THE EFFECT OF UTERINE ENVIRONMENT ON SPERM CELLS

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CHAPTER I

INTRODUCTION

A considerable amount of research has been conducted in the past twenty years to determine what changes, if any, take place in sperm cell morphology when they are in the environment of the female reproductive system. Most of this work has been conducted with rabbits, rats, and hamsters. However, in the last several years similar work has appeared with sheep, and to an even more limited extent some work has been done with the bovine. Much of the problem encountered with the larger species is due to the great expense of maintaining them and the lack of a suitable technique for studying a population of sperm cells in the intact animal.

When the literature is reviewed it can be seen that there is a definite need for spermatozoa to be present in the female reproductive tract for a period of time that is unique for that species in order for complete functional competance to be imparted to the spermatozoa. This phenomena has been well established for rabbit, rat, and hamster. It has also been recently established for sheep, and it is still debated whether such a phenomena exists in cattle. The empirical evidence would favor such a phenomena existing. Primarily this evidence consists of the optimal breeding times that have been observed in the bovine. It has been well established that the optimum time to breed cows is approximately 16-24 hours before ovulation, and that the closer or

farther away from ovulation that breeding takes place poorer conception rates are the result. However, it has not been clearly demonstrated that fertility of bovine spermatozoa is dependent on some period of residence in the uterus.

There is also the process of sperm cell aging or deterioration that takes place in the female reproductive tract. At various times this process has been confused with the capacitation process. However, at the present time it would appear that no structural changes are associated with capacitation, and structural changes that have been observed are a result of the aging process, or deterioration of the sperm cell due to other processes.

If the changes that take place in the sperm cell in the female environment could be better understood, this could lead to improved recommendations to cattlemen concerning the optimum time to breed their cattle. The research that is reported herein was conducted to determine the effect of uterine environment on livability and acrosomal status of bovine sperm cells.

CHAPTER II

LITERATURE REVIEW

The Morphology of the Head Cap

The acrosome is a membrane system that comprises a double membrane enclosing an electron dense material (Saacke and Almquist, 1964; Blom and Birch-Anderson, 1965). This membrane system is situated external to the nuclear membrane and internal to the cell membrane.

Hancock (1952) stated that the galea capitis is a loose cap investing the anterior half of the head and overlying a second more compact cap, the acrosome which is loosely applied to the head. Fawcett (1958) in describing this same region observed a similar arrangement of the membranes but observed that the outer "head cap" or "galea capitis" was continuous with the inner "acrosome". The same observations prompted Blom and Birch-Anderson (1965) to describe the acrosome as a double walled sac.

Much of the confusion concerning the arrangement of the acrosomal membranes centers on the equatorial segment. Hancock (1952) in his description of the acrosome stated that the boundaries of the equatorial segment were determined by the galea capitis anteriorly and the posterior boundary of the acrosome posteriorly. However, Nicander and Bane (1962) considered the equatorial segment as being bounded by common membranes that were closer together than in other regions of the acrosome. This arrangement was confirmed by Blom and Birch-Anderson (1965)

and by Saacke and Almquist (1964). The latter authors considered the formation of the structure to be due to alteration of the head cap, as did Hancock (1952), who stated that its formation was a post-mortem change, or at least a change in structure immediately preceding death.

Along the anterior margin of the acrosome is a thickening that is commonly called the apical body. Blom and Birch-Anderson (1961) described this structure as a closed fold and a thickening in which a narrow cylindrical body or vacuole is situated.

Function of the Acrosome

The role of the acrosome in some lower animals has been well defined (Colwin and Colwin; 1961 and 1963). However, the functional significance of the acrosome in mammals is not as well defined. Some attempt is usually made to equate the role of the acrosome in fertilization in lower animals to what might possibly occur in higher animals. Colwin and Colwin (1965) have suggested that the acrosome reaction as described by Blom (1963), and that observed by Piko and Tyler (1964) was similar to the reactions noted in Hydroides and Saccoglossus. They further suggest that on sperm cells that have completed the acrosome reaction, the inner acrosomal membrane corresponds to the acrosomal tubule that provides the mechanism of sperm attachment as described for Hydroides and Saccoglossus. Hadek (1962) speculated that the gradual denudation of the head and unsheathing of the apical body could be interpreted as the physiological equivalent of the acrosome reaction in the Annelida.

Early theories regarding the function of the mammalian acrosome usually involved a mechanical function of the acrosome. However, these

theories have long since been rejected. The function of the acrosome is now regarded as providing a surface that is capable of attachment to the egg and of yielding enzymes that enable the spermatozoan to enter the egg (Bishop and Austin, 1957). This is in agreement with the observations made by Bedford (1966) that capacitation of rabbit spermatozoa invests spermatozoa with the competence to establish contact with the ovum as well as endowing them with the ability to penetrate the zona pellucida during the fertile life of the ovum after ovulation.

Dickman (1964) proposed that the spermatozoa attaches itself to the surface of the zona pellucida and while attached it secretes a substance which conditions the zona pellucida so that the spermatozoan can pass through it. Uncapacitated spermatozoa are capable of attachment to the zona pellucida, but because they have not synthesized the appropriate substance, or cannot release such, the spermatozoa is unable to penetrate the substance of the zona pellucida.

McClean and Rowlands (1942) found that hyaluronidase from several sources was capable of liquifying the viscous gel cementing the follicle cells around freshly ovulated rat ova causing denudation of the egg. They proposed that the gel consisted of hyaluronic acid, and that the function of seminal hyaluronidase was to bring about denudation of the egg.

Bishop and Austin (1958), Austin (1960), and Hathaway and Hartree (1963) indicated that the acrosome was the carrier of hyaluronidase because when the membranes of the acrosome are disrupted hyaluronidase is released. Austin (1960) postulated that capacitation causes release of hyaluronidase, through change in the properties of the acrosome. Piko (1964) states that the acrosome, because of its staining proper-

ties, seems to be composed of at least two components; a hypothetical "zona lysin" and probably hyaluronic acid. The hyaluronidase would dispense the cumulus oophorus, and the "zona lysin" would possibly be involved in the passage of the sperm cell through the zona.

Allison and Hartree (1970) are in agreement with this proposal. They state that the hyaluronidase would be released at the vesiculation stage as the spermatozoa moves through the cumulus oophorus, while enzymes such as protease would be bound to the inner acrosomal membrane to become available to facilitate penetration of the zona pellucida.

It should be noted that in domestic ungulates the cumulus oophorus is absent at the time of ovulation. The exception to this would be the pig (Hancock, 1962). This leaves open the question of the functional significance of hyaluronidase in these species, because hyaluronidase is present in comparable concentrations as to those found in the rabbit.

Smithberg (1953) working with mouse ova observed that trypsin, chymotrypsin, and ficin digested the zona pellucida at temperatures of 22° to 23° or at 37° C, and at a pH of 6.5 to 7.6.

Chang and Hunt (1956) also observed that the enzyme trypsin was the most effective in dissolving the zona pellucida in all species that were tested (rabbit, rat, and hamster). In this same experiment hyaluronidase was not capable of digesting the zona pellucida.

Srivastava, et al. (1964) extracted a cell-free acrosomal lipoglycoprotein from ram, bull, and rabbit spermatozoa that was capable of dispersing both the cumulus and corona cells whereas hyaluronidase only dispersed the cumulus. It was also observed in some of the experiments that the acrosomal lipoglycoprotein removed the zona pellu-

cida. If this lipoglycoprotein was capable of dissolving the zona pellucida it would have to contain some enzyme other than hyaluronidase because this enzyme is not capable of dissolving the zona pellucida.

In addition to the presence of hyaluronidase, Stambaugh and Buckley (1969) have extracted an enzyme from the rabbit acrosome that is capable of dissolving the zona pellucida. This enzyme had an optimum pH of 8.2 and had many of the enzyme characteristics of pancreatic trypsin. This extract when incubated with denuded rabbit ova at 37.5°C was capable of complete dissolution of the zona pellucida in 1½ hours. The authors concluded that the penetration of the zona pellucida by the spermatozoan is an enzymic process involving mainly the trypsin-like enzyme of the acrosome.

Allison and Hartree (1970) suggested that the acrosome could be regarded as a specialized lysosome which evolved to facilitate fertilization in multicellular organisms. Several enzymes that are present in lysosomes have also been found or are thought to be present in the acrosome. Enzymes that have been found in lysosomes include acid phosphatase, neuronidase, proteases, aryl sulphatase, B-N acetylglucosaminidase, phospholipase A, and hyaluronidase. All of these enzymes occur in fresh ram semen, and present evidence indicates that neuronidase and hyaluronidase is present in the acrosome (Hartree and Scrivastava, 1965; Srivastava, et al., 1965).

