EFFECT OF HIGH AND LOW DOSAGE OF FRESH AND FROZEN SEMEN ON ACCESSORY SPERM NUMBER, FERTILITY AND EMBRYO QUALITY IN ARTIFICIALLY INSEMINATED CATTLE

by

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(ABSTRACT)

This study was designed to :1) Determine the effects of fresh vs frozen semen at a high inseminate dosage ($100x10^6$sperm) contrasted to their effects at a conventional dosage ($20x10^6$ sperm) on accessory sperm per ovum and 2) Evaluate the relationship between accessory sperm number per embryo/ovum and fertilization status/embryo quality if accessory sperm number were affected by treatment. In this study semen from four bulls routinely giving a minimum of 70% morphologically normal and 60% motile sperm cells were used. Ejaculates of these bulls were split and prepared for use as fresh and frozen semen at either $100x10^6$ or $20x10^6$ cells per dose in .5 mL French straws. Half of the total semen filled straws were frozen in liquid nitrogen at -196°C and half were stored at 5°C for 4 days after collection and used as unfrozen. Cows in standing heat were inseminated with fresh or frozen semen at either high ($100x10^6$ sperm) or conventional dose ($20x10^6$ sperm). Ova/embryos were recovered non surgically on day 6 after breeding. Accessory sperm were counted in the recovered embryos/ova after partial digestion with Pronase followed by compression of the embryo/ovum with a cover slip. From 129 inseminations to normally cycling cows, 98 embryos/ova were recovered. To reduce male effects, embryos/ova used were randomly balanced across treatments, by ejaculate within bull for evaluation of frozen vs fresh semen ($n = 80$) and by bull for evaluation of high vs low dosage treatments ($n = 76$). No difference ($P > 0.05$) in accessory sperm was observed for fresh vs frozen semen at either the high or low dosage. The mean accessory sperm values for fresh high dose ($n = 21$), frozen high dose ($n = 21$), fresh low dose ($n = 19$), and frozen low dose ($n = 19$) were 26.81 ± 30.23 (SD), 36.05 ± 44.74 (SD), 29.37 ± 55.97 (SD) and

30.16± 70.18 (SD) respectively. When data for embryos/ova resulting from fresh and frozen semen were pooled within dosage, a significant difference was observed between the median accessory sperm values for high and low doses of semen ($P < .05$). Mean ± SD and median values for accessory sperm were: 37.8± 38.3 and 27.5; 28.9± 62.8 and 3.0, for the high and low dose, respectively. Increasing accessory sperm number by the higher dosage improved the fertilization status/embryo quality ($P < .05$). Percentage unfertilized ova, degenerate embryos and embryos classified poor to fair and good to excellent were: 3, 5, 24, 68; and 21, 16, 18, 45, for the high and low dose, respectively. Overall, embryos/ova classified good to excellent, poor to fair, degenerate and unfertilized had median accessory sperm values of 18, 9.5, 5.5 and 0, respectively. However, the lack of accessory sperm in unfertilized ova was significantly different from excellent-good quality embryos ($P < .05$).

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Introduction

It is commonly known that one major cause of fertilization failure is the lack of a sufficient number of sperm cells at the site of fertilization. Studies have shown that a large population of sperm is lost exteriorly due to retrograde flow from the genital tract of a cow after artificial or natural breeding, while some are phagocytized by leukocytes or move into the peritoneum. Also, the inseminate is divided, as each part goes into one of the uterine horns, further reducing the number of sperm cells at the site of fertilization in monotocous species such as cattle.

It has been reported that the population of sperm which actively colonize the lower oviductal isthmus under the sustained phase of sperm transport provides the fertilizing sperm (Hunter et al., 1982, Hunter and Nichol, 1983; Hunter and Wilmut, 1984). It is also clear that viability and morphology of this population are improved relative to the inseminate (Overstreet et al., 1978; Larsson and Larsson, 1986; Mitchell et al., 1985). Thus, for successful fertilization, an inseminate containing high quality sperm (motile and morphologically normal) is necessary.

Freezing of semen has been shown to adversely affect sperm motility (Saacke, 1982) and sperm transport as well as retention in all segments of the female reproductive tract in cattle (Lineweaver et al., 1970), sheep (Mattner, 1969) and swine (Pursel et al., 1978).

In addition to the quality of semen used, another major factor affecting fertilization is the number of sperm in the inseminate which also affects the number of sperm reaching the site of fertilization (Chen et al., 1989). A positive correlation has been shown between sperm numbers in the inseminate and sperm numbers retained in various compartments of the reproductive tract post insemination (Morton and Glover, 1974). By using very high numbers of sperm per inseminate in pigs (Weitze et al., 1988) and cattle (Hawk et al., 1988), numbers of sperm which gained access to the eggs and fertilization rates were improved when compared to that attained using normal sperm dosage used in artificial insemination of these species.

Studies have shown a positive correlation between the accessory sperm number and fertilization rate (Hunter and Wilmut, 1983; Weitze et al., 1988). Dejarnette et al. (1992) have also shown that accessory sperm number is positively correlated to both fertilization rate and embryo quality when judged 6 d post insemination.

Unsuccessful attempts have been made to increase accessory sperm number in ewes (Hawk and Conley, 1971) and cows (DeJarnette et al., 1992) by blockage of retrograde flow of sperm from the genital tract post breeding as well as increasing the sperm dosage from $20x10^6$ to $40x10^6$ sperm (Dejarnette et al., 1992).

The present experiment represents another effort to increase accessory sperm number. The experiment was designed to determine if very high doses of either fresh or frozen semen ($100x10^6$ vs $20x10^6$sperm) could increase accessory sperm number and, if so, influence fertilization status and embryo quality as judged on d 6 following insemination.

Experimental Rationale

Evidence exists which shows that fertilization failure frequently occurs due to insufficient availability of sperm at the site of fertilization. Accessory sperm have been shown to be related to the number of sperm at the site of fertilization or available for penetration of the egg. Also, low accessory sperm numbers have been correlated to poorer embryo quality which could translate into lower reproductive efficiency. Experiments have been conducted to increase the number of accessory sperm in several species by blocking the retrograde flow of sperm out of the tract or by increasing sperm concentration in the inseminate. However, initial efforts have not been successful with cattle. The current experiment was designed to push the limits, by more sharply increasing the number and quality of sperm, comparing fresh and frozen semen at very high dosage (100 $x10^6$ sperm) and at conventional dosage ($20x10^6$ sperm). This range of treatments should be sufficiently broad to determine the value of accessory sperm in evaluation of bovine reproductive capability using artificial insemination. A lack of an effect here would seriously question the value of accessory sperm in assessing the value of quality or quantity of inseminates used in artificial breeding.

Literature Review

Sperm Reservoirs in the Female Genital Tract

Although many millions of spermatozoa are deposited in the reproductive tract of the female during breeding, very few reach the site of fertilization. Many spermatozoa are retained or inhibited at selective points, e.g., cervix, uterotubal junction and isthmus of the oviduct. Thus, each segment of the reproductive tract may not only act as a reservoir of sperm for the next segment (Morton and Glover, 1974) but also a selective barrier for progression of fertile sperm.

Cervix

The cervix is a sphincter like structure that lies between the vagina and body of the uterus. The structure of the cervix differs in detail among farm animals. For the cow, Mullins and Saacke (1989) have provided the most recent information, showing the cervix of the dairy cow to consist of longitudinal crypts and folds that extend from the external cervical os over the annular rings, to the cervico uterine junction. They further reported that in areas in the cervix where longitudinal

sections through a groove contained sagitally sectioned spermatozoa, the spermatozoa were frequently observed to be unidirectionally aligned close to the epithelial surface and travelling in opposition to the apparent direction of ciliary beat. El -Banna and Hafez (1970) reported that in heifers, following artificial insemination at the external os (with a known volume and concentration), a large quantity of mucus in the upper vagina often contained numerous spermatozoa. The percentage of total spermatozoa decreased with time in the vagina, cervix and uterus. They concluded that cells might be lost either to the outside by retrograde flow, or phagocytized by leukocytes or released into the peritoneum. The percentage of sperm recovered in this study was 81% from vagina, 13% from the cervix and 5.1% from the uterus at 16 h post insemination. In rabbits, Morton and Glover (1974) suggested that cervical mucus helped direct the sperm into the cervical crypts forming reservoirs.

Morton and Glover (1974) found maximum numbers of spermatozoa (approximately $10x10^6$) in the rabbit cervix 2 h after natural mating. This peak of sperm cells was followed by a gradual decline over the next 72 h. A similar kind of study was conducted by Hawk and Conley (1975) in which ewes were killed at 2 h and at 22 h after mating. The cervix was divided into three segments for the localization of sperm. At 2 h after mating the sperm were found in all segments but 22 h there was a decrease in the numbers of sperm in the posterior and middle segments of the cervix. Mattner (1973) reported a rapid decrease in cervical sperm between 4 and 24 h post mating in ewes, and Dobrowolski and Hafez (1970) recovered 3 times less sperm from the cervix at 8 h than at 1 h after breeding in heifers.

In addition, the cervix may act as a selective barrier against non viable or morphologically abnormal sperm because mucosal folds are very complex within the cervix. Experiments have shown that the cervix may act not only as a sperm reservoir (Morton and Glover, 1974) to prolong the life of sperm, but may also selectively retain viable sperm cells. Mattner and Braden (1963) reported that dead spermatozoa are rapidly eliminated from the cervix of the ewes and suggested that they were carried in the mucus as it passed into the vagina. Karabinus and Saacke (1987) found improvement in sperm population morphology after cervical passage in Holstein cows. Based on their results,

they suggested that this enrichment of normal and viable sperm may have occurred due to cervical passage, cervical filtration, differential survival of normal vs abnormal sperm or preferential elimination of sperm from the uterus by phagocytosis or retrograde removal. Thus, the role of the cervix as an active filter for sperm motility or flagellation appears real; however, filtration based on sperm morphology per se is still speculative.

Oviductal Isthmus

The area of transition between the oviduct and the uterine horn is commonly referred to as the uterotubal junction in bovine. It acts as a barrier against morphologically abnormal spermatozoa, especially against severely abnormal ones in the rat (Krzanowska, 1974). From the standpoint of sperm transport in most species the uterotubal junction and lower isthmus of the oviduct are considered together. Larsson and Larsson (1986), while studying the localization of spermatozoa within the oviducts of artificially inseminated cattle after synchronization of estrus with prostaglandin injection, recovered more sperm from the uterotubal junction of artificially inseminated cattle than from the ampullary isthmus junction at 2 and 12 h after artificial insemination. Eight animals were artificially inseminated by intra uterine deposition of frozen-thawed semen. The inseminated dose was either $20x10^6$ or $200x10^6$ sperm frozen in French mini straws. Four animals were inseminated at fixed time intervals (48 or 72 h) after Cloprostenol injection. The remaining animals were inseminated during spontaneous estrus. Recovery of sperm was reduced when low sperm numbers ($20x10^6$) were used and also when Cloprostenol was used to synchronize estrus. However, more sperm were recovered from the lower (uterotubal junction, isthmus and ampullary isthmus junction) than the upper (ampulla) parts of the oviducts after artificial insemination.

