SPERM DISCHARGE AND RETENTION BY THE REPRODUCTIVE TRACT OF THE COW FOLLOWING ARTIFICIAL INSEMINATION

By

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CHAPTER I

INTRODUCTION

Fertility is a non-yield trait which has great economic importance in dairy herds (Jansen, 1985). Gerritts et al. (1979) came to the conclusion that if services per conception could be reduced from 2.0 to 1.5, it would equal 20 million dollars per year to U.S. dairymen. Reduction in fertility leads to more services per conception, prolonged calving intervals and enhanced culling. Fertilization failure has been reported to occur in up to 16% of apparently healthy females (Freeman, 1983). This number increases to 40% for cows that fail to conceive after several inseminations (Boyd et al., 1969).

Sperm transport depends upon a coordinated series of physiological events that appear to be susceptible to malfunction or to interference, or both by stress factors, physiological and anatomical changes. Therefore, it is no surprise that studies on this topic have proliferated since the late 19th century, not only because of a fundamental interest in the transport and migration of gametes but more recently as an approach towards a potential means of reducing infertility.

Wiley et al. (1982) questioned whether failure of sperm

transport could account for fertilization failure of superovulated cows since sperm numbers in the oviducts did not differ significantly between superovulated and nonsuperovulated cows. However, their data did reveal that cows with ampullary sperm had a higher fertilization rate (14 of 23 ova fertilized, 61%) compared to cows in which no sperm were found in the ampulla (3 of 12 ova fertilized, 25%). This suggested that less efficient sperm transport to the oviduct results in fertilization failure.

Sperm traverse the tract in two phases. The first is termed the rapid phase, in which sperm can be recovered from the oviducts within minutes after insemination. However, most of these sperm are dead which questions if they are actually involved with fertilization. Hafez (1973) theorized that these sperm may stimulate the release of products into the lumen of the oviduct to facilitate maturational changes in gametes or prepare the phagocytotic system for arrival of greater sperm numbers. The second phase of transport termed sustained or prolonged phase, involves a combination of factors which slowly release the spermatozoa from selective reservoirs within the tract for movement towards the site of fertilization. Factors involved in this stage of transport include smooth muscle contractions, sperm motility, cervical mucus characteristics, ciliary action, and fluid currents all in concert.

Structural obstacles are present within the

reproductive tract which allow sperm reservoirs to form limiting the number of spermatozoa reaching the site of fertilization. These sperm provide a source to maintain adequate oviductal numbers for fertilization. The cervix is a barrier consisting of a complex network of crypts and folds entrapping spermatozoa and delaying their forward passage. The caudal isthmus of the oviduct functions to slowly release sperm into the site of fertilization, restricting numbers of spermatozoa which pass. The uterotubal junction serves as a barrier in the pig and rat, as sperm have been found to pass in this area by virtue of their own motility (Hunter, 1980; Gaddum-Rosse, 1981). Sperm barriers have been found to be of importance in other species as sperm numbers in these areas are related to those in the oviduct. These data suggest that the degree of sperm retention in the reproductive tract influences fertilization rates.

Only a small portion of inseminated sperm is actually retained by the reproductive tract. Mitchell et al. (1985) indicated 60% of the inseminated sperm are lost to the exterior by 12 hours after insemination. This limits the number of sperm available for transport to the oviduct. At this point, it is unclear whether or not substantial sperm discharge occurs with conventional artificial insemination, as these investigators used 20 to 50 times the normal number of sperm used in artificial insemination. In addition, Mitchell et al. (1985) used sperm which were abnormal and

not cryopreserved. Potential improvements in fertilization rates have been found in other species by increasing the retention of spermatozoa the reproductive tract by decreasing retrograde sperm loss (Pursel, 1982; Hawk & Cooper, 1978; Hawk & Cooper, 1984).

Various methods have been employed by investigators to improve the retention of spermatozoa in the reproductive tract of the cow (Hawk, 1983). It has been suggested that site of semen deposition substantially increases fertilization rates when placed in the uterine horn as compared to the uterine body or cervix (Senger, 1987). In addition, clitoral stimulation in the beef cow (Short, 1979) and stimulation of the reproductive tract by natural means (Bedford, 1971) have been found to improve fertilization rate. Several drugs and hormones are effective (in other species) in increasing sperm transport to the oviduct when given at certain dose ranges (Hawk & Cooper, 1975; 1976; 1978). Prostaglandin E_1, prostaglandin $F_{2\alpha}$, oestradiol 17-β carbocholine, oxytocin, ergonovine, acetylcholine, GnRH, and purified FSH have been utilized for sperm retention which several of these may have potential value in the cow (Hawk, 1987).

Importance of improving sperm transport by artificial or natural means has been demonstrated in other species. Therefore, it can be inferred that a real potential exists for the improvement of fertilization by reducing the large retrograde discharge which occurs in cattle after

insemination. Perhaps, through the administration of drugs or hormones, fertilization failure can be reduced in cattle by improving sperm retention. However, it must first be determined whether or not substantial sperm loss occurs with typical doses of sperm used in artificial insemination of the bovine.

CHAPTER II

REVIEW OF LITERATURE

Significance of Sperm Transport Failures in Farm Animals

Fertilization failure can account for a significant reduction of reproductive efficiency of livestock (Hawk, 1983). In the dairy industry, fertilization failure is equivalent to embryonic mortality as a major cause of infertile services (Hawk, 1983) and accounts for about 15% of all infertile services (Diskin, 1980; Roche, 1981). Failure of fertilization occurs in 10% or more of the ewes bred (Blockey, 1975). Gilts and sows that have been mated naturally or artificially inseminated with fresh semen, average only 5% fertilization failure (Hawk, 1983). However, the percentage of gilts with no fertilized ova or with some unfertilized ova, increased substantially when frozen semen is used (Polge, 1978). In swine, high rates of fertilization failure is associated with a lack of sperm in the oviducts near the time of ovulation (Pursel et al., 1978). Hawk (1983) concluded that fertilization failure can result from several factors which include: 1) structural barriers to the union of sperm and ova 2) the inability of sperm to penetrate ova or the unfertilizability of the ova

and 3) failure of sperm transport in the female reproductive tract. Therefore, it can be theorized that the frequent absence of sperm in the oviducts around the time of ovulation and the associated reduction in fertilization rates are related.

Resultant reproductive wastage due to fertilization failure is a major source of economic loss in animal production. In cattle, this loss can be through extended calving seasons, lower production, and increased cost in artificial insemination due to increased services per conception (Roche, 1981). Gerritts et al. (1979) calculated a gain of 20 million dollars per year to U.S. dairymen if services per conception could be reduced from 2.0 to 1.5.

One possible method to help lessen the percentage of fertilization failure in cattle was suggested by King et al. (1965). These investigators developed a program for re-training artificial insemination technicians. Procedures used by technicians is an important factor in increasing reproductive efficiency of cattle after A.I. The technician's consistency in placing the semen in the uterine body was found to be 25% before retraining was initiated. By the conclusion of the retraining period the consistency of the technicians had improved to near 80%. These technicians obtained a repeatably high fertility rate when they returned to the field. Therefore, fertilization failure can be decreased to some degree through the employment of capable, well-trained technicians as well

as through the use of proper equipment and the application of the latest technical information and methods. Technician competence can be a major factor affecting fertilization rate after artificial insemination. However, even with the best possible artificial insemination methods, fertilization failure stills occurs in 13 to 15% of dairy cattle (Hawk, 1983).

Dynamics of Sperm Transport

Rapid Transport of Sperm to the Oviducts

Various mechanisms of sperm transport exist in female mammals. The actual transport of spermatozoa following mating is usually described as occurring in sequential phases. The initial phase of transport transfers spermatozoa from the site of insemination to the oviducts at much faster rates than can be attained by the propulsive activity of the sperm flagellum (Hafez, 1973). Transport of sperm to the oviducts within a few minutes has been termed the rapid phase of transport. The rapidity of sperm transport is thought to be primarily due to the increase in contractile activity of the myometrium and myosalphinx occurring during coitus. This activity may be due to the release of oxytocin from the female neurohypophysis by the stimulus of coitus (Hafez, 1973).

Several investigators recovered sperm from oviducts of farm animals within a few minutes after mating or insemi-ation. Vandemark & Moeller (1951) found sperm in oviducts

of cows a few minutes after natural mating or artificial insemination into the cervix. Mattner (1963) detected large numbers of sperm in oviducts of ewes killed 5 or 10 min after mating. First et al. (1968) recovered sperm from oviducts of sows 15 min or less after insemination.

Live sperm are not essential for rapid transport to the oviducts. Mattner & Braden (1963) recovered killed ram sperm from oviducts of ewes within a few minutes after insemination in the anterior vagina. First et al. (1968) recovered killed sperm from oviducts of sows 30 minutes after insemination into the uterus. The fact that killed sperm are cleared quickly from the reproductive tract may be relevant because motile sperm may adhere to the epithelium or enter folds of the cervix and uterus during rapid transport and thus, avoid rapid propulsion through the tract (Hawk, 1983). However, dead and dying sperm that are transported rapidly could release factors into the tubal lumen to help facilitate maturational changes in gametes, or stimulate the influx of leukocytes for phagocytosis of the greater numbers of motile spermatozoa (Overstreet & Cooper, 1978).

After natural mating of females, in which semen is deposited into the anterior vagina, a portion of the semen enters the cervix almost immediately and small numbers of sperm are apparently propelled rapidly to and through the oviducts by contractions of the reproductive tract. Most of the sperm that pass into and through the oviducts of rabbits

during the rapid transport phase are nonmotile, have damaged membranes and are probably dead (Overstreet & Cooper, 1978). It has not been ascertained whether or not sperm that appear in the oviducts of farm animals soon after mating are viable, nor is it clear why sperm recovered from oviducts of rabbits soon after mating are dead or damaged. "Either the sperm that move all the way to the oviducts are damaged during their rapid ascent, or only dead or damaged sperm are subject to rapid transport all the way through the reproductive tract" (Hawk, 1983).

Many investigators have found little or no evidence for a rapid phase of sperm transport in ewes. In numerous studies, few sperm were recovered from oviducts of ewes at 30 min or at 1 or 2 h after mating or artificial insemination, and no sperm were recovered from oviducts of a high proportion of the ewes (Hawk & Conley, 1975; Hawk et al., 1978; Hawk & Cooper, 1977; Hunter et al., 1980). In all of these studies, it is possible that sperm had been transported rapidly to the ampulla after mating or artificial insemination and moved into the peritoneal cavity by the time of necropsy. It is more likely that a rapid phase of sperm transport in sheep often may be omitted or suppressed. The inconsistent finding of a rapid phase of sperm transport in sheep simply may reflect variation of effectiveness of physiological mechanisms responsible for the rapid phase of transport. Stress on the ewe suppresses rapid transport of sperm to the oviducts. Mattner (1963)

found ewes conditioned to being handled had large numbers of sperm in the oviducts at 15 min after mating, whereas unconditioned ewes had relatively few sperm in the oviducts. When the conditioned ewes were disturbed deliberately, sperm were not in the oviducts at 15 min after mating. Thibault & Winterberger-Torres (1967) reported a similar effect of stress. In the work of Mattner (1963), stress did not reduce the number of sperm in the cervix at any time after mating (15 min, 4, 24 or 48 h) and did not reduce the number of sperm in the oviducts at 24 or 48 h.

In contrast to rapid transport of damaged and dead sperm transported to oviducts of rabbits, sperm capable of fertilizing ova can be transported to oviducts of gilts as early as 30 minutes after mating. Hunter (1981) ligated and transected oviducts of gilts above or below the uterotubal junction at 30, 45, and 60 min after mating. Nearly 50% of the ova were able to be fertilized when transection above the junction at occurred 30 minutes after mating. The percentage increased with transection below the junction at 30 min or delay in transection at either site until 60 min.

Sustained Phase of Sperm Transport

After ejaculation, spermatozoa gradually invade the complex cervical crypts, mucus in the cervical canal, endometrial glands, endometrial fluid, uterotubal junction, and oviductal fluid. The orientation of micelles of cervical mucin leads from their point of secretion, the

cervical crypts and the cervical epithelium (Hafez, 1973). These orientation lines tend to guide a majority of motile spermatozoa to the mucosa and into the cervical crypts. Motile spermatozoa distributed throughout the cervical mucin, the villi of the mucosa, and the cervical crypts persist there for long periods of undetermined duration. This period is known as the colonization of reservoirs. The cervical crypts and uterotubal junction constitute the major selective barriers for spermatozoa. After adequate reservoirs of spermatozoa are established within the reproductive tract, the spermatozoa are released gradually from the selective barriers to the site of fertilization. This slow release which involves the innate motility of spermatozoa and contractile activity of the myometrium and myosalphinx, continues for a prolonged period.

It is doubtful that the initial rapid phase of sperm transport in some animals is totally separate in time from the sustained phase. Initial stages of the prolonged phase of sperm transport probably begins within a few minutes or even seconds after insemination. Mattner (1963) found large numbers of sperm in the cervix of the ewe at 15 min after mating. It is likely these sperm were at least a portion of those that penetrated the cervical folds and crypts to establish a reservoir to support prolonged transport to the oviducts (Hawk, 1983). Schott & Phillips (1941) indicated that a high percentage of ewes had sperm in the anterior segment of the cervix or in the uterine horns at 15 to 40

min after mating. Similarly, Overstreet & Cooper (1978) reported more sperm in the upper ampulla, fimbriae, and on the ovaries of rabbits than in the lower segments of the oviduct at 1, 15 and 90 min after mating. Sperm reaching the oviducts within a few minutes, at least in sheep and rabbits, represent a small portion of the inseminate that was transported rapidly through the tract while the main pool of sperm remained in the vagina and cervix (Hawk, 1983).

