THE EFFECT OF BOVINE FOLLICULAR FLUID ON BOVINE SPERM

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CHAPTER I

INTRODUCTION

It has been established for several species that sperm cells undergo some unique and necessary alterations in the environment of the female reproductive system before they have the capability to penetrate the egg cell. These alterations in the sperm cells have been called the capacitation process. Experiments studying the fertilization and early developmental processes have been limited due to the inability to achieve the sperm cell changes required for fertilization.

Considerable research conducted with laboratory animals has tested the effect of follicular fluid upon the sperm cells of various species. Some findings indicate that follicular fluid can cause capacitation of sperm cells in _in vitro_ systems. However these findings can only concretely be applied to the laboratory animals which were used in the experiments. Studies on _in vitro_ fertilization in farm animals are few and, so far, not encouraging. Additional work is needed with bovine gametes to define the problems and processes involved in the capacitation and fertilization process.

Considerable controversy exists about morphology of the acrosome and the changes it undergoes before, during, and after capacitation. The use of the Wells-Awa stain for assessing the state of the acrosome in this study may better define the morphological changes characteristically caused by follicular fluid.

Recent research indicates that the sperm cells of laboratory animals can be capacitated with bovine follicular fluid and that these cells are capable of *in vitro* fertilization. This research to be discussed later indicates that the sperm cells are affected in such a way that allows them to remain viable for much longer periods of time than was previously considered possible.

If these phenomena do indeed occur they could be of far reaching impact upon the livestock industry. These characteristics would have considerable use in the collection and distribution of semen. They could be utilized in the storage of semen to achieve more extensive utilization of superior bulls. With the increasing use of ova transfer and embryo transplant in animal husbandry, the *in vitro* capacitating factors could be isolated and utilized to obtain greater utilization of these techniques. There is also the possibility that if the sperm cells could be capacitated prior to placement in the female, a different insemination time could be utilized in artificial insemination to achieve higher conception rates.

CHAPTER II

LITERATURE REVIEW

Introduction

The objective of this research was basically two-fold, (1) to evaluate the stimulatory effect of bovine follicular fluid upon sperm cells, and (2) determine the morphological effect of follicular fluid on sperm cells. These two phases have been dealt with to a limited extent by previous workers. Much of the work has been accomplished utilizing laboratory animals, especially the work done in the morphology area. Some of the work has been done using follicular fluid from a species the same as the spermatozoa, but most of the work has dealt with the effect of follicular fluid from a species different than that from which the sperm cells were collected.

This review will deal first with the studies which worked primarily in the area of the stimulatory action of follicular fluid, and then with those studies which dealt with both the stimulatory action and the morphological effect or the morphological effect alone. Because of the nature of the work and the different methods used by different workers, it is deemed desirable to deal with some of the studies in depth.

Stimulatory Effect of Follicular Fluid

Some of the earliest work done in the area of the stimulatory effect of follicular fluid was done by Olds and Vandermark (1957).

They reported that spermatozoa survived longer in follicular fluid than in any other fluid collected from the genital tract of the cow. On the average, under relatively anaerobic conditions at 37°C, spermatozoa remained motile about nine hours in cervicovaginal mucus, seven hours in uterine fluid, 12 hours in oviduct fluid, and 19 hours in follicular fluid. The rate at which spermatozoa penetrated the genital tract fluids was determined at room temperature. In general the spermatozoa progressed at the rate of about three to four millimeters per minute in mucus and oviduct fluid and about six millimeters per minute in uterine and follicular fluid. There appeared to be little difference in the rate of travel in mucus or follicular fluid due to stage of cycle. However, the rate appeared to be markedly faster in uterine and oviduct fluid from cows near estrus.

Oxygen uptake by the spermatozoa was greatest in follicular fluid and lower in oviduct fluid, mucus, and uterine fluid, respectively. This is the same decreasing order shown by these fluids in maintaining the viability of spermatozoa. It is apparent from these data that the metabolic behavior of the sperm varied greatly in the different fluids. Glucose utilization was enhanced in the presence of follicular fluid and was reduced in uterine and oviduct fluids.

In this same study another experiment was conducted in which follicular fluid was dialyzed against distilled water and then against either saline or saline plus glucose. Follicular fluid retained its stimulatory action regardless of whether it was undialyzed or dialyzed against either saline or saline plus glucose. This would indicate that the effect on sperm metabolism is due to nondialyzable substances, perhaps enzymes, proteins or large molecules.

Agglutination of spermatozoa was frequently noted in all of the genital tract fluids studied, but most frequently in follicular fluid. No variation in the occurrence of agglutination was apparent when fluids used were recovered from the reproductive tract at different times of the estrous cycle. In follicular fluid, agglutination of spermatozoa was observed to occur as early as ten minutes at room temperature in some samples, while in others it occurred only after three to four hours at 37°C. The spermatozoa aggregated in clusters of varying numbers and the tails remained motile. There were always some free spermatozoa not involved in the agglutination.

Whole porcine follicular fluid contains a dialyzable, heat stable agent which is effective at concentrations as low as 5% in stimulating porcine sperm respiration (Grotjan et al., 1974). Fresh or frozen whole bovine follicular fluid was compared with Krebs-Ringer phosphated buffer with 1% glucose added (Grotjan et al., 1975). Both had detrimental effects on respiration and motility of sperm. Severe head to head agglutination involving a large percentage of sperm was observed for both fresh and frozen whole bovine follicular fluid. The effects of various forms of bovine follicular fluid were also tested. The expected inhibitory response was noted for 20% whole bovine follicular fluid. Spermatozoa suspended in 20% dialyzed bovine follicular fluid had overall mean respiration rates significantly greater than spermatozoa suspended in whole bovine follicular fluid, but significantly less than those suspended in Krebs-Ringer phosphate controls. Spermatozoa suspended in 20% heated bovine follicular fluid exhibited higher overall mean respiration rates than those for either of the other two treatments. All forms of bovine follicular fluid reduced motility

scores and caused agglutination. Motility scores for spermatozoa suspended in either heated bovine follicular fluid or dialyzed bovine follicular fluid were greater than for whole bovine follicular fluid. This would indicate that some of the toxic effects of whole bovine follicular fluid on porcine spermatozoa are removed by dialyzing or heating bovine follicular fluid.

In Vivo Changes in the Acrosomes

Yanagimachi and Noda (1970) recovered sperm from the oviducts of golden hamsters and observed that the acrosome cap of motile sperm in the cumulus cells were either swollen, wrinkled or totally absent as determined by phase-contrast microscopy.

Bedford (1963) concluded from in vivo work with the rabbit that visible acrosome changes seen in a proportion of uterine spermatozoa, using rose-bengal stain, probably did constitute a morphological concomitant of the capacitation process. He felt the evidence suggested that the changes in the acrosome might be a morphological manifestation of a senile state in a certain proportion of the sperm population. His work also displayed fairly conclusively that the acrosome cap is shed from rabbit spermatozoa by the time the perivitelline space is reached but the exact moment at which the acrosome is discharged from these spermatozoa remained uncertain.

The acrosome can be discharged from the sperm head in the course of either a "true" or a "false" acrosome reaction. The "true" or physiological acrosome reaction, described by Barros et al., (1967) involved progressive breakdown and fusion between the plasma membrane and outer acrosome membrane which together form a series of vesicles.