The oviductal isthmus is the narrow constricted portion of the oviduct proximal to the uterotubal junction. The fertilizing sperm migrate from the uterotubal junction into the isthmus, and have been found to remain stored in the isthmus until near ovulation. This phenomenon of sperm storage was observed in rabbit (Harper, 1973; Overstreet and Cooper, 1978), sheep (Hunter and Nichole, 1983) and cattle (Hunter and Wilmut, 1983). Harper (1973) transferred unfertilized rabbit oocytes to the oviduct of naturally mated recipients, or recipients artificially inseminated with 200 million sperm. He found that 40% of such eggs were penetrated in 1.5 h following transfer to mated recipients at 9 to 9.75 h after mating, 58 and 66% were penetrated during the same period if transfers were delayed until 13.75 to 14.25 and 19.5 to 20.0 h after mating, respectively. By 24 h after mating the percentage of eggs penetrated had fallen again to 43%. He also showed by ligation of the oviduct at the uterotubal junction that adequate sperm were in the oviduct by 9.25 to 9.75 h after insemination to ensure this enhanced fertilization following transfer at 14 or 17 h but not 20 h after insemination. Further experiments in which ligation was done at the ampullary-isthmic junction or the middle of the isthmus showed that sperm were retained in the proximal isthmus just above the uterotubal junction for several hours. When whole ovidcuts were fixed and serially sectioned and the number and distribution of sperm was determined, it was found that at 9 h after insemination few sperm were at the site of fertilization. In the oviduct of animals killed at 14 or 20 h after insemination, the sperm in the oviduct had become redistributed with many more found at the site of fertilization. He concluded that more eggs were penetrated in a short period of time (1.5) followed late transfers because there was the immediate possibility of egg and sperm meeting. Hunter and Wilmut (1983) found that a slow progression and displacement of viable spermatozoa occurred in the oviduct. These authors obtained highest fertilization results 12 h after estrus, indicating that a functional sperm reservoir at 12 h post estrus is in the oviductal isthmus rather than the cervix of the cow. Hunter (1981) and Hunter et al. (1982) concluded that 1 or 2 h in pig and 8 h in sheep is the time required to establish a sperm reservoir in the oviduct of these animals.

The optimum time for the establishment of a functional population of spermatozoa in the oviduct has been the subject of investigation in many species. Wilmut and 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. A mechanism in the rabbit may release sperm from the isthmus to the ampulla near the time of ovulation (Overstreet et al., 1978). Hunter et al. (1980) proposed that a population of spermatozoa competent to promote fertilization in the ewe was first found in the oviducts approximately 8 h after mating.

Sperm Transport in the Female Reproductive Tract After Breeding

It has been shown experimentally in cattle, by inseminating live or dead sperm or by natural breeding, that sperm traverse the female reproductive tract from the site of semen deposition to the site of fertilization rapidly in one phase (VanDemark and Moeller, 1951). Later, Overstreet and Cooper (1978) reported that in the rabbit sperm traverse the reproductive tract in two phases, i.e.,

1) Rapid phase of sperm transport and 2) Sustained phase of sperm transport.

Rapid Phase of Sperm Transport

During the rapid phase of sperm transport, which begins immediately after natural breeding or artificial insemination, sperm reach the oviduct within a few minutes. Sperm need not be alive to be transported rapidly to the oviducts. Mattner and Braden (1963) recovered killed sperm from oviducts of ewes within a few minutes after artificial insemination and First et al. (1968) recovered killed sperm from oviducts of sows 30 min after insemination in the uterus. Spermatozoa involved in the fertilization process are not likely to be included in these early arriving spermatozoa which

are more likely to be voided to the peritoneal cavity or phagocytized by the leukocytes. The increased contractile activity of the uterus and oviducts in response to breeding are the most likely to play a major role in this rapid phase of sperm transport.

Overstreet and Cooper (1978) conducted an experiment in which twenty New Zealand white does were mated with one of three stud bucks of proven fertility. The oviducts of five does were flushed for sperm recovery at 1 ,15, 90 min and 4 h after mating. Sperm morphology and viability was also studied. In one out of seven animals examined at 1 to 15 min post coitum, motile spermatozoa were recovered from the ampulla and of these, only 8% were motile. A median of 98% of sperm recovered from ampulla at 1 to 15 min post coitum had visible disruptions of the membranes over the acrosomal region of the head. Most spermatozoa were cleared to the peritoneal cavity between 15 min and 4 h post coitus. They concluded that the early arrival of these sperm cells might be due to uterine contraction. The same mechanism of sperm transport was studied in dairy cows by VanDemark and Moeller (1951). Eighteen cows were inseminated with motile and non motile spermatozoa deposited in the cervix during estrus. Non motile spermatozoa were recovered from the oviduct 4 min post insemination, suggesting that uterine activity may be involved. First et al. (1968) recovered spermatozoa from the oviducts of sows 30 min after insemination of live or dead spermatozoa and found non significant differences in number of sperm recovered from either the upper or lower isthmus. The role of uterine motility in the rapid phase of sperm transport was later studied by VanDemark and Hays (1952) in cows. Four cows which had completed one or more gestations were used during estrus, and again subjected to the same stimuli of mating at several stages of the estrous cycle and the uterine responses were recorded. By a water filled balloon placed in the uteri connected to a kymograph, records have been made showing uterine responses to mating. The presence of the bull, muzzling by the bull and non copulatory mounting and mounting followed by copulation and ejaculation all served as stimuli which resulted in increased uterine tone and uterine contraction within a few seconds. Copulation and ejaculation produced the greatest uterine response which was characterized by tetanic contractions. The uteri during estrus and post estrus responded similarly. These results suggested that uterine activity evoked by

mating probably plays a role in the rapid transport of spermatozoa in the cow. The involvement of uterine contraction in sperm transport was indirectly studied by Lightfoot and Restall (1971). They reported that relaxation of genital tracts of ewes after halothane anaesthesia caused a complete inhibition of cervical and uterine contractions. In this study, ewes were inseminated 5 min after establishing anaesthesia and then killed. The quantitative passage of spermatozoa to the uterus was depressed by halothane anaesthesia. Similarly, ovine uterine contractions involved in sperm transport were studied by Hawk (1970). Hawk observed uterine contractions to be moving towards the oviducts in controls ewes, but towards the cervix in ewes fitted with an intrauterine device. He concluded that lower fertility due to an intrauterine device might be explained by interference with sperm transport.

Sustained Phase of Sperm Transport

The sperm cells that take part in fertilization are derived from the sustained phase of sperm transport into the oviduct. Most of these sperm cells remain in the oviductal isthmus and a majority of them ascend to ampulla during the periovulatory period (Overstreet and Cooper, 1978).

After adequate reservoirs (cervix, uterotubal junction and lower isthmus) of spermatozoa are established within the selective points of the reproductive tract, the spermatozoa are released gradually from these points. This slow release involves the innate motility of spermatozoa and could involve the contractile activity of the musculature. Overstreet and Cooper (1978) localized spermatozoa in the reproductive tract of female rabbit at 1.5, 4, 6, 8, 10, 12, and 16 h post coitum and suggested that sperm transport in the female rabbit proceeded by a sequential build up of cells in ascending regions of the tract. The tubal isthmus rather than the uterotubal junction was shown to be the most important region restricting (controlling release of sperm cells) sperm ascent to the site of fertilization. Significant numbers of sperm did not accumulate in the isthmus until 4 h post coitum and, in this sustained phase, there was no migration of sperm beyond the isthmus until 6 h post

coitum. This transtubal migration is of limited duration and has ended by the time fertilization is completed. Hunter and Wilmut (1983) mated heifers with mature bulls. Following natural services, the uterotubal junctions of these heifers were ligated under local anaesthesia at 6, 8, or 12 h post mating. No ova were found to be fertilized following ligation at 6 h. Fertilization rate increased after ligation at 8 or 12 h after mating. These results suggested that fertilizing spermatozoa were present in the oviduct at 8 and 12 h after mating. Dobrowolski and Hafez (1970) compared the number of sperm cells recovered from the uterus of heifers slaughtered at 1, 8, and 24 h post insemination. They recovered maximum number of sperm from the uterus at 24 h. On the other hand, they recovered less sperm cells from the oviduct at 1 or 24 h compared to 8 h post insemination. It would appear that the number of spermatozoa retained in the oviducts increases gradually up to 8 h and then declines. El-Banna and Hafez (1970) killed New Zealand white rabbits at 1, 4, 8, 12, 20, 24, 36, 48 and 72 h after insemination. They reported maximum numbers of spermatozoa recovered from the oviducts ranged from 13,320 to 20,292 and occurred from 8 - 12 h, while the total number of sperm recovered from the whole tract decreased over the experimental period. After reaching a peak 12 h after insemination, the number of spermatozoa in the oviducts declined sharply.

Transport of Sperm in the Oviduct

The oviduct has the unique function of conveying spermatozoa and eggs in the opposite direction. Ciliary currents contribute to sperm movement in some mammals like pigs and rabbits which have pro ovarian ciliary beats but for others, such as cow and ewe they may function to transport the egg. In guinea pig and rat there is little ciliary function due to the scarcity of the cilia in these species (Gaddum-Rosse and Blandau, 1976). Cyclic variation in patterns of oviducal motility have been observed and compared with blood progesterone level (Bennett et al., 1988). These workers found

that luteal phase motility patterns were of low amplitude and frequency and the amplitude of motility increased 3-5 days before estrus (circulating progesterone level < 1.0 ng/mL plasma). In addition, Ruckebusch and Bayard (1975) recorded sporadic weak potentials of contraction near the uterotubal junction during diestrus in cows, while the amplitude and frequency increased progressively during proestrus. At estrus and metestrus, the activity became continuous and involved the middle portion of the oviduct in cows (Bennett et al., 1988).

Despite transporting eggs and sperm in opposite direction, the oviduct has the unique property of retaining sperm cells in the isthmus before ovulation. Sperm have been observed binding to the apical surface of the epithelium lining the oviduct. Suarez et al. (1991) studied the role of oviduct as a sperm reservoir. These workers observed the attachment of boar sperm within minutes to mucosal explants of isthmus and ampullary regions of gilt oviducts at the beginning of the incubation but after 24 h of incubation the number of oviductal bound sperm dropped significantly but the motility of the bound sperm did not. In addition to the binding of sperm to the epithelial surface, sperm were also trapped in a mucous material that floated in patches above some epithelial surfaces. These workers indicated that sperm sticking to ciliated cells and mucus could create a sperm reservoir in the isthmus.

Progression of ram spermatozoa within the sheep oviduct was studied in relation to the time of ovulation. Viable spermatozoa are apparently sequestered in the caudal isthmus for as long as 17 to 18 h and redistribution of spermatozoa occurred near ovulation. This phenomenon of transient storage of spermatozoa and then redistribution has been studied in sheep (Hunter and Nichol, 1983) where fertilization rate was zero when transection of the oviduct just above the uterotubal junctions was done up to 21 h after mating but the fertilization rate increased from 6.1 to almost 81.3 % when the transection time increased from 22 to 26 h after mating. Moreover, a high average of accessory sperm were recorded at 26 h (0-26). The most important finding in this study was that after traversing the cervix and uterus, viable ram spermatozoa did not progress onward through the oviduct after mating early in estrus but rather arrested in the caudal isthmus. They remained restricted in this portion of the oviduct until very close to the moment of ovulation.