In swine (Hunter, 1981), doe rabbits (Overstreet et al., 1978), and cattle and sheep (Hafez, 1973), sperm are retained in the caudal segment of the isthmus until about the time of ovulation when small numbers move to the site of fertilization. In gilts mated early in oestrus, sperm were held in the caudal isthmus for 36 h or more before they progressed toward the ampulla (Hunter, 1984).

Sperm are transported into the oviducts of pigs faster after mating close to the time of ovulation than after mating earlier in oestrus (Hunter, 1981). Also, sperm move into the oviducts most rapidly near ovulation in the hamster (Battalia & Yanagimachi, 1979), guinea-pig (Yanagimachi & Mahi, 1976), and rats (Shalgi & Kraicer, 1978). In contrast, sperm transport was less efficient in ewes mated late in oestrus, near the time of ovulation, than in ewes mated early in oestrus (Mattner & Braden, 1969).

The time for sperm to pass through the reproductive tract and into the oviducts of sheep may vary with stage of

oestrus. Mattner & Braden (1969) and Killeen & Moore (1970) inseminated ewes artificially early or late in oestrus. More sperm were recovered in the oviducts at 4, 6 and 24 h after insemination and higher proportions of ova were fertilized in ewes inseminated early compared to those inseminated late in oestrus. Great variation among animals is always found in numbers of sperm in oviducts, and animals which contain no oviductal sperm (Hawk, 1983). Further, total number of sperm found in the oviduct at various times after insemination (most of the sperm presumably in the caudal isthmus) may not accurately predict whether sperm will be present at the time and site of fertilization and are actually capable of fertilizing ova.

In swine, the boar ejaculate fills the uterine lumen of the female. Edematous polypoid processes at the utero-tubal junction prevent entrance of semen directly into the oviducts (Hunter, 1975). Seminal plasma in the uterus, along with most of the sperm, disappears within 2 h, probably resulting from drainage to the exterior (Hunter, 1975). After most of the ejaculate has disappeared, the greatest concentration of sperm are found in the uterotubal junction and the isthmus of the oviducts (Einarsson, 1980; Hunter, 1975; Hunter, 1981).

Transport of Sperm in the Cervix

The cervical mucosa is composed of complex cervical crypts lined with secretory and ciliated epithelial cells.

The ciliated cells beat toward the vagina exhibiting an effective stroke followed by a recovery stroke (Hafez, 1973).

Various factors are involved in sperm transport in the cervix, such as the contractile activity of the vagina and cervix, the biophysical and biochemical properties of cervical mucin, the innate directional motility of the spermatozoa, and the possible role played by the orgasm of the female in altering the mechanical interactions of the cervix with the uterus. The importance of these factors varies with species.

When cervical mucin and semen are placed in opposition *in vitro*, phase lines immediately occur between the fluid. After time, sperm phalanges appear and then develop marked degrees of arborization. The terminal aspects of the arborization consists of channels through which only one or two spermatozoa can pass. Overstreet & Cooper (1978) found sperm progress through cervical mucus parallel with the direction of alignment of glycoprotein molecules and are then directed to the cervical mucosa.

Mullins & Saacke (1982) provided information that may be highly relevant to the movement of sperm into and through the cervix of cattle. These authors found that the lining of the cervix consisted of longitudinal crypts and folds that extended from the external cervical os over the annular rings to the cervicouterine junction. This anatomical structure would enable sperm to swim along the base of the

folds and enter the uterus without being exposed to the main stream of mucus flowing into the vagina. Because of retrograde movement of the sperm deposited in the uterus, this apparent safe pathway for sperm migration may function as a reservoir even after artificial insemination in the uterine body.

The concentrations of oestrogens and progestins, which vary throughout the oestrous cycle, affect the quantity and the biophysical and biochemical properties of the cervical mucus. These cyclic changes in the mucin affect the pattern and rate of sperm migration. Hunter (1982) suggested pre-ovulatory secretion of progesterone may act progressively to relax the cervix, particularly to form a more fluid central channel in the cervical canal due to increased drainage of uterine secretions. The cervical mucin secreted at the time of ovulation provides a suitable environment for the maintenance of spermatozoan metabolic activity (Hafez, 1973). In addition, the pH of the cervical mucin is important for sperm transport. Spermatozoa are susceptible to changes in the pH of the cervical mucus and maintain their activity best in a pH of 7 to 8.5. This is the pH range of normal mid-cycle cervical mucus.

The role of sperm passage into the cervix after coitus in the rabbit has been studied by Bedford (1971). He found that there are sufficient vaginal spermatozoa for normal fertility in the cervix within five minutes after coitus. In addition, he also indicated the early passage of

spermatozoa into the cervix was enhanced by a second successive coital stimulus. These studies emphasize the importance of the early transport of spermatozoa into the cervix.

Hawk & Conley (1975) and Hawk et al. (1978) studied sperm recovery from various regions of the reproductive tract in the ewe. Each ewe was mated to two rams within a short period of time. The cervix was divided into anterior, middle, and posterior thirds before the recovery of sperm. Sperm were then recovered at distinct intervals of time after mating. These investigations indicated that by 2 h after insemination, more sperm were moving retrograde from the cervix to the vagina than were entering the cervix from the vagina. The number of sperm decreased in the posterior third of the cervix between 2 and 8 h and in the middle third of the cervix between 8 and 22 to 24 h. Declining sperm numbers in posterior and middle segments of the cervix presumably reflect some movement of sperm anteriorly into the uterus and oviducts. Most sperm lost from the cervix probably moved into the vagina. The authors speculated that sperm numbers in the anterior third of the cervix may have increased from 2 to 8 h after mating with a subsequent decline 22 to 24 h. Sperm numbers remained reasonably constant until at least 22 to 24 h after mating, which was shortly after ovulation. Numbers of sperm in the uterus and oviducts gradually increased, with sperm numbers in the oviducts increasing most markedly after 8 h. This increase

is in accord with data of Hunter et al. 1980, which indicated that sheep ova were consistently fertilized after transection was delayed until 10 h postmating.

Sperm Transport In the Uterus

Little is understood about the pattern of sperm distribution in the uterine lumen. However, it is known that spermatozoa invade some of the endometrial glands (Hafez, 1973). Sperm phagocytosis by leukocytes in the lumen also occurs. Howe & Black (1963) stated that leukocyte invasion occurred between four and eight hours after vaginal insemination. They further suggested that leukocytes are not the primary way of removing sperm cells. Similarly, Mattner (1968) indicated that the presence of spermatozoa in the genital tract resulted in increased leukocyte numbers in the lumen of the uterus and cervix. This author also found leukocytes were separated from the spermatozoa in the cervix because viable sperm were located in the crypts of the cervix. This is an important factor in the survival of an adequate population of spermatozoa in the cervix of ruminants after mating.

Sperm are rapidly lost from the uterine lumen. Lovell & Getty (1968) suggested several factors that may be involved in the relationship of sperm to the uterine wall and the rapid disappearance of sperm from the lumen. Phagocytosis, clumping of sperm, cilia in glandular tubules, adherence on the surface of the epithelium near the utero-

tubal junction and to epithelial cells, could be primary factors. Sperm tended to persist in the region of the uterotubal junction which may be due to the presence of cilia on the surface epithelium of the uterus near the UTJ. The authors suggest that perhaps there is an antigenic or immunochemical relationship between morphologically similar flagellum of the spermatozoa and the cilia of the epithelial cells that could influence motility, clumping, lysis or phagocytosis of sperm.

Transport of Sperm in the Oviducts

After sperm have passed the uterotubal junction, they are held in the caudal segment of the isthmus for some time. Restriction of sperm to this part of the isthmus may be due to a combination of anatomical and physiological characteristics of the isthmus which include edema, a restricted lumen, propagation of smooth muscle contractions toward the uterus, and also the potential temporary depression of sperm motility (Hunter & Nichol, 1983). Temperature gradients in the oviducts may be involved in the reduction of sperm motility (Hunter & Nichol, 1986). Thermistor probes placed in the proximal ampulla and caudal isthmus of oestrus gilts before and after ovulation revealed that before ovulation the temperature was .69 C lower in the isthmus than in the ampulla of mated gilts and .43 C lower in unmated gilts. After ovulation the isthmus was only .1 C lower than the ampulla. The authors hypothesized changes in activity of

the vascular and lymphatic systems or perhaps contractile activity might cause temperature variations. In turn, lowered isthmic temperature might be responsible for reduced sperm motility.

The oviduct has the unique function of conveying spermatozoa and eggs in opposite directions almost simultaneously. It is assumed that sperm transport within the oviduct is controlled primarily by muscular contractions in the oviductal wall (Blandau & Gaddum-Rosse, 1974), and possibly upon activated motility of sperm (Cummins, 1982).

The effects of cilia induced fluid currents in the oviduct during the oestrous cycle on sperm transport was investigated by Gaddum-Rosse and Blandau (1976). They found particles applied to the mucosa of either the ampulla or isthmus were consistently transported toward the uterine end of the oviduct. After finding variation among several species in the direction or existence of ciliary currents, these authors concluded that sperm transport through the isthmus was probably independent of ciliary action and pro-ovarian contractions in the isthmus. However, ciliary currents and contractions are even more evident at the time of ovulation in the pig, and are more likely to be involved in sperm transport to the ampulla.

Hunter & Nichol (1986) suggested that the small portion of sperm reaching the site of fertilization may be due to the fact that ad-ovarian progression of spermatozoa is tightly controlled immediately after ovulation. This may

indicate a prolonged arrest of viable sperm in the caudal quarter of the oviductal isthmus after mating at the onset of oestrus, followed by a discrete and regulated release initiated close to the time of ovulation.

Apparently the sperm transported to the oviducts soon after insemination, even sperm that may be alive, are not involved in fertilization. Hunter et al. (1980) ligated and transected the oviducts of cattle, sheep, and swine at various intervals after mating and various intervals relative to ovulation. Their purpose was to determine the time by which sperm that fertilized ova had passed the point of ligation and transection. In heifers mated early in oestrus (Wilmut & Hunter, 1984), only 1 of 11 ova was fertilized when the oviduct was transected adjacent to the uterus at 6 h after mating. Transection at 8 or 10 h resulted in fertilization of about 40% of the ova and at 12 h 70%. Sperm entering the oviduct soon after mating either did not remain in the oviduct, or if still present at ovulation, were not capable of fertilizing eggs. In a subsequent study, Hunter & Wilmut (1984) ligated and transected oviducts 2 cm cranial to the uterotubal junction of heifers mated at the beginning of oestrus. Transection between 16 and 31 h after mating resulted in only 3 of 14 ova (21%) being fertilized in animals that had not ovulated at the time of transection. In animals that had ovulated before transection, 7 of 8 ova were fertilized (88%). The results of these two studies indicate that sperm capable of

fertilizing ova reached the oviduct about 8 h after mating, at least in heifers mated early in oestrus. Sperm are held in the caudal end of the isthmus of the oviduct for as long as 18 h or more until about the time of ovulation, and sperm then progress toward the site of fertilization near the isthmic-ampullary junction.

Considerable numbers of sperm have sometimes been found in the oviducts after mating or insemination of cattle. Dobrowolski & Hafez (1970) counted thousands of sperm in flushings of the oviducts of Hereford heifers at 1, 8 and 24 h after depositing 2 billion sperm against the external cervical os. Although these sperm could have been remnants of an earlier rapid phase of transport, they were probably the beginning of the prolonged phase because the uterus and uterotubal junction were populated with sperm. El Banna & Hafez (1970) quantified an average of about 17,000 sperm in the oviducts of Hereford heifers at 16 h after similar inseminations with somewhat fewer sperm, and Mattner (1968) found an average of 7210 sperm in the oviducts of Hereford heifers at 1 and 4 h after insemination at the external os of the cervix with 32 to 34 million sperm. The relatively low number of sperm in the oviducts could have been a function of either time post-insemination or number of sperm in the inseminate.

First et al. (1968) examined the reproductive tract of swine at 15 min and at 2, 4, 8 and 24 h after insemination and found similar numbers of sperm in oviducts each time.

Sperm in the oviducts at 15 min might have been a different population from sperm in the oviducts at later times.

Formation of Sperm Reservoirs

Each segment of the reproductive tract must act, at least temporarily, as the reservoir from which sperm pass to the next anterior segment of the tract. Certain segments of the tract, are believed to provide reasonably safe havens for sperm. Mattner (1963; 1966; 1968) has suggested the cervix of cattle and sheep serves as the main sperm reservoir from which sperm can move continually into the uterus. The cervix supplies an area in which large numbers of motile sperm are maintained. Allison & Robinson (1972) indicated that adequate progesterone priming of the ewe is a necessary prerequisite for a pattern of production of cervical mucus which favors the establishment and subsequent survival of a population of sperm in the cervix.

How many sperm ever leave the crypts and folds of the cervix of cattle and sheep to move into the uterus is not known. After flushing the cervix, Mattner (1966) found large numbers of sperm remaining in the strands of mucus located between the microvilli and in the tubular or simple saccular, cervical glands. In addition, he found stress, when imposed upon the cervical mucus, causes an alignment of mucoid molecules along the lines of strain. A majority of spermatozoa are constrained to follow these pathways of least resistance. These sperm then progress immediately to

the wall of the cervix rather than directly to the lumen of the uterus. Morton & Glover (1974) suggested that the cervical mucus helps direct the sperm into the cervical crypts where subsequent reservoirs are formed. In addition, the cervix may act as a reservoir because the mucosal folding is very complex within the cervix and leukocytes are found to be far less numerous in the cervix compared to that of the uterus or vagina, which suggests less phagocytic removal of sperm.