The points of fusion were presumed to provide ports through which a progressive loss of acrosomal contents probably occurs after the onset of vesiculation. The "false" reaction describes the changes in the acrosome which are usually seen as a feature of degeneration breakdown. In contrast to the true reaction, the false reaction is seen as a random loss of the plasma membrane and outer membrane of the acrosome as separate entities (Bedford 1970).

Ericsson (1967) found no evidence in his work with bulls and rabbits that capacitation involved removal of the acrosome. The morphological changes were attributed to aging rather than to their capacitated or non-capacitated state. Using fluorescence microscopy he found no difference in the fine structure of capacitated or uncapacitated sperm.

Blom (1963) using thin sectioning techniques with bull sperm electron microscopy, postulated a possible function of the acrosome cap and the apical body. In close proximity of the egg a hypothetical enzyme or similar agent in the apical vacuole of the sperm cell would interact with some agent secreted by the egg or its covering and would dissolve or break the very thin cell membrane. This would allow the apical fold to open and the acrosome cap to move forward and become loosened from its close contact with the nucleus. The double membrane of the acrosome cap would break into two halves, give off their depots of hyaluronidase and the sharp front edge of the nucleus is then free and ready to penetrate the ovum. Although hypothetical and unproven this theory was prompted by the fact that bull sperm cells possessing abnormal apical bodies are infertile.

In Vitro Acrosome Changes

Franklin *et al.*, (1970) described the "normal" acrosome reaction of the golden hamster from observations of sperm incubated with eggs in tubal fluid under conditions known to be conducive to sperm penetration of ova *in vitro*. Vesiculation of the surface of the acrosome occurred anterior to the equatorial segment and the anterior portion of the acrosome separated from the sperm head. The vesicles were formed from the outer acrosomal membrane and overlying plasma membrane. The more centrally located uubstances of the acrosome dispersed, but a peripheral layer of acrosomal material detached along with the vesiculated membranes. The result was a detached portion of the acrosome that is expanded in size but still retained a broad cap-like shape. It was their conclusion that the acrosome reaction occurs both as a normal prerequisite to sperm penetration of the zona pellucida and as a degenerative change and these two types of acrosomal changes are difficult to distinguish.

In a paper presented by Barros (1967a) sperm cells were incubated in Eagles Tissue culture medium with various reproductive tract fluids added to epididymal sperm incubated at 37°C for several hours. Observations were made with a phase contrast microscope of 100 mature vigorously motile sperm cells chosen at random to determine if the acrosome reaction had occurred. Follicular fluid from ripe follicles and oviductal fluid with ova was very efficient at inducing the acrosome reaction. Oviductal fluid from the castrated side of unilaterally castrated females was about 50% as efficient at inducing the acrosome reaction as the fluids containing follicular components. The acrosome

reaction involves a change in optical contrast and eventual detachment of the acrosome (Barros and Austin 1967b). The reaction seems to take place rapidly as very few intermediate changes were noted. Practically no reacted acrosomes resulted until four hours after incubation and agglutination of spermatozoa occurred only in aqueous humor. No acrosome reaction was seen when spermatozoa were suspended in medium 199, in aqueous humor or in fluid hen egg white, despite good survival, as shown by continuation of active progressive motility for six hours or more. A high proportion of spermatozoa with reacted acrosomes (84%, range 54-100%) was seen with tubal fluid containing eggs, and the frequency dropped considerably (47%, range 11-85%) when the tubal fluid was obtained on the side lacking the ovary in a unilaterally ovariectomized hamster. This would indicate that the follicular contents contribute to the ability of tubal fluid to cause the acrosome reaction. When tubal eggs were washed and suspended in medium 199, the preparation was found to have very poor reaction-invoking properties (8% range 3-17%). The most consistently effective medium for inducing the acrosome reaction turned out to be follicular contents from immediately preovulatory follicles (9-10 hours after HCG injections), the incidence was 97%, range 90 to 100%. When the follicular contents were obtained from the half grown follicles, four hours after HCG injection, the reaction percentage was lower (43%, range 4-89%).

Tubal fluid with eggs from rats and mice clearly supported the development of the acrosome reaction in hamster spermatozoa, but at a rather low frequency (30%, range 10-52% and 40%, range 17-80%, respectively). Concurrent with the acrosome reaction work, Barros and Austin (1967b) also reported that 66.5% of follicular eggs incubated

with epididymal sperm for 6-10 hours were fertilized (confirmed by the presence of two polar bodies together with one or more sperm tails and two or more developing pronuclei). Treating tubal eggs similarly resulted in 49% fertilization while washing tubal eggs with medium 199 three times brought fertilization down to 4.6%.

Yanagimachi (1969a) used follicular fluid from various species and tested its effect on in vitro capacitation of hamster spermatozoa. When hamster epidiymal spermatozoa were incubated in vitro in the presence of hamster follicular fluid in Tyrodes solution for three hours or more, they became fully capacitated. The majority of the capacitated cells had lost the acrosome cap as determined by phase contrast microscopy of motile cells, and displayed extremely vigorous motility. When placed in contact with freshly ovulated eggs in vitro the spermatozoa started to enter the zona pellucida of the eggs as early as 10 minutes after insemination. Sperm penetration through the zona pellucida, however, occurred most often between 30 and 50 minutes after insemination. The sperm capacitating potency of follicular fluid was greatly reduced when diluted with a large volume of Tyrodes solution. Cumulus oophorus cells, their matrix, corona radiata cells and egg cells lacked the ability to induce functional capacitation of the spermatozoa. Follicular fluid of four different species was tested. In respect to their potency to capacitate hamster spermatozoa, the fluid of three species could be arranged in the following order; hamster greater than the mouse, mouse greater than rat. Rabbit follicular fluid was totally ineffective on hamster sperm.

Mukherjie and Lippes (1972) studied the effect of human follicular fluid and tubal fluids on rat, mouse and man spermatozoa. Sperm

samples were incubated at 37°C with the following fluids for up to four hours: 1) follicular fluid without heat inactivation; 2) follicular fluid-heat inactivated; 3) a mixture of heat inactivated follicular fluid:tubal fluid (1:2); 4) tubal fluid; 5) normal saline. A single solution procedure was used to stain the acrosome of the sperm cells (Cassarette 1953). The stain consisted of an aqueous solution containing 5% aniline Blue and 5% Eosin. The acrosome stained blue and sperm nucleus red with presence or absence of stained acrosomes being interpreted as the completion or lack of the acrosome reaction. In direct preparation, with no pretreatment by the human fluids, all sperm maintained the acrosome staining. Very little change was noted in acrosome staining on sperm exposed to untreated follicular fluid (human 7% and rat 3.5%). However in the mouse, 20.5% of the sperm lost the acrosome staining potential when exposed to untreated follicular fluid. Treatment with tubal fluid alone led to a loss of acrosome staining in human (58.6%) and rat sperm (40.2%) whereas in the mouse the effect was comparable to untreated follicular fluid (26% versus 20.5%).

Since untreated follicular fluid led to the immobilization of the sperm, heat inactivation of this fluid was used to counteract this effect. Treatment of sperm with heat inactivated follicular fluid caused considerable loss of acrosome staining in all species (human, 67.6%; rat, 43.6%; mouse, 32.0%). Although heat inactivated follicular fluid as well as nontreated tubal fluid alone led to the loss of acrosome staining, the most effective treatment in all three species was a combination of the two. Such treatment resulted in the loss of acrosome staining in a higher percentage of cells (human, 80%; rat, 74%; and mouse, 50.3%).