Wilmut and Hunter (1984) suggested that the population of spermatozoa capable of fertilization was established in the oviduct over a period of not less then 6 h and probably more than 12 h in heifers. These workers ligated and sectioned the caudal isthmus of the oviduct at 6, 8, 10 or 12 h after mating. Fertilization rate was 9% if the oviduct was ligated and cut 6 h after mating and accessory sperm were few. But as the time after mating increased to 8, 10, and 12 h, the proportion of fertilized eggs increased to 40%, 42% and 70% respectively. Hunter and Wilmut (1983) indicated that a slow progression and displacement of viable spermatozoa occurred in the oviduct. These authors obtained highest fertilization results 12 h after mating (matings were made within 8 h of the detected onset of estrus). Hunter and Dziuk (1968) demonstrated that boar spermatozoa could reach and penetrate the zona pellucida of pig eggs within 2 h of insemination. Hunter (1984) reported that transection of the pig oviductal isthmus 1.5 to 2.0 cm proximal to the uterotubal junction at intervals from 3 to 36 h after mating prevented fertilization in 269 of 270 eggs, whereas 98% of 223 eggs were fertilized in the control oviducts. He further reported that transection at 38 (pre ovulatory), 40 (peri ovulatory), and 42-44 h (post ovulatory) after mating yielded, respectively, 5%, 40% and 100% fertilization. Hunter indicated a pre ovulatory arrest of viable spermatozoa in the caudal region of isthmus for 36 h or more followed by an active ad-ovarian distribution.

Sperm Loss After Breeding From the Female Genital Tract

During natural breeding a large number of spermatozoa are deposited in the female genital tract and only a few reach the site of fertilization. The number of sperm reaching the ovum may be even more critical during artificial breeding because of frozen semen and reduced numbers of sperm cells per inseminate. It has now been shown that a large number of sperm cells are lost externally through the vagina or phagocytized while a small portion is transported through the infundibulum into the pelvic cavity.

Various experiments have been conducted to measure the percent of spermatozoa which are lost and the percent which remain in the various segments of the genital tract after breeding. Dobrowolski and Hafez (1970) inseminated heifers with $2x10^9$ sperm cells. Heifers were slaughtered at either 1, 8, or 24 h post insemination and immediately after slaughter the reproductive tracts were removed and each segment of the tract was flushed for sperm recovery. These workers observed sperm recovery rates of 13.4%, 3.8% and 0.9% of the total inseminated spermatozoa at 1, 8 and 24 h respectively. Lightfoot and Restall (1971) recovered only 15% of the inseminate from the reproductive tract of ewes 2 h after insemination. First et al. (1968) recovered approximately 40% of the total spermatozoa inseminated from the uteri of the sow at 15 min after insemination of 40 billion spermatozoa. Morton and Glover (1974) reported that if $50x10^6$ sperm cells were deposited during mating in rabbits, only 20% could be recovered 30 min later and concluded that most of the sperm had been lost by drainage to the exterior. Larsson and Larsson (1985) studied the distribution of spermatozoa in the female genital tract of heifers after artificial insemination of frozen semen. Eight heifers were artificially inseminated in the uterine body with $160x10^6$ spermatozoa frozen in French mini straws. These heifers were slaughtered at 2 and 12 h after insemination. The percent of the inseminate recovered from all segments (vagina, cervix, uterus, uterotubal junction, isthmus, ampulla and infundibulum) after flushing was 14.6% at 2 h and 0.6% at 12 h. From the uterus there was a slight decline in the number of spermatozoa recovered at 2 ($151x10^3$) compared to 12 h ($79.2x10^3$) post insemination. The number of spermatozoa recovered from oviducts were similar at 2 ($89.6x10^3$) and 12 h ($71.5\ x10^3$) after insemination. At 2 h, spermatozoa were found in all parts of the oviduct with the majority located in the uterotubal junction, whereas at 12 h, most were recovered from the isthmus.

Hawk and Conley (1971) determined the percent of spermatozoa that are lost due to retrograde flow. These workers divided ewes into two groups. In the treatment group the reproductive tracts of ewes were ligated at the vulvovaginal junction immediately after insemination. In the control group, the vulvovaginal junction of the reproductive tract was not ligated. Ewes were slaughtered 24 h later and the number of spermatozoa in each segment of the genital tract was determined by

leucocyte counting chamber. Sixty -two percent of the total inseminate was recovered from the ewe with ligated tracts 24 h following insemination. Less than 1% of the inseminate was recovered from the control ewes. Despite the apparent increase in sperm retention, fewer spermatozoa were found in the oviducts of ligated tracts than those of the controls. They suggested that this reduction in numbers of sperm at the site of fertilization could possibly be due to a shift in the direction of uterine contractions caused by the ligature itself. They compared these results with those of Hawk (1970), where insertion of a plastic spiral, or intra uterine device into the ovine uterine body caused contractions to be propagated towards the cervix rather than the oviducts, totally inhibiting sperm transport to the oviducts. Mitchell et al. (1985), while studying the distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the female bovine genital tract, observed that only 6.3% of the inseminate could be recovered 12 h following artificial insemination. A second experiment was conducted to determine the extent of retrograde sperm loss from the female reproductive tract following artificial insemination. Three pairs of Holstein cows were artificially inseminated with 420 million sperm in a 0.5 mL dose of fresh semen. The inseminate was deposited in the uterine body. All discharged mucus and urine was collected for 12 h following insemination and examined for presence of spermatozoa. At 12 h after insemination, cows were slaughtered, genital tracts were ligated and removed, and sperm retention and distribution evaluated. An average of 73% of the total number of spermatozoa inseminated were recovered from each cow. Approximately 61% of the inseminate was recovered from discharged mucus or 83% of the total number of sperm recovered. Cumulatively, this loss was nearly complete within 8 h of insemination and greater than half of the total number of spermatozoa were recovered from cervical mucus within 6 h of insemination. Only 6.5% of the inseminate was actually retained in the reproductive tract 12 h following insemination. When distribution of spermatozoa retained in the tract was evaluated, Mitchell et al. (1985) observed that greater than 90% were found in the mucus or vagina. With respect to site of semen deposition being the uterine body, this marked proportion in the mucus and vagina again suggested that large numbers of spermatozoa were eliminated from the gamete transport mechanisms of the female due to retrograde sperm loss. Overall, results from this experiment suggested that as much as 90% of the artificially inseminated spermatozoa are lost from the

female reproductive tract as a result of retrograde flow. On the other hand, Tilbrook and Pearce (1986) recovered 82% of the inseminate from the genital tract of ewes at 3 h following insemination but noted that losses increased rapidly after this time with only 18% and 10% being recovered at 9 and 12 h, respectively. They also observed that the proportion of sperm lost from the vagina due to retrograde flow was not affected by stage of estrus, sperm motility, sperm concentration or inseminate volume.

Phagocytosis

Substances of extra cellular origin that enter the cell by endocytosis do so by membrane bound bodies. The cytoplasmic vacuoles formed by engulfing extracellular material may contain fluid with materials in solution or suspension. This process is called pincytosis. If a relatively large solid material such as a microorganism or sperm is included in the vacuole then the process is called phagocytosis. Most of the sperm cells which are not lost by drainage or expulsion to the exterior are probably phagocytized by leukocytes (Mitchell et al., 1985). Hafez (1973) reported that phagocytosis was less efficient in the progesterone dominated phase of the estrous cycle than in the follicular phase.

Effect of Different Compounds on Sperm Transport

Various experiments have been conducted, primarily in rabbits and ewes, to improve the sperm transport in the female genital tract from the site of semen deposition to the site of fertilization. The effects of several compounds on sperm transport have been studied (Equist et al., 1975; Mandl,

1972 and Hawk and Cooper, 1978). These compounds were either injected before or after breeding (Hawk and Cooper, 1978) or added to the inseminated semen (Mandl 1972). Some of these compounds have given hopeful results in certain species of animals and none in other. These compounds include prostaglandin $F_{2\alpha}$, prostaglandin E_1, oxytocin, estradiol, phenylephrine and ergonovine. Mandl (1972), while studying the effect of prostaglandin E_1 on sperm transport in doe rabbits killed at either 1.5 or 2 h after insemination, recovered 1.8 and 18 times more sperm, respectively, from the uteri and oviducts of treated rabbits compared to controls.

Prostaglandin $F_{2\alpha}$: Hawk and Cooper (1977), while studying sperm transport in the genital tracts of naturally- mated ewes after regulation of estrus with prostaglandin $F_{2\alpha}$ (dose 10 mg) administered on day 10 or on day 16 of the estrous cycle observed significantly fewer sperm in the anterior and middle cervices of ewes treated on day 10 of the estrous cycle than in unregulated control ewes. On the contrary, for ewes treated with prostaglandin $F_{2\alpha}$ on day 16 of the estrous cycle, the average number of sperm in each segment was similar to that for control ewes. Significantly more sperm were recovered from each cervical segment of the cervix in the ewes treated with prostaglandin $F_{2\alpha}$ on day 16 than in the ewes treated on day 10 of the estrous cycle. The number of cells in the uteri and oviducts of these three groups (control, d 10 and d 16) did not vary significantly. Thus these workers suggested that the amount and ratios of ovarian steroids influencing the reproductive tract during prostaglandin induced luteolysis and the ensuing estrus may differ from the amounts and ratios influencing the tract during natural luteolysis and estrus. In another experiment Hawk et al. (1982) studied the effect of prostaglandin $F_{2\alpha}$ on sperm motility, response to live dead staining and acrosomal morphology of the sperm. Ewes in either natural or prostaglandin $F_{2\alpha}$ (dose 11 mg) regulated estrus were bred either by natural mating or by artificial insemination and necropsied 2 h after breeding. The reproductive tract was excised and the cervix was cut transversely into the anterior, middle, and posterior segments. Sperm were recovered from these segments and their viability was examined. In the posterior segment, the percentages of unstained and normal acrosomal sperm were each significantly lower in prostaglandin $F_{2\alpha}$ regulated estrus than natural ewes. Despite

studying the viability of sperm recovered from the genital tract of prostaglandin $F_{2\alpha}$ regulated estrus in ewes, Hawk and Conley (1985) also studied the effect of prostaglandin $F_{2\alpha}$ on the uterine contractions. These workers reported increased frequency of uterine contractions in treated ewes and suggested that this increased frequency could be one of the causes of reduced sperm transport. In contrast to sheep, Hawk et al. (1982) reported increased sperm numbers in the oviducts, uterus and cervix of prostaglandin $F_{2\alpha}$ treated rabbit does at 2.5 h following injection of 0.75 mg prostaglandin $F_{2\alpha}$. Equist et al. (1975) reported that when prostaglandin $F_{2\alpha}$ either added to ram semen or injected into ewes, the average number of sperm recovered from the oviducts and uterus could be increased as well as increasing the rate of sperm transport from the posterior cervix to the oviducts. These workers recovered $6.4x10^3$ and $13.9x10^3$ sperm from the oviducts and uteri, respectively, of ewes inseminated with semen containing prostaglandin $F_{2\alpha}$ and $10x10^3$ and $33.5x10^3$ sperm respectively, from the oviducts and uteri of the prostaglandin injected ewes. On the other hand, they recovered $0.4x10^3$ and $2x10^3$ from the oviducts and uteri of control ewes, respectively. All ewes in this experiment were inseminated with $300x10^6$ frozen sperm.