The anterior third of the cervix of the ewe contains a reasonably constant sperm population until after ovulation (Hawk, 1983). The establishment of a sperm population of normal size in the anterior third of the cervix of the ewe soon after mating or insemination is essential for subsequent movement of normal numbers of sperm to the oviducts. (Hawk & Conley, 1975). The number of sperm in the anterior third of the cervix at 2 h after mating or artificial insemination is closely related to the number in the oviducts at 22 to 24 h, which is near the time of ovulation (Croker et al., 1975). Low sperm numbers in the anterior segment of the cervix at 2 h after insemination reduces the number of sperm in the oviducts around ovulation and reduces fertility (Hawk & Conley, 1975). This relationship does not necessarily mean that the cervix serves as a long-term reservoir. However, it is clear that failure in establishment of a normal population of sperm in the anterior cervix soon after mating precludes the transport of

normal numbers of sperm to more anterior segments of the tract of the ewe.

The time to establish a functional population of spermatozoa in the oviduct has been a subject of investigation in many species. Wilmut & Hunter (1984) found that the population of spermatozoa capable of fertilization is established in the oviduct over a period of not less than 6 h, and probably more than 12 h, in cattle. Hunter & Wilmut (1983) indicated that a slow progression and displacement of viable spermatozoa occurs in the oviduct. These authors obtained highest fertilization results 12 h after oestrus, indicating the functional sperm reservoir at 12 h post oestrous is the oviductal isthmus rather than the cervix in the cow. Hunter et al. (1982) concluded that 8 h is an estimate of the time required to establish a sperm reservoir in the sheep oviduct.

A population of spermatozoa sufficient to give maximum fertilization in the pig is established in the oviduct within 1 to 2 h of mating, which thereby affords protection from the uterine invasion of polymorphonuclear leukocytes (Hunter, 1981). In the rabbit, however, Mortimer (1977) concluded that a cervical reservoir is established within 5 min of coitus. Overstreet et al. (1978) indicated that the rabbit may have physiological mechanisms which restrict sperm to the isthmus of the oviducts for several hours and also regulate the time of movement of sperm to the ampulla to a period near ovulation. Hunter et al. (1980) proposed

that similar mechanisms in the ewe may release sperm from the isthmus to the ampulla near the time of ovulation, suggesting a population of spermatozoa competent to promote fertilization in the ewe is first found in the oviducts approximately 8 h after mating. They hypothesized that, if an interval of 8 to 10 h is needed to establish a functional group of spermatozoa, then this questions the theory of rapid sperm transport providing sperm for fertilization.

Several investigators obtained data implying that the uterotubal junction and the isthmus of the oviduct act as sperm reservoirs in the cow, pig, and some other species (Hunter, 1975; Hunter et al., 1980; Rigby, 1966). These suggestions are based upon prolonged maintenance of high sperm numbers in these areas. Quinlivan & Robinson (1969) concluded, based on sperm counts obtained from ewes between one and 48 h after insemination, that the isthmus might serve as a sperm reservoir for the ampulla. Smith et al. (1987) implied that the lower segments particularly the caudal isthmus appear to be acting as a reservoir since no sperm reached the cranial isthmus or ampulla until the commencement of ovulation. Sperm are held in the caudal isthmus of cattle, sheep and swine from several hours after mating until ovulation, at which time some of the sperm move to the ampulla (Hunter, 1981; 1984; Hunter et al., 1982; Hunter & Nichol, 1983; Hunter et al., 1980; Hunter & Wilmut, 1984). These workers indicated the establishment of a sperm reservoir in the caudal isthmus that is normally maintained

for many hours. In artificially inseminated cattle, it is not known whether the cervix or any other segment of the reproductive tract, except presumably for the caudal isthmus, serves as a long term sperm reservoir.

Mechanisms of Sperm Transport

Sperm Motility Influence on Sperm Transport

Sperm motility is apparently critical to certain phases of sperm transport and, therefore, must be considered in the discussion of sperm transport mechanisms. It appears that motility of sperm is important to population of the cervical crypts with normal numbers of sperm. Flagellation may also be helpful or essential for sperm to pass through the uterotubal junction, within the oviduct, and ultimately to penetrate the ova. Baker & Degan (1972) in a study of live and dead spermatozoa in the reproductive tract of gilts found that the transport of dead sperm was less efficient than that of live sperm. This suggests the lack of normal motility may act as a selection factor against retention of these sperm in the female tract.

Mattner (1968) found that more than half of the sperm in the cow cervix at 19 and 22 h after mating remained in the cervix after the cervical lumen was flushed. Apparently, most of the sperm in the cervix were located in the cervical crypts and were not lying free in the cervical lumen, suggesting that their own motility enabled them to become embedded in the crypts. Mattner & Braden (1969) and

Lightfoot & Restall (1971) inseminated ewes in the external cervical os with motile or killed sperm. Within a few hours large numbers of motile sperm but only a few killed sperm were found in the cervix. Killed spermatozoa only were located in the central area of the cervical lumen, whereas most of the motile sperm resided in the cervical folds. The killed spermatozoa, which remained in the lumen of the cervix, disappeared through drainage to the exterior. Passage of spermatozoa through the cervical mucus to the cervical mucosa appeared to be effected by their own motility.

Overstreet & Tom (1982) inseminated rabbits intra-vaginally and found that transport of sperm through the cervix, uterus and oviducts in 15 min required either seminal plasma or motile sperm. Killed sperm suspended in saline were not even transported into the cervix. However, sperm motility was not required when the killed sperm were suspended in seminal plasma. The constituents of seminal plasma may initiate rapid transport by stimulation of vaginal contractions. However, motile sperm suspended in artificial medium can swim into the cervix.

Passage through the cervical mucus is critical to allow sperm to migrate from the site of deposition to the point of fertilization. Kumar et al. (1983) found that the penetration rate of sperm in the oestrous mucus of cattle depended on the tonicity of the uterus and the level of electrolytes in the mucus. Cryopreserved spermatozoa

penetrated at a greater rate than those in chilled semen, perhaps due to the fact of greater motility. Memon & Gustafsson (1984) found after 180 min, sperm motility and the number of sperm decreased during in vitro migration in the cervical mucus of ewes, but the distance over which the sperm migrated in vitro increased. In addition, Selavive-Villarroel & Kennedy (1983) concluded that the migration rate of spermatozoa was not different in cervical mucus samples from young or mature ewes. However, they did find the migration rate was significantly slower in mucus collected 10 and 16 h after the onset of oestrus compared to that collected within the first 5 h of oestrus.

When swimming in cervical mucus, sperm follow the lines of strain (Tampion & Gibbons, 1962) leading into the cervical crypts (Mattner, 1966). Overstreet & Cooper (1978) suggested that during the sustained phase of sperm transport sperm progress through the cervical mucus parallel with the direction of alignment of glycoprotein molecules and are thereby directed to the cervical mucosa. Spermatozoa accumulate in the crypts between the mucosal villi and form a "reservoir", from which they are presumed to gain release for subsequent upward migration (Mattner, 1966; 1968). It seems justified to conclude that dead or immotile sperm remain in the mainstream of mucus to be carried posteriorly into the vagina and ultimately to the exterior.

Gaddum-Rosse (1981) made direct observations of sperm moving through the uterotubal junction in the rat. Only

motile sperm and not immotile sperm or dye, could pass through the junction. She theorized that sperm motility is important or essential for sperm entry into the oviduct of the rat. Cummins (1982) suggested that hyperactivation, which is characterized by increased flexion of the flagellum, increased amplitude of the flagellar waves, and marked asymmetry of beat, may be an aspect of the prefertilization behavior of mammalian spermatozoa that is of advantage to the fertilizing sperm in the oviduct. In addition, the proportions of motile sperm exhibiting hyperactivation were greatest in the ampulla. Hyperactive motility of sperm may assist in the movement of sperm from the isthmus to the site of fertilization, and may be essential for penetration of the zona pellucida (Bedford, 1983).

Various investigators have indicated that sperm motility is essential for adequate numbers of spermatozoa to be found at the site of fertilization. Vandemark & Moeller (1951) stated that the transport of both motile and nonmotile spermatozoa in the reproductive tract of the oestrous cow takes place rapidly to the oviduct. Katz & Yanagimachi (1980) found activated hamster spermatozoa confined by the solid boundaries of the ampulla lumen appeared to be able to migrate and gain distance by virtue of their own motility which might be of significance in the final transport of fertilizing spermatozoa. Hunter (1982) indicated that sperm motility is critical in the uterotubal

junction and spermatozoa gain access to the isthmus by virtue of their own motility. Fertilization resulting from intraperitoneal insemination was studied by Hunter (1978). In order to result in success, the sperm, by means of their own motility, had to travel to the infundibulum and ampulla of the oviduct. Fertilization was never comparable to natural mating due to the absence of significant sperm reservoirs and that timing of insemination was then critical.

Reproductive Tract Motility Influence on Sperm Transport

The female reproductive tract possesses the physiological mechanisms, at least immediately after insemination, to transport sperm to the oviduct without the active participation of the sperm themselves. Killed sperm are transported to the oviducts of the ewe (Mattner, 1963), the gilt (First et al., 1968; Baker & Degan, 1972), and the cow (Vandemark & Moeller, 1951) within a few minutes after insemination. Rapid transport is almost certainly accomplished by contractions of the female reproductive tract. The number and strength of uterine contractions in the cow increase for a few minutes after mating (Vandemark & Hays, 1953). Hawk (1975) hypothesized that uterine contractions originate either in the body of the uterus or cervical musculature. While some uterine contractions are occur through longitudinal muscle fibers which then straighten the

entire uterine horn, most contractions appear as moving constrictions propagated lengthwise along the uterine horn. Hawk (1975) and Ruckebusch & Bayard (1975) concluded that the number, and especially the strength, of contractions on the reproductive tract of cattle and sheep, increase around oestrus. Ruckebusch & Bayard (1975) indicated that the contractile activity of the reproductive tract during oestrus becomes synchronized between the oviduct and the myometrium, at which time both the amplitude and the frequency of the contractions is the greatest. However, the last remaining zone of activity, near the end of oestrus was the uterotubal junction in cows (Ruckebusch & Bayard, 1975). These contractions seemed to be directed toward the cervix at this time.

Similar changes occur in the origin and direction of uterine contractions of sheep during oestrus. Hawk (1975) indicated that early in oestrus, the majority of contractions originate in the cervix or posterior segments of the uterus and move anteriorly. Late in oestrus, the majority of contractions originate near the uterotubal junction and move posteriorly. It appears that contractions of the reproductive tract move spermatozoa into the anterior cervix and sperm motility moves spermatozoa from the cervical lumen into the cervical crypts. In recovering fluid containing spermatozoa from the uterus by cannulation, Baker & Degan (1972) observed that the fluid flowed in a pulsating manner, suggesting the major forces of sperm

transport are pressures exerted during insemination and by uterine contractions. This pressure and contractions allowed spermatozoa to reach the upper half of the oviduct within 15 min of mating. Overstreet & Cooper (1978), in a study of the rapid phase of sperm transport, indicated initial coordinated contractions may be what propel uterine sperm to the peritoneal cavity, as these contractions cease two to five minutes post coitus.

Several investigators have suggested other major motility factors that enable spermatozoa to be transported within the reproductive tract. In 1955, Rowson speculated that the ascent of fluid in the reproductive tract is mechanical and found that an injection of oxytocin caused a more rapid ascent. Viring et al. (1980) reported that transuterine transport of small and medium molecules had begun by 5 min after deposition into the uterus. They further indicated that this labeled substance moved to the anterior segments of the oviduct, indicating uterine motility was essential. Mattner & Braden (1963) discovered that mating caused a reflex release of oxytocin which increased the motility of the uterus. The mechanisms of sperm transport in the pig oviduct were studied by Blandau & Gaddum-Rosse (1974). Strong ciliary currents were found that could transport particulate matter upward, toward the ovarian end of the ampulla, throughout the length of the isthmus and into the region just anterior to the ampullary-isthmic junction. At this location, the upward and downward

streams merged. These data suggest the pattern and direction of ciliary beat in the isthmus may help spermatozoa move toward the ampulla by either direct ciliary action or fluid currents within the narrow lumen.

The relationship between uterine contractions and efficiency of sperm transport is not clear. Vandemark & Hays (1953) found the presence of a bull, nuzzling by the bull, noncopulatory mounting, and mounting followed by copulation and ejaculation all served as stimuli which resulted in increase uterine tone and contractions. Cooper et al. (1985) concluded that clitoral stimulation caused an immediate single uterine contraction, and this could be repeated at 2.5 min intervals. Fright in ewes inhibited uterine contractions, although the inhibited uterine contractions did not impair sperm transport (Lehrer et al., 1978; 1979). In retrospect, the impaired sperm transport in artificially inseminated ewes may be explained by the observation that uterine contractions are reversed by the insertion of the insemination equipment which ultimately may disturb the formation of cervical sperm reservoirs.

Several compounds have been tested in rabbits (Hawk, 1983), and ewes (Hawk & Conley, 1985) for effects on sperm transport and uterine contractions. The ability of a compound to increase the number and strength of contractions was not always found to be related to its ability to increase the retention and transport of sperm. Other investigators have also studied the effect of various com-

pounds on uterine motility. Hunter et al. (1982) suggested that the major increase of prostaglandin $F_{2\alpha}$ and prostaglandin E shortly before ovulation lead to greater contractile activity, especially of the myometrium. Compared to the controls, Hawk (1973) reported prostaglandin $F_{2\alpha}$ treated ewes had significantly fewer uterine contractions moving toward the oviducts, suggesting that sperm transport may be more susceptible to disturbances in sheep than in cattle. Thibault & Winterberger-Torres (1967) concluded, that although oxytocin increased the magnitude of contractions of the ewe myometrium, it is doubtful that the endogenous oxytocin secreted at the time of mating has any action. This would be due to the fact that the transport from the vagina to the isthmus requires more than one hour. Croker & Shelton (1973) indicated there was an increased proportion of contractions moving towards the body of the uterus which favored expulsion rather than retention in progesterone treated ewes. The administration of oestradiol by Hawk (1975) 20 to 40 h after the beginning of oestrus prevented the change in direction of contractions implying that endogenous oestrogen initiates the type of uterine motility seen during early oestrus. Therefore, declining oestrogen secretion is responsible for the change in direction of contractions during oestrus. These results indicate factors other than number and strength of uterine contractions help to determine the efficiency of sperm

transport, at least when experimental treatments were imposed.