In addition to the loss of acrosome staining, capacitation was tested in the mouse by *in vitro* fertilization using sperm treated with various fluids and their combinations. The only fluids resulting in fertilized oocytes were tubal fluid with 15% of the embryos going to two cell state and 50% of these developing to normal blastocysts in three days *in vitro* growth. The use of heat inactivated follicular fluid alone resulted in two cell embryos in 21.3% of the oocytes with 65.2% of these developing to blastocysts. The most effective treatment was a 1:2 mixture of inactivated follicular fluid and tubal fluid yielding 59.5% two cell embryos of which 77.7% developed to blastocysts. Attempts to achieve *in vitro* fertilization of human ova with sperm treated with a 1:2 mixture of heat inactivated follicular fluid and tubal fluid proved unsuccessful.

Biologic evidence of capacitation giving rise to normal embryos developing to blastocysts was observed only when sperm capacitation was complete (after four hours). This was also noted to be the same time when the acrosome was no longer staining blue.

Yanagimachi (1972) incubated guinea pig eggs in Biggers, Whitten and Whittingham solution and observed that only those spermatozoa without acrosomal caps had attached to the zona pellucida (Biggers *et al.*, 1971). The spermatozoa with intact acrosomal caps, either agglutinated or nonagglutinated, came into contact with the zona surfaces, but none made permanent attachment. Sperm heads lacking acrosomal caps could be seen within the zona pellucida as early as 10-15 minutes after insemination. These observations led the workers to assume that the acrosome reaction occurs either in the final stage of capacitation or as a sequential event following the completion of capacitation.

Gwatkin and Andersen (1969) indicated that sperm capacitation does not seem to be necessarily a function of acrosome loss. Motile sperm collected directly from the epididymis of hamster possessed acrosomes and failed to fertilize ova within a four hour incubation period. Incubation for six hours in medium 199 caused only a few sperm to lose their acrosome (2%) and only a small proportion of eggs were fertilized (12%) by the resulting sperm population. Hamster follicular fluid (9-10 hours post HCG), however, markedly increased both the proportion of sperm losing their acrosome (65%) and the percentages of eggs fertilized (21%). In both respects hamster follicular fluid was as effective as oviduct fluid with or without eggs in the cumulus cells. Bovine follicular fluid, pooled from several ovaries collected at the abbattoir and from follicles ranging in diameter from 2mm to 20mm, consistently arrested sperm motility within a few minutes. However, when this toxicity was removed by heating for one hour at 60°C the acrosome removing and capacitating effects were comparable with those of the hamster preparations. The effect of diluting the bovine follicular fluid on both acrosome removal and capacitation was studied. Acrosome loss declined with increasing dilution, but there was no comparable decline in the apparent capacitation of the sperm population as judged by the proportion of eggs which were fertilized. These experiments demonstrated that detoxified bovine follicular fluid has the same capacitating effect as hamster ovarian and genital tract secretions on a population of hamster sperm. They concluded that it was apparent that this effect occurs at a dilution which has a barely detectable effect on acrosome removal. Therefore it seems unlikely that the sperm which fertilize the ova within four hours, (that is, become functionally

capacitated) are those which have lost their acrosomes, rather the capacitated sperm must be those in a stage earlier than this terminal state. In this study some sperm were capacitated in medium 199 alone, indicating that follicular factors are not uniquely essential for the capacitation process, however they obviously accelerate the process.

Iwamatsu and Chang (1969) carried out an experiment which demonstrated that mouse sperm can be capacitated in vitro in the presence of bovine follicular fluid. They incubated mouse eggs, a suspension of epididymal sperm, and one drop of heated bovine or human follicular fluid at 37°C in an atmosphere of 5% carbon dioxide and 95% oxygen for 1-6 hours. At the end of the period of incubation the proportion of motile sperm and sperm with or without acrosomes was determined with a phase contrast microscope. Eggs were considered to have been penetrated only when there was an enlarged sperm head or a male pronucleus in the vitellus with a fertilizing sperm tail in or around the vitellus.

The proportion of motile sperm with and without acrosomes, at any time between one hour and six hours after insemination varied from 43% to 64% in any preparation. The pattern of motility appeared to be different between those with an acrosome and those without. In the intact sperm, the midpiece was sharply bent during movement. Sperm without an acrosome were propelled by a wave of beats from the midpiece to the top of the tail. Although numerous sperm were attached to the zona pellucida within 15 minutes of insemination, the detachment of the acrosome and sperm penetration through the zona was not observed less than two hours after insemination. In the presence of bovine follicular fluid, the proportion of motile sperm without acrosomes increased from

7.6% two hours after insemination to 28% after six hours. This increase correlated approximately with the proportion of eggs penetrated.

In the absence of bovine follicular fluid and especially after the egg clot had been washed in Tyrodes solution the proportions of active sperm without an acrosome and penetrated eggs increased only slightly between two and five hours after insemination. There was a significant difference in the proportion of eggs that had been penetrated four hours after insemination between preparations with and without bovine follicular fluid present, which led them to conclude that heated bovine follicular fluid has a beneficial effect on the capacitation of mouse sperm.

Yangimachi (1969b) reported that bovine follicular fluid, like rabbit follicular fluid, is very toxic to hamster spermatozoa, but can be readily detoxified by moderate heat treatment. Such detoxified follicular fluid could efficiently induce the acrosome reaction and capacitation of hamster spermatozoa. Determination of the effects of follicular fluid and its fractions on the motility and acrosome reaction of hamster spermatozoa was made. In each observation over 100 strongly motile spermatozoa were examined. The spermatozoa were recorded as "reacted" when their acrosome caps (1) had completely disappeared or (2) had undergone profound modification or disintegration. The majority of the reacted spermatozoa in the study fell in category (1).

Bovine follicular fluid which was not subjected to heat-treatment (56°C, 30 minutes) was very harmful to hamster spermatozoa. When an epididymal sperm suspension was mixed with an equal volume of unheated follicular fluid, 100% of the spermatozoa became immotile within 3-4 minutes. When follicular fluid was diluted with Tyrodes solution

(1:2, 1:4, 1:8) and mixed with an equal volume of sperm suspension, all the spermatozoa were immobilized in ten minutes, 30 minutes, and two hours, respectively. In no case did the spermatozoa agglutinate. The heated follicular fluid, unlike unheated fluid, was very favorable to hamster spermatozoa. It sustained the viability of spermatozoa for more than five hours. A conspicuous phenomenon noticed during observations was head to head agglutination of active spermatozoa. The agglutination appeared soon after spermatozoa were mixed with follicular fluid and remained prominent during the first two hours of incubation.

Yanagimachi (1969b) also tried to determine what components in the heated follicular fluid are responsible for the stimulation and the acrosome reaction of spermatozoa. His study indicated that a dialyzable fraction of the follicular fluid was the prime factor responsible for sperm stimulation, and a nondialyzable fraction was the potential acrosome reaction-inducing factor. Additional work in his study indicated that successful fertilization occurs only in the medium in which sperm acrosome reaction is induced, and only after the sperm acrosome reaction has been complete for some period of time.

CHAPTER III

MATERIALS AND METHODS

Semen Collection and Handling

The semen used in this experiment was collected from four different dairy bulls housed in individual pens at the Oklahoma State University dairy barn. The bulls were on routine maintenance rations with no other experimental regime applied. The bulls had been collected twice weekly for several months prior to the experiment.

The semen was collected using an artificial vagina with water temperature between 52°C and 54°C. An estrus cow was used, if available, for the teaser animal. Normal collection procedures were used to insure a clean, high quality sample. The bull was restrained behind the teaser animal and allowed several false mounts before the actual collection was made.