Morphological Changes of the Acrosome in the Female Environment

Morphological changes of the acrosome have been observed in a number of species after the spermatozoa have reached the ampulla of the fallopian tube (Austin and Bishop, 1958). These structural changes are

usually associated with the loss of the acrosome prior to penetration of the zona pellucida or with changes in the optical properties indicating the release of acrosomal material or a change in the composition of the acrosome. Whether the morphological changes that have been observed are required for the spermatozoon to complete its function of fertilizing the ovum is debated. Several researchers feel that no structural changes are involved in the physiological change in the spermatozoa whereby it gains the competence to complete fertilization. These researchers feel that the usual structural changes observed are concerned primarily with aging of the spermatozoa in the female reproductive tract.

Ericson (1967) concluded that sperm cells of both rabbits and cattle did not exhibit morphological changes that could be associated functionally with the sperm cells ability to complete fertilization. He further stated that the observed changes were probably associated with sperm cell aging.

Austin and Bishop (1958) stated that removal of the acrosome is a prerequisite of fertilization in golden hamster, guinea pig, and Libyan jird. In no instance was a spermatozoan that retained its acrosome found within any part of the egg. Bedford (1963) and Chang (1962) doubted that acrosomal alteration was involved before the sperm cell was capable of completing fertilization. Bedford (1964) stated that changes observed in rabbit spermatozoa were probably associated with some degenerative process of the sperm cell. Austin (1961) is in agreement with the above observations concerning the rabbit. However, he further states that there are pre-fertilization changes in the acrosome of certain rodents. Blom (1963 and 1964) has outlined a process

of acrosome detachment from the sperm head of bulls. His theory involved: (1) an interaction with an agent secreted by the egg or its surroundings, breakage of the sperm cell membrane at the front edge of the post nuclear cap, breakage of the outer membrane of the acrosome along the front edge of the equatorial segment causing the outer membrane to be lifted thus exposing any enzymes carried by the acrosome; (2) the outer membrane breaks into two halves shaped like bathing-caps (sometimes the two halves will remain together). The inner membrane of the acrosome, including its front protrusion, the perforatorium now forms the front part of the sperm head and most likely will play an important role in the penetration process. This theory, though not tested, cannot be entirely ruled out as it is in agreement with what has been observed in several other species. If Blom's theory is correct, and Mahajan and Menge's observations (1966) are correct what would appear to be the acrosome reaction in bull spermatozoa would also be a rapid process. This would be in agreement with Barros and Austin's (1967) observations that the acrosome reaction was a rapid process, because of a lack of intermediate changes that were observed.

In three species (guinea pig, hamster, jird), Austin and Bishop (1958) noted that the acrosome remained intact until the sperm head reached the zona pellucida, and that loss of the acrosome probably occured at the surface of the zona. An intact acrosome was never observed on a sperm cell in the substance of the zona pellucida or in the perivitelline space. Yanagimachi and Chang (1964) observed in hamsters that the acrosome must be removed before the spermatozoa can penetrate the zona pellucida. The loss of the mammalian acrosome in this situation is usually equated with the acrosome reaction, or more

precisely, with the loss of the outer acrosomal and overlying plasma membrane together with peripheral acrosomal contents which appear to be relatively insoluble.

Yanagimachi (1969a) reported that hamster spermatozoa incubated in hamster follicular fluid for 1½ to 2 hours were extremely active and that 1.3 per cent of all the motile spermatozoa had no visible acrosome. After 3½ to 6 hours even more spermatozoa were showing this extreme activity, and most of these extremely active spermatozoa had no visible acrosome. This same pattern of extreme motility and lack of the acrosome was evident through 12 hours. Yanagimachi concluded that such complete disintegration of the outer acrosome membrane and overlying cell membrane and the exposure of the inner acrosome membrane was an essential preliminary to sperm passage through the zona pellucida of the egg. He further stated that the change represented a final stage of sperm capacitation. Yanagimachi (1969b) in a related study, in which hamster spermatozoa were incubated in heat treated bovine follicular fluid, obtained similar results as those above. The number of vigorously motile spermatozoa were incubated in heat treated bovine follicular fluid, obtained similar results as those above. The number of vigorously motile spermatozoa reached a maximum of 60 per cent at 4 hours, the acrosome reaction was first apparent at 2 hours after incubation, and the number of reacted spermatozoa reached a maximum in the next 2 hours. It was also noted that reacted spermatozoa have to stay on the surface of the zona pellucida approximately 30 minutes before they actually pass through the membrane substance.

Barros, et al. (1967) have described a process of vesiculation in the hamster spermatozoa as being involved in the acrosome reaction.

The vesiculation process involves a fusion of the acrosome and the cell membrane followed by the formation of vesicles. The vesiculation process involves all of the outer acrosome membrane except that portion covering the equatorial segment. This vesiculation would allow the slow release of any enzyme carried by the acrosome that would aid in the passage of the spermatozoan through the granulosa cell layer of the egg. The authors further stated that the process of vesiculation is well advanced by the time the spermatozoa reaches the zona pellucida.

Franklin, et al. (1970) in studies with the Golden Hamster sperm observed that most of the acrosome becomes detached from the sperm head prior to penetration of the zona pellucida, and the authors further stated that this is a "normal and necessary change". The gross features of the process involved in the loss of the acrosome, as described by the authors, consisted of swelling, and the beginning of detachment at the uncurved side of the sperm head, at the anterior limit of the equatorial segment. Detachment progressed along the anterior margin of the equatorial segment--across the dorsal and ventral surface of the sperm head and terminated at the curved top of the acrosomal region. The detached acrosome maintains it cap-like shape. At the submicroscopic level the outer membrane of the acrosome and the cell membrane undergo vesiculation and are eventually lost together. The process is completed when the entire sperm cell separates from the acrosome.

Bedford (1970) observed that rabbit spermatozoa undergo a similar acrosome reaction when they are in the vicinity of the egg and that the spermatozoa had to be in the female tract a minimum of 12 hours before the reaction occured. No sperm that had not undergone the acrosome

reaction was found deep in the zona even though there were sperm cells that had not undergone the reaction laying on the zona or in its periphery. Hadek (1963) observed that spermatozoa in the process of penetrating through the zona pellucida appeared to be in various stages of denudation.

Colwin and Colwin (1965) suggested that the vesiculation of the acrosomal membranes observed by Saacke and Almquist (1964) included the overlying cell membrane. These membranes fuse and eventually disperse leaving the inner acrosomal membrane exposed which then becomes the apical covering of the spermatozoa.

The acrosome reaction also occurs in immotile sperm, probably as a postmortem change (Austin and Bishop, 1958; Franklin, et al., 1970). This "false acrosome reaction", which is the result of degenerative changes, involves a random loss of the outer acrosomal membrane in contrast to the progressive fusion and breakdown as described by Barros, et al. (1967). At the light microscope level the physiologically normal acrosome reaction can be distinguished from loss of the acrosome as a postmortem change only by the continued motility of the normally reacted sperm.

Distribution of Spermatozoa in the Female Reproductive Tract

Rapid transport of spermatozoa throughout the entire reproductive tract of the female has been reported for various species. However, the rates of distribution vary with different species.

Adams (1956) estimated that spermatozoa pass through the uterus at the rate of 0.5 to 3.3 mm per minute. He also concluded that the motility of the spermatozoa is largely responsible for their reaching

the site of fertilization, and the action of the uterus was of secondary importance in rabbits.

The speed of transport of hamster spermatozoa to the site of fertilization has been estimated to be approximately 30-60 minutes (Chang and Sheaffer, 1957).

In a review Olds and VanDemark (1957a) stated that spermatozoa have been found to reach the oviduct of rats within 15 minutes after copulation. However, the number of spermatozoa in the oviduct continues to increase for several hours.

The time required for rabbit spermatozoa to reach the upper part of the oviduct has been reported to be five minutes to three hours. The latter figure is the one that is now generally accepted.

The speed of spermatozoan transport in cattle was studied by VanDemark and Moeller (1951) and they found sperm cells in the ovarian portion of the oviduct in as little time as 2.5 minutes post-insemination. It was also observed that nonmotile spermatozoa were also transported into the ovarian portion of the oviduct in 4.3 minutes in one cow, and 12 minutes in another. Studies of the speed of spermatozoan motility indicated that the fastest moving sperm would require 30 minutes to traverse the distance from the cervix to the ovarian portion of the oviduct if they traveled at the rate displayed in vitro. The author's concluded that factors other than sperm motility were more important in the transportation of spermatozoa to the site of fertilization.