Measurements of Sperm Transport

"Fertilization can result from several factors including structural barriers to the union of sperm and ova and, at least theoretically, to the inability of sperm to penetrate ova or to the unfertilizability of ova. However, there is reason to believe that failure of sperm transport in the female reproductive tract accounts for most fertilization failure" (Hawk, 1983). An indirect measure of sperm transport is the microscopic examination of ova within the first week after oestrus. Separate studies in cattle have indicated counts of accessory sperm in the zona pellucida help to signify the magnitude of sperm transport. Hawk & Tanabe (1986) and Sergson & Libby (1982) have concluded that accessory sperm embedded in or attached to the zona pellicuda of the fertilized ova generally number between 5 and 50. However, accessory sperm can range from none to a few hundred.

Hunter et al. (1980) found that transection and ligation of the oviduct after natural mating or artificial insemination in the ewe, followed by subsequent examination of the ova for fertilization, provided information on the time required for a functional population of fertilizing sperm to have passed the point of closure of the oviduct and be available for fertilization. Results of this method

could be influenced by the effects of surgery or manipulation and handling of the oviduct. These events could then affect the distribution of the spermatozoa in the oviduct.

Actual counting of sperm numbers in the various tract regions of the female, especially at different time periods following insemination (Dobrowski & Hafez, 1970; El-Banna & Hafez, 1970; First et al., 1968; Hunter, 1981; Larsson, 1986), provide information on the time required for spermatozoa to pass through the reproductive tract and form an adequate fertilization pool in the oviduct. These data are useful in determining the overall distribution and fate of the sperm cells within the reproductive tract. It is important to note that sperm numbers within the reproductive tract vary considerably and large numbers of animals are often required to provide reliable relationships. Additional factors contributing to the variation among studies of sperm transport in cattle are different methods of insemination, variation of thoroughness in sperm recovery, small animal numbers, differences in lengths of time between insemination and necropsy, and manipulation induced redistribution of sperm (Hawk, 1987).

Fate of Sperm Deposited in the Female Reproductive Tract

In many instances, only a small portion of the sperm inseminated into cattle (Dobrowolski & Hafez, 1970), sheep

(Quinlivan & Robinson, 1969), swine (First et al., 1968), and rabbits (Hawk & Cooper, 1979) could be recovered from the reproductive tract. The loss of sperm was generally attributed to phagocytosis or voidance to the exterior. After inseminating heifers with 2 billion sperm at the external os of the cervix, Dobrowolski & Hafez (1970) recovered an average of only 13.4% from the entire reproductive tract 1 h later. The percentage then dropped to 3.8% at 8 h and to 0.9% at 24 h. After inseminating ewes with 500 million sperm in the external cervical os, Quinlivan & Robinson (1969) recovered only about 3% of the sperm from the tract one hour later. By 12 and 24 h, the proportion recovered averaged about 0.25%. A high proportion of the inseminate was normally lost to the exterior. First et al. (1968) deposited semen in the uterus of sows and 15 min later recovered from the uterus less than half of the inseminated sperm. At 30 min after artificially inseminating rabbits, Morton & Glover (1974) could recover only 20% of the sperm in the inseminate from the entire reproductive tract and concluded that most of the sperm had been lost by drainage to the exterior.

The appearance of polymorphonuclear leukocytes is known as the leukocytic response to the presence of semen and results in phagocytosis. Phagocytized sperm are often seen in the flushings of the reproductive tract (Mitchell et al., 1985). Most of the sperm not lost by drainage or expulsion to the exterior are probably phagocytized. Active

phagocytosis is only noted 8 to 16 h after spermatozoal deposition, and maximal leukocytic response is slower in the oviduct (12 to 16 h) than in the uterus (6 to 8 h) (Mitchell et al., 1985). Phagocytosis is less efficient in the progesterone-dominated phase of the oestrous cycle (Hafez, 1973). The length of time sperm survive and remain in the crypts of the cervix, in which they are partially protected from phagocytosis in cattle and sheep, is apparently not known. Sperm have been found in the reproductive tract of farm animals as long as 72 h after insemination (Hawk, 1983).

Very small percentages of the inseminate are lost through additional avenues. Unknown numbers, undoubtedly a small proportion of the inseminate, pass through the oviducts and into the body cavity. Sperm may also be incorporated into cells lining the reproductive tract (Larsson & Larsson, 1985).

Bedford & Witkin (1983) depleted doe rabbits of complement by treatment with anticomplement cobra venom factor. Depletion of complement resulted in higher numbers of sperm in flushings of the oviduct at 12 and 13 h after mating and in more sperm attached to ova. Complement, perhaps acting in conjunction with leukocytes or antibodies, may normally be involved in reducing the number of sperm that reach the oviducts of rabbits. Fahmi & Hunter (1985) determined that follicular fluid of cows in estrus contained higher amounts of complement than did follicles of cows at

other stages of the oestrous cycle. These authors speculated that follicular fluid complement entering the oviduct at ovulation may have some role in removal of sperm from the female.

Distribution and Loss of Spermatozoa

Although many millions of spermatozoa are deposited into the reproductive tract of the female, only very few ever reach the egg at the site of fertilization. Most spermatozoa are retained at the selective barriers; the cranial portion of the cervix, the uterotubal junction, and the isthmus of the oviduct. Spermatozoa also undergo phagocytosis by the leukocytes which invade the endometrial cavity. A continuous loss probably occurs as spermatozoa move randomly back into the vagina (Hafez, 1973).

Apparently, the first experiment to quantitate sperm in the reproductive tract in cattle was done by Mattner (1968). He mated 5 dairy cows twice to a bull and sacrificed the cows 19 to 22 h later. He flushed the lumen of the cervix, uterus and oviducts and counted sperm in the flushings. A gradient in sperm numbers was apparent through the reproductive tract. Numbers decrease from several million in the cervix to several thousands in the oviducts.

Two years later, Dobrowolski & Hafez (1970) deposited two billion sperm into the external cervical os of Hereford heifers and subsequently sacrificed the heifers at 1, 8 and 24 h post insemination. They separated the entire repro-

ductive tract into its various segments and counted sperm in the flushings of each segment. Their results also indicated sperm numbers decrease drastically from the vagina to the oviducts in which a rapid and sustained decline was apparent in the total number of sperm throughout the entire tract. Of the total sperm deposited, only 13.4% were recovered at 1 h, 3.8% at 8 h, and 0.9% at 24 h. In addition, the maximum numbers of sperm in the oviduct coincided with the time of ovulation. Motile sperm remained in the cervix longer than in other regions of the reproductive tract. In heifers which had ovulated, vaginal numbers were maximum one hour after insemination and then decreased while uterine numbers of sperm increased between 1 and 24 h. Oviductal numbers reached their maximum at 8 h after A.I., indicating the number of sperm in the oviduct increases gradually and then declines.

El-Banna & Hafez (1970) reported that maximum recovery from the oviduct occurred 16 hours after insemination in heifers inseminated at the external os of the cervix. These authors also indicated that the quantity of sperm recovered from the oviduct does not necessarily give an accurate number, since this represents sperm that are in-transit. Therefore, sperm in the oviduct must be continually replenished to ensure viable sperm for fertilization. The percentage of sperm recovered in this study was, 81% from the vagina, 13% from the cervix, and 5.1% from the uterus at 16 hours post insemination.

An experiment was done in dairy cattle to study the distribution of spermatozoa after inseminating approximately 300 million sperm in the uterine body (Suga & Higaki, 1971). Cows were slaughtered between 3 min and 5 h after insemination. Although more than likely all of the sperm were not recovered from the tract, as sperm were recovered from all regions of the reproductive tract by scrapping the lining of the reproductive tract with the edge of a glass slide, their results did indicate changes in sperm distribution. The investigators concluded that by 30 to 60 min most of the sperm had moved posteriorly and were recovered from the vagina and cervix with only a few thousand remaining in the uterus.

Hawk & Conley (1971) studied the loss of spermatozoa from the reproductive tract of the ewe by ligating the vulvovaginal junction to prevent loss of spermatozoa by drainage. They were able to recover 62% of the inseminated sperm from ewes in which the vulvovaginal junction was ligated. Less than 1% was recovered from unligated ewes indicating a large loss of spermatozoa by drainage to the exterior.

More recent studies in cattle, have also demonstrated the retrograde movement of sperm from the site of deposition. Two hours after depositing 160 million frozen thawed sperm in the uterine body, Larsson and Larsson (1985) recovered 14.6% of the inseminate from the entire reproductive tract; 98.5% of the sperm recovered were in the

vagina and cervix. At 12 h after insemination, 0.6% of the inseminate was recovered and 73.7% of the recovered sperm were in the vagina and cervix.

In the first experiment done by Mitchell et al. (1985), the vast majority of sperm deposited in the uterine body were not recovered. Therefore, the investigators conducted another experiment to determine the routes by which sperm were lost from the reproductive tract. The authors deposited an average of 420 million sperm in the uterine body. Of the total inseminate, 70% of the sperm were abnormal. The investigators collected all mucus and urine discharged by the cows, aspirated mucus from the vagina every 2 h, and sacrificed the cows at 12 h post insemination. Sperm that remained in various segments of the reproductive tract and sperm that were lost in mucus, urine, or on equipment used for insemination or aspiration were counted. The investigators accounted for 73% of the inseminate. However, 6.5% of the inseminate remained in the cow and 60.7% was found in discharged mucus, and more than 95% of the sperm recovered were in the vagina and cervix. A few percent of the inseminate was found in mucus aspirated from the vagina, on equipment, or in the urine. The most important finding of the experiment was the extent to which sperm were lost from the cow in mucus discharged to the exterior. Of the sperm recovered in discharged mucus, 21% of the inseminate was calculated to have been discharged by 2 h after insemination, 28% by 4 h, 52% by 6 h, and about

60% by 8, 10 and 12 h. Thus, by 6 h over half of the inseminate had been lost. Of the 27% of inseminated sperm not found, perhaps most of the missing sperm, were phagocytized, as partially phagocytized sperm were seen in all parts of the tract but were not included in the sperm counts.

Tilbrook & Pearce (1986) studied the pattern of loss of spermatozoa from the vagina of the ewe after artificial insemination in the vagina. They found at 3 h after insemination, 82% of the inseminate remained in the vagina, after which time losses were much greater. After 9 h 18% could be recovered from the vagina and at 12 h 10% was recovered from the vagina. The loss from the vagina was not a linear rate but followed a pattern of gradual decline continued by a longer period of rapid loss where most of the spermatozoa disappeared.

The distribution of fresh and frozen thawed sperm in the reproductive tract of gilts after A.I. was investigated by Pursel et al. (1982). They documented that more sperm were recovered from the uterotubal junction and uterus of gilts inseminated with fresh semen than from gilts inseminated with frozen semen. The investigators also indicated that the motility of the frozen spermatozoa was decreased which was theorized to be critical in the cervix and the uterotubal junction of the gilt by Hunter (1980, 1982). Motility is also important and probably essential for sperm distribution and entry into the oviduct of the

rat. Gaddum-Rosse (1981) revealed that motile sperm emerged, usually individually, at the uterotubal junction.

Although most sperm deposited in the uterine body after artificial insemination soon pass to the cervix and vagina, it appears that some sperm are continually present in the uterus. However, no information is available on the turnover of sperm in the uterus and oviduct or the extent to which sperm in these segments, at any one time, move posteriorly into the cervix and vagina to be replaced by other sperm moving anteriorly into the uterus.

Treatments and Mechanisms of Inhibiting Sperm Transport

Sperm transport and survival in the female reproductive tract is sometimes reduced drastically. Most of the information has been acquired from sheep, an animal in which mechanisms of sperm transport may be more susceptible to disruption than in cattle or swine. Sperm transport in ewes can be severely disrupted by a variety of experimental treatments or management conditions. Mattner (1963) found that stress in ewes decreased the number of sperm in the oviduct 15 min after coitus but did not influence the transport of spermatozoa into the cervix, the distribution of spermatozoa within the genital tract at 4, 24 and 48 h, the number of sperm on the zona pellucida of recovered eggs, or the proportion of eggs fertilized.

Cows may be more resistant to disruption of sperm

transport by experimental treatments than are ewes. Regulation of oestrus in cattle with progestogen reduced the fertilization rate in some experiments (Hill et al., 1971) but not in others (Wishart, 1977). Regulation of oestrus with prostaglandin $F_{2\alpha}$ generally has not reduced fertility when cattle were inseminated in conjunction with detected oestrus (Macmillian & Day, 1982).

An IUD (plastic intrauterine device) in one horn of cattle inhibits sperm transport only locally, probably by physical interference with the passage of sperm (Hawk, 1983). In the ewe, the insertion of an IUD into the lumen of the uterus before oestrus reduces drastically both the number of sperm transported to the oviducts and ultimately fertilization rate (Hawk, 1969).

