]. Martinus Nijhoff, Boston, pp.135-141.
- Wilmut, I. & Hunter, R.H.F.**(1984) Sperm transport into the oviducts of heifers mated early in estrus. *Reproduction and Nutrition Development* 24: 461-465.
- Wilmut, I. & Sales, D.I.**(1981) Effect of asynchronous environment on embryonic development in sheep. *Journal of Reproduction and Fertility* 61: 179-184.
- Wilmut, I., Sales, D.I. & Ashworth, C.J.** (1985) Physiological criteria for embryo mortality: Is asynchrony between embryo and ewe a significant factor? In: *Genetic Reproduction in Sheep* [eds. R.B. Land & D.W. Robinson]. Butterworths, London, pp. 275-289.

- Windsor, D.P.**(1993) Transcervical insemination of Merino ewes. *Australian Society of Reproductive Biology. Proceedings of the 25th Annual Conference*, The University of Otago, Dunedin. p.74.
- Windsor, D.P.**(1994) Artificial insemination of sheep. *Western Australia Journal of Agriculture* 33: 145-147.
- Windsor, D.P.**(1995) Factors influencing the success of transcervical insemination in Merion ewes. *Theriogenology* 43: 1009-1018.
- Windsor, D.P., Szell, A.S., Buschbeck, C., Edward, Y., Milton, J.T.B. & Buckrell, B.C.**(1994) Transcervical artificial insemination of Australian Merino ewes with frozen-thawed semen. *Theriogenology* 42: 147-157.
- Zaicev, V.V., Varnavskaya, V.A. & Varnavskij, A.**(1989) Diluents for freezing of ram semen. *Ovtsevodstvo* 6: 34-35 in Eppleston, J., Salamon, S., Moore, N.W. & Evans, G.(1994) The depth of cervical insemination and site of intrauterine insemination and their relationship to the fertility of frozen-thawed semen. *Animal Reproduction Science* 36 (1994): 211-225.