VanDemark and Hays (1952) demonstrated that the rapid transport of spermatozoa could be due to the motility of the uterus at or soon after mating. This phenomena has been demonstrated in both the estrous

and the postestrous cow. It was also demonstrated that epinephrine will inhibit this activity. This could possibly prevent or delay spermatozoa being transported into the oviducts, and thus could result in impaired fertility.

Noyes, et al. (1958) concluded that dead spermatozoa usually do not pass the cervical barrier, although conditions are favorable for the passage of live spermatozoa. A suspension of spermatozoa that contained 90 per cent dead sperm cells and 10 per cent live sperm cells was inseminated into does that had been mated by a vasectomized buck. When spermatozoa were recovered from the uterine horns in subsequent hours the percent of motile cells was consistently between 70 and 90 per cent.

Mattner and Braden (1963) confirmed earlier work that indicated that spermatozoa can be found in the fallopian tubes of ewes within 30 minutes after coitus. This rapid transport in the ewe is also attributable to the motility of the tract itself and not to the spermatozoa. It was also found that inert particles could be found in the uterus 15 minutes after being deposited in the vagina. The authors also observed that spermatozoa may be transported to the fallopian tubes in untreated ovariectomized ewes, though in somewhat smaller numbers in ewes in which estrus was induced.

Mattner (1963) observed that when sheep became frightened or excited at mating rapid transport of spermatozoa was inhibited. It was found that the stress inhibits the initial rate of spermatozoan transport to the fallopian tubes. However, stress did not influence the passage of spermatozoa from the vagina into the cervix. It was further stated that rapid transport is of short duration and that slow

transport appears to occur irrespective of the occurrence of failure of rapid transport and probably continues as long as spermatozoa are present in the cervix. Because ovulation in sheep occurs near the end of estrus and the ovum is capable of being fertilized during the next 12-15 hours it is unlikely that rapid transport of spermatozoa to the fallopian tubes is essential to fertilization in ewes that are mated during estrus.

The transportation of spermatozoa through the female reproductive tract results in a concentration gradient of spermatozoa that varies with time (Braden, 1963; El Banna and Hafez, 1970; and Dobrowolski and Hafez, 1970).

El Banna and Hafez (1970) found that the average number of sperm recovered from the oviduct of the rabbits 8 hours after insemination was 13,320 and 12 hours after insemination it was 20,292. The average number of sperm cells recovered from the cattle 16 hours after insemination was 17,025. These numbers declined, 36 to 40 hours after insemination, to 981 in the rabbit and 4,230 in cattle. They also found that percentage of sperm recovered was 50, 45, and 4.5 per cent from the vagina, cervix, and uterus respectively at 16 hours post-insemination. The distribution at 36-40 hours after insemination was 85, 15, and 0.5 per cent respectively. In cattle, respective percentages recovered from the same regions were 81, 13, and 5.1 at 16 hours and 47, 34, and 16 at 36-40 hours. It was also found in both species that the maximum number of sperm cells was found in the oviduct at the time of ovulation.

Rigby (1964) found that whole semen could be recovered from sows only up to 1 hour after insemination. At later periods no appreciable amount of fluid could be recovered from the tract and the per cent of

sperm cells recovered was greatly reduced. It was also found that the number of spermatozoa recovered from the lower part of the uterine tube was directly related to the number at the uterotubal junction. From this it appears that the concentration of spermatozoa at the site of fertilization is likely to be related to the number of spermatozoa persisting at the uterotubal junction.

Braden (1953) observed that only one of every 10,000 to 100,000 sperm cells ejaculated attains the ampulla of the fallopian tube in rabbits. The number of sperm cells recovered from the upper halves of the uterine horns one hour after mating was 0.052×10^6. The number at 2 hours was similar but between 2 and 4 hours it rose to 0.42×10^6 after which there was no significant increase up to 28 hours. Sperm cells were not found in the fallopian tubes until 3 hours after mating, when the mean number present was 56 per animal. In the upper third of the tubes, the mean number of sperm cells post-insemination increased from 0 at 3 hours to 38 at 4 hours, 84 at 6 hours, and 249 at 10 hours, which is the approximate time of ovulation in the rabbit.

Female Environmental Factors that Influence Spermatozoa

The environment required to bring about changes in the functional status of the acrosome seems to be varied. Claims have been made for in vitro capacitation (Yanagimachi, 1970; Kirton and Hafs, 1965), and it has been claimed that sperm cells have been capacitated in such diverse locations as the isolated intestine, eye, and bladder (Noyes, et al., 1958; Bedford, 1967).

Olds and VanDemark (1957b) have reviewed some of the aspects of the environment of the female reproductive tract. The pH of the var-

ious parts of the reproductive tract has been found to be dependent upon the species concerned and the stage of the estrous cycle. The vaginal fluid of rats is about 7.2 except during estrus when the pH falls to 4.4. In ewes, the pH was found to be 6.69 during diestrus and 6.6 during estrus. In cattle the pH has been observed to be 6.5-7.0 during diestrus and 7.6 to 8.1 during estrus.

The uterine fluid pH in cows has been reported to vary from 5.8 to 7.0 and varied little during the estrous cycle, but the pH of the uterine fluids of both rats and mice rose during estrus.

The pH of the ewe's oviduct has been found to be 6.0 to 6.4 during diestrus, 6.4 to 6.6 during proestrus, and 6.8 to 7.0 during estrus and metestrus. The pH of the bovine oviduct has been reported as 6.72.

The pH of bovine follicular fluid has been reported in the range of 7.3 to 7.8.

A great variety of ions have been demonstrated in the oviducts of rabbits and cattle (Hamner and Williams, 1965; Olds and VanDemark, 1957b). Of the ions thus far elucidated, it appears that bicarbonate could be of particular importance. Hamner and Williams (1965) found it in concentrations of 1.1 to 2.2 mg per milliliter in rabbit oviduct fluid. This ion has been found to increase the oxygen uptake of rabbit, human and rooster spermatozoa (Hamner and Williams, 1964). Other ions present are potassium, phosphate, chloride, sodium, and calcium. Hamner and Williams (1965) found that the concentrations of potassium, phosphate, and chloride were similar to the concentrations found for these ions in cattle by Olds and VanDemark, 1957, but that sodium and calcium were different.

Dukelow, et al. (1966) determined that rabbit uterine fluids con-

tain amylase activity that is four times as great as the activity found in blood serum. Further support of the importance of β-amylase in the uterine and tubual fluids could be indicated by the work of Kirton and Hafs (1965). These workers were able to capacitate spermatozoa by incubating the spermatozoa in vitro with β-amylase. Williams, et al. (1965) found that β-amylase destroyed a capacitation blocking factor found in seminal plasma. The above findings have led Weiman and Williams (1967) to postulate that the removal of spermatozoa from the presence of this factor is part of the capacitation process.

Chang (1958) found that the ability of spermatozoa to become fully capable of fertilizing the ovum while residing in the uterus or in the oviduct seemed to be independent of gonadotropic or estrogenic stimulation of the uterus, but that this process was inhibited by progesterone. Essentially no fertilization took place when sperm cells were incubated in pseudopregnant rabbits. However, 63 per cent of the eggs were fertilized when the sperm cells were incubated in estrous rabbits. When estrous rabbits were treated with progesterone, only 2.1 per cent of the ova were fertilized. When immature or ovariectomized rabbits were treated with estrogen no improvement in fertilization rate was observed. However, when these rabbits were treated with progesterone the fertilization rate decreased.

Hamner, et al. (1968) also observed that rabbit spermatozoa cannot attain complete functional competence in the environment of the pseudopregnant uterus. The highest rates of fertilization were obtained when the spermatozoa were incubated in intact estrous does. Incubation of sperm in ovariectomized does treated with estrogen did not enhance their fertilizing capacity over untreated ovariectomized does.

Progesterone treatment of ovariectomized does prevented them from being able to prepare sperm to fertilize. The authors concluded that a specific hormonal balance must prevail for maximum activation of the spermatozoa.

Bedford (1967) observed that the pseudopregnant uterus is capable of instilling a limited functional competence in the sperm cell. However it was also found that if the spermatozoa were injected into the uterus 0 to 1 hours prior to ovulation the fertilization rate was 63.5 per cent as compared to 7.7 per cent when the spermatozoa were injected about 2 hours after ovulation.

Hamner and Williams (1963) noted a fourfold increase in respiration of rabbit spermatozoa when incubated in the rabbit uterus. Bishop (1956) demonstrated that the rabbit tubal lumen was an aerobic environment. The oxygen pressure within the tubal lumen was evaluated during estrus, and in the first three postovulatory days. The pO_2 remained high, at approximately 60 mm of mercury. It can readily be seen that the environment of the female reproductive tract is capable of maintaining spermatozoa at these increased rates of respiration.