Estradiol: The effects of estradiol on sperm transport was studied in various species of animals and encouraging results have been obtained. Hawk et al. (1982) inseminated rabbit does either while in estrus (d 0) or one day later (d 1 after the induction of ovulation). Does inseminated on day 0 were given an intramuscular injection of either 1 mL of corn oil (control) or 3 μg of estradiol in 1 mL of corn oil followed 1 h later by an intravenous injection of 100 IU of human chorionic gonadotrophin and artificial insemination. Does inseminated on day one were given human chorionic gonadotrophin on day 0 and corn oil or estradiol 23 h later followed by insemination 1 h after injection of estradiol. Does were inseminated with 0.2 mL of semen containing 30 million sperm. Does were necropsied and sperm recovered 2.5 h after insemination. The number of sperm recovered from any segment of the reproductive tract did not differ significantly between day 0 and day 1. When data of both day 0 and day 1 were combined, estradiol increased the number of sperm recovered from the oviducts by about five fold and by three fold for the entire reproductive tract.

Similarly Hawk and Cooper (1975) conducted an experiment to test a range of doses of estradiol for effects on sperm transport. Eighty ewes were given subcutaneous injection of 0 (corn oil only), 5, 10, 30, or 100 μg of estradiol in 2 mL of corn oil. Half of the ewes given each amount of estradiol were treated 4 h before mating and the other half were treated at the time of mating. Each ewe was mated 4 h after estrus. The ewes were necropsied 20 h after mating, and the reproductive tracts were removed and flushed. The number of sperm recovered from the oviducts, uterus, and cervix differed significantly among groups given different amounts of estradiol. The 10, 30, and 100 μg doses of estradiol significantly increased, by about three fold over controls, the number of sperm cells recovered from the cervix. The 30 μg dose significantly increased, by more than 10 fold over controls, the number of sperm cells recovered from the uteri and oviducts.

Hawk and Cooper (1978) recovered more sperm from the entire tract of estradiol -treated does (30 μg) inseminated with $130x10^6$ sperm than does inseminated with $37x10^6$ sperm cells. These workers further reported that of the total number of sperm in the inseminate ($37x10^6$), 12.4% was recovered from the control and 52.4% from the treated does. Similarly 14.6% and 28.2% were recovered from the control and treated does that were inseminated with $130x10^6$ sperm. Fateh EL_Bab et al. (1983) inseminated does with a high dose ($60x10^6$) and low dose ($1x10^6$) of sperm cells. Treated groups were injected with diethylstilbestrol (0.1 mg for 3 days subcutaneously). All does were sacrificed 10 h after artificial insemination. Of the total number of sperm in the inseminate, 11.99% and 27.64% were recovered from the control and treated does, respectively, that were inseminated with $1x10^6$ sperm. When inseminated with $60x10^6$, 11.93% and 55.60% were recovered from the control and treated does, respectively.

Progestogen(s): The effect of progesterone administration during synchronization of the female on gamete transport has also been studied by several workers. Quinlivan and Robinson (1969) recovered significantly fewer sperm cells from the various segments of the reproductive tract of progestagen treated ewes than from untreated ewes. Similarly Hawk and Conley (1971) recovered

fewer spermatozoa and a higher proportion of tailless spermatozoa from medroxyprogesterone sponge treated ewes than from control ewes. However, when two steroids (estrogen and progesterone) were given together in a post insemination injection, an increase in the number of sperm retained in the reproductive tract of ewes was found (Allison and Robinson 1972).

Oxytocin: Lightfoot and 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. 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 and Fitzpatrick (1974) reported that when 80 mu oxytocin were injected into the doe rabbit, mass transport of semen occurred into the uterus. They suggested a coordinated response of the genital tract to a critical oxytocin stimulus which may facilitate mass transcervical passage of fluids in the ovariectomized estrogen-treated doe. Hays and VanDemark (1953) reported that oxytocin increased the uterine motility in intact cows.

Accessory Sperm Number and Their Relationship to Fertility

Accessory sperm are those sperm which reach the site of fertilization and initiate ovum penetration but are finally trapped in the zona pellucida of the egg due the zona reaction. Studies have shown a positive correlation between the accessory sperm number and the fertilization rate (Hunter and Wilmut, 1983; Weitze et al., 1988). DeJarnette et al. (1992) have shown that accessory sperm are positively correlated to both fertilization rate and embryo quality when bovine embryos are judged on day 6 post insemination. Saacke et al. (1988b) reported that both fertilization failure and early

embryonic death are related to low numbers of accessory sperm per ovum or embryo. These workers also theorized that the vestments of the egg provide the final selective barrier to sperm penetration. Normal sperm will penetrate more frequently if sufficient sperm are present at the site of fertilization. On the other hand, when sperm numbers at the site of fertilization are low, the incompetent abnormal sperm will be able to fertilize the egg more frequently due to less competition. It is speculated that this could lead to early embryonic death and lower fertility. Research has shown that normal sperm are more efficient in penetration of the ovum than abnormal sperm has been recorded recently (DeJarnette et al., 1992).

In swine, Weitze et al. (1988) reported a highly significant increase in accessory sperm per embryo due to addition of seminal plasma to diluted semen. Thirty six gilts were divided into two groups, 24 being inseminated with $2x10^9$ and 12 with $0.5x10^9$ sperm. Each of these gilts was inseminated with 60 mL seminal plasma or 60 mL semen extender (control) before insemination. The average number of accessory sperm per embryo was higher in the gilts pretreated with seminal plasma than in the control group. Mean number of accessory sperm per embryo was 85.2 and 25.9 for the seminal plasma pre treated and non treated high dose groups, and 69.6 and 8.6 for the low dose, respectively. In another experiment, Weitze et al. (1988) used 24 gilts. These gilts were heat checked twice a day and inseminated 24 h after the beginning of standing heat with a single dose of $5 x10^9$ frozen sperm. In addition, animals were divided into 3 experimental groups and received the following intra cervical treatment before insemination: eight gilts received 50 mL dilution medium, eight, 50 mL frozen-thawed seminal plasma and eight, 50 mL dilution medium containing 2 μg estrone and 4.5 μg estrone sulfate and 5 μg 17 β estradiol. Three to five days later embryos were recovered from the uterus and evaluated under the stereomicroscope. Fertilization rates were 33.3, 89.5 and 64.3 %, for the three groups, respectively. Also, a significant increase in accessory sperm number per embryo was observed in gilts with additional seminal plasma added before insemination as compared to estrogen or dilution medium treated gilts.

Hawk and Tanabe (1986) discovered that first service cows had significantly higher numbers of accessory sperm per ovum (Mean = 40) when compared to repeat breeders (Mean = 19). Ninety eight

percent of ova recovered from the first service heifers contained accessory sperm and 77% in repeat breeders. The range of accessory sperm was 0 to 334 in the first service heifers and 0 to 124 in repeat breeders. The fertilization rate in first service cows was 74% and for the repeat breeders 43%. The proportion of cleaved ova that contained accessory sperm was also somewhat higher in the first service cows (18%) than in repeat breeders (11%). This study suggests a strong correlation between accessory sperm number and fertilization rate and that at least part of the repeat breeder syndrome is related to inadequate sperm transport.

Segerson and Libby (1981) studied the effects of selenium and Vitamin E treatment on fertilization rate and accessory sperm number in Charolais cows. Cows were allotted by age, weight and reproductive performance to either a control (saline) or selenium and vitamin E treated group. Control and treated cows were fed the same selenium deficient diet. Treated cows received an initial injection of 40 mg selenium and 544 IU vitamin E. In the control group 8 ml 0.9% saline solution was injected. No difference was observed in the proportion of fertilized ova recovered from the two groups. However, the cows that received selenium and vitamin E-treatment had a higher mean accessory sperm number per ovum (35.6 ± 7.2) than did controls (24.8 ± 7.7.).

Hawk et al. (1988) reported an improved fertilization rate of 93% in superovulated cattle when they used a high dose of fresh semen (4.4 billion sperm) compared to a fertilization of only 53% when lower doses of frozen semen (70 million sperm) were used. The proportion of embryos containing accessory sperm was also found to be greater in cows inseminated with high doses of fresh semen (32%) than with the lower dose of frozen semen (12%).

Fresh and Frozen Semen Dosage and Their Relationship to Fertility

Lineweaver et al. (1970) recovered more sperm cells from the reproductive tract of the heifers 4 h after insemination with fresh semen then frozen semen was used. Eighteen Hereford heifers were inseminated with 1 mL of semen 8 to 12 h after estrus detection. The semen was deposited midway through the cervix in all heifers. Six heifers were inseminated with 1 mL of frozen semen ($3.4x10^7$) and slaughtered 4 h later. Twelve were inseminated with 1 mL of fresh semen ($3.2x10^7$), and then slaughtered in two groups of six at 1 and 4 h after insemination. The reproductive tracts were removed immediately after slaughter, clamped at the infundibulum, 1 cm above and below the uterotubal junction, and at the anterior vagina. They were then placed in a water bath until flushing was complete. The oviducts, uterotubal junctions and uterine horns were flushed individually using physiological saline at 37°C and the number of sperm cells were measured using the hemocytometer. When 1 mL of frozen or fresh semen was deposited midway in the cervix of the heifers, an average of 191 sperm per heifer was found in the oviducts 4 h after insemination with frozen semen, as opposed to an average of 458 sperm per heifer 4 h after insemination with fresh semen. When the oviduct was flushed 1 h after insemination, 469 sperm were observed, as opposed to an average of 458 sperm per heifer flushed 4 h after insemination with fresh semen. The average number of sperm recovered from the uteri of heifers flushed 4 h after insemination with frozen semen, 1 h after insemination with fresh semen and 4 h after insemination with fresh semen were 733, 3,955 and 1,550 respectively.

Pursel et al. (1978) inseminated 68 gilts intracervically with equal numbers (7.69×10^9) of fresh or frozen sperm. Sperm were recovered from various segments of the reproductive tracts of gilts killed at 2, 8 and 24 h after insemination. Significantly more sperm were recovered from the uterotubal junctions and uteri of gilts inseminated with fresh semen than gilts inseminated with frozen semen

at all times of insemination. In the oviducts, no differences were found between fresh and frozen semen in the number of sperm recovered from gilts killed at 2 or 8 h. However, at 24 h markedly fewer sperm were recovered from the oviducts of gilts inseminated with frozen semen than gilts inseminated with fresh semen. In another experiment, Pursel (1982) prevented the retrograde sperm expulsion from the uteri of gilts after insemination of frozen boar sperm to increase the sperm retention time in order to enhance fertilization rate. Twenty five gilts were surgically inseminated intra uterine after laparatomy with $500x10^6$ sperm. After insemination, one uterine horn in each gilt was randomly selected for ligation at the anterior border of the intercornual ligament. Of the total number of sperm in the inseminate, 32.2 and 11.4% were recovered from the ligated and unligated uterine horns, respectively. Significantly more fertilized ova were recovered from the ligated than from the unligated horn of the reproductive tract (87 vs 64%). Likewise, the number of accessory sperm attached to zona pellucida of the fertilized ova was significantly higher for the ligated than for the unligated uterine horns (18.8 vs 3.7).