Removal of the corpus luteum-bearing ovary of ewes in midcycle causes drastic disruption of sperm transport mechanisms when the ewe is in oestrus 2 days later (Hawk et al., 1981). Removal of the CL-bearing ovary, especially on day 10 of the cycle, decreased the number of sperm recovered, particularly in the anterior one third of the cervix. Such treatment probably changes the balance of ovarian hormones that influence the reproductive tract at oestrus. In the doe rabbit, Fateh El-Bab et al. (1983) found unilateral ovariectomy significantly reduced the total numbers of sperm recovered compared to that in intact does.

In addition to regulation of estrus with progestogen or prostaglandin $F_{2\alpha}$, four other treatments disrupt sperm

transport mechanism in the ewe. Small amounts of oestradiol are found to be detrimental to sperm transport (Noyes et al, 1959). Lightfoot et al. (1967) found lower fertilization rates and fewer sperm in the cervix, uterus and oviducts of ewes grazing on subterranean clover with high oestrogen content than in those grazing oat pasture, which served as a control. Sperm did not enter the cervix in adequate numbers in the ewes grazing clover. The authors speculated there was a change in the nature of the cervical environment. Likewise, administration of 25µg of oestradiol per day for 14 d before oestrus in the ewe reduced sperm numbers in the middle and anterior thirds of the cervix at 2 h after insemination and in the oviducts at 24 h (Croker et al., 1975). In an additional experiment using 30µg compared to 90µg of oestradiol, it was found that at 1, 12 and 24 h, in each region of the cervix, fewer spermatozoa were recovered from ewes treated with 30µg than in those of the other group. The higher dose of oestrogen may have facilitated passage of spermatozoa through the cervix.

Presently, little is known about the site or cause of apparent sperm transport problems in cattle with fertilization failure (Hawk, 1987). It is unclear whether insufficient numbers of sperm are retained in the female tract, or whether the sperm transport mechanism fails at one or more sites in the tract. This problem may be located in the cervix and uterus, as in sheep, or in some other site of the reproductive tract. Perhaps sperm fail to populate the

caudal isthmus in normal numbers or the sperm in the isthmus fail to move to the ampulla (Hawk, 1987). One or more of the aforementioned mechanisms may account for the apparent sperm transport problems in cattle.

Influence of Site of Semen Deposition After A.I.

Many investigators have sought to determine whether or not variation in the placement of semen in the reproductive tract influences sperm transport within the uterus and ultimately fertility. Hawk & Tanabe (1986) deposited semen deep in one uterine horn approximately 15 to 20 cm anterior to the uterine body in both single ovulating and superovulating cows. Some cows in each group were infertile repeat breeders. Cows were necropsied and ova recovered approximately 3 d later. In single ovulating cows, semen was deposited in the uterine body, uterine horn adjacent to the side of impending ovulation or uterine horn opposite the side of impending ovulation. No significant differences were found among sites of semen deposition in fertilization rate or number of accessory sperm. In 28 superovulating cows, semen was deposited deep in one horn and a total of 490 intact ova were recovered. The fertilization rate was significantly higher (74%) on the side of semen deposition than on the opposite side (58%), but the difference between sides of the tract was due almost entirely to four cows in which the fertilization rate was 93% on the side of

insemination and 19% on the opposite side. The overall results suggest that in most cases sperm deposited in one uterine horn fertilize ova on both sides of the reproductive tract. Transuterine migration may occur by passing through the uterine body or perhaps entering the cervix or even the vagina and into the opposite horn.

Killeen & Moore (1970) compared transport and fertilization in the ewe following cervical and uterine insemination, early and late in oestrus. They found fertilization was directly related to the number of spermatozoa recovered from the oviducts. Early and late uterine insemination gave high fertilization rates. Insemination late in oestrus at the cervix resulted in a significantly lower number of spermatozoa in the oviducts and significantly lower fertilization rates. In addition, they suggested that the ewe possesses an effective mechanism, operating through the transportation of spermatozoa, that inhibits fertilization of aged eggs and consequent reproductive wastage arising from the abnormalities associated with late fertilization.

In a more recent study, Larsson (1986) deposited semen deep in one uterine horn of 8 heifers, sacrificed the heifers 2 hours later, flushed the various segments of the reproductive tract and counted sperm in the flushings. Sperm were recovered from the uterus and oviducts on both sides of the tract, with as many or more sperm being recovered from the side opposite to insemination as from the adjacent side.

Other investigators have also studied the effect of cornual insemination on fertilization rates. Senger et al. (1987) compared conception rates to artificial insemination services of either the uterine body or uterine horn. They observed a 50% conception rate for the uterine body depositions compared to 61% for the cornual inseminations. Similar results were found by Williams et al. (1987) who indicated that cervical insemination conception rates (40%) were significantly less than rates resulting from insemination in the uterine body (48.4%) or cornual (50.4%) insemination. Perhaps retraining the inseminators to more accurately place the semen in the uterine body may have resulted in improved fertilization rates as was shown in an earlier study (King & Macpherson, 1965). However, an investigation by Seguin (1984) found no significant effect of insemination in the uterine horn as compared to the uterine body.

All in all, these results of more recent studies tend to indicate that deposition of semen in the uterus may offer an advantage in terms of fertility over deposition of semen in the anterior cervix. However, sperm that is deposited in one horn of the uterus seem to become distributed between both sides of the reproductive tract in most cows.

Treatments and Mechanisms of Improving Sperm Transport

Numerous investigators have attempted to improve sperm

transport to the oviducts. Successes reported in literature may be selective because unsuccessful attempts are less likely to be reported by the investigators. This issue is indeed of concern considering approximately one insemination of every seven results in fertilization failure and the fertilization rate has often been lower in cows with histories of being repeat breeders than in other cattle (Tanabe & Almquist, 1953; Hasler et al., 1983). However, the technology and means are currently available to improve fertilization rate to some degree.

Retraining of inseminators has generally improved fertility, and the improvement may be due at least in part to improvement in the placement of semen and better semen handling techniques (King & Macpherson, 1965). Variation in site of semen deposition has been considered to be responsible for at least part of the variation in fertility among artificial inseminators (Peters et al., 1984). In view of experimental results, which indicate sperm deposited deep in one uterine horn are soon distributed throughout both sides of the reproductive tract (Larsson, 1986) and fertilize ova on both sides of the tract (Hawk & Tanabe, 1986), improvement of fertility after retraining inseminators may be due more to such factors as improved handling of semen or to penetration of the cervix of a higher proportion of cattle than to improved precision in placing semen in an exact location in the uterus.

Several compounds, when added to semen used for

insemination or injected into females near insemination, have increased the number of sperm recovered later from the oviducts. These compounds include prostaglandin E_1, or prostaglandin $F_{2\alpha}$ added to rabbit semen or injected into does. Hawk & Cooper (1979) found that injection of $PGF_{2\alpha}$ in the rabbit immediately before insemination increased at least three fold the number of sperm recovered from the oviducts, uterus, cervix and vagina as well as the total number recovered from the tract. In addition, the $PGF_{2\alpha}$ treatment reduced the normal loss of sperm from the tract during the first 3 h after insemination. Mandl (1972) stated that prostaglandin E_1 stimulates the contraction of the caudal part of the oviduct in the doe rabbit. This author suggested a suction effect could be created by the inhibition of uterine activity and stimulation of the caudal portion of the oviduct. Also, PGE relaxes the cervix which may allow for increased sperm transport to the uterus. Edquist et al. (1975) found when prostaglandin E_1 and $F_{2\alpha}$ were combined and added to ram semen or injected into ewes, the average number of sperm recovered from the uterus and oviducts were increased as well as increasing the rate of transport from the posterior cervix to the oviducts.

Oestradiol 17-β is another compound which has been injected into females. Hawk & Cooper (1975), concluded that oestradiol 17-β injected in the ewe prior to insemination significantly increased, by more than two fold, the number of sperm cells recovered from the cervix and increased, by

more than eight fold, the number of sperm cells from the uterus and oviducts. Exogenous oestradiol given to the doe rabbit increased the number of sperm cells in the oviducts and uterine horns as early as 2 h after administration of the steroid (Hawk & Cooper, 1976). In 1978, the same two investigators found administration of oestradiol to the doe rabbit, particularly 30μg, resulted in an increased number of sperm in the oviducts and other segments of the reproductive tract and delayed a loss of sperm from the reproductive tract. Greater sperm numbers were found in some segments of the reproductive tract within 3 h of when oestradiol was injected several hours after insemination.

Another compound which affects sperm transport was found to be carbocholine, a drug known to affect contractions of smooth muscle. Carbacholine was inseminated in 100ml of semen in the gilt. This treatment significantly increased the number of sperm in the oviducts 16 h after insemination and increased the conception rate (Baker et al., 1968). Thibault & Winterberger-Torres (1967) found oxytocin injected after mating speeds up transport of sperm in the uterus as this hormone has a stimulatory effect on smooth muscle. However, since oxytocin is destroyed quickly by the female system, one may doubt whether the endogenous oxytocin secreted at the time of mating has any action at all.

It is unfortunate that each compound, as indicated above, seemed to be maximally effective only in a narrow

dose range (Hawk, 1983). Such was the case with prostaglandin $F_{2\alpha}$ (Hawk et al. 1982) and oestradiol (Hawk & Conley, 1978). Prostaglandin $F_{2\alpha}$ administered at the time of insemination and at a dose of 0.75 mg, was found to increase sperm numbers in the oviducts, uterus, and cervix of the rabbit. However, if the dose used was 0.15 mg or 3.75 mg, no significant increase was found. When oestradiol was given to oestrus rabbits prior to mating, compared to 0.3µg, 1µg and 10µg, three µg of oestradiol resulted in the highest number of sperm to be recovered from the oviducts and uterus.

Several compounds have been inconsistent in their effects on fertility, fertilization rate, or number of sperm in the reproductive tract. The addition of amylase or glucuronidase to bull semen has improved fertility significantly in some, but not in all experiments (Foulkes & Goodey, 1980). Any beneficial effect of these enzymes has been ascribed to effects on sperm or on the diluent rather than to effects on sperm transport. Administration of oestradiol to ewes near mating increased the number of sperm in the oviducts or anterior cervix in some experiments. Hawk et al. (1978) gave two oestradiol injections before and after mating. The injection of oestradiol before mating increased sperm numbers significantly in the anterior one third of the cervix; however, the second oestradiol injection after mating had no apparent effect. Lightfoot & Restall (1971) concluded that the injection of oxytocin into ewes near

insemination had no significant effect on the number of sperm in the uterus and oviducts at 30 min or 2 h after insemination or on the number of sperm in the zona pellucida. However, injection of oxytocin into gilts at insemination improved the rate of fertilization and consequently, litter size (Stratman et al., 1959). Likewise, in the rabbit, Morton & Fitzpatrick (1974) suggested that when 80 iu oxytocin were given, mass transport of semen occurred into the uterus. This suggests a coordinated response of the genital tract to a critical oxytocin stimulus which may facilitate mass transcervical passage of fluids in the ovariectomized oestrogen treated doe.

Other compounds have been investigated for their possible effects on sperm transport. Phenylephrine, which is an alpha adrenoceptor agonist, increased numbers and strength of uterine contractions in rabbits under oestrogenic influence (Hawk & Cooper, 1984). Hawk et al. (1982) found phenylephrine increased sperm numbers in the oviducts by 50 fold and in the uterus 10 fold. These investigators also found the compound ergonovine, a muscle stimulator, increased sperm numbers by 10 fold in the oviducts and uterus. Hawk et al. (1982) indicated that the administration of acetylcholine increased the number of sperm recovered one hour later from the vagina and cervix but not from the oviducts. This compound was found to increase the frequency and amplitude of uterine contraction.

Two steroids when given together, oestrogen and progesterone, have been found to increase sperm numbers. However, this response was dependent upon the dose of each compound given (Allison & Robinson, 1972).

Administration of gonadotropin-releasing hormone (GnRH) to cattle at the time of insemination has improved fertility in some experiments, especially at repeat services (Stevenson et al., 1984; Lee et al., 1983), but has not improved fertility in other experiments (Anderson & Malmo, 1985). Treatment with GnRH during oestrus may affect time of ovulation, corpus luteum development, progesterone secretion, and embryonic survival. The GnRH likely is acting through its affects on the release of FSH and LH. Lee et al. (1983) suggested that GnRH administration induced an additional surge of LH that enhanced active lutenization of granulosa cells to insure adequate production of progesterone for pregnancy when successful fertilization occurs. The endogenous release of LH may then coordinate a series of events that culminate in ovulation. This may also cause the cervical mucus to change in its consistency and environment. Uterine motility would also decline due to increasing levels of progesterone and decreasing oestrogen. The GnRH could then theoretically affect sperm transport and fertilization rate.

Donaldson et al. (1986) removed LH from a commercial FSH preparation by column chromatography and used the purified FSH to superovulate cattle. The purified FSH, when

compared with the commercial preparation, increased the fertilization rate from 52% to 74% and the number of transferable embryos per donor cow from 2.9 to 6.3. Adding LH to purified FSH reduced the fertilization rate and number of transferable embryos. Treatment of cows with purified FSH was associated with a reduction in blood progesterone concentrations during oestrus compared with progesterone in cows treated with unpurified FSH. It then appears that LH in FSH preparations has a deleterious effect, perhaps by stimulating progesterone secretion, on some phase of sperm survival or transport in the reproductive tract of superovulating cows (Hawk, 1987).

Clitoral stimulation has been studied by a number of investigators as a means of improving sperm transport. Randel et al. (1975) and Lunstra et al. (1983) both found clitoral stimulation at the time of artificial insemination was an effective means of increasing pregnancy rates in beef cows but not in heifers. Clitoral stimulation may affect sperm transport through uterine contractions (Cooper et al., 1985), or time of ovulation (Hawk, 1987) in the dairy cow as evidence from the beef cow indicates that this may be true. Conversely, Foote et al. (1984), reported clitoral massage causes an immediate uterine contraction but does not cause a release of oxytocin. In addition, this technique was not found to have any effect on the non-return rate of dairy cows and heifers.