In view of what is known concerning the state of the acrosome in the female environment of certain laboratory animals it would be of interest to determine if the same situation exists in the domestic animals commonly used for agricultural purposes.

This preliminary study was initiated to investigate the effect of residence in the bovine uterus on certain characteristics of sperm cells. The objectives of this study were:

1. To develop a technique for repeated samplings and characterization of populations of sperm cells in the bovine uterus.

2. To determine the effect of short-term residence in the uterus on the status of the acrosome.
3. To determine the effect of short-term residence in the uterus on sperm cell livability.

CHAPTER III

MATERIALS AND METHODS

Semen Collecting and Handling

The sperm cell populations used in this study were secured from four dairy bulls from the Oklahoma State University dairy herd. The bulls were housed in the dairy bull barn in individual pens with adjacent runs and were on a once weekly collection regime.

All semen was collected on the day it was to be used. A cow was restrained in the collection chute for use as a teaser animal. The bulls were led to an adjacent chute where the preputial area was cleaned with warm water and dried. The bull was then led to the collection stall and restrained behind the cow for 5-8 minutes. The bull was allowed to serve the artificial vagina filled with water which ranged between 130to 140°F. Routine procedures were followed to prevent cold shock of the sperm cells. The graduated tube of the artificial vagina was protected by a plastic tube containing water at 95-100°F with the collecting funnel and tube being further protected by an insulated jacket.

The ejaculates were protected aginst cold shock until insemination by placing the collecting tube containing the semen in water at 95-100°F. This period prior to insemination was no longer than 20 minutes. During this period the initial ejaculate characteristics were determined by procedures that will be described in the staining

procedure. The semen was then drawn up into a breeding catheter and the catheter was placed in a protective jacket until the time of insemination.

Semen for a preliminary study with rabbits was collected from two bucks that were on a routine schedule of two collections per week. An artificial vagina temperature of 100°C was used for the collection procedure. After collection, the centrifuge tube containing the semen was placed in a beaker of water at 37°C. Initial ejaculate characteristics were determined as described for the bulls.

Insemination and Recovery of the Sperm Cells

Recovery of Sperm Cells from Rabbits

Ten does were artificially inseminated, each with approximately 0.5 ml of semen. These does were then sacrificed in pairs at intervals of 3, 6, 9, and 12 hours by injecting 2 cc of Buthanasia into the heart. The reproductive tracts were removed, excess tissue trimmed off, and the uterus and oviduct were then separated. Each oviduct was flushed with 2 ml of sodium citrate, and each uterine horn was flushed with 3.0 to 5.0 ml of sodium citrate. All of the samples were centrifuged to concentrate the sperm cells. The supernatant was poured off and the sperm cells were resuspended in four drops of sodium citrate.

Slides were then prepared for the determinations of the per cent live cells and the condition of the acrosome on the spermatozoa recovered from the uterus.

Later work with the rabbits involved inseminating larger volumes of semen (1.0 ml) to insure that larger numbers of spermatozoa would be recovered from the oviduct.

Recovery of Sperm Cells from Cows

Five normal cows from the Oklahoma State University dairy herd were used as the test units. These cows received routine management and were in varied stages of the estrous cycle, with only two of the cows being in heat. The rectovaginal technique was used to deposit a volume of undiluted semen, no less than 5 ml with an average of 1 billion sperm per ml, into the uterus. The uterine horn into which the semen was deposited was carefully determined to aid in the recovery procedure.

The semen was recovered with a stainless steel breeding catheter that had four holes drilled in its anterior end. These holes were located two on each side of the anterior end of the catheter. A 2 cc syringe which was used to provide a partial vacuum to pull the semen into the catheter was connected to the catheter with a two-inch piece of clear plastic tubing. The catheter was carefully inserted into the uterine horn with gentle vacuum being applied via the syringe as the catheter was inserted progressively deeper into the uterine horn. When the sperm cell-uterus fluid mixture was visible in the plastic tube, the catheter was withdrawn and wrapped in a protective jacket to protect the cells against cold shock.

It should be pointed out that the above described recovery and study procedure is unique in that it is a repeated sampling of the same population of cells within the same uterine environment. Other studies, as reviewed, employed slaughter of the test animals with only one observation per animal being possible. No attempt was made to flush the sperm cells from the uterus because it was felt that this would alter the uterine environment and thus make later samplings and conclusions meaningless. However, flushing would probably be a distinct aid in

recovering sperm cells after the first few hours post-insemination.

Samples of the population of sperm cells in the uterus were removed at 0.5, 1.0, 2.0, and 4.0 hours post-insemination. These collection periods allowed the evaluation of changes in the population of sperm cells relatively soon after insemination. Evaluation of the sperm cells at these short intervals is important because there is evidence that if any changes in the sperm cells do take place it is relatively rapid (Mahajan and Merge, 1966).

This technique of recovery worked relatively well. However, a problem that was frequently encountered was the inability to recover sperm cells at certain recovery periods. This was particularly a problem as the interval between insemination and recovery increased. Excellent success was encountered up to one hour, beyond this time it became increasingly difficult to consistently recover sperm cells.

An attempt was made to recover sperm cells from the oviducts as well as the uteri of four ovarectomized heifers that were slaughtered in pairs at 8 and 16 hours after insemination. Frozen semen was used that had a concentration of approximately 100 thousand sperm cells per ml. Four ampules of semen were used to inseminate each heifer.

During one phase of this study 0.5 ml of semen was removed from each of two ejaculates for in vitro incubation. These samples were placed in 2 cc vials and incubated at 37°C in a water bath. They were sampled at the same time intervals as the in utero incubations and comparisons of the two treatments were made to determine if there was any difference in the rate of change of the morphological characteristics being evaluated.

Determination of Ejaculate Characteristics

Before an ejaculate was used the quality of the ejaculate was determined using the following procedures:

a) Semen volume: It was desired to use at least 5 ml of semen. If the first ejaculate yielded less than 5 ml of semen a second ejaculate was obtained and the two were combined.

b) Sperm concentration: Ejaculates were judged to be of adequate concentration if they had a creamy white color.

c) Sperm motility: The motility of the ejaculates was evaluated microscopically and assigned a value on a 0-4.0 rating system. Ejaculates used in this study had to have a rating of 3.0 or better.

d) Per cent live spermatozoa: This was determined for each ejaculate using the nigrosin-eosin live-dead differential stain.

e) Acrosome characteristics: Acrosome characteristics were determined for each ejaculate using the Wells-Awa acrosome stain (Awa, 1968).

Staining Procedure

Following recovery of the sperm cells from the uterus and oviduct the concentration of the sperm cells was first determined to judge whether the centrifuging procedure would have to be used. Slides were then prepared to evaluate the per cent of live spermatozoa and the condition of the acrosome.

The sperm cells were removed from the catheter and placed in 0.5 to 1.0 ml of 2.9 per cent sodium citrate. If much mucus was present it was carefully agitated with a glass stirring rod to loosen the sperm

cells from the mucus. This suspension was then centrifuged at a slow speed for 5 to 10 minutes and the pellet was resuspended in 0.5 ml of sodium citrate. Centrifuging was not done if an adequate concentration of sperm cells was present initially. One drop of sperm cells was then placed in one drop of nigrosin-eosin live-dead stain, prepared and used as described by Hancock (1952). The dead sperm cells take up the eosin due to changes in the cell membrane permeability, while the live cells take no stain. The sperm cells were allowed to stain for one to three minutes, then, one drop of sperm cell-stain mixture was withdrawn, placed on a clean microscope slide, smeared, and dried on a warm stage at 37°C. Duplicate smears were made and the percentage of live cells was determined by counting the stained and unstained sperm cells. A minimum of 100 sperm cells were counted and when possible at least 200 sperm cells were counted. Partially stained sperm cells were considered dead.