Roche et al. (1968), while studying the competitive fertilizability of mixtures of equal number of motile fresh and aged (kept for 24 h at 5°C) spermatozoa of rabbit bucks of two different breeds (New Zealand vs Dutch Belted), observed a significantly smaller proportion of offsprings produced by the insemination ($15x10^6$sperm) of does with aged spermatozoa of either buck than fresh spermatozoa. Thus these workers suggested that the differences between fresh and aged spermatozoa could be due to the faster penetration of eggs by the fresh spermatozoa than the aged spermatozoa.

Lightfoot and Salamon (1970) reported that the use of frozen semen as compared with fresh semen, resulted in lower proportion of fertilized eggs following cervical (45% vs 70%) and cervical traction (42 % vs 68%) insemination in merino sheep. However, both fresh and frozen semen were of equal fertility (91% vs 87% percentage of fertilized eggs) when inseminated into the uterus. Ewes in this experiment were artificially inseminated with fresh or frozen semen collected from two rams and

diluted to a concentration of $1.6x10^9$ sperm per mL and embryos were collected surgically 48 to 60 h after insemination following mid-ventral laparotomy.

In another experiment Lightfoot and Salamon (1970) examined the effects of type of semen (fresh vs frozen) and concentration of motile spermatozoa per mL in frozen or fresh semen on the number of spermatozoa recovered from the fallopian tubes 24 h after cervical insemination. Forty two sheep were divided into three experimental groups, each of fourteen sheep. Group 1 was inseminated with frozen semen, 0.8 x 10^9sperm per mL, group 2, frozen semen, $1.6x10^9$ sperm per mL and group 3, with fresh semen, $1.6x10^9$ sperm per mL. Each ewe was inseminated once (cervical insemination) with 0.1 mL of semen 1-16 h after the onset of estrus. Spermatozoa were recovered from the fallopian tubes of eighteen (64.3%) of the twenty eight ewes examined 24 h after insemination with frozen semen, whereas all fourteen ewes inseminated with fresh semen yielded spermatozoa. The mean number of spermatozoa recovered were 7741 and 575 for ewes inseminated with fresh and frozen semen respectively. These workers observed no effect due to concentration of motile spermatozoa in the frozen semen. These authors also reported the relationship between the percentage of eggs recovered with spermatozoa in the zona pellucida and type of semen: frozen, 0.8x 10^9, 22.2%; frozen, $1.6x10^9$, 37.5%; fresh, $1.6x10^9$, 62.5%. Baker et al. (1968) reported that insemination of gilts 8 h (ovulation controlled with HCG) before ovulation with $5x10^9$ or 10 $x10^9$sperm (fresh semen) resulted in a significantly greater number of sperm in the oviducts and a higher proportion of eggs fertilized than insemination with $1x10^9$ sperm at the same time. These workers further reported that as the number of sperm retained in the female gilts reproductive tract increased after insemination, the number of sperm attached to zona pellucida and their recovery from the oviduct also increased significantly.

Materials and Methods

Semen Collection and Initial Evaluation

Four mature bulls housed at Virginia Polytechnic Institute and State University Dairy Farm Center were selected based on their ability to routinely provide a minimum of 70% morphologically normal spermatozoa and 60% progressively motile sperm. Two successive ejaculates were collected from each bull at one week intervals by artificial vagina. Bulls were given two minutes active restraint between two false mounts prior to collection of each ejaculate.

Immediately following collection of each ejaculate, volume and percent progressive motility were recorded. Motility of the neat semen was estimated to the nearest 10% from wet smears examined at 100X under a phase contrast microscope equipped with a heated stage (37 °C). The semen smear was prepared by thoroughly mixing neat semen with a drop of egg yolk citrate extender on a microscopic slide and carefully placing a cover slip on the mixture.

Ejaculates were then combined, thoroughly mixed and 1 mL of semen was removed for subsequent determination of sperm concentration, using a spectrophotometer (Spectronic-20) calibrated for

bovine sperm. The remaining portion of the pooled ejaculate was extended (1:1) with Fraction A of the freezing extender (see appendix D for detail)

Semen Processing

Semen Dilution

As mentioned earlier, a volume of Fraction A equal to the semen volume was added immediately following collection. The remainder of Fraction A was added to the semen after the determination of sperm concentration (from 1 mL of the pooled ejaculate separated before dilution) approximately 15 to 20 minutes post collection according to one of the two experimental doses (see Figure 1 on page 28 for Experimental Design). After glycerolation and filling of straws (see appendix D for adding Fraction A, Fraction B, and Antibiotics), the straws were divided into two groups: those to be used for the fresh semen treatment and those designed for the frozen semen treatment. Straws to be used as fresh semen were maintained at 5 °C and used for insemination over a period of 4 days. Straws for the frozen semen treatment were frozen, stored in liquid nitrogen and used over a period of one month beginning 2 weeks after freezing.

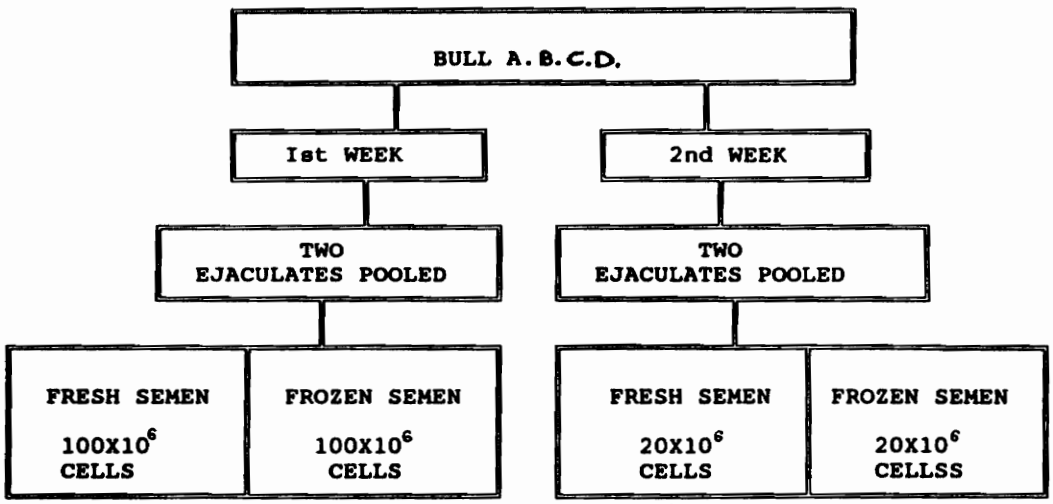

Figure 1. Experimental Design

Breeding of Experimental Cows

Estrus Detection

Sixty mature, nonlactating Holstein cows declared free of genital tract anomalies by rectal palpation were included in the present study. These animals were repeatedly used for artificial breeding and non surgical embryo recovery 6 days later. Cows were observed for signs of estrus for 30 minutes approximately at 12 h intervals (6 A.M. and 6 P.M.). Cows were subjected to breeding if they showed the primary sign of estrus, standing estrus. Cows observed in standing estrus were inseminated 8 to 12 h later.

Experimental Design

The pooled ejaculates (see Figure 1 on page 28) of each bull were alternately (each week) assigned to be prepared for insemination at the low dose (20 x 10^6 sperm) or high dose (100x10^6 sperm). Prior to freezing, the straws were divided, half being frozen and stored at -196°C, the other half maintained at 5°C. Inseminations were carried out first with the fresh semen of a given bull's ejaculate, the number of inseminations dependent upon the extent that cows from the 60-cow herd showed natural heats within 4 days of semen collection. Frozen semen of the same ejaculate was used over a one month period (beginning 2 weeks after semen collection) until the number of ova recovered were equal to those for the fresh semen of the same pooled ejaculate. This provided assurance that the performance of one male or ejaculate would be the same for both frozen and fresh semen at a specific dosage. Also, semen of each bull was used at both dosage levels within the same season (approximately a 1.5 to 2-month period) with semen prepared at high and low dose levels on alternate weeks. To evaluate dosages, inseminations were balanced across bulls such that each

bull had equal numbers of low and high dose inseminations from which ova/embryos could be recovered. Whenever there were excess ova/embryos for any given bull to a specific dose they were eliminated by random removal of ova/embryos resulting from fresh and frozen inseminations to that dose.

Embryos/Ova Collection and Recovery

Embryos/ova from single ovulating cows were collected on day 6 or 7 following the day of insemination using a nonsurgical method. Before placing the Foley catheter in position for flushing, an epidural anaesthesia was given taking all aseptic precautions. The skin above and surrounding the sacrococcygeal joint was clipped and scrubbed with Betadine (Purdue Fredrick Co, Norwalk, CT). The needle was inserted between last sacral and first coccygeal vertebrae or between first and second coccygeal vertebrae. During epidural anaesthesia, 3.5 to 4 mL Lidocaine (Buttler Co, Columbus, OH) was injected. The volume was varied in some cases according to the size of the animal and duration of anaesthesia required. Both ovaries were palpated for the presence of a functional corpus luteum in order to determine the side of ovulation. Using the rectovaginal technique, a 20 gauge Foley catheter with a 5 mL cuff was passed through the cervix. The cuff was inflated with 6 to 8 mL air inside the body of uterus of the cow. After inflation of the cuff, withdrawal of the catheter was attempted to assure its firm placement during flushing. Approximately 500 ml of Dulbeco 's phosphate buffered saline (flushing medium prepared at laboratory supplied by Gibco Laboratories Grand Island, N.Y.) containing 5 mL Fetal Calf Serum and 1 mL antibiotic -antimycotic (10,000 units/mL Penicillin G Sodium, 10,000 mcg/mL Streptomycin sulfate and 25 mcg/mL amphotericin B) was used for the genital tract flushing (see " Appendix A.Composition of Antibiotic-Antimycotic used in Phosphate Buffered Saline (flushing medium)" on page 60 for detail). The volume was delivered and recovered in increments of 50 to 100 mL. Fluid was directed to either horn by occluding the opposite horn by pressing it against the pelvic bone. The horn

ipsilateral to the ovary containing the corpus luteum was flushed with 300 mL medium. The contra lateral horn received the remaining 200 mL. The first 100 mL (50 mL increments) was placed in the horn ipsilateral to the ovary containing the corpus luteum and after recovering the fluid, 50 mL was delivered to each horn alternately. Following the flushing procedure at least 100 mL air was infused to aid in recovering the remaining few ml of flushing medium. The recovered flushing medium was filtered through an EM-CON mesh filter (Immune System Inc., Spring Valley, WI.) and the final sediment along with approximately 3 mL of the recovered flushing medium was placed in a petri dish and searched for the ovum/embryo using a dissecting stero microscope. Once located, the embryo/ovum was transferred to a petri dish (35x10mm) containing fresh (PBS with 1% fetal calf serum) for evaluation and accessory sperm quantification.