Alterations to the reproductive tract can also affect

sperm transport. Pursel (1982) conducted an experiment to determine whether the prevention of retrograde sperm expulsion from the uterus would enhance ovum fertilization by frozen-thawed boar sperm. Earlier work by Pursel et al. (1978) showed frozen thawed sperm to be lost from the uterus more rapidly then fresh sperm. He ligated one uterine horn of gilts inseminated with frozen thawed boar semen to prevent expulsion of sperm. Four hours after ligation and insemination he recovered significantly more sperm from the ligated horn than from the unligated uterine horn. In addition, more ova were fertilized and more accessory sperm were attached to the fertilized ova on the side of the ligated horn. Whether treatments can be devised for cattle reducing the loss of sperm to the exterior remains to be determined.

Conclusion

Fertilization failure accounts for 15% of all infertile services under optimal breeding techniques (Freeman, 1983) in dairy and beef cows, and is second only to embryonic mortality as a cause of infertile services (Hawk, 1979). Substantial losses of spermatozoa occur within the first few hours after artificial insemination (Mitchell et al., 1985; Suga & Higaki, 1971; Larsson & Larsson, 1985). In other species such as the pig (Pursel, 1982), ewe (Hawk & Cooper, 1984), and rabbit (Hawk & Conley, 1979), reducing retrograde loss of spermatozoa results in a higher percentage of

fertilized ovum due to more sperm in the oviducts. Mitchell et al. (1985) reported significant positive correlations for sperm numbers between the vagina with the uterus and the vagina with the oviduct. Hawk & Conley (1975) found the oviductal sperm numbers at 22 h to be positively related to numbers of sperm in the anterior cervix 2 h after mating. These results suggest that a real potential may exist to determine sperm numbers in various regions of the reproductive tract based on those discharged from the vagina. Perhaps termination of the cow for this area of research can be avoided if oviductal sperm numbers can be predicted in this manner. In addition, if decreasing the retrograde loss of sperm in the cow leads to higher sperm numbers in the more anterior regions of the tract, this may reduce fertilization failure in the cow. However, knowledge of sperm distribution among the tract regions and discharge of sperm is needed before methods of improving sperm retention can be developed.

CHAPTER III

SPERM DISCHARGE AND RETENTION BY THE REPRODUCTIVE TRACT OF THE COW FOLLOWING ARTIFICIAL INSEMINATION

Summary

Of the artificial inseminated cryopreserved sperm, 72% ±4.2% from 13 cows could be recovered 12 hours after artificial insemination. The highest proportion 68.4% of recovered sperm (95%) was obtained prior to slaughter in discharged mucus and mucus flushed from the vagina. Percentage of inseminated sperm remaining in the tract was dramatically lower (2.7%) and decreased with progressively more anterior tract regions. The oviduct contained only 0.071% of the inseminate 12 h after breeding. A negative partial correlation existed for sperm recovered from discharged mucus with sperm not discharged from the vagina. The majority of the inseminated sperm were moved retrograde from the site of insemination to the exterior. Reducing retrograde discharge of sperm may provide more sperm for transport to the oviduct and increase fertilization rates in artificially inseminated dairy cattle.

Introduction

Fifteen percent of reproductively healthy cows experience fertilization failure (Hawk, 1983; Diskin & Sreenan, 1980; Roche, 1981). This is second only to embryonic mortality as a cause of conception failure assuming optimal breeding techniques. Hawk (1983) suggested that insufficient sperm transport to the oviduct may be a major cause of fertilization failure. Because reports of sperm absence at the site of fertilization (Hawk et al., 1982; Wiley et al., 1982), fertilization failure in cattle likely results from failure of sperm to contact ova rather than from the unfertilizability of the ova. Work of Hawk & Tanabe (1986) and Gustafsson (1985), suggest that fertilization rates approach 100% if sperm capable of fertilization reached the ampulla.

A high percentage of artificially inseminated sperm are lost from the cow reproductive tract within a few hours after insemination (Dobrowolski & Hafez, 1970; Suga & Higaki, 1971; Larsson & Larsson, 1985; Mitchell et al., 1985). In addition, only a few thousand sperm could be recovered from the oviducts. Research in other species has been done to determine the magnitude of retrograde sperm loss and the effect of reducing such on fertilization rate. Ligation of the vulvovaginal junction of the ewe (Hawk & Conley, 1971) resulted in 62% recovery of the inseminate being recoverable from the vagina. Also placing a ligature around the uterine horn in the sow after insemination with

frozen semen increased fertilization rates due to improved sperm numbers in the oviduct (Pursel, 1982). Cryopreservation of boar sperm normally causes depressed fertilization rates as greater proportions of sperm are lost in retrograde discharge than is the case with fresh semen (Pursel et al., 1978). Stimulating sperm transport with $PGF_{2\alpha}$ (Hawk & Cooper, 1979) and oestradiol (Hawk & Cooper, 1978) has increased sperm numbers in the oviducts and other tract regions in the rabbit. Oestradiol (Hawk & Cooper, 1978) has also has been found to increase fertilization rates in the doe. Smooth muscle stimulants, ergonovine and phenylephrine, have also been reported to increase sperm number in the oviducts and the fertilization rates of ewes (Hawk & Cooper, 1984).

Reducing retrograde sperm loss as a means of decreasing fertilization failure has not been attempted in cattle. Prior to conducting such research, knowledge of sperm distribution within the tract around the time of fertilization and sperm discharged prior to this time is needed. This information, while available for high concentration of fresh or abnormal sperm, or both, has not been provided for a more normal dose of cryopreserved bovine semen. Answering this question would indicate whether or not potential exists for decreasing fertilization failure through increased sperm retention within the reproductive tract. Therefore, our objectives were to determine the distribution of a normal artificial insemination dose of cryopreserved sperm within

the reproductive tract and discharged mucus of the cow, and to examine relationships among the sperm numbers in the major tract regions with those of the discharged mucus.

Materials and Methods

A more detailed outline of the methods is presented in appendix table 10. Seven primiparous and ten multiparous cows at least 60 days postpartum with normal reproductive tracts (as determined by rectal palpation and breeding records) were used in this study. Oestrus was induced in diestrus cows with $PGF_{2\alpha}$ or cloprostenol. With frequent oestrus detection, cows were bred 8 to 16 h after the onset of oestrus and 12 hours prior to slaughter. A pool of high quality cryopreserved semen from at least 5 bulls was used throughout the experiment. The initial post thaw percentage motility, percentage intact acrosomes as classified by Saacke & White (1972) and percentage normal sperm were 50%, 96%, and 79% respectively. Two 0.5 ml straws of semen were thawed and pooled. An aliquot of the semen was loaded into a standard 0.5 ml straw and approximately 75 X 10^6 sperm in 0.48 ml was deposited in the uterine body by an experienced inseminator. The volume deposited was determined by measuring the actual volume delivered from the insemination system. Sperm concentration in the remainder of the semen pool was quantified with a hemacytometer so that actual sperm numbers inseminated were known.

Collection of Discharged Mucus

Immediately after insemination, a latex funnel was attached to the vulva of the cow with an industrial adhesive (3M Scotch Grip #4799) to facilitated clean separation of feces, urine and mucus. Mucus and urine that were discharged during the 12 h between insemination and slaughter were collected. Twenty to 40 min prior to slaughter the cow was induced to urinate by stroking the ventral vulva and fecal material was physically removed from the rectum to reduce the potential contamination of recovered fluid. The vagina was flushed with 1 liter of 2.9% (v/v) sodium citrate solution (37 C) to remove the majority of the vaginal mucus. This prevented potential loss of sperm rich mucus during the recovery process and provided a more accurate indication of sperm numbers recoverable without sacrificing the cow.

Tract Removal and Preparation

A modification of the technique described by Mitchell et al. (1985) was utilized for the removal of reproductive tract and recovery of sperm. Urination was stimulated immediately prior to stunning to reduce the chance of urine reflux contamination of the tract. After stunning, the hindquarters were elevated at a modest angle to avoid inducing anterior flow of mucus within the tract while allowing sufficient exsanguination. During exsanguination, the vulva was closed with a cyanoacrylic adhesive and surgical staples to prevent contamination and subsequent

loss of mucus. Simultaneously, the tract and bladder were located through a mid-ventral incision. A ligature was immediately placed around the urethra to prevent urine reflux. Hemostats were applied at the infundibulum and 0.5 cm posterior to the utero-tubal junction (UTJ). A ligature was also placed around the uterine body. Six to 10 min was required after stunning to isolate the tract regions.

Sperm Recovery

Both oviducts including the UTJ were removed and flushed through the ostium with 30 ml of sodium citrate. The flushings were combined. The vagina was irrigated three times (120 ml each) and then excised from the cervix. Vaginal mucosa was exposed and covered with 50 ml of 0.5 N NaOH for 30 min. The liquified mucus was removed by aspiration from the surface of the vagina. Cervical irrigation was repeated 3 times (60 ml each) using a blunt edge 16 gage 3 cm needle. A foley catheter was then induced through the cervix into the uterine horns. Each horn was flushed two times with 120 ml of sodium citrate. The uterine body was irrigated with an additional 120 ml of sodium citrate. Uterine flushings were combined. Uterine and cervical mucosa were exposed and covered with 0.5 N NaOH for 30 minutes to liquify any sperm-containing mucus for recovery.

Concentration of Sperm for Counting

The mucus from each sample was dissolved by increasing the flushing volume by 50% with 1N NaOH. A minimum of 24 h at 5 C was allowed to insure complete liquification. Prior to centrifugation, the pH of each sample was adjusted to about pH 7 with acid (1N HCL). Successive centrifugation steps at 1500 X g for 20 min were used to concentrate the sperm into reduced volumes suitable for hemacytometer counts of the tract regions. The concentrated samples were stored at 5 C until sperm quantification was complete. The same two observers conducted the hemacytometer counts throughout the study. The most efficient laboratory sperm recovery method was determined by an experiment examining procedures for mucus dissolution, time, and centrifuge tube composition (Appendix Tables 4 & 5). Sperm recovery was determined to be best around 1500 X g for 20 to 60 min. In addition, using polycarbonate as opposed to polypropylene tubes yielded higher sperm recovery percentages. Sodium hydroxide as compared to alpha amylase was found to be more efficient in mucus liquification.

Determination of Recovery Efficiency

Efficiency of sperm recovery in the laboratory was determined with 4 cows that were not inseminated prior to slaughter. Twenty-four ±4 h after the onset of oestrus the cows were slaughtered and their reproductive tracts removed. This time frame insured that the cow's reproductive tract was in the same phase of the oestrous cycle as our sperm

recovery cows. Each region was flushed as previously described. The flushings were inseminated with 10×10^6 sperm in the laboratory. Sperm were concentrated as per procedures used for inseminated cows. Our recovery efficiency was determined to be: vagina 86%; cervix 58%; uterus 56%; and oviduct 63%. These were used as correction factors to determine our overall recovery of sperm in the laboratory.

Statistical Analysis

Data were analyzed using General Linear Models procedures from Statistical Analysis Systems (SAS, 1982). Included in the model for analysis were all cow by reproductive tract location interactions. Actual sperm numbers recovered from the genital tract regions and percent of the inseminate recovered from each tract region were transformed by determining the log base 10 and arc sine square root values respectively prior to analysis. The model error term used was the cow by reproductive tract region interaction. Partial correlations were examined for the numbers of inseminated sperm recovered from different tract regions and discharged mucus. Correlations were considered significant at $P \leq 0.10$. Sperm numbers in various tract regions were considered to be different if the value ± 2 times its S.E.M. did not overlap with values for each other tract region ± 2 times their S.E.M.

Results

The average number of sperm inseminated was 72 X 10^6 and distribution of sperm among the tract regions (Table 1) varied ($P \leq 0.01$ Appendix Table 8). Most of the sperm (49 X 10^6) recovered prior to slaughter were present in a combination of discharged mucus and lavaged mucus (range 26 X 10^6 to 66 X 10^6 Appendix Table 6). Of the sperm remaining in the reproductive tract after slaughter, the greatest numbers were recovered from the uterus, vagina and cervix. Numbers were of similar magnitude and ranged widely from 2.6 X 10^4 to 2.5 X 10^6, 6.4 X 10^4 to 4.6 X 10^6 and 1.3 X 10^4 to 3.3 X 10^6 for the uterus, vagina and cervix, respectively. The least amount of inseminated sperm was recovered from the oviducts which did not differ statistically from sperm numbers in the cervix. The oviduct values also varied considerably from 2.9 x 10^3 to 1.3 X 10^5 sperm.

Percentage of inseminated sperm distributed throughout the female reproductive tract (Table 2) differed between the recovered mucus and tract regions ($P \leq 0.01$ Appendix Table 9). Of the inseminate, 68% was available from the discharged mucus and vagina before slaughter. Although the actual percentages of sperm recovered from the uterus, vagina and cervix varied, they were not different from each other. The oviduct was similar in magnitude to the cervix however it differed statistically from the uterus and vagina. All were less than the percent of the inseminate recovered from the vagina prior to slaughter and in

Table 1. Sperm numbers recovered from the cow reproductive tract 12 hours after artificial insemination

Tract location	Sperm numbers (x 10^6)	Observations	S.E.M.(x 10^6)
Inseminate	72.6	17	± 0.930
Recovered* vagina	49.3[a]	14	± 3.20
Vagina	0.770[b]	16	± 0.280
Cervix	0.341[bc]	17	± 0.190
Uterus	0.971[b]	17	± 0.160
Oviduct	0.052[c]	16	± 0.0093

[a,b,c]Means in a column with no superscript in common differ from other means by two standard errors.