The acrosome staining technique used was developed by Wells and Awa (1968). The stain is prepared by combining one volume of a one per cent solution of water soluble eosin B (total dye content 88 per cent) with one volume of a one per cent solution of water soluble fast green FCF (total dye content 90 per cent). To this is added 1.7 volumes of ethyl alcohol. Sperm cell smears were prepared by placing one drop of semen in two drops of the stain. If the concentration of sperm cells was great a dilution was made by placing one drop of semen in 5-10 drops of 2.9 per cent sodium citrate to obtain a desirable concentration for staining. From this one drop would be removed and placed in 2-4 drops of the acrosome stain. The sperm cells were allowed to stain for 3-10 minutes depending on the ability of the sperm cell to take the

stain. One drop of the sperm-stain suspension was then withdrawn, smeared on a prewarmed microscope slide, and air dried at 37°C. The condition of the acrosome was determined by counting a minimum of 100 sperm cells and when possible 200 sperm cells were counted. A nonaged sperm cell was one that displayed a uniformly smooth acrosome that was closely adherent to the nucleus, and was free from any evidence of aging. Various morphological changes were considered indicative of some stage of aging. These are as follows:

a) Elevated or thickened acrosome: This is characterized by a partial or complete swelling of the outer membrane of the acrosome cap and thus an enlargement in acrosomal size,

b) Wrinkled acrosome cap: This is characterized by a wrinkled outer edge either over the anterior portion of the cap or spreading over the entire surface of the acrosome,

c) Enlarged equatorial segment: This is characterized by the typically half moon shape of the equatorial segment becoming exagerrated as the acrosome becomes loosened from the sperm cell head.

d) Detaching acrosome cap: Observed as a loose structure in the process of being removed from the sperm head, and eventually leading to the state of capless sperm,

e) Disintegrating acrosome cap: This indicates the later stage of aging,

f) Detached acrosome: This is characterized by the lack of the acrosome and is considered the terminal stage of the aging and/or deterioration sequence. The upper portion of the nucleus stains a light pink instead of the typical green when the

acrosome is present.

In the results the classification, "total aged acrosomes", consists of all sperm cells that display evidence of aging. The classification "aging acrosomes" consists of those sperm cells that display evidence of aging, but still have an acrosome. "Capless" sperm cells are those that lack the acrosome and are considered to be in an advanced stage of aging. No genetic acrosomal abnormalities were observed in the ejaculates of the bulls used in this study.

Differences among cows and time for each characteristic studied was examined for significance with an analysis of variance procedure using a randomized complete block design. To detect differences between time periods Duncan's new multiple range test was used. A total of 8 replications were conducted. Cells could not be consistently recovered in all the time periods in all replications. Subsequently, statistical analysis was limited to only those replications with complete data.

CHAPTER IV

RESULTS AND DISCUSSION

Familiarization studies were conducted using 10 doe rabbits as the experimental units to determine what problems might be encountered when sperm cell recovery was attempted in cattle.

It became immediately apparent that large numbers of sperm cells would have to be used if adequate numbers of sperm cells would be recovered to make quantitation of the acrosomal condition possible. Very few sperm cells were recovered from the reproductive tracts when artificial insemination techniques were used to breed the does. Sperm cells were recovered upon flushing the excised systems. However, at no time was an adequate number of sperm cells recovered from any reproductive tract to quantitate the condition of the acrosome or the percentage of live cells. This may have been due to inadequate sperm cell numbers at insemination or perhaps due to the fact that the does were not in estrus at the time of insemination. Surgical techniques would probably yield more favorable results in insuring adequate numbers of sperm cells for quantitation. This phase of the study did aid substantially in working out the procedure for recovering sperm cells from dilute concentrations.

The procedure devised to recover sperm cells at various time intervals from the uterus of the cows without slaughtering them worked reasonably well. In the early phases of the study trouble was encoun-

tered with mucus and to a greater extent with blood. The problem with the mucus continued to be a problem throughout the course of the study. However, the problem with the blood was alleviated by using a smaller syringe to provide a vacuum. A 5 cc syringe was used in the early phases of the study, but it was felt that this syringe was creating too much vacuum and as a result the mucosa of the uterus was being irritated more than was desired. When a 2 cc syringe was used this problem was greatly reduced, but not eliminated. What little blood was encountered after the smaller syringe was put into use did not interfere with the procedures used to evaluate the sperm cells. Some problem was also encountered in recovering sperm cells at the 2 and 4 hour post-insemination collection period. Recovery of the sperm cells at the 4 hour post-insemination period was especially difficult. However, this problem may not have been the result of any inadequacy in the recovery technique. It is more likely that this problem was the result of sperm cells being removed from the uterus either by transport to other areas of the tract or possibly by phagocytic engulfment (Austin, 1957 and Bedford, 1965). When sperm cells were recovered at this time period the concentration of the sperm was almost invaribly dilute.

In the cattle the concentration of spermatozoa recovered was highly variable. However, in 5 cows, the concentration of recovered spermatozoa at all recovery periods was adequate so that the percentage of live spermatozoa and the condition of the acrosome could be quantitated. Figure 1 graphically presents the average change in the percentages of live spermatozoa during the four hour sampling period. A significant ($P<.05$) change in the percentage of live spermatozoa,

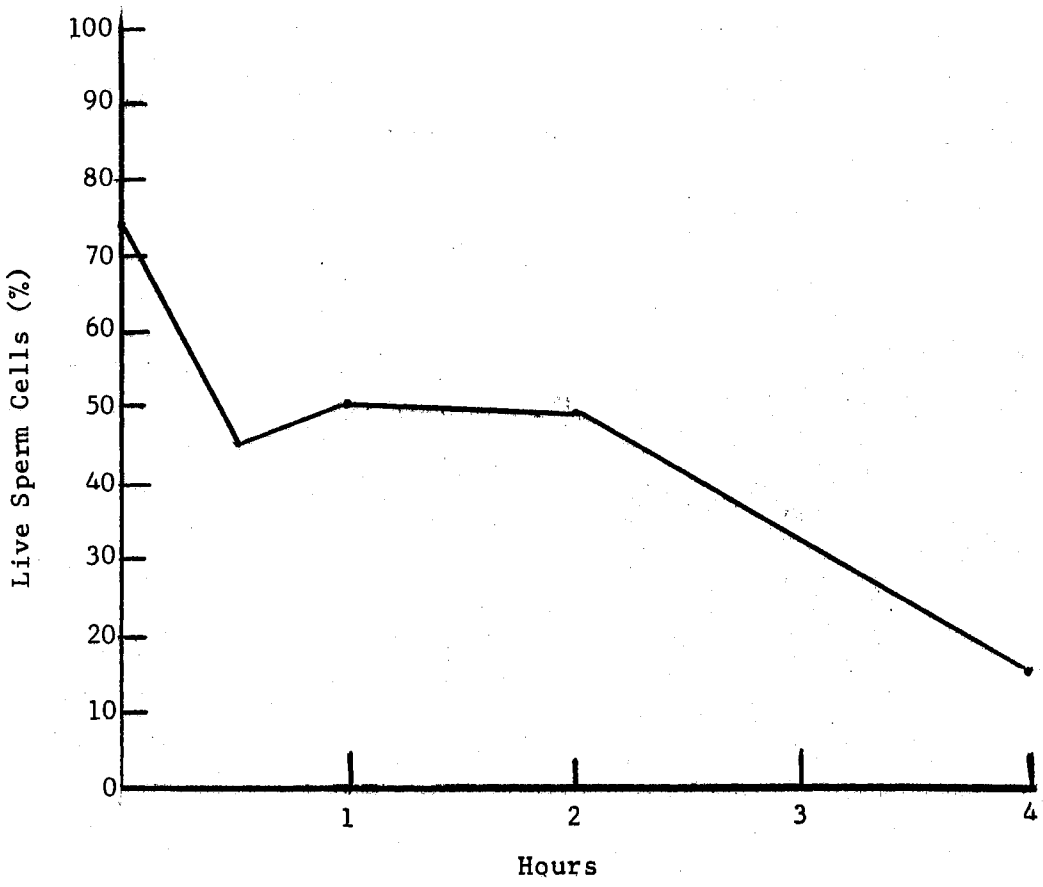

Figure 1. The Effect of Uterine Environment on the Percentage of Live Sperm Cells.*

*Each plot is the average of 4 observations. See appendix Table I.

from initial ejaculate characteristics of 74.6 per cent to 45.1 per cent, occured in the first 30 minutes of the sampling period. In the following sampling periods at one and two hours post insemination the percentage of live spermatozoa was 50.4 and 49.7 per cent respectively. These percentages of live spermatozoa were not significantly different from the original ejaculate characteristics. This could possibly be explained on the basis that the reproductive tract, by some mechanism, was selectively removing dead spermatozoa from the uterus. The percentage of live spermatozoa at 4 hours post insemination was significantly different ($P<.05$) from the percentages at 0.5, 1.0, and 2.0 hours post insemination. The difference from the initial percentage live was highly significant ($P<.01$). This drop in viability of spermatozoa in the uterus is similar to the findings of Olds and VanDemark (1957). These authors noted that spermatozoa maintained in the uterus at 37°C were not viable after 5.5 hours residence in the uterus of cattle that were 11 to 17 days postestrus. They also observed that spermatozoa lived longer in uterine fluid from cows in or near estrus than in uterine fluids from other stages of the estrous cycle.