The semen was collected in 15 ml plastical conical collection tubes and placed in a water bath maintained at 35°C to 37°C. The collection procedure was repeated until all four bulls were collected. The semen utilized in this study were collected in four different weeks at least six days apart.

Evaluation of Sperm Characteristics and Morphology

The following procedures were used to evaluate the characteristics

of the cells of the ejaculate prior to imposing treatments:

a) Sperm concentration: A spectrophotometer (Spectronic 20), previously calibrated with hemocytometer sperm cell counts, was used to determine the concentration of cells in the initial ejaculate. This information was used in achieving twenty million live cells per ml of incubation media.

b) Motility: One drop of raw semen or incubated cells was placed on a prewarmed clean glass slide with one drop of 2.9% sodium citrate. A clear cover slip was placed over the suspension and the slide examined under a light microscope (430X). The percentage of motile cells was estimated in units of five from 0 to 100. The rate of progressive motility was scored on a scale from 0 to 4, 0 displaying no movement, and rates of 2, 3, and 4 indicating increasingly vigorous movement.

c) Percent normal cells: The percentage of normal cells was determined from the Wells-Awa stained slides (Wells and Awa 1970). The determinations made were head abnormalities, including enlarged heads, free heads, pinheads and pyriform heads, or tail abnormalities including broken, bent, hooked or coiled tails. The Wells-Awa stain was prepared according to the following procedure. One volume of a 1% solution of water soluble eosin B and one volume of a 1% solution of water soluble fast green FCF was mixed with 1.7 volumes of 95% ethyl alcohol. All stains, solutions and glassware were prewarmed on a slidewarmer. Smears from the samples being evaluated were prepared by mixing one drop of raw semen diluted 1 to 10 with 2.9% sodium citrate with three to four drops of the

Wells-Awa stain and allowed to stain for two to four minutes in a glass vial. A drop of this mixture was then placed on a clear glass slide and another slide used to distribute the stained cells over the slide.

d) Percent altered acrosomes: This was also determined using the Wells-Awa stained slides. There were several stages of alteration of the acrosome observed. They can be outlined as follows;

1) Elevated or thickened acrosome: This is characterized by a partial or complete swelling of the outer membrane of the acrosome and thus an enlargement of acrosome size;

2) 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;

3) 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 a capless sperm;

4) Disintegrating acrosome cap: The partial removal of and overall destabilization of the acrosome cap;

5) Detached acrosome: This is characterized by the lack of the acrosome. The upper portion of the nucleus stains a light pink instead of the typical blue-green when the acrosome is present;

6) Unaltered acrosome: Characterized by the tightly adhering smooth appearance of the blue-green staining acrosome membrane system.

Two hundred cells were counted from each slide and the percent normal cells and percent altered acrosomes within each cell type were calculated from these observations.

Follicular Fluid Recovery

The ovaries used in this study were obtained from tracts of cows being slaughtered at a large packing house and represented a sample of cows of varying stages of pregnancy and the estrous cycle. The follicular fluid was harvested from follicles ranging in size from 2mm to 20mm. An effort was made to avoid abnormal ovaries. Due to normal follicular growth, one could deduce that the larger contribution to the total volume was made by mature follicles. The ovaries were excised from the tracts and the follicular fluid withdrawn with a needle and syringe and placed in a glass tube immersed in an ice water bath. This was accomplished within 15 to 20 minutes of death of the animal. This may be important since work summarized by Edwards (1974) indicated a difference in glucose and lactic acid contents of follicular fluid extracted from intact ovaries versus that extracted from ovaries excised and held for a time before removal of the fluid. The follicular fluid was transported to the laboratory in the ice water bath, filtered through a Kimble 40-60 micron fritted glass filter and frozen in 20 ml snap cap plastic vials at -5°C. The individual vials were thawed when needed at room temperature of approximately 25°C and then warmed to 37°C by placing in a beaker of warm water.

Incubation Media Preparation

The treatments used consisted of combinations of Krebs-Ringer phosphate buffer containing 0.5% fructose prepared according to the method of Umbreit (1957) and follicular fluid. The follicular fluid and Krebs-Ringer phosphate buffer were placed in glass screw cap vials in the following ratio of volume to volume: (100% follicular fluid: 0% Krebs-Ringer phosphate), (50% follicular fluid: 50% Krebs-Ringer phosphate), (25% follicular fluid: 75% Krebs-Ringer phosphate), (0% follicular fluid: 100% Krebs-Ringer phosphate). The 100% Krebs-Ringer phosphate media served as the control. These combinations of follicular fluid and Krebs-Ringer phosphate buffer were utilized to determine the effects of bovine follicular fluid on sperm stimulation and the acrosome characteristics.

Incubation and Sample Recovery Procedure

Twenty million live sperm cells per ml were added to screw cap vials containing three to five ml of the different media and the vials were then placed in a warm water bath at 37°C and incubated for nine hours under aerobic conditions. At three hours, six hours, and nine hours of incubation a small drop of each sperm cell suspension was placed on a clear glass slide and a cover slip placed on top of the drop and the slide placed under a phase contrast microscope (430X). Determinations of type of motility, percent motile cells and the presence of cell agglutination were made. In addition one-half ml was removed at each time interval and centrifuged at approximately 1700 x G for two minutes, the supernatant poured off and the pellet again

resuspended in one-half ml of 2.9% sodium citrate. The centrifugation was necessary to wash off the follicular fluid as preliminary research had indicated that it interfered with the staining properties of the spermatozoa. One drop of resuspended spermatozoa was then placed in glass vials containing four drops of Wells-Awa stain and allowed to remain in the vial for three-four minutes to facilitate staining. A drop of the stained spermatozoa was then placed on a slide and another slide was used to make a thin layer smear. The slides were utilized to make the determinations of normality and acrosome status described earlier in this section.

Statistical Analysis

The data was analyzed as a split-split-plot blocking on bulls with week of collection as the main unit treatment, percent follicular fluid as the sub-unit treatment and length of incubation as the sub-sub-unit treatment (Snedecor and Cochran 1967). In addition, the linear, quadratic, and cubic effects and the interactions of these factors were partitioned out. The means will be presented and discussed in tabular form as well as in response surfaces.

CHAPTER IV

RESULTS AND DISCUSSION

The results presented and the discussion concerning these results are from data collected during investigations conducted with 16 ejaculates collected from four bulls in four different weeks. The initial ejaculate characteristics in the tables and in the figures at "0" time were not included in the statistical analysis of the data but are presented to indicate the characteristics of the population of cells immediately prior to being placed in the various media.

Three dimensional plots are utilized to aid in discussion of the data. These plots are constructed from the overall means secured from each incubation time and follicular fluid concentration.

Sperm Stimulation

The most obvious effect of follicular fluid on the spermatozoa was observed when microscopic evaluation for type of motility was made. Appendix Table VI shows that the rate of motility of sperm cells was stimulated by all concentrations of follicular fluid. The sperm cells displayed a very accelerated and exaggerated type of movement, a type of movement far beyond the scope of the normal scale of 0 to 4 commonly utilized in describing rate of motility. Yanagimachi (1970) reported a similar description of this type of movement in capacitated hamster sperm cells. Grotjan et al. (1974) reported a similar increase in rate

of motility of porcine sperm cells when incubated in porcine follicular fluid. Their work indicated a correlated increase in respiration of the stimulated sperm cells. Olds and Vandemark (1957) reported higher rate of movement of bovine sperm cells in follicular and uterine fluid as well as a marked increase in oxygen consumption.