*Spermatozoa recovered from discharged mucus and the vagina prior to slaughter.

Table 2. Percentage inseminated sperm recovered from the cow reproductive tract 12 hours after artificial insemination

Tract location	% Inseminated sperm	Observations	S.E.M.(%)
Recovered* vagina	68.4[a]	14	± 4.28
Vagina	1.07[b]	16	± 0.392
Cervix	0.470[bc]	17	± 0.262
Uterus	1.35[b]	17	± 0.235
Oviduct	0.071[c]	16	± 0.013

[a,b]Means in a column with no superscript in common differ from other means by two standard errors.

*Spermatozoa recovered from discharged mucus and the vagina prior to slaughter.

Table 3. Partial correlations between the transformed sperm numbers of the inseminate recovered from the various cow reproductive tract regions

	Vaginal discharge[b]	Not discharged from the vagina[c]	Vagina	Cervix	Uterus	Oviduct
Recovered[a] vagina	-.06	-.33	-.37	-.16	-.27	-.34
Vaginal discharged		-.71*	-.30	-.05	-.35	.15
Not discharged from the vagina			.16	.06	.15	-.007
Vagina				.08	.27	.12
Cervix					.28	.32
Uterus						.08

n=13-17.
*$P \leq 0.01$.
[a]Sperm recovered prior to slaughter in discharged mucus and from the vagina.
[b]Sperm recovered via mucus drainage over 12 h.
[c]Sperm not discharged by the cow prior to slaughter (includes sperm recovered prior to slaughter).

discharged mucus. Ranges of values for these tract regions are shown in Appendix Table 7.

Partial correlations were used to examine the potential relationships between the numbers of inseminated sperm recovered in discharged mucus alone or including sperm lavaged from the vagina prior to slaughter with sperm numbers found in the isolated tract regions (Table 3). An inverse correlation existed between the proportion of sperm in the discharged mucus after insemination and sperm not discharged from the vagina which includes sperm flushed from the vagina prior to and after slaughter. Also, there were no apparent relationships among other tract regions for numbers of sperm recovered.

Discussion

When comparing our study with previous research (Dobrowolski & Hafez, 1970; El-Bana & Hafez, 1970; Suga & Higaki, 1971; Larsson & Larsson, 1985; Mitchell et al., 1987) it is important to be mindful of the aspects of such experiments that are less relevant to the sperm distribution within the tract and discharge from the tract after insemination of normal doses of cryopreserved sperm. Previous studies utilized fresh semen and inseminated 10 to 50 times more sperm than that which is typical of artificial insemination. At most, the sperm numbers we used were 2 to 4 times that of a normal dose of semen. Mitchell et al. (1985) directed their research at the retention and

discharge of a highly abnormal sperm population. By terminating the cow 12 h after insemination, sufficient time was allocated in our study for the establishment of sperm reservoirs within selective tract regions that are necessary for fertilization (Wilmut & Hunter, 1984). Previous work terminated experiments five or less hours post insemination (Larsson & Larsson, 1985; Suga & Higaki, 1971; Dobrowolski & Hafez, 1970) which did not accurately represent the status of sperm populations within the tract near the time of ovulation.

Our overall recovery of 72% of the inseminated sperm was similar to an earlier study by Mitchell et al. (1985). A number of possibilities exist for our inability to account for 28% of the inseminated sperm. Although urine expelled between insemination and slaughter was saved, a large precipitate of silica and struvite crystals made sperm quantification after centrifugal concentration impossible. Silage was the principle source of these urine contaminates. Separation of urine from discharged mucus was at least as good as in the work of Mitchell et al. (1985) who found minor numbers of sperm in the urine (0.06% of $.42 \times 10^9$ inseminated sperm).

Most sperm are probably lost in the laboratory due to inadequate flushing procedures. Mattner (1968) found more than one half of the sperm remained in the crypts and folds of the cervix after flushing. Sperm have also been found in the lining of the reproductive tract of the cow but not in

large quantities (Larsson & Larsson, 1985). Techniques that would improve recovery of sperm from mucosal surfaces probably need further development.

Phagocytosis by epithelial cells and leukocytes also contributes to the loss of spermatozoa from the reproductive tract. The crypts and folds of the cervix afford protection of the spermatozoa from attack by leukocytes and the leukocytic response is less in the oviducts than in the uterus (Hafez, 1973). However, although some sperm are lost to phagocytosis (Mitchell et al., 1985; Hawk, 1983), leukocytes are not a major cause of sperm loss because the leukocytic invasion does not begin until 4 to 8 h after insemination.

Another potential loss or alteration of sperm distribution, or both, was immediately after stunning prior to the isolation of the tract regions. The potential redistribution at this time has been demonstrated in the rabbit by Overstreet et al. (1978). However, manipulation and isolation of tract regions was accomplished in 6 to 9 min, about one-fourth of the time required by Mitchell et al. (1985).

The variations in the sperm concentration procedures of Mitchell et al. (1985) were modifications in G-force, centrifuge tube composition and pH of the centrifuged fluid. These changes were found to improve recovery. As an additional modification, in difference from previous studies (Mitchell et al., 1985; Larsson & Larsson, 1985; Suga &

Higaki, 1971; El-Bana & Hafez, 1970; Dobrowolski & Hafez, 1970), the proportion of sperm lost due to centrifugal concentration techniques was determined. With such as a correction factor, at least the efficiency of the sperm concentration steps could be accounted for.

Of the inseminated sperm which can be accounted for, 95% were either discharged or recovered from the vagina of the cow within 12 h after insemination. While El-Bana & Hafez (1970) found 81% of the sperm recovered to be in the vagina, Larsson & Larsson (1985) and Mitchell et al. (1985) accounted for 90% and 95% respectively, of the total recovered sperm, in the vagina and cervix. Of the sperm remaining in the reproductive tract and recovered after slaughter, the total recovery of sperm amounted to 3% of the inseminate. This percentage is lower than that reported by Mitchell et al. (1985) who found 6.5% of the inseminate in the tract after slaughter. This may be due to their insemination of 420 X 10^6 sperm compared to our use of 75 X 10^6 sperm. The rather progressive decline in cryopreserved sperm numbers recovered in tract regions anterior to the cervix in the present study was also common in previously reported research using fresh semen, (Mitchell et al., 1985; Larsson & Larsson, 1985; Suga & Higaki, 1971; El-Bana & Hafez, 1970; Dobrowolski & Hafez, 1970; Mattner, 1968). Of the sperm remaining in the reproductive after slaughter, the uterus contained the highest percentage. The oviduct, however, was considerably lower at 2.4% of the sperm

recovered. Thus, a significant proportion of the inseminated cryopreserved sperm deposited in the uterine body are lost to the exterior.

Predictable relationships between the proportion of sperm in various tract regions and discharged mucus have been observed by other investigators. Hawk & Conley (1975) found a positive relationship between sperm numbers in the anterior cervix at 2 h after mating and sperm numbers in the oviduct at 22 h in the ewe. Although such was not apparent in our study, there was a significant negative relationship between sperm numbers discharged and sperm remaining in the vagina. This agrees with Pursel (1982) and Hawk & Conley (1979) who found that decreasing sperm numbers discharged resulted in increased sperm numbers in the regions of the reproductive tract. Mitchell et al. (1985) found significant positive correlations for sperm numbers between the vagina with the uterus and the vagina with the oviduct. However, in their study, they compared only those sperm which could be recovered from the vagina after slaughter with other tract regions. Based on these correlations, Mitchell et al. (1985) concluded that the vagina acts as a sperm reservoir to the oviduct. The correlations demonstrated by Mitchell et al. (1985) did not occur in our study. This may be due to extreme variation in sperm numbers recovered in the discharged mucus which has the reciprocal effect of increasing the variation of sperm numbers not discharged from the vagina. This variation

developed as some cows were not recumbent for sufficient time to allow mucus discharge after insemination.

The percentage of sperm discharged from the cow soon after insemination may not be related to cryopreservation of the semen as has been indicated in the pig. In the gilt, more sperm were recovered from the oviducts and uterus following insemination with fresh semen than in those gilts inseminated with frozen semen (Pursel et al., 1978). However, in our experiment the proportion of cryopreserved sperm discharged were not different from the numbers of fresh as well as abnormal sperm discharged in an earlier study (Mitchell et al., 1985). Previous studies have also used 10 to 50 times more the typical numbers of sperm used in artificial insemination (Mitchell et al., 1985; Larsson & Larsson, 1985; Suga & Higaki, 1971; El-Bana & Hafez, 1970) and recovered similar numbers in the discharged sperm and within the tract regions as were recovered in this study. Therefore, sperm discharge and distribution appears to be independent of cryopreservation and numbers of sperm inseminated in the bovine.

In other species such as the pig (Pursel, 1982), ewe (Hawk & Cooper, 1984) and rabbit (Hawk & Cooper, 1979), when retrograde loss was reduced more ovum were fertilized. Hawk & Cooper (1984) increased by more than two times the number of eggs fertilized when more sperm were retained within the reproductive tract of the ewe. A greater percentage of ova fertilized were recovered when the uterine horn was ligated

and cryopreserved sperm were retained within the uterus of the sow (Pursel, 1982). Cryopreservation of sperm normally depresses fertilization rates in the sow as a much greater proportion of these sperm are discharged compared to fresh semen (Pursel et al., 1978). Considering that much of the inseminated sperm were discharged soon after insemination in the cow, it is also possible that increased retention could improve fertilization rates.

In other species various hormones have been used to increase sperm retention. Hawk & Cooper (1979) found that the injection of $PGF_{2\alpha}$ in the rabbit immediately before insemination, increased by at least three fold the number of sperm recovered from the oviducts, uterus, cervix and vagina as well as the total number recovered from the tract. In addition, this increased retention caused increased fertilization rates. Prostaglandin E_1 (Mandl, 1972), oestradiol (Hawk & Cooper, 1975, 1976, 1978) and oxytocin (Morton & Fitzpatrick, 1974), have all been found to increase sperm retention. However, a limited dose range was effective.

Certain drugs, which are smooth muscle stimulants, have been used successfully in other species to increase sperm transport. Phenylephrine increased sperm numbers in the oviduct by 50 fold in the rabbit (Hawk et al., 1982). Sperm numbers were elevated 10 fold in the oviducts and uterus of the doe rabbit after administration of ergonovine (Hawk et al., 1982). Carbocholine enhanced sperm numbers in the

oviduct and increased conception rate in the gilt (Baker et al., 1968).

Clitoral stimulation might affect fertilization rate through improved sperm transport perhaps by uterine contractions (Cooper et al., 1985) or the timing of ovulation (Hawk, 1987). However, Foote et al. (1984), reported that while clitoral massage caused an immediate uterine contraction it does not cause a release of oxytocin to further stimulate contractions. In addition, this technique was not found to have any effect on the non-return rate of dairy cows and heifers. However, in the rabbit Bedford (1971) found a second coital stimulation to be beneficial for increasing sperm numbers available for fertilization.

Hawk (1983) stated that insufficient sperm transport to the site of fertilization may be the major cause of fertilization failure in farm animals; however, nothing is known about the site or cause of apparent sperm transport problems in cattle. Since heifers have a relatively high fertilization rate (Gustafsson, 1985; Bearden et al., 1956; Boyd et al., 1969; Kidder et al., 1954), it would appear that pregnancy and parturition are situations that reduce the efficiency of sperm transport and retention. Substantial discharge of sperm occurs within the first 12 h after insemination even with a relatively normal dose of cryopreserved sperm. Thus, even after normal artificial insemination, sperm are available in the discharged mucus

for transport to the oviduct to improve fertilization rates. Reducing retrograde discharge of sperm may be beneficial to the reproductive performance of artificially inseminated dairy cattle.

CHAPTER IV

ADDENDUM TO THE DISCUSSION

Sperm numbers recovered from each region of the reproductive tract may be less than actual due to inefficiencies in the recovery technique as 28% of inseminated sperm were not accounted for. Mattner (1968) found more than one-half of the sperm remained in the crypts and folds of the cervix after flushing in cattle. Even with the best possible methods of recovery, sperm also were lost to glassware and centrifugation steps. In addition, an attempt was made to evaluate the effectiveness of tract flushing procedures with oestrous phase reproductive tracts in the laboratory. However, sperm recovery after insemination at the various tract regions was much less than from in vivo inseminated cows. Apparently sperm were absorbed to the mucosal surface and less available for recovery from dead tracts. Sperm were perhaps better incorporated into the mucus and fluids of live tracts than in dead tracts. Recovery of sperm was better in live tract due to the fact that the fluids and mucus were more readily flushed from the tract. Knowledge of recovery efficiency from the tract would improve the quality of sperm transport research.

In our study, as well as others (Mitchell et al., 1985; Larsson & Larsson, 1985) a very low percentage of the sperm that remain in the tract are actually found in the oviduct. This percentage, however, may not give an accurate indication of the number of sperm reaching the oviduct as this number could represent sperm in transit (El-Bana & Hafez, 1970). Therefore, this concept supports the theory that the caudal isthmus serves as a sperm reservoir (Wilmut & Hunter, 1984; Hunter & Barwise, 1982). If these sperm are in transit, then a small percentage pass though the oviduct and into the peritoneal cavity (Larsson, 1986) and are unavailable.