At first evaluation, these changes in percentages indicate a very rapid die-off of sperm cells in the uterine environment. This may well be the case. However, there is the possibility that the viable cells of the total population are migrating with time to other areas of the female tract and the predominantly dead and weaker cells remain in the uterus. Other areas of the reproductive tract were not sampled in this study. It would be interesting to determine whether there is a different livability pattern in the oviducts and cervix. Olds and VanDemark, 1957; Mattner, 1968; and El-Banna and Hafez, 1970

suggested that these areas were more favorable for maintenance of sperm cell viability.

In two replicates, 0.5 ml of the ejaculates used for inseminating the cows was incubated at 37°C in a water bath and sampled at the same time interval as the portion placed in the cows. Figure 2 presents the average changes observed in the population of cells subjected to these two environments where the time and temperature elements were the same. Although the numbers of observations are limited, there is again support of either rapid loss of viability in the uterus or movement of viable cells to other areas of the reproductive system.

The average changes in percentage of total aged acrosomes is represented in Figure 3. The greatest change in acrosomal status occured in the first 30 minutes. During this time a significant change ($P<.05$) from 33.5 to 52.4 per cent occured. The only other change of note occured at the next sampling period, one hour post-insemination. During this period the percentage of total aged acrosomes increased from 52.4 to 67.5 per cent. During the following collection periods at 2 and 4 hours post-insemination, the acrosome status remained relatively stable. This indicates that rather large changes occur during the first hour the cells are in the uterus. Again, it cannot be stated with certainty whether these changes are characteristic of the total population or of the portion of the original population remaining in the uterus.

Figure 4 also presents the average changes in levels of capless spermatozoa. None of the changes were of such magnitude to be statistically significant. Changes ranging from 5-10 per cent were typical. However, more observations would assist in determining if such changes

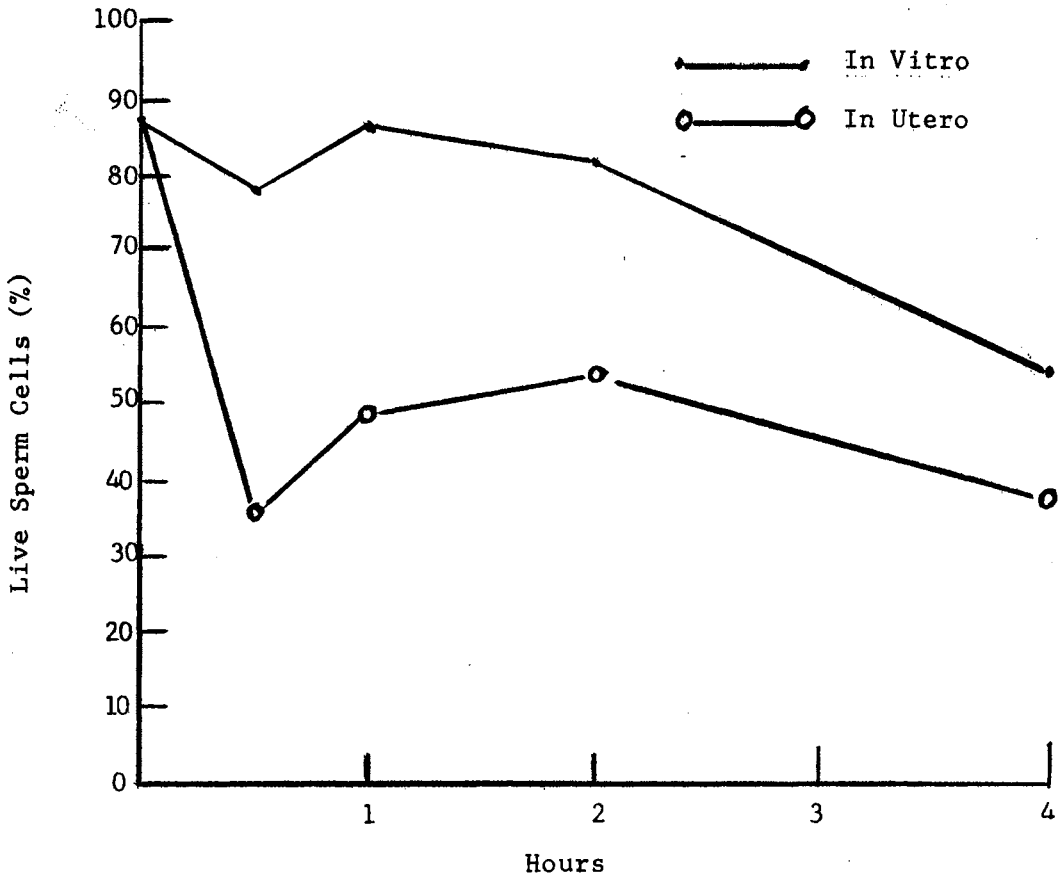

Figure 2. Comparison of the Percentage of Live Sperm Cells Incubated at 37°C and In Utero.

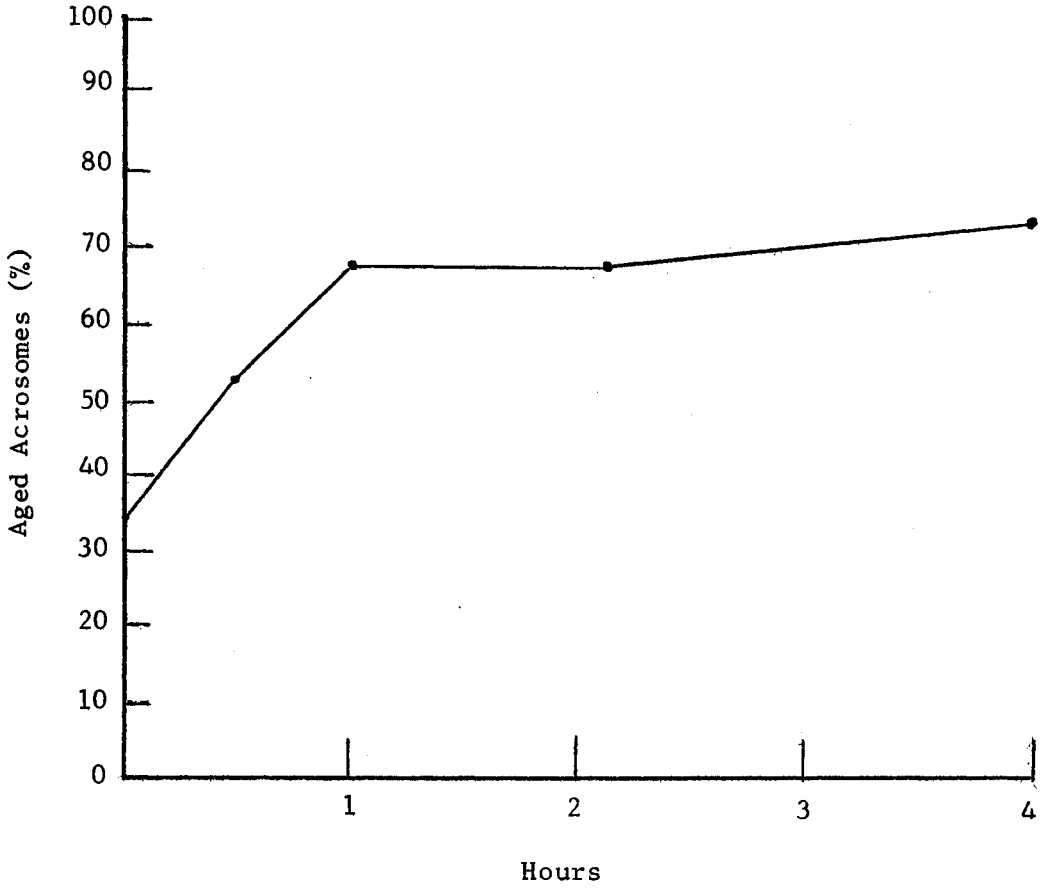

Figure 3. The Effect of Uterine Environment on the Level of Total Aged Acrosomes.*

* Each plot is the average of 5 observations. See appendix Table IV.