Evaluation of Fertilization Status and Embryo Quality

Once located, the embryo was classified as fertilized or unfertilized. Fertilized embryos were graded as excellent, good, fair, or poor, using the classification of (Lindner and Wright, 1983). This evaluation considered compactness and homogeneity of the cell mass. An unfertilized ovum (UFO) or degenerate embryo was confirmed following the evaluation of DeJarnette et al. (1992). This evaluation considered an unfertilized ovum as one which showed no sign of cleavage or when two to six blastomeres or fragments failed to reveal nuclei when examined in smears at 400X under differential contrast optics. Embryos that had blastomeres with nuclei but were too underdeveloped or retarded to be considered viable embryos under the classification of Lindner and Wright (1983) were designated as degenerate.

Zona Pellucida Digestion and Quantification of Accessory Sperm

To reveal accessory sperm, the procedure of DeJarnette et al. (1992) was employed. This technique involve partial digestion of the zona pellucida of the embryo or ovum in a hanging drop slide containing 3μL of phosphate buffered saline with 0.5 % Pronase (Behring Diagnostics, La Jolla, CA.). The edge of the coverslip holding the ovum/embryo was previously coated with a light smear of the pertoleum jelly to prevent evaporation during digestion. Zona digestion was monitored at approximately 2 min intervals using phase contrast optics (400X) and was considered to be ready for smearing when the zona pellucida became poorly delineated or irregular in appearance (4 to 10 min). At this time the cover slip with the embryo or ovum was removed from the hanging drop slide and lowered onto a siliconized slide containing a 5μL drop of phosphate buffered saline. This process was carried out using a steromicroscope (10X). The drop with the ovum was permitted to contact the drop on the siliconized slide. The coverslip was then released, gently flattening the zona pellucida and extruding the blastomeres or vitellus. The area containing the flattened zona was denoted with a water proof marker to aid in later relocation using phase contrast or differential interference contrast microscopy. Using either of these optics at 400X magnification, accessory sperm number was obtained by direct count.

Fresh and Frozen-Thawed Semen Viability Tests

Percent Motility of Fresh and Frozen-Thawed Semen

Percent motility of fresh semen was recorded on day 1, (next day after semen collection) and day 4 after semen collection. During this study three fresh semen straws were randomly selected from the total straws resulting from the pool of paired ejaculates of each bull. The straws were dried completely to avoid the mixing of water drops with the semen, the contents pooled in a 1.5 mL Eppendorf tube (Sarsted,West Germany). Two small drops of this mixed fresh semen was put separately on a microscopic slide previously warmed to 37°C. The remaining semen was then incubated for 3 h at 37°C in dry bath for later evaluation. Each semen drop was mixed on the slide with an applicator stick and the cover slip applied in such a way as to evenly spread the drop. The smears so prepared were observed using a phase contrast microscope equipped with a heated stage at 37°C for percent motile cells at 100X magnification. At least 10 regions were observed and then an estimate of percentage of motile cells (nearest 10%) was recorded. Percent motility was again determined after three hours of incubation by preparing a similar smear from the same semen sample. On day 4 after semen collection the same test was repeated for the fresh semen. Frozen semen was evaluated like the fresh semen for percent motility but after storage at -196°C for two weeks in liquid nitrogen and thawing at 37°C for 45 seconds.

Percent Intact Acrosome Quantification of Fresh and Frozen-Thawed Semen

The smear prepared for the examination of percent motility was also (from pooled contents of the same three straws prepared for sperm motility evaluation) observed under the differential interference contrast microscope at 1000X magnification using oil immersion lens for percent intact acrosomes at 0 h and 3 h after incubation in dry bath at 37°C. Intact acrosomes were based on the

presence the apical ridge (Saacke and Marshall, 1968). One hundred cells from each of the two smears were counted and from that the percent intact acrosomes were averaged. On day 4 of semen collection the same test was repeated at 0 h and after 3 h of incubation using the smear prepared for percent motility. After thawing, frozen semen stored in liquid nitrogen at -196 °C was evaluated in the same manner as the fresh semen.

Morphological Examination of Frozen -Thawed Semen

Frozen - thawed semen was used for the morphological examination after incubation in liquid nitrogen for two weeks at -196°C. Three straws of frozen semen of each ejaculate were thawed in water at 37°C for 45 seconds and then these straws were evacuated into a 1.5 mL Eppendorf tube. A drop of 1 M sodium fluoride was placed on each end of a microscope slide and then a drop of frozen-thawed semen was mixed with each one of them separately. The slide, so prepared after the application of cover slips, was observed under oil immersion lens at 1000X magnification using an interference contrast microscope. The following categories of cells were recorded: percent normal sperm, percent abnormal sperm head, percent droplets (proximal, translocating, and distal) and percent tail abnormalities, percent detached head and percent broken neck.

Statistical Analysis

All statistical analyses were conducted with programs available through the Statistical Analysis System (SAS, 1985). DeJarnette et al. (1992) revealed the skewed distribution and high heterogeneity of variance associated with accessory sperm data in cattle and recommended that

median rather than mean accessory sperm values were the most important physiologically. On this basis, numbers of accessory sperm per ovum/embryo were analyzed for differences due to freezing and dosage using the Wilcoxon two-sample test (SAS, 1985) for medians. Treatment means are presented with standard deviation and standard error and were tested using t test procedure (SAS, 1985) Also, because differences among treatments in variance of accessory sperm number may be physiologically important, accessory sperm data (numbers of sperm per ovum/embryo) were first normalized using the equation: log10 (X + 1), where X is the direct count of accessory sperm. Treatment differences in Variances were then evaluated for significance by the F test. Percent ova/embryos having accessory sperm and percent fertility were tested using Chi-square contingency tables.

Due to the subjective nature of judging embryo quality and to a lesser extent fertilization status and degenerate embryos, embryos/ova were grouped into four categories for analysis: unfertilized ova, degenerate embryos, poor to fair embryos and good to excellent embryos. Chi-square contingency tables were used to detect changes in fertilization status/embryo quality distribution in response to frozen vs fresh semen and high vs low dosage. Differences among bulls regarding accessory sperm were detected by using a nested design.

A nested design was considered to be the appropriate model to determine the effect of bull, semen and dose on accessory sperm. The model used was as follow

$Yijk = U + Bi + Dj(Bi) + Sk(Bi) + Sk*Dj(Bi) + Eijk$

Yijk = Accessory sperm

U = Over all mean

Bi = Effect of bull ($i = 1,2,3,4$)

Dj(Bi) = Effect of dose nested within bull ($j = 1,2$)

Sk(Bi) = Effect of semen nested within bull ($k = 1,2$)

Sk*Dj(Bi) = Interaction between semen and dose

Eijk = Random error

Results

Semen Quality

The mean and range in progressive motility of fresh and frozen semen at the two dosage levels is presented in Table 1. Although the number of ejaculates were too small for meaningful statistical analysis, as expected, motility of frozen semen appeared less than that for fresh semen. Also, the motility of semen prepared at the high and low dosages appeared comparable and reasonably narrow in range among ejaculates (50 to 80%). Additional semen data, (Acrosomal integrity and morphology) are presented in Appendix C.

Table 1. ESTIMATE OF THE PERCENTAGE PROGRESSIVELY MOTILE SPERM IN THE INSEMINATES

	$20x10^6$sperm			$100X10^6$sperm		
Semen	n	Mean	Range	n	Mean	Range
Frozen	5	60.0	50-70	7	60.0	50-80
Fresh*	5	68.0	60-80	7	70.0	60-80

*Fresh semen was evaluated after 1 d storage at 5 °C.

Efficiency of Embryo Recovery and Their Distribution Among Treatments

A total of 98 embryos/ova were recovered non surgically from a total of 129 inseminations on day 6 (efficiency of 76%). The distribution of these embryos/ova by treatment was 22, 28, 21 and 27 for the fresh semen/high dose, frozen semen/high dose, fresh semen/low dose, and frozen semen/low dose, respectively. From the pooled and split ejaculate of each bull, an effort was made to recover equal numbers of embryos/ova resulting from use of fresh vs frozen semen to minimize the influence of a specific bull or ejaculate within a treatment. Since this number was limited by the number recovered from cows inseminated with the fresh semen portion of the split sample, regardless of number of embryos/ova recovered for the frozen semen counterpart, only the number equalling the fresh semen embryos/ova for that same ejaculate were used for the evaluations. On this basis, 80 of the 98 embryos/ova recovered qualified for the assessment of accessory sperm parameters for fresh vs frozen semen, 42 at $100x10^6$ and 38 at $20x10^6$ sperm per dose.

Effect of Semen Freezing and Dosage on Accessory Sperm Parameters

The distribution of embryos/ova against each treatment, and the effects of fresh vs frozen-thawed semen at each dosage on accessory sperm parameters and fertilization rate are presented in Table 2. Freezing semen had no significant effect on fertility, nor any of the accessory sperm parameters measured at either dosage. It was evident however, that potential differences existed between the

Table 2. EFFECTS OF LOW ($20x10^6$CELLS) AND HIGH ($100x10^6$CELLS) DOSES OF FRESH AND FROZEN-THAWED SEMEN ON ACCESSORY SPERM (AS) PARAMETERS AND FERTILIZATION RATE

Semen	n*	Mean No AS ± SEM	SD	Median	Range in AS per Embryo/Ovum	Percent Embryos/Ova with AS	Percent Fertilization
Fresh $100x10^6$	21	26.81 ± 6.6	30.23	13	0-104	81	86
Frozen $100x10^6$	21	36.05 ± 9.77	44.74	19	0-157	90	95
Fresh $20x10^6$	19	29.37 ± 12.87	55.97	3	0-224	68	68
Frozen $20x10^6$	19	30.16 ± 16.13	70.18	3	0-291	63	84

*Number of embryos/ova. Within dosage, fresh and frozen treatments were the result of split ejaculates from four bulls.

high and low dosages with respect to number of accessory sperm per embryo/ovum. Since freezing had no effect on accessory sperm parameters, embryos/ova recovered from insemination with fresh and frozen semen were combined within dose and the data were reexamined for the effect of dose on accessory sperm. Again, to avoid the potential influence of any one of the four bulls, excess embryos/ova from a specific bull at either dosage were randomly removed such that the number of embryos/ova derived from insemination to any one of the four bulls was the same at both dosages. The effect of dosage on fertility and accessory sperm number is presented in Table 3. Seventy-six embryos/ova qualified for comparison of dosages. A mean accessory sperm value of 37.82±38.3 SD (n = 38) for high dose and a mean accessory sperm value of 28.92±62.8 SD (n = 38) were recorded for low dose. Differences due to dosages for mean accessory sperm were not significant ($P > .05$). However, the Median accessory sperm values revealed a nine-fold difference in favor of the high dosage ($P < .05$). The median accessory sperm values for the high dose was 27.5 and 3 for the low dose. The high standard deviation associated with the low dose was not different from that of the high dose, but it did approach significance at $P = .10$.

Bulls Effect

A significant difference was observed among bulls regarding mean accessory sperm values ($P < .05$)(Table 4). Appendix C (Table 7 for frozen semen, Table 8 for fresh semen) shows the viability parameters and appendix C (Table 9) shows the morphological characteristics of sperm of each bull. It appears from these data (Table 4) that bulls differ in the number of accessory sperm they achieved, most shown notable by the high median values for accessory sperm resulting from matings to bull A relative to the other bulls.