There was no relationship between percent follicular fluid in the incubation media and the rate of movement of the cells. Accelerated movement was observed in all samples containing follicular fluid at all time periods but never in the Krebs-Ringer phosphate controls.

Agglutination

Another phenomena apparent under microscopic evaluation was the presence of head to head agglutination of the sperm cells in samples containing follicular fluid. This agglutination was observed at all time intervals but not in all samples (Appendix Table VI). More cells appeared to be involved in the agglutination in media containing a higher percentage of follicular fluid. Head to head agglutination has been reported by various other workers when using follicular fluid in the incubation of sperm cells (Grotjan et al. 1974 and 1975, Olds and Vandemark 1957, Yanagimachi 1969b). It apparently is beneficial in at least some cases as Saacke (1973) reported that motility was maintained at a higher rate in samples displaying agglutination, which was generally true in our observations also. There is a possibility that all cells may be involved at one time or another in agglutination, even though when observations were made it was apparent there were many free cells. These free cells may represent cells from previously agglutinated spermatozoa that have broken their attachment from one another. They

may also represent cells that will at a future time agglutinate. The increased rate of motility discussed earlier was displayed both by agglutinated cells and free cells. It is also possible that the two phenomena, agglutination and accelerated motility, are independent of each other.

The agglutination may represent a phenomena necessary for capacitation of sperm cells. Some type of substance or enzyme may be exchanged or mutually shared during agglutination or it may be simply a group approach to overcoming the obstacles in the female reproductive tract.

Motility

The percent motile cells for each treatment and incubation time are presented in Table I. The analysis of variance (Appendix Table V) indicates that both treatment and incubation had highly significant effects ($P < .01$) on percent motile cells. Higher percentages of motile cells were maintained in those preparations containing follicular fluid than in the controls, as was true with work by Olds and Vandemark (1957). The response surface (Figure 1) shows the detrimental effect of 100% follicular fluid and that motility was higher with intermediate percentages of follicular fluid. The 50% and 25% follicular fluid media maintained motility at a significantly higher level ($P < .05$) at nine hours than did either of the other media. The Krebs-Ringer phosphate buffer was the least effective in sustaining motility. The seemingly synergestic action of follicular fluid and Krebs-Ringer phosphated buffer is most probably due to the contribution of a sperm stimulatory

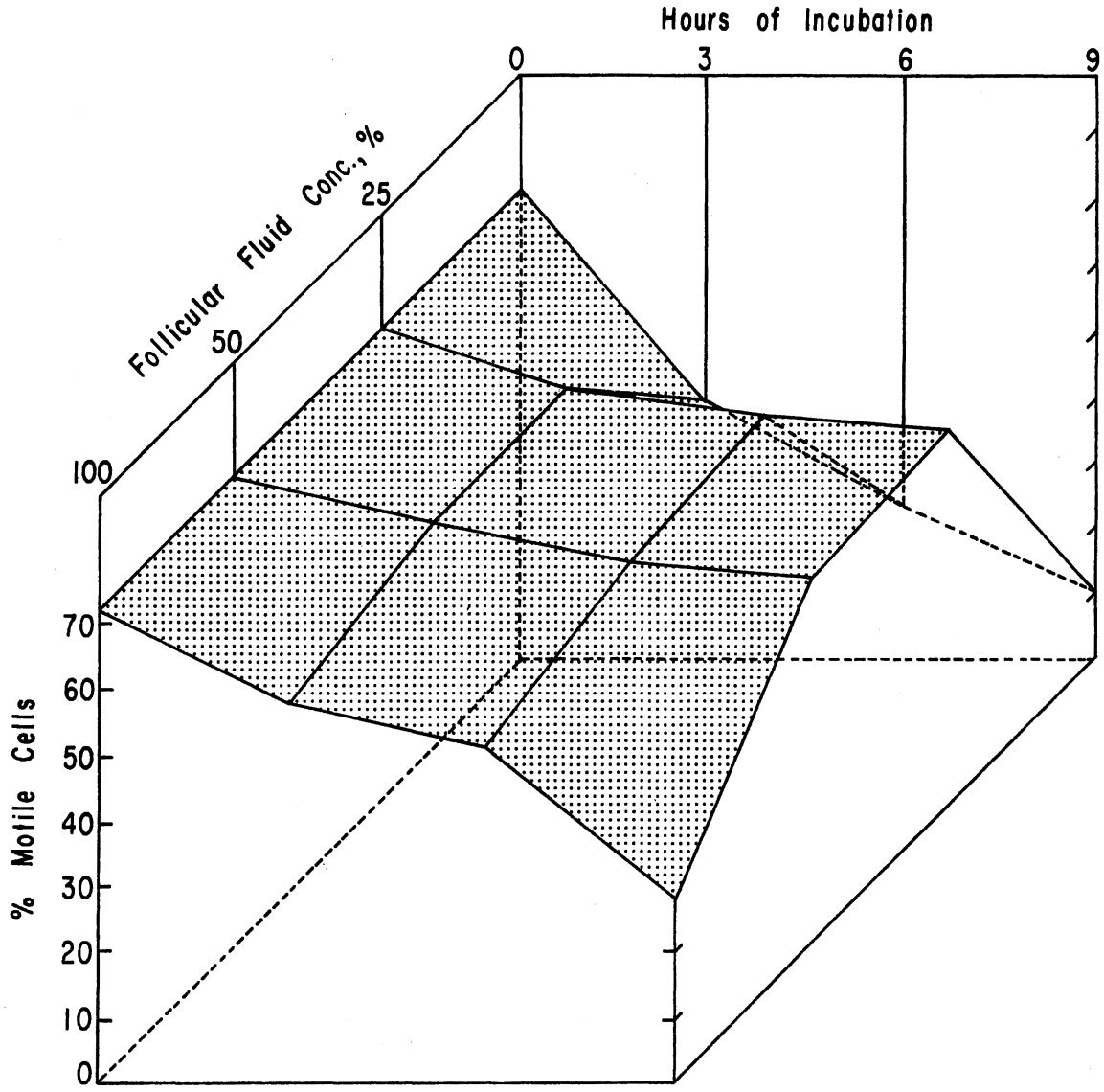

Figure 1. The Effect of Follicular Fluid in the Incubation Media and Length of Incubation on the Percent Motile Cells

substance by follicular fluid and the buffering capacity of the Krebs-Ringer phosphated buffer.

TABLE I

MEANS OF PERCENT MOTILE CELLS AVERAGED OVER WEEK OF COLLECTION AND BULLS

	Percent Follicular Fluid			
	0%	25%	50%	100%
Initial Ejac.	72.1	72.1	72.1	72.1
3 Hours	40.3*	64.0	64.0	58.7
6 Hours	24.6	60.9	58.1	51.8
9 Hours	11.7	58.1	56.2	28.7

*The S.E. for comparing means within rows is ± 3.4.

*The S.E. for comparing means within columns is ± 2.88.

As length of incubation increased the percent motile cells decreased regardless of the type of incubation media, but more drastically in those media containing no follicular fluid and 100% follicular fluid. Analysis indicated that the response to increasing percent follicular fluid was linear, quadratic and cubic in nature (Appendix Table V). The contribution of linear effects could be expected. However, the cubic effects may be due to the lack of equal gradations in the percentages of follicular fluid from 0% to 100% as the cubic trend is not

present in the means or response surface. The linear response of motility was significant indicating a linear decrease depicted by the response surface (Figure 1). There was a significant interaction ($P < .01$) between quadratic treatment effects and linear time effects, indicating that the percent motile cells observed decreased in a linear fashion changing from one time period to another, but more drastically at at least one of the concentrations of follicular fluid than the linear decrease would indicate (Figure 1).