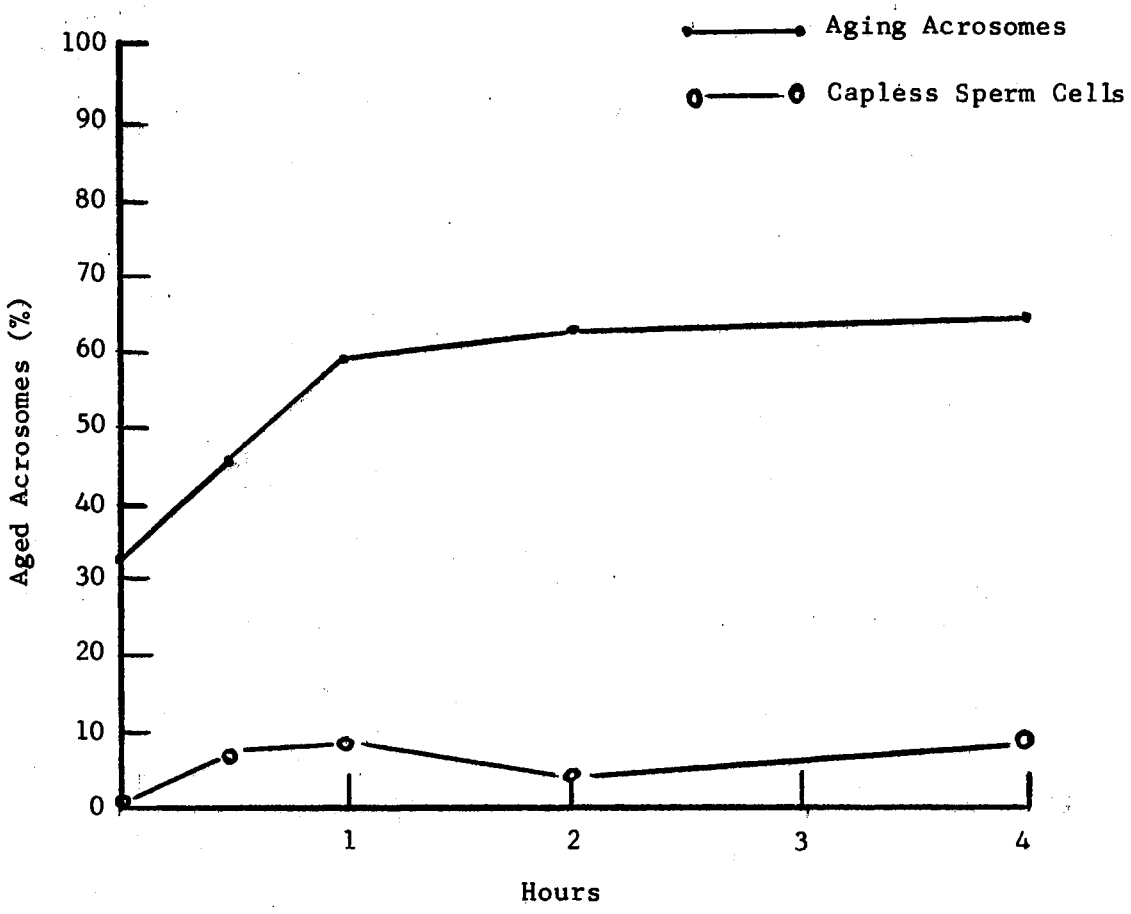

Figure 4. The Effect of Uterine Environment on the Level of Aging Acrosomes and Capless Sperm Cells.*

* Each plot is the average of 5 observations. See appendix Tables II and III.

were of significance. During the first 30 minutes there was an increase in the percentage of capless spermatozoa. The initial ejaculate level was 0.7 per cent capless spermatozoa. This percentage increased to 7.1 per cent after 30 minutes. There was little change in the percentage of capless spermatozoa from 30 minutes to one hour. However, from one to two hours there was a change in the percentage of capless spermatozoa from 8.4 to 4.2 per cent. Following this period there was an increase to 8.3 per cent at 4 hours post-insemination. The data indicates that the aged acrosomes are not advancing to the capless stage, which is considered to be an advanced stage of aging, at least during the first four hours.

Figures 5 and 6 present a comparison of acrosome status of cells incubated in vitro at 37°C and in utero. The percentage of total aged acrosomes exhibited a similar pattern as the per cent of live spermatozoa. During the first 30 minutes there was a difference of 25.0 per cent between the in utero and in vitro incubation. At 1.0, 2.0, and 4.0 hours these differences were 50.0, 31.2, and 35.3 respectively for each time period. It is also of interest to note that essentially no detached acrosomes were observed in the in vitro incubations, but there were moderate percentages of detached acrosomes in the in utero incubations. This would possibly indicate that the aging conditions observed in the acrosome were not a function of temperature only, but that some factor or factors present in the environment of the female reproductive tract in some way influenced the aging process of the acrosome. What these factors might be is not known at this time, and the functional significance of the observations is not clear. The phenomena observed could possibly be a degenerative process, or it

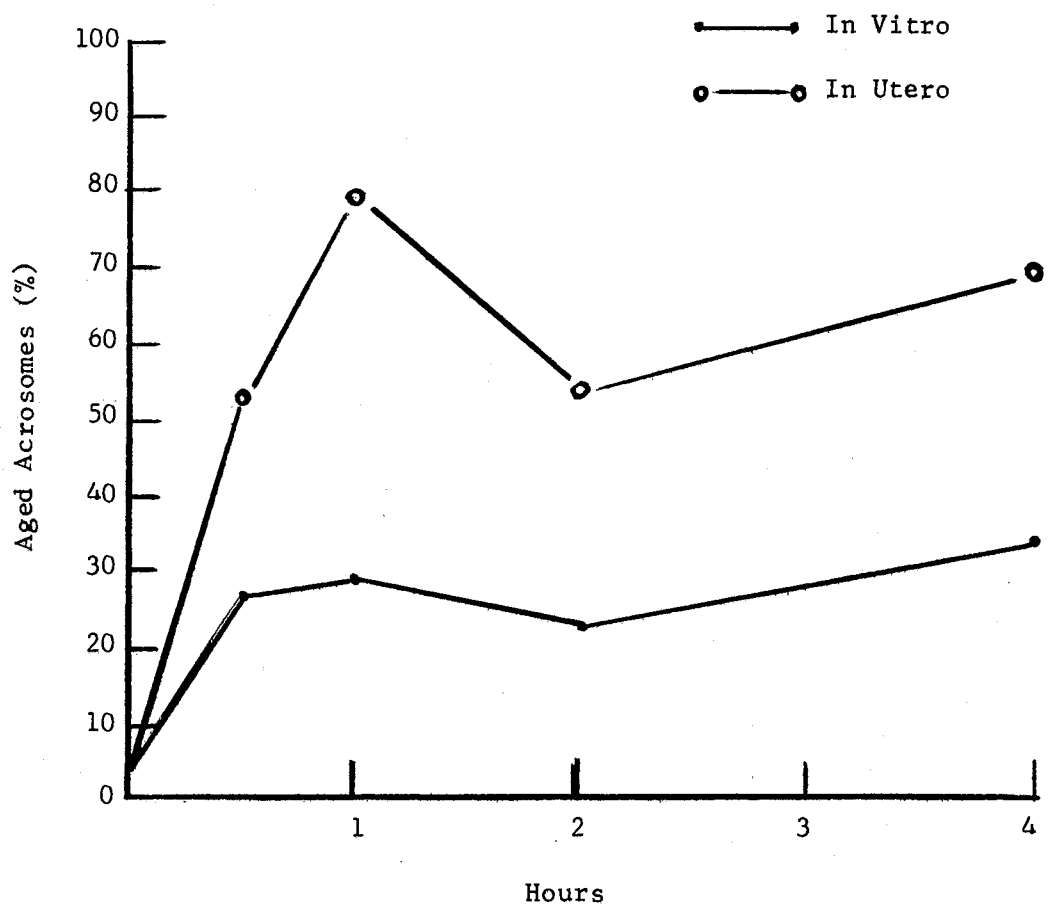

Figure 5. Comparison of the Percentage of Total Aged Acrosome Incubated at 37°C and In Utero