Table 3. EFFECTS OF LOW ($20x10^6$CELLS) AND HIGH ($100x10^6$CELLS) DOSES OF SPERM ON ACCESSORY SPERM (AS) PARAMETERS AND FERTILIZATION RATE

Semen	n	Mean N0 AS ± SEM	SD	Median	Range in AS per Embryo/Ovum	Percent Embryos/Ova with AS	Percent Fertilization
$100X10^6$	38	37.8 ± 6.22^a	38.3	27.5^b	0-157	92^d	97^d
$20X10^6$	38	28.76 ± 10.16^a	62.81	3^c	0-291	79^e	79^e

n = number of embryos/ova, across dosages equally represented by ejaculate of 4 bulls.
a,b,c = column values with different superscripts differ, ($P < .05$).
d,e = column values with different superscripts differ, ($P < .10$).

Table 4. EFFECTS OF EXPERIMENTAL BULLS ON ACCESSORY SPERM (AS) PARAMETERS

Bull	n	Mean N0 AS ± SEM	SD	Median	Median*	n	Mean No AS ± SEM	SD	Median	Median*
		$100x10^6$Sperm Cells**					$20x10^6$Sperm Cells***			
A	13	49.38 ± 11.12^a	40.17	45	45(13)	12	57.33 ± 24.46^a	84.51	34.5	44(9)
B	24	17.8 ± 4.39^b	21.52	10.5	18 (18)	13	11.46 ± 6.93^b	25.02	3	4 (9)
C	6	52.5 ± 22.71^a	55.65	32	32 (6)	10	25.9 ± 22.08^{ab}	69.80	2	4 (7)
D	7	23.86 ± 12.04^{ab}	31.90	6	22(5)	13	4.15 ± 2.29^b	8.26	0	8 (5)

*Median accessory sperm (AS) value per embryo/ovum for only those embryos/ova with accessory sperm.
**Fresh and Frozen-thawed high dose ($100x10^6$) pooled data across all the experiment.
***Fresh and Frozen-thawed low dose ($20x10^6$) pooled data across all the experiment.
a,b = columns values with different superscripts differ, ($P < .05$).

Sperm Dose and Its Relationship to Fertilization Status and Embryo Quality

Sperm Number per Inseminate

Fertilization status/embryo quality was influenced positively ($P < .05$) by sperm dosage, but not by freezing. Fertilization status/ embryo quality for the two sperm dosages is presented in Figure 2. Use of the high dosage of semen resulted in a shift in fertilization status/embryo quality toward the viable embryo (classified excellent to poor), at the expense of the unfertilized ova and degenerate embryos.

The relationship between mean, standard deviation and median values for the accessory sperm number per embryo/ovum and fertilization status/embryo quality is presented in Table 5. There was an apparent but non significant increase in the mean as well as in the median values for the accessory sperm in excellent-good quality embryos group (38.8 ± 57.6) compared to fair-poor (20.2 ± 22.5), degenerate (23.9 ± 40.30) groups. Unfertilized ova had basically zero mean accessory sperm value and were statistically different ($P < .05$) from excellent-good quality embryos. Similarly, a no difference ($P > .05$) was observed regarding the median accessory sperm value for the embryos classified excellent-good, fair-poor and degenerate. Excellent-good embryos had the highest median accessory sperm values of 18.0, fair-poor 9.5, and degenerate group 5.5 while unfertilized ova had the lowest median accessory sperm value of zero. Only the unfertilized ova and excellent- good embryos were different in median accessory sperm ($P < .05$)

The embryos/ova were also divided into two groups. One group (viable) consisted of excellent, good, fair and poor embryos while the other group (non viable) consisted of degenerate and unfertilized embryos/ova as shown in Table 6. The mean, and median accessory sperm values for the embryos classified viable were compared to the mean and median accessory sperm values for the

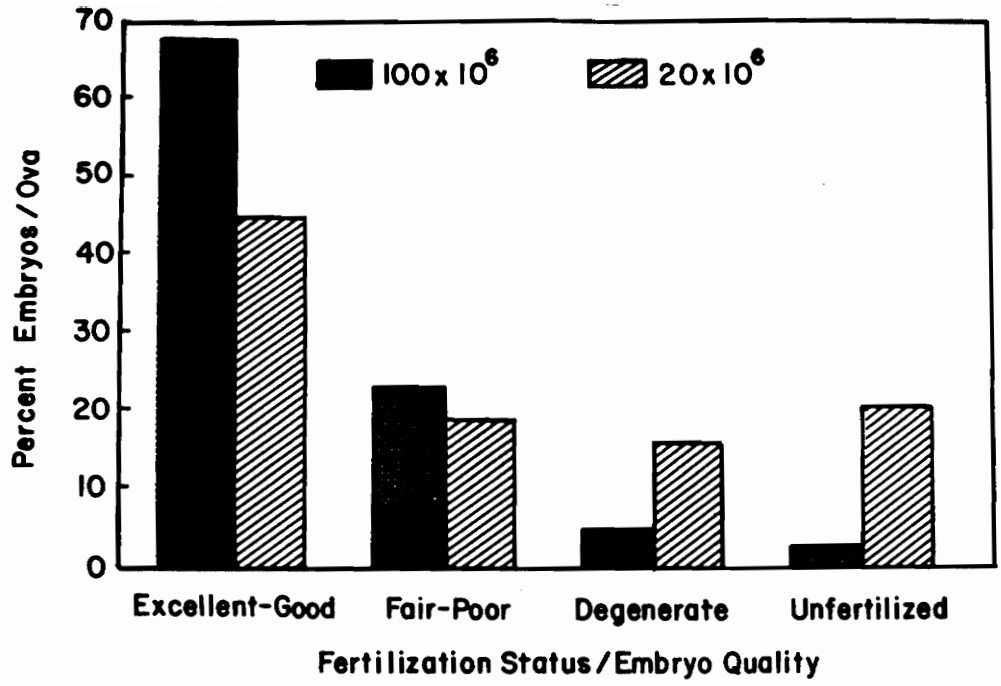

Figure 2. Frequency Distribution of Embryos/Ova by Semen Dose

Table 5. RELATIONSHIP BETWEEN ACCESSORY (AS) SPERM NUMBER AND FERTILIZATION STATUS/EMBRYO QUALITY

Fertilization Status/ Embryo Quality	n	Mean No AS ± SEM	SD	Median
Excellent-Good	53	38.79 ± 7.90^{a}	57.58	18^{a}
Fair-Poor	20	20.2 ± 5.03^{ab}	22.49	9.5^{a}
Degenerate	10	23.90 ± 12.75^{ab}	40.29	5.5^{a}
Unfertilized	15	0.13 ± 0.13^{b}	0.52	0^{b}

a,b = column values with different superscripts differ, ($P < .05$).

non viable group. The viable group had the highest mean accessory sperm values of 33.70±50.97 (n = 73) and the median value of 14 while the non viable group had the mean value of 9.64±27.39 (n = 25) and median accessory sperm value 0. These values for the means and medians for both groups were significantly different ($P < .05$).

Table 6. RELATIONSHIP BETWEEN ACCESSORY SPERM (AS) NUMBER AND FERTILIZATION STATUS/EMBRYO QUALITY

Fertilization Status/ Embryo Quality	n	Mean No AS ± SEM	SD	Median
Excellent-Good Fair-Poor	73	33.70 ± 5.97^a	50.97	14^a
Degenerate Unfertilized	25	9.64 ± 5.48^b	27.39	0^b

a,b = column values with different superscripts differ, ($P < .05$).

Discussion

Fresh vs Frozen Semen

Based on the semen freezing methods used in this study, semen freezing had no effect on accessory sperm number (see Table 2 on page 39), while increasing the dosage of semen from $20x10^6$ to $100x10^6$ cells resulted in a nine - fold increase in median accessory sperm per embryo/ovum (see Table 3 on page 41). The lack of an effect due to semen freezing was unexpected since sperm from fresh inseminates have been shown to be preferentially retained and transported to the oviduct when compared to sperm from frozen inseminates in a variety of species including: cattle (Lineweaver et al., 1970), sheep (Mattner et al., 1969) and swine (Pursel et al., 1978). However, since the time of these studies when 20 to 50% loss of sperm viability due to freezing was common, semen freezing methods have improved, particularly through cryopreservation methods associated with the development of the straw as the major package for semen in cattle and sheep. Thus, the disparity in motility of 8 to 10% between the fresh and frozen semen in the current study (see Table 1 on page 37) may have been too small to be reflected in sperm transport or accessory sperm number difference.

Sperm Dose per Inseminate

Evidence exists which shows that the total sperm retained and reaching the oviduct post-mating appears to be quite highly dependent upon the sperm or semen dosage. Larsson and Larsson (1988) compared ($200x10^6$ vs $20x10^6$) sperm per inseminate and found that only cows inseminated with the higher dosage provided sufficient numbers of recovered sperm in the oviduct (> 50) for evaluation of sperm distribution in this organ within 12 h of insemination. They also reported that the animals in which more than 50 spermatozoa were found, the distribution varied in an unpredictable manner both between animals and between oviducts within the same animal. Morton and Glover (1974) observed a positive correlation between the number of sperm in the inseminate and regional counts in rabbits, including the oviducts. They observed no difference between $0.5x10^6$ and $1.0x10^6$sperm cells after insemination of rabbit and quantifying the sperm in various regions of the reproductive tract. However, they recovered more sperm cells from the reproductive tract by inseminating $50x10^6$ and $500x10^6$ sperm cells per inseminate. Their results could be compared to the results of this experiment in which a low median accessory sperm value was recorded for low dose and high median accessory sperm value for high dose. Also 92% of (see Table 3 on page 41) total embryos/ova contained accessory sperm for high and only 79% for the low dose. Hawk et al. (1988) increased the proportion of ova with accessory sperm in superovulated cows from 12% to 32% and fertilization rate, from 53% to nearly 100%, by increasing the dosage of sperm in the inseminate from $70x10^6$ to 4.4×10^9. Although the present experiment showed a marked increase in median accessory sperm using $100x10^6$ sperm compared to $20x10^6$ sperm, a previous study in our laboratory comparing $40x10^6$ with $20x10^6$ sperm per inseminate yielded no difference, indicating it was an insufficient increase (DeJarnette et al., 1992). Present results are also in agreement with the results of Baker et al. (1968). They recovered a higher proportion of fertilized eggs from gilts after insemination of either $5x10^9$ or $10x10^9$ sperm than insemination with $1x10^9$ sperm. These workers also reported that as the number of sperm retained in the female reproductive tract after insemination increased, the number of sperm attached to zona pellucida increased sig-

nificantly ($P < .01$). In swine, Weitze et al. (1988) using frozen semen, showed that accessory sperm numbers could be increased by either sperm dosage or addition of seminal plasma. Thus, it is also possible that seminal plasma, in addition to sperm numbers, may have been important to the current results as well as those of Larsson and Larsson (1988) and Hawk et al. (1988), and perhaps to the lack of response in the results of DeJarnette et al. (1992) where the increase in semen was much more modest.