Table V (Appendix) indicates that week of collection was significant ($P < .01$) in its effect on percent live cells. This agrees well with other research (Wells, et al. 1972) which indicates that there is significant week to week variation in the percent live cells in ejaculates of bulls.

The purpose of this study was not to identify the substances responsible for sperm stimulation. Yanagimachi (1969b) indicated that the sperm stimulating substances in bovine follicular fluid was a heat-stable substance of low molecular weight since it was a dialyzable substance and withstood heat treatment of 90°C. He stated from unpublished work that it was neither glucose nor bicarbonate. His work was with hamster cells, so it cannot be stated with finality that the substances responsible for stimulation in his work are the same substances responsible for the stimulation and maintenance of motility in bull spermatozoa described in the present study.

Normality

Table II presents the percent normal cells determined for each combination of follicular fluid and incubation time. Follicular fluid

in any concentration did not significantly affect percent normal cells. As the incubation period increased the incidence of normal cells increased slightly and then decreased regardless of the type of incubation media used. There were significant differences ($P < .01$) in percent normal cells among weeks as well as a significant week by incubation time interaction. The week by time means (Table III) indicate that in the first two weeks of collection there was no significant difference in the percent normal cells at the three, six and nine hour periods. However, in the last two weeks, the percent normal cells were reduced significantly at the nine hour period of incubation. The contribution of week of collection to the variation is also in agreement with Wells et al. (1972).

TABLE II

MEANS OF PERCENT NORMAL CELLS AVERAGED OVER WEEK OF COLLECTION AND BULLS

	Percent Follicular Fluid			
	0%	25%	50%	100%
Initial Ejac.	90.0	90.0	90.0	90.0
3 Hours	94.9*	94.9	94.7	95.1
6 Hours	94.3	95.5	94.9	95.5
9 Hours	90.6	92.2	90.6	90.7

*The S.E. for comparing means within rows is ± 1.21.

*The S.E. for comparing means within columns is ± 1.21.

TABLE III

MEANS OF PERCENT NORMAL CELLS AVERAGED OVER FOLLICULAR FLUID CONCENTRATION

Week	Hours of Incubations			
	0	3	6	9
1	91.0	97.8*	97.2	96.0
2	92.2	95.3	97.7	97.5
3	92.5	93.7	91.8	86.7
4	84.6	92.8	93.4	83.9

*S.E. for comparing means in rows and columns is ± 1.21.

The three-dimensional response surface for percent normal cells is presented in Figure 2. Partitioning of effects indicated that both linear and quadratic effects of time were significant ($P < .01$). This is indicated by the level response in normality between three and six hours and subsequent decrease at nine hours. The occurrence of increased percent normal cells after three and six hours can perhaps be due to artifacts caused by conditions unique at collection of cells and the subsequent incubation periods. It would not be logical that treatments or incubation could change the gross morphological characteristics of the cells from an abnormal to a normal status.

Table II shows that normal cells comprised a very large percentage (90 to 95%) of the population of cells in any incubation media at any time. Therefore, the gross morphological condition of the cell will

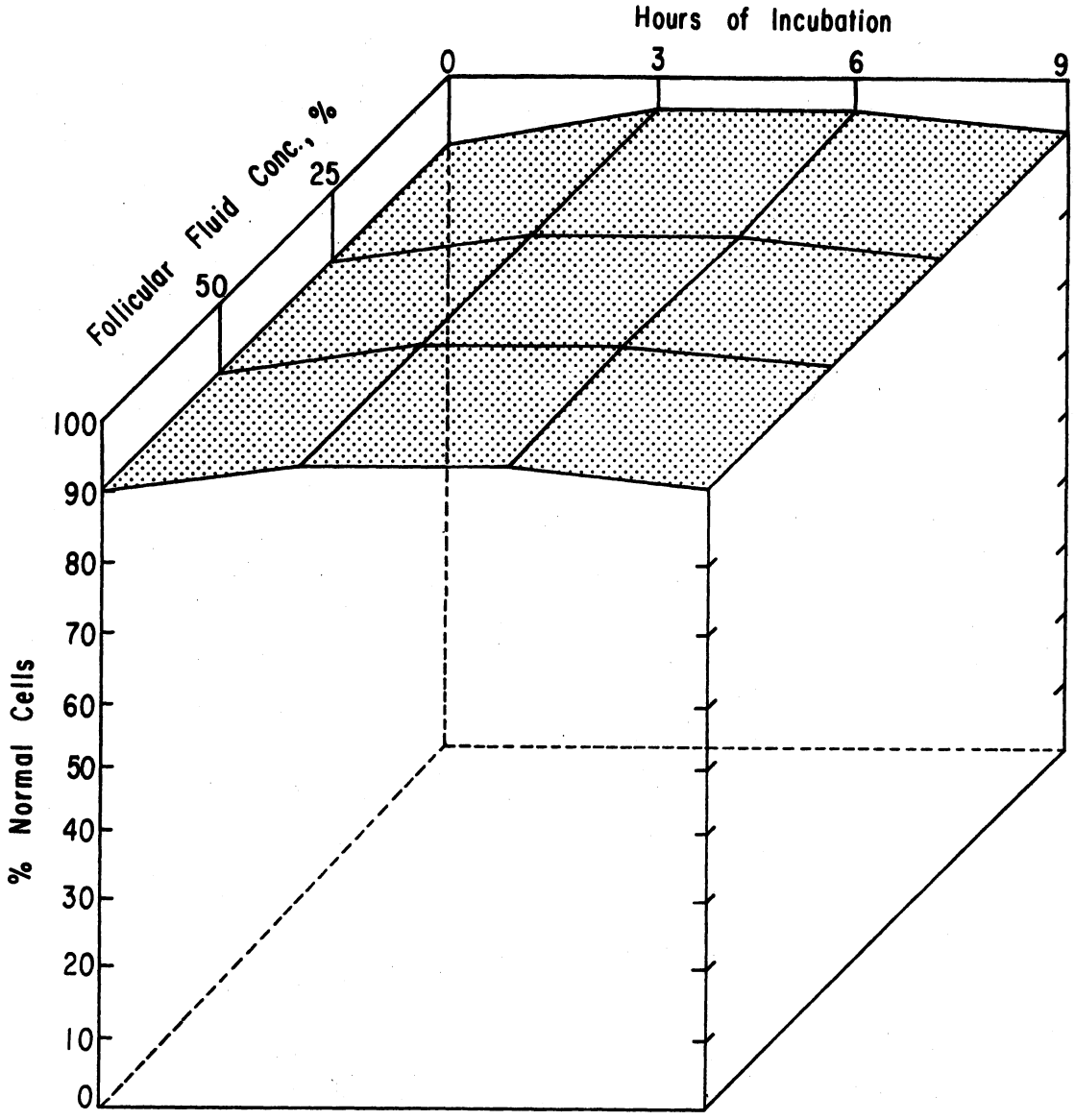

Figure 2. The Effect of Follicular Fluid in the Incubation Media and Length of Incubation on Percent Normal Cells

be disregarded when dealing with the state of the acrosome.