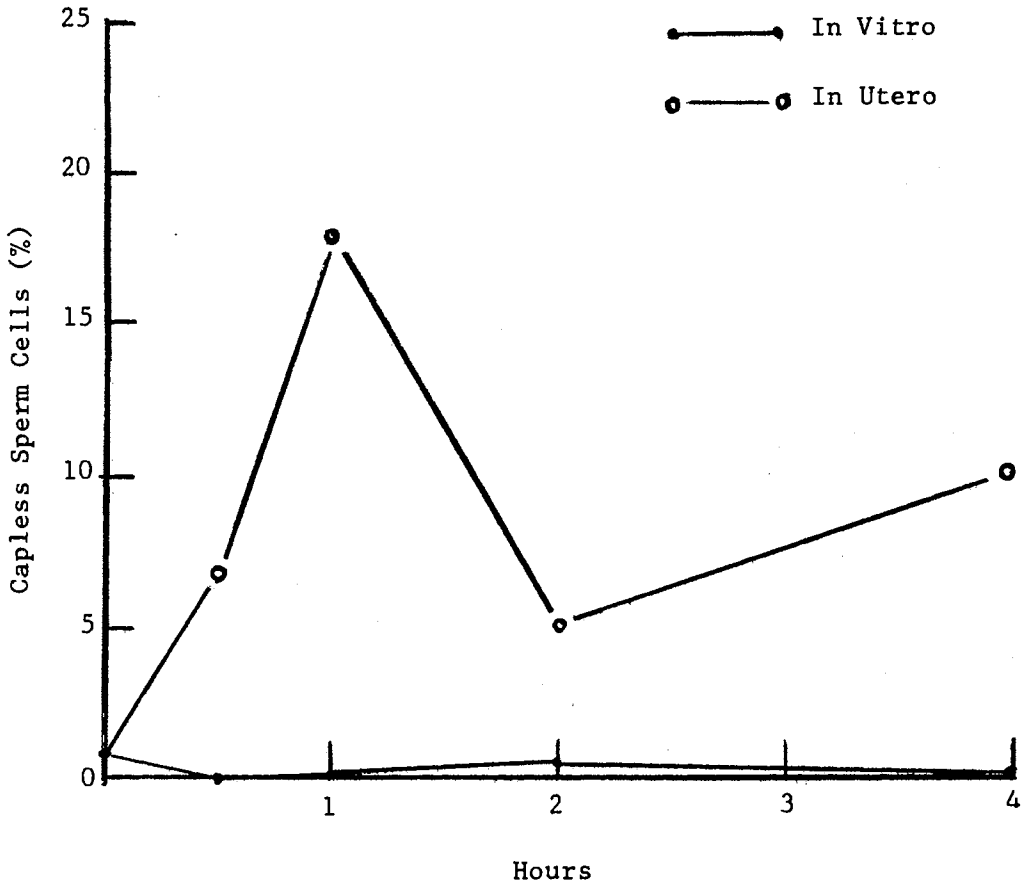

Figure 6. Comparison of the Percentage of Capless Sperm Cells Incubated at 37°C and In Utero.

could have some functional significance in the reproductive process. It should be pointed out that any change in the shape of the acrosome was classified for the purpose of this study, as aged. It may well be that the greater percentage changes observed in utero represent, at least in part, normal and necessary changes in the state of the acrosome. Further research is obviously necessary to clarify these observations.

There is also an indication in the in utero incubations that the percentage of live sperm cells is changing more rapidly than the aging process of the acrosome. This can be readily observed between two and four hours when the percentage of live sperm cells makes its second dramatic drop. During this same period the acrosome remains relatively stable. This is also evidenced in the first 30 minutes of the incubation. However, at this early period the differences in rates of change of the percentage of live sperm cells and the acrosomal aging process is not as great as between two and four hours. These observations would possibly indicate that the changes observed in the acrosome could be a post mortem change as reported by Hancock (1953), Bedford (1970), Saacke (1964), and Saacke and Marshall (1968).

No sperm cells were recovered from the oviducts of the heifers that were slaughtered at 8 and 16 hours. This was somewhat surprising since at least 100 million sperm cells were placed in each heifer. Several possible explanations exist. The cells may have been poorly placed, i. e., not in the uterine horn. Too, the time intervals may have been so long that the cells drained from the uterine, were phagocytized (Austin, 1957; Bedford, 1965), or were embedded in the uterine lining (Lovell and Getty, 1968).

One could only speculate on what effects the oviducts would have on the percentage of live spermatozoa, and on the acrosome. The possibility exists that the environment of the oviduct would be more favorable to the sperm cell as Olds and VanDemark (1957) have indicated.

CHAPTER V

SUMMARY AND CONCLUSIONS

Five cows were used to study the effect of the uterine environment on spermatozoa. The cows were inseminated with 5 ml of semen, and then sperm cells were recovered from the uterus of these cows without slaughtering them at post-insemination intervals of 0.5, 1.0, 2.0, and 4.0 hours.

The technique devised for recovering sperm cells from the uterus worked reasonably well. Some difficulty was encountered with the recovery periods at 2.0 and 4.0 hours, however, the major problem was at the 4.0 hour recovery period.

The analysis of the data revealed a significant ($P<.05$) change in the percentage of live spermatozoa during the first 30 minutes and the last two hours of the collection period. The final change in the percentage of live spermatozoa resulted in a highly significant ($P<.01$) difference from the initial ejaculate characteristics. At the 1.0 and 2.0 hour time periods there was little change in the percentage of live spermatozoa. This possibly could have been due to removal of sperm cells from the reproductive tract.

Rapid changes in the acrosome were also evident during the first 30 minutes of incubation. During this time a significant ($P<.05$) increase in the total per cent of aged acrosomes took place. Further changes were observed at the one hour period of incubation with little

change occuring thereafter.

The percentage of sperm cells that exhibited an advanced stage of age (i.e., capless) also changed significantly ($P<.05$) during the first 30 minutes of incubation.

The in vitro incubation of the sperm did not result in such profound changes in the sperm cell as did the in utero incubation. In all criteria evaluated the changes were always less than for those in the in utero incubation.

This study indicated that the uterine environment has a profound effect on the sperm cell. The factors involved seem to act rather rapidly, and the incubation study would indicate that the changes brought about in the sperm are not entirely due to temperature, or the sperm cell itself. It should also be noted that the animals used in this study were not in estrus and that the observations made here may not be the same as would be typical for cows in estrus.

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APPENDIX

TABLE I

SUMMARY OF PER CENT LIVE BY REPLICATES AND TIME PERIODS

	Post Insemination Recovery Periods (Hrs.)				
Cow	0.0[a]	0.5	1.0	2.0	4.0
	(%)				
1	63.5	50.0	61.0	16.0	
2	85.0	77.0	81.0	53.0	
3[b]	67.0	67.0	32.0	62.4	7.5
4[b]	66.5	28.0	37.6	66.8	10.0
5	66.0	44.5	6.0		11.0
6[b]	81.0	33.0	70.5	13.0	22.0
7[b]	84.0	52.5	61.5	56.5	22.0
8	90.0	18.0	36.0		54.0

[a] Initial ejaculate characteristics
[b] Cows used for analysis

TABLE II

SUMMARY OF PER CENT AGING ACROSOMES

BY REPLICATE AND TIME PERIODS

	Post Insemination Recovery Periods (Hrs.)				
Cow	0.0[a]	0.5	1.0	2.0	4.0
	(%)				
1	23.5	58.0	50.0	54.3	
2	10.5	31.0	51.0	71.0	
3[b]	60.0	65.5	73.5	50.3	47.0
4[b]	12.0	34.0	37.0	52.8	60.0
5[b]	57.5	37.5	77.0	81.0	93.0
6[b]	24.5	42.5	45.0	80.0	66.0
7[b]	10.0	47.0	63.0	49.0	56.6
8	16.5	43.5	60.5		63.0

[a] Initial ejaculate characteristics
[b] Cows used for analysis

TABLE III

SUMMARY OF PER CENT CAPLESS SPERM CELLS

BY REPLICATES AND TIME PERIODS

	Post Insemination Recovery Periods (Hrs.)				
Cow	0.0[a]	0.5	1.0	2.0	4.0
	(%)				
1	0.0	9.0	6.0	28.5	
2	1.0	24.0	4.0	9.0	
3[b]	0.0	9.5	0.5	3.0	17.0
4[b]	0.0	8.5	11.0	10.0	4.5
5[b]	1.5	5.0	4.0	1.0	3.0
6[b]	1.0	7.5	15.0	2.0	6.0
7[b]	1.0	5.0	11.5	5.0	11.0
8	0.5	8.5	24.0		10.0

[a] Initial ejaculate characteristics
[b] Cows used for analysis

TABLE IV

SUMMARY OF PER CENT TOTAL AGED ACROSOMES

BY REPLICATES AND TIME PERIODS

	Post Insemination Recovery Periods (Hrs.)				
Cow	0.0[a]	0.5	1.0	2.0	4.0
	(%)				
1	23.5	67.0	56.0	82.8	
2	11.5	65.0	55.0	79.0	
3[b]	60.0	75.0	74.0	53.3	64.0
4[b]	12.0	42.5	48.0	62.8	64.5
5[b]	59.0	42.5	81.0	82.0	96.0
6[b]	25.5	50.0	60.0	82.0	72.0
7[b]	11.0	52.0	74.5	54.0	66.0
8	17.0	52.0	84.5		73.0

[a] Initial ejaculate characteristics
[b] Cows used for the analysis

VITA

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