Hormonal stimulation has had varied effects on sperm transport. Oestradiol has been found to be deleterious to the development of adequate sperm numbers in the cervix of the ewe (Croker et al., 1975). However, this effect was dose related. In an experiment using 30µg compared to 90µg at 1, 12, 24 h in each region of the cervix, fewer spermatozoa were recovered from ewes treated with 30µg than those treated with 90µg. The higher dose of oestrogen may have facilitated passage of spermatozoa through the cervix. Oxytocin has also been deleterious to sperm transport in the ewe in some experiments (Lightfoot et al., 1971). However, Winterberger-Torres (1967) doubted whether the endogenous oxytocin secreted at the time of mating has any action at all since oxytocin is destroyed quickly by the female system. Foote et al. (1984) studied the possibility of an

oxytocin release due to clitoral stimulation. These investigators did find clitoral stimulation to cause an immediate uterine contraction; however, this did not cause a release of oxytocin.

Sperm transport in superovulation may be affected by the preparation of FSH used. Donaldson et al. (1986) removed the LH from the commercial FSH preparation and found this purified FSH to increase fertilization rates from 52% to 74%. It appears that the LH in the FSH preparations may stimulate progesterone secretion and consequently affect sperm survival or transport in the reproductive tract of cattle. The influence of endocrine imbalances on sperm transport could be at least part of the explanation for less than optimal fertilization rates in dairy cows.

Time of insemination may also affect the numbers of spermatozoa transported and retained within the reproductive tract. Mattner & Braden (1969) found that insemination late in oestrous resulted in fewer spermatozoa in the cervix, uterus, and oviducts and consequently a lower fertilization rate in the ewe. This reduction may be due to changes which occur in the origin and direction of uterine contractions. Early in oestrus the majority of the contractions originate in the cervix or posterior segments of the uterus and move anteriorly, whereas late in oestrus, the majority of contractions originate near the uterotubal junction and move posteriorly. If this is true in the cow, approaches developed to improve sperm retention may be

directed at preventing this directional change or increasing the magnitude of the contractions earlier after insemination. Not only would fertilization rates be improved, but the period of time during oestrous when insemination is most effective may be increased.

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APPENDICES

APPENDIX A

OUTLINE AND RESULTS OF PRELIMINARY RECOVERY EXPERIMENT

Table 4. Effect of pH, tube composition, NaOH and alpha amylase on the recovery of spermatozoa in the laboratory

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Objective: To determine if centrifuge tube composition and if the medium to liquify oestrous mucus samples affected sperm recovery in the laboratory.

Protocol:

I. Five samples were done in duplicate.

II. Each sample was centrifuged at 1500 X g for 20 min.

III. The first sample consisted of 8 ml cow cervical mucus liquified with 32 ml NaOH. These samples were neutralized to a pH of 7 and inseminated with 10 X 10^6 sperm and centrifuged in a polypropylene tube.

IV. The second sample was identical to the first however it was centrifuged in a polycarbonate tube.

V. The third sample consisted of 8 ml of mucus liquified with 32 ml NaOH however the pH was allowed to remain at 12. This sample was inseminated with 10 X 10^6 sperm and centrifuged in a polypropylene tube.

VI. The fourth sample consisted of 8 ml cow cervical mucus liquified with 32 ml 100 mg% alpha amylase at a pH 7. This sample was inseminated with 10 X 10^6 sperm and centrifuged in a polycarbonate tube.

VII. The fifth sample consisted of the same components as the fourth however this sample was centrifuged in a polypropylene tube.

VIII At the end of centrifugation, the supernate of each sample was drawn down to 2 ml and sperm numbers quantified by use of a hemacytometer.

IX. Quantification was done by the same 2 observers throughout the experiment. The mean of the observations from each observer was used as the actual percentage recovery.

X. It was noted that complete liquification by alpha amylase was difficult and resulted in clumping of sperm.

Table 4. (Continued)

XI. An aliquot of inseminated semen was saved in order to accurately predict the numbers of sperm each sample was inseminated with.

XII. Results indicate the NaOH at pH 7 in polycarbonate tubes yielded the highest recovery. This was used to determine other aspects of an optimal sperm recovery system in the laboratory.

XIII Results are presented in Table 5.

Table 5. Effect of pH, tube composition, NaOH and α amylase on the recovery of spermatozoa in the laboratory

Solubilizing agent	pH	Tube composition*	Mean % recovered**	S.D.
NaOH	7	PC	95.10	± 6.93
NaOH	7	PP	77.20	±17.68
NaOH	12	PP	72.60	± 0.56
α-amylase	7	PC	86.05	± 1.48
α-amylase	7	PP	50.40	±26.02

* PP=polypropylene.
PC=polycarbonate.

**Mean percentage of the duplicates.

APPENDIX B

STATISTICAL TABLES

Table 6. Range of sperm numbers recovered from the various tract regions

		$(x10^6)$			
Region	n	Minimum	Maximum	Mean	S.D.
Inseminate	17	67.0	85.2	72.6	± 3.84
Total recovery	13	28.0	67.1	52.8	±11.1
Recovered* vagina	14	26.2	66.4	49.3	±12.0
Vaginal** discharged	14	0.004	65.7	37.3	±20.2
Vagina	16	0.065	4.64	0.77	± 1.12
Cervix	17	0.013	3.26	0.34	± 0.78
Uterus	17	0.026	2.51	0.971	± 0.670
Oviducts	16	0.0029	0.133	0.052	± 0.037

* Sperm recovered in the discharged mucus and from the vagina prior to slaughter.

**Sperm in discharged mucus.

Table 7. Range of percentages of the inseminated sperm numbers recovered from the various tract regions

		-----------------%			
Region	n	Minimum	Maximum	Mean	S.D.
Total recovery	13	40	92	72	±15
Recovered* vagina	14	37	91	68	±16
Vaginal** discharged	14	0.01	91	51	±27
Vagina	16	0.09	6.5	1.1	±1.6
Cervix	17	0.02	4.5	0.5	±1.1
Uterus	17	0.04	3.7	1.35	±1.0
Oviduct	16	0.004	0.185	0.071	±0.052

* Sperm recovered in the discharged mucus and from the vagina prior to slaughter.

**Sperm in discharged mucus.

Table 8. Analysis of variance of sperm numbers recovered from each tract region[a]

Source	Degrees of freedom	Mean square	F value
Cow	16	0.33503	1.61*
Location[b]	4	20.31417	97.40**
Error	59	0.20856	

* P≤0.10.

**P≤0.01.

[a]Statistical analysis was performed on transformed (log 10) values.

[b]Location of recovered sperm within the tract.

Table 9. Analysis of variance for the percentage sperm in the inseminate recovered from each tract region[a]

Source	Degrees of freedom	Mean square	F value
Cow	16	0.00528	0.72
Location[b]	4	2.29285	312.82*
Error	59	0.00733	

*$P \leq 0.01$.

[a]Statistical analysis was performed on transformed (arc sine square root) values.

[b]Location of recovered sperm within the reproductive tract of the cow.

APPENDIX C

OUTLINE OF THE EXPERIMENTAL PROCEDURE FOR SPERM RECOVERY COWS

Table 10. Outline of the experimental procedure for sperm recovery from cows.

I. Prior to and at the Onset of Estrus

A. Cows were accustomed to a halter and long term restraint to reduce stress.

B. Cows were palpated prior to injection of $PGF_{2\alpha}$ for reproductive soundness and presence of a responsive corpora lutea.

C. Candidates were injected 60 h prior to the time needed for the onset of oestrus.

** Careful observation was employed to insure that the onset of oestrus was accurately detected.**

D. Prepare barn and laboratory.

II. Prior to Insemination

A. Preparation of the Cow

1. The cow was induced to urinate.

2. The rectum was cleaned of feces.

3. The hair was trimmed on and around the vulva.

4. The vulva cleaned thoroughly with betadine scrub.

5. The tail was restrained with cotton twine.

6. Fly dust was applied if necessary.

B. Preparation of Semen and Insemination

** Prior to insemination of the cow the actual volume of semen deposited by the A.I. gun should be known. In the laboratory the volumetric discharge was checked using the balance. The straws were then marked to indicate the correct volume.**

1. Two straws of semen were thawed and pooled.

Table 10. (Continued)

2. The marked straw was loaded with the correct volume of semen.

3. The additional quantity of that semen was saved for quantification to determine actual sperm numbers inseminated.

4. The same experienced inseminator was used for each insemination.

5. The semen was inseminated in the uterine body.

III. After Insemination

A. The vulva was thoroughly washed and dried.

B. The vulva was carefully wiped with the adhesive solvent to remove skin oil residues.

C. A latex funnel was applied with 3-M industrial adhesive (#4799).

1. The funnel was constructed using latex artificial vagina liners and adhering them together in the shape of a funnel with rubber cement.

2. The funnel was secured by applying the adhesive to the outside rim of the funnel and labia of the vulva and sealing all spaces.

E. The adhesive was allowed to dry thoroughly.

IV. Collection of Discharged Mucus and Urine

A. The funnel was rinsed often with distilled water or sodium citrate (2.9%) to avoid contamination of the discharged mucus.

B. In between rinsing, the funnel was closed with a paper clip to avoid dripping.

C. All urine was saved. One to four liters of urine could be discharged at a time.

D. When the cow laid down:

1. An unbreakable beaker was placed under the funnel.

Table 10. (Continued)

2. Particular care was taken to avoid any any contamination at this time.

V. Prior to Slaughter

A. The cow was prepared for flushing the vagina 30 min prior to slaughter.

1. The cow was induced to urinate.

2. The rectum was cleaned of remaining feces.

3. The funnel and rectum were washed and dried.

4. Two liters of 2.9% sodium citrate were warmed.

5. A latex extension was attached on the funnel to facilitate collection of the flushing.

B. The vagina of cow was flushed with one liter of 2.9% sodium citrate.

1. This flushing was kept separate from the sample collected over the previous 12 h..

2. The use of a stomach tube calf feeder with a lateral opening in the last 4 cm of the tube facilitated flushing.

C. Just prior to stunning the cow was induced to urinate to avoid contamination of the reproductive tract.

VI. After Stunning

A. The cow was elevated to a horizontal position for exsanguation.

B. After exsanguation the hindquarters were lifted to a 30 to 45 degree angle with the floor.

1. Steps A & B were to reduce the gravity and visceral displacement induction of sperm redistribution in an anterior direction from the vagina towards the oviduct.

Table 10. (Continued)

C. Through a midventral incision the tract regions were isolated.

1. A ligature was placed at the urethra.

2. Hemostats were placed at the infundibulum and 1/2 cm posterior to the utero-tubal junction.

3. A ligature was placed at the uterine body.

D. At the same time, the vulva was closed with a cyanoacrylic adhesive and surgical staples.

E. During evisceration the reproductive tract was carefully separated from the rectum.

VII. Sperm Recovery

A. The reproductive tract was carefully trimmed of extra tissue.

B. A second hemostat was placed on the uterine side of the utero-tubal junction hemostat and the oviducts were then removed.

1. Each oviduct including the uterotubal junction was flushed from the ostium with 30 ml of 2.9% sodium citrate (3 X 10 ml) with a blunt edge 3 cm 16 gage needle and a 10 cc syringe. The samples from both oviducts were then pooled.

C. The vagina was flushed with 360 ml (3 X 120 ml) of 2.9% sodium citrate. These samples were pooled.

D. The cervix was irrigated with 180 ml (3 X 60 ml) of 2.9% sodium citrate with a blunt edge 3 cm 16 gage needle and a 60 cc syringe.

E. A foley catheter was introduced through the lumen of the cervix and into a uterine horn. Each horn was then flushed through the anterior tip of the horn with 240 ml (2 X 120 ml) of 2.9% sodium citrate. An additional 120 ml of sodium citrate was passed through the uterine body. These samples were all pooled.

Table 10. (Continued)

F. The vagina, cervix, uterus were cut to expose the mucosa which was covered with 50 ml 1/2 N NaOH and allowed to soak for 30 min after which time the liquified mucus was aspirated. These samples were each kept separate from their respective flushings.

G. Each sample's volume was increased by 50% with 1 N NaOH and stored at 5C for 24 h to allow complete liquification of the mucus.

VIII. Sperm Recovery

A. Prior to centrifugation each sample was neutralized with 1 N HCL.

B. The samples were centrifuged in polycarbonate tubes for 20 minutes at 1500 X g.

C. After centrifugation the supernate was disguarded.

D. Additional dilute sample was added to the tube along with the previously concentrated pellet of sperm.

E. When the sample beaker was empty, it was thoroughly rinsed with distilled water and the surface cleaned with a rubber policeman.

F. The same procedure as (E.) was followed when eliminating tubes in the centrifugation series.

G. A constant number of initial tubes were used for concentration of each sample throughout the experiment:

Sample	50ml Tubes Used
Vagina	10
Uterus	8
Cervix	5
Oviduct	3
Discharged Mucus	10
Lavaged Mucus	10
Vaginal Mucosal Recovery	2
Uterine Mucosal Recovery	2
Post Slaughter Rinsing of Funnel	2

Table 10. (Continued)

H. At the final centrifugation step for each sample, a 15 ml polypropylene screw top conical tube was used. After centrifugation the supernate was drawn off to a volume which best allowed for accurate quantification by use of a hemacytometer.

I. The samples were stored in the 15 ml polypropylene tubes at 5 C until quantification was completed.

IX. Quantification

A. The same two observers were used throughout the experiment.

B. Each sample was warmed to room temperature and vortexed thoroughly prior to counting.

C. A 0.1 mm (red blood cell) or 0.2 mm deep (white blood cell) hemacytometer were used to determine spermatozoa concentration.

D. If dilution was necessary for counting, this was done with distilled water.

E. The inseminate sample was diluted into 2 samples at a 1:50 dilution. The average of these 2 samples was used to determine the actual total number of inseminated sperm following correction for insemination volume.

F. After quantification by both observers the samples were frozen.

VITA 2

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