Acrosome Status

The state of the acrosome was drastically changed by incubation in media containing follicular fluid. The alteration of the acrosome in sperm in the follicular fluid media was very pronounced being of the disintegrating, detaching or detached category while the small amount of alteration seen in the Krebs-Ringer phosphate control was of the slightly thickened or wrinkled category. There was a tremendous change from the initial ejaculate acrosome state (5% altered) to the three hour state (39.27% altered) in the cells exposed to follicular fluid, while the alteration in the control media rose to only 15.65% in the same time period. This was the most drastic change that the acrosome was to undergo. It should be noted that the alteration observed in the controls was virtually complete at the end of three hours of incubation in the controls while it continued to change in the media containing follicular fluid. The controls never approached the alteration present in the follicular fluid media. The increase in the alteration of the acrosome was of much greater magnitude than was the decrease in motility for this same time period, indicating that this change in the acrosome was taking place in motile cells.

As length of incubation and concentration of follicular fluid increased the percent altered acrosomes increased (Figure 3 and Table IV). The increased alteration of the acrosome with increasing amount of follicular fluid is in agreement with findings of Yanagimachi (1969b).

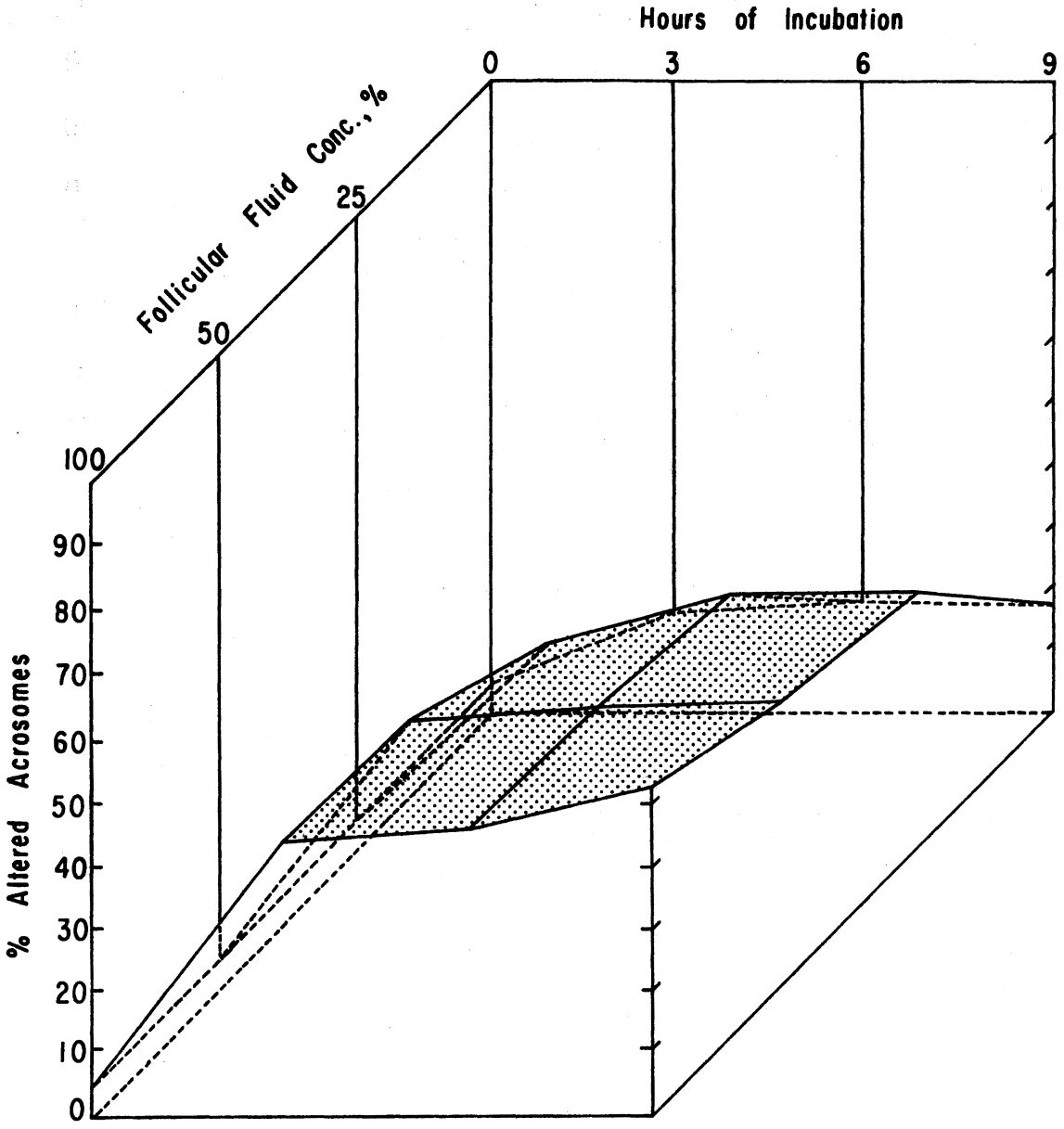

Figure 3. The Effect of Follicular Fluid in the Incubation Media and Length of Incubation on the Alteration of the Acrosome

TABLE IV

MEANS OF PERCENT ALTERED ACROSOMES AVERAGED OVER WEEK OF COLLECTION AND BULLS

	Percent Follicular Fluid			
	0%	25%	50%	100%
Initial Ejac.	5.0	5.0	5.0	5.0
3 Hours	15.6*	32.7	41.4	43.7
6 Hours	17.6	40.5	43.6	45.3
9 Hours	17.1	40.8	44.5	52.5

*The S.E. for comparing means within rows is ± 2.72.

*The S.E. for comparing means within columns is ± 2.53.

The effect of follicular fluid concentration on percent altered acrosomes was highly significant ($P < .01$). The linear, quadratic and cubic effects of percent follicular fluid were all significant ($P < .01$). However, the cubic effect is not shown in the means (Table IV) or the surface response curve (Figure 3). Again the lack of a 75% concentration of follicular fluid was probably responsible for the cubic effect being significant. The quadratic effect on altered acrosomes was displayed in a linear fashion by the drastic increase in altered acrosomes as the percent follicular fluid increased to 25%. However once the 25 and 50% concentrations were reached, the rate of increase in altered acrosomes was more reduced (Figure 3). The effect of length of incubation was significant ($P < .01$) with the linear

effects being significant ($P < .01$). This is seen in the response curve (Figure 3) as a gradual increase in percent altered acrosomes across the three, six and nine hour time periods.

The change in the acrosome may be indicative of either a past or present state of capacitation (Yanagimachi 1969a and 1969b, Iwamatsu and Chang 1969, Gwatkin and Andersen 1969, Barros and Austin 1967b, Yanagimachi and Noda 1970). If the elevation, loosening or disintegration of the acrosome that we observed and designated as an altered acrosome is in fact the uniform vesiculation of the outer acrosomal membrane and the plasma membrane described by Barros et al. (1967), then certainly the substance responsible for induction of this change would be quite beneficial to the industry. One can only suppose at this point that this is the "true" acrosome reaction from its similarity to work published by the previously cited authors.

However, this may be merely a degenerative change (Saacke 1970 and 1971, Wells and Awa 1970) due to either nonphysiological or environmental conditions (Blom 1946, Clermont et al. 1955, Hathaway et al. 1963, Iype et al. 1963, Marx 1964). This would mean that the change in the acrosome observed in this study was merely the random loss of the outer acrosomal membrane described by Bedford (1970).