Bulls Effect

Although semen viability (motility) differences among bulls were small (% motility 50 to 80), a significant difference ($P < .05$) was found among bulls regarding accessory sperm numbers in the zona pellucida (see Table 4 on page 42). It is possible that semen quality factors other than the conventionally monitored motility and morphology could be responsible for this difference and thus affect male fertility. One major semen component which should be considered is seminal plasma, which could provide the necessary factors regarding sperm transport/zona penetration. Recently, Killian (1992) has shown by a modified competitive in vitro fertilization assay that cauda sperm collected from the vas deferens cannula of bulls were relatively less likely penetrated zona free bovine oocytes than vas cannula-derived sperm mixed with accessory sex gland fluid. Killian (1992) also compared the protein composition of seminal plasma collected from high and low fertility bulls. He reported that two proteins (protein I and protein II) were associated with seminal plasma of very low fertility bulls compared with high fertility bulls.

Effect of Dose on Accessory Sperm Number and Fertilization Status/Embryo Quality

The effect of dosage on fertility and accessory sperm number is presented in Table 3 on page 41. Median accessory sperm values revealed a nine-fold difference in favor of the high dosage ($P < .05$). Also, fertilization status/embryo quality was influenced positively ($P < .05$) by sperm dosage (Figure 2).

Across the experiments, embryo quality and fertilization status was associated positively with increased accessory sperm number. In other words, excellent to good quality embryos had the highest mean accessory sperm number while the unfertilized ova had basically zero accessory sperm and these values were significantly different (see Table 5 on page 45) ($P < .05$) from one another. Degenerate and fair to poor embryos were intermediate between the unfertilized eggs and excellent to good embryos in accessory sperm number, although not significantly so. Nevertheless, the positive relationship between accessory sperm and fertilization status/embryo quality established by DeJarnette et al. (1992) appeared to be further confirmed in the current experiment. Excellent to good embryos, fair to poor, degenerate and unfertilized ova had median accessory sperm values of 18.0, 9.5, 5.5 and 0, respectively. More important, the improvement in accessory sperm number from 3.0 to 27.5 as a result of raising the dosage ($20x10^6$to $100x10^6$) was simultaneously accompanied by an improvement in fertilization status/embryo quality ($P < 0.05$) as predicted by DeJarnette et al. (1992). When viable embryos (excellent-good & fair-poor) were compared to degenerate and unfertilized ova across the experiment, the viable embryos had the highest accessory sperm number (see Table 6 on page 47). These results are in agreement with research of Saacke and Co—workers (1986) where a similar relationship was observed between accessory sperm numbers and fertilization status/embryo quality in superovulated cows.

Data from this study would suggest that factors used in predicting male or inseminate fertility, particularly within the first 6 d of gestation, include both embryo quality and fertilization status since the impact of the male seems to be important to both. Degenerate embryos (as classified in this study) would be as unlikely as unfertilized ova to result in a pregnancy. In addition, Lindner and Wright (1983) have already shown that embryos classified fair to poor have only half the chance of surviving following transfer as do embryos classified excellent to good. Thus, differences in the se two categories due to the male may also represent a loss to be considered. If only the degenerate embryos and unfertilized ova were considered unable to result in pregnancy, the pregnancy rates for the high and low dosages used in this study would have been 92 and 63%, respectively, and a difference upon which post-implantation losses have yet to be applied.

The reason that increasing numbers of accessory sperm results in higher quality embryos is still unclear. However, in a reverse manner, Setchell et al. (1988) showed that reducing sperm numbers inseminated in rats through unilateral vasectomy of the mating males resulted in increased embryonic mortality. Also, there is evidence that abnormally shaped sperm capable of reaching the ovum can bind to and penetrate the ovum but do less efficiently than normal (Krzanowska and Lorenc, 1983; Kot and Handel, 1987; Marsh et al., 1987). Based on these findings Saacke et al. (1991) proposed the explanation that if sperm transport is inadequate i.e. less sperm at the site of fertilization at the appropriate time, there should be more unfertilized ova and more ova fertilized by potentially abnormal sperm since competition with normal would be reduced. Thus lower numbers of accessory sperm per ovum would there be associated with a higher proportion of ova being fertilized by abnormal sperm. This concept was further strengthened by the results of the present experiment where numbers of accessory sperm positively associated with embryo quality, most notable where the results of the high and low inseminate dosages were compared.

Implications

This study shows that increasing sperm number or semen dosage results in greater numbers of accessory sperm with resulting improvement in fertilization status/embryo quality. It is anticipated from these data and previously published data that other efforts resulting in an increase in the median accessory sperm number to matings would also improve reproductive capacity by increasing both fertility and embryo quality. Finally, accessory sperm number in embryos/ova of single-ovulating cows appears to be a valid method of monitoring sperm transport in the female.

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Appendix A.Composition of Antibiotic-Antimycotic used in Phosphate Buffered Saline (flushing medium)

(Gibco Laboratories, Grand Island, N.Y., Cat. No.600-5245AE).

Each mL contains

Penicillin G sodium	=	10,000 units
Streptomycin sulfate	=	10,000 mcg
Amphotericin B as Fungizone	=	25 mcg

Appendix B. Embryo Quality Evaluation Procedures

Lindner and Wright (1983)

Excellent: An ideal embryo, spherical, symmetrical with cells of uniform size, color and texture.

Good: Trivial imperfections such as a few extruded blastomeres, irregular shape, few vesicles.

Fair: Definite but not severe problems, presence of extruded blastomeres, vesiculation, few degenerate cells.

Poor: Severe problems, numerous extruded blastomeres, degenerate cells, cells of varying sizes, large numerous vesicles but a viable-appearing embryo mass.

Table 7. POST THAW SEMEN VIABILITY CHARACTERISTICS OF CRYOPRESERVED SEMEN COLLECTED FROM FOUR EXPERIMENTAL BULLS

Bull	Ejaculate No	Percent Progressive Motility (0h)	Percent Progressive Motility (3h)	Percent Intact Acrosome (0h)	Percent Intact Acrosome (3h)
A	3	58	33	74	66
B	5	56	38	75	60
C	2	60	45	74	55
D	2	75	35	75	71

Table 8. VIABILITY CHARACTERISTICS OF FRESH SEMEN KEPT AT 5 °C FOR 4 DAYS AFTER COLLECTION FROM FOUR EXPERIMENTAL BULLS

Bull	Ejaculate No	Day (0)				Day (4)			
		% Progressive Motility		% Intact Acrosome		% Progressive Motility		% Intact Acrosome	
		(0 h)	(3 h)	(0 h)	(3 h)	(0h)	(3h)	(0 h)	(3 h)
A	1	70	50	83	68	60	0	75	56
B	4	68	58	79	69	53	35	70	60
C	2	60	50	77	69	40	30	72	70
D	2	80	35	87	85	45	30	81	80

Table 9. MORPHOLOGICAL CHARACTERISTICS OF CRYOPRESERVED SEMEN COLLECTED FROM FOUR EXPERIMENTAL BULLS

Bull	Ejaculate N0	%Normal Heads	%Abnormal Heads	%Protoplasmic Droplet	%Tail Abnormalities	%Detached Heads	%Broken neck
A	3	90	4	0	2	2	2
B	5	82	9	0	4	4	1
C	2	76	13	1	4	4	2
D	2	89	2	0	5	2	2

Appendix D.Semen Processing

Concentration: The concentration of sperm cells per mL of the ejaculate was determined using a Spectronic - 20. Before the determination of sperm cells concentration, the instrument was turned on for 15 minutes and later standardized with (7.9 mL) 2.9% Sodium Citrate (w/v) at a wave length 550 um. In a glass cuvette 100 μ L semen and 7.9 mL Sodium Citrate was thoroughly mixed. The cuvette was dried, cleaned with kimwipes (Kimberley- Clark Corporation, Roswell) and placed in the Spectronic-20 and the reading was recorded.

Semen Processing

Semen Dilution

As mentioned earlier a volume of Fraction - A equal to the semen volume was added immediately following collection at the bull barn before shifting to the laboratory for further dilution. The remainder of Fraction -A was then added at the laboratory after the determination of sperm concentration. High (100×10^6) and low (20×10^6) doses were prepared from each ejaculate separately. The extender was consisted of two fractions i.e. Fraction - A (without glycerol) and Fraction - B (with glycerol). The extender was prepared one day before semen collection and kept in a cold room at 5°C over night.

Composition of Fraction - A (To make 100 mL)

i) Egg Yolk (unfertilized egg)	=	20 % (v/v)
ii) Isotonic Sodium Citrate Dihydrate (2.9% w/v) ph. 6.8 adjusted with 1 M Citric acid mono hydrate.	=	78 % (v/v)
iii) Antibiotics	=	2 % (v/v)

Preparation of Antibiotics

Both Tylosine (Elanco Product Company 740 S. Alabama Street Indianapolis,IN) and Gentamycin (Schering Animal Health 1011 Morris Avenue Union, NJ) (Powder) were dissolved in distilled water so that the total volume of Tylosine, Gentamycin and Linco-Spectin (UpJohn Company Kansas City MO)(available in liquid form, used as such) was not exceeded more than 2 mL (for 100 mL Fraction - A) and each mL of Fraction -A received the following amount.

a) Tylosine =	100 μg/mL
b) Gentamycin =	500 μg/mL
c) Linco-spectin =	300 /600 μg/mL

Composition of Fraction - B (To make 100 mL)

i) Glycerol =	14% (v/v)
ii)2.9 % Sodium Citrate (w/v) =	66% (v/v)
iii) Egg Yolk =	20% (v/v)

Procedure for Adding Fraction - A

Part of Fraction- A was added to raw semen just after collection at the barn in order to maintain ph as well as to provide essential nutrients to sperm cells. After determination of sperm cell concentration, the remaining Fraction -A was added in the laboratory at room temperature. The flask containing partially diluted (Fraction-A) semen was placed in a beaker containing 150 mL tap water at room temperature. The beaker with flask containing semen was then placed in the cold room at 5°C. The temperature of the water in the beaker was recorded after each half an hour until it reached to 5°C. This took approximately 3 to 4 hours. The flask containing diluted semen was removed from the beaker at 5°C (water temperature in the beaker) and permitted to remain in the air at 5°C (temperature of cold room). Semen was then further diluted with an equal volume of glycerolated extender (Fraction -B) according to the prescribed treatment.

Procedure for Adding Fraction - B

Fraction - B (glycerolated Fraction) was added to Fraction - A (with semen) in four volumes i.e. 10%, 20%, 30%, and 40% at 10-minute intervals. The extended semen was left over night in the cold room at 5°C. Then the next morning after semen collection, the semen was placed in 0.5 mL French Straws labelled for high (100×10^6) or low (20×10^6) dose bull and date of semen collection. Half of the total number of semen loaded straws were subjected to freezing in liquid nitrogen. The remaining half were kept at 5°C for 4 days and used to inseminate as fresh semen. For freezing, straws were held 8 cm above the surface of liquid nitrogen in vapors for 10 minutes prior to being plunged in to the liquid nitrogen.

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Publications

Saacke, R. G., J. M. DeJarnette, R. L. Nebel and S. Nadir.1991. Assessing bull fertility. Society for Theriogenology. Proc. for Annual Meeting. San Diego Ca. Page 56-69.

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Professional Societies

Society for the Study of Reproduction

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