The acrosome changes observed in this study cannot be clearly defined at this time. It may be that follicular fluid greatly speeds up the aging process that all cells undergo. However, the changes may be a morphological indication of capacitative changes and can possibly be characteristic of those changes occurring in the oviduct prior to fertilization. Yanagimachi (1969b) reported that the factors in bovine follicular fluid responsible for the acrosome reaction are (1) the

sperm stimulatory substance discussed previously and (2) an undialyzable substance or substances of high moelcular weight. Both the sperm stimulatory and undialyzable fractions had to be present to induce the acrosome reaction in hamster cells. This would indicate that if the change in the acrosome is merely degenerative one could isolate the factors responsible for sperm stimulation and utilize it without damage to the acrosome.

CHAPTER V

SUMMARY AND CONCLUSIONS

Bovine spermatozoa were incubated in bovine follicular fluid to study the effects of follicular fluid upon sperm cells. The cells were incubated for nine hours in 0%, 25%, 50% and 100% solutions of bovine follicular fluid extended with Krebs-Ringer phosphated buffer. The effects of these different dilutions of follicular fluid upon rate of motility, percent motile, normality and the state of the acrosome were studied.

An accelerated rate of motility was exhibited by cells incubated in all concentrations of follicular fluid. Head to head agglutination was also observed to occur in cells incubated in follicular fluid.

The motility of spermatozoa was best maintained by 25% and 50% follicular fluid media. The linear effects of length of incubation and quadratic effects of percent follicular fluid on motility were significant ($P < .01$). The most dramatic decrease in motility was observed in the 0% and 100% follicular fluid media at the six and nine hour times.

Normality of the cells was not affected by follicular fluid but was affected by length of incubation ($P < .01$). There was a tendency for the normality of the cells to increase at the three and six hour periods and then decrease at nine hours. There were never less than 90% (average) normal cells in any of the media at any time.

The acrosome was affected by both incubation and percent follicular fluid present with incubation effects being significantly linear ($P < .01$) and percent follicular fluid having significant quadratic effects ($P < .01$). The greatest alteration in the acrosome was exhibited between initial ejaculate characteristics and the three hour observations. The alteration of the state of the acrosome in follicular fluid media was much more pronounced and much greater than in the controls.

It appears that there are substances in follicular fluid that can cause significant changes in bovine spermatozoan characteristics in in vitro systems. These changes in other species have been shown to be intimately involved in the fertilization process. It can be projected that the changes observed in this study may assist in arriving at a better understanding of the fertilization process in farm animals.

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TABLE V

ANALYSIS OF VARIANCE TABLE

Source	DF	MS		
		Motility	%Normal	Altered Acrosomes
Bull (blocks)	3	403.7	15.0	328.5
Week of Collection	3	1064.9*	686.9*	142.9
Bull × Week (Error a)	9	207.4	8.6	467.4
Treatment (Follicular Fluid Conc.)	3	12906.5*	8.2	8796.4*
Treatment Linear	1	4565.5*	1.4	19171.7*
Treatment Quadratic	1	29688.1*	1.3	6464.1*
Treatment Cubic	1	4466.1*	22.0	753.4*
Week × Treatment	9	142.0	11.8	403.5
Bull × Treatment	9	223.0	21.0	101.9
Bull × Treatment × Week	27	117.4	9.0	66.9
(Bull × Treatment) + (Bull × Week × Treatment) = (Error b)	36	143.8	12.0	75.6
Time (Hours of Incubation)	2	5257.2*	329.0*	482.4*
Time Linear	1	10458.1*	478.5*	943.4*
Time Quadratic	1	56.2	179.4*	21.3
Week × Time	6	219.3	125.1*	166.5
Treatment × Time	6	791.6*	3.6	90.6
Treatment Linear × Time Linear	1	206.1	1.5	131.4
Treatment Linear × Time Quadratic	1	499.5	6.6	146.9
Treatment Quadratic × Time Linear	1	3612.0*	3.8	.4
Treatment Quadratic × Time Quadratic	1	160.2	.2	46.3
Treatment Cubic × Time Linear	1	220.5	9.8	182.9
Treatment Cubic × Time Quadratic	1	51.7	0.0	35.8
Week × Treatment × Time	18	77.4	7.0	27.9
Bull × Time	6	91.2	26.8	85.9
Bull × Week × Time	18	95.0	13.9	76.1
Bull × Treatment × Time	18	70.2	13.7	39.5
Bull × Week × Treatment × Time	54	53.5	8.6	43.0
(Bull × Time) + (Bull × Week × Time) + (Bull × Treatment × Time) + (Bull × Week × Treatment × Time) = (Error c)	96	66.8	11.7	51.2

*($P < .01$)

TABLE VI

AGGLUTINATION AND TYPE OF MOTILITY SCORES*

Week	Bull	Time	Percent Follicular Fluid			
			0	25	50	100
1	1	3	0 -	1 +	1 +	3 +
		6	0 -	1 +	1 +	2 +
		9	0 -	1 +	1 +	1 +
	2	3	0 -	1 +	1 +	3 +
		6	0 -	1 +	1 +	2 +
		9	0 -	1 +	1 +	1 +
	3	3	0 -	1 +	1 +	3 +
		6	0 -	1 +	1 +	2 +
		9	0 -	0 +	0 +	1 +
	4	3	0 -	1 +	1 +	3 +
		6	0 -	1 +	1 +	2 +
		9	0 -	1 +	1 +	1 +
2	1	3	0 -	1 +	1 +	1 +
		6	0 -	1 +	1 +	1 +
		9	0 -	1 +	1 +	1 +
	2	3	0 -	1 +	1 +	1 +
		6	0 -	1 +	1 +	0 +
		9	0 -	1 +	1 +	0 +
	3	3	0 -	1 +	1 +	1 +
		6	0 -	1 +	1 +	1 +
		9	0 -	1 +	1 +	1 +
	4	3	0 -	0 -	1 +	2 +
		6	0 -	1 +	1 +	1 +
		9	0 -	1 +	1 +	1 +

TABLE VI Continued

Week	Bull	Time	Percent Follicular Fluid 0	25	50	100
3	1	3	0 -	1 +	1 +	1 +
		6	0 -	0 +	0 +	1 +
		9	0 -	1 +	1 +	1 +
	2	3	0 -	1 +	1 +	1 +
		6	0 -	1 +	1 +	1 +
		9	0 -	1 +	1 +	1 +
	3	3	0 -	1 +	1 +	1 +
		6	0 -	0 +	1 +	1 +
		9	0 -	0 +	1 +	1 +
	4	3	0 -	0 +	1 +	1 +
		6	0 -	0 +	1 +	1 +
		9	0 -	1 +	1 +	1 +
4	1	3	0 -	1 +	1 +	1 +
		6	0 -	0 +	0 +	0 +
		9	0 -	0 +	0 +	0 +
	2	3	0 -	1 +	1 +	1 +
		6	0 -	1 +	1 +	3 +
		9	0 -	0 +	0 +	3 +
	3	3	0 -	0 +	0 +	0 +
		6	0 -	0 +	0 +	0 +
		9	0 -	0 +	0 +	0 +
	4	3	0 -	1 +	0 +	2 +
		6	0 -	0 +	1 +	3 +
		9	0 -	0 +	1 +	3 +

*0 - indicates no agglutination observed.
1 - indicates 2 to 4 cell agglutination.
2 - indicates 5 to 8 cell agglutination.
3 - indicates massive agglutination.
(+) - indicates presence of accelerated motility
(-) - indicates absence of accelerated motility.

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