THE ROLE OF CHOLESTEROL IN THE ACROSOMAL RESPONSIVENESS OF SPERM

BY

RENEE JEAN ZARINTASH

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Thesis Approved:

Nicholas L. Cross
Thesis Adviser

Charlotte L. Olsby

Jeff Hardin

Thomas C. Collins
Dean of the Graduate College

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background Information

Sperm are highly specialized cells that have essentially one important function: fertilization of an egg. To fertilize an egg, however, sperm cells must perform many tasks as they travel through the male and female reproductive tracts.

Sperm mix with seminal and epididymal fluids during their journey through the male reproductive tract. All fluids and sperm cells together at ejaculation are deposited in the lower female reproductive tract and must travel though the cervix, uterus, and oviducts where they reach the vicinity of the released egg. There are several barriers around the egg that the sperm must push through. The first barrier is the cumulus matrix, composed of cumulus cells and hyaluronic acid. Immediately surrounding the egg is the zona pellucida which the sperm cell must press through before it reaches the egg plasma membrane.

As the sperm reaches the zona pellucida, it undergoes the acrosome reaction, an exocytotic event that involves fusion of the outer acrosomal membrane and plasma membrane on the anterior portion of the sperm head.

The acrosome reaction is considered a final step in capacitation: physiological, cellular and biochemical changes that render the sperm capable of fertilizing an egg. The acrosomal contents are released and the sperm penetrates the zona pellucida, crosses the perivitelline space and binds to the egg plasma membrane. Soon, the entire sperm has entered the egg cytoplasm, and an egg is fertilized (reviewed by Yanagimachi, 1994).

In many cases, a sperm is not able to fertilize an egg. There exists several known causes, and several unknown causes, for such cases of infertility. Clinical tests can sometimes find the factor causing the infertility, such as extremely low numbers of sperm, immotile sperm, morphologically abnormal sperm, or sperm that fail to penetrate eggs. In some instances, these problems can be cured, but many times clinicians cannot pinpoint the problem. A possible cause attributed to sperm unable to penetrate eggs is high sperm membrane cholesterol levels. This cholesterol could originate from seminal plasma or epididymal fluids, or just be a characteristic of that individual's lipid make-up. It is possible that sperm from these unexplained infertile men may not have the ability to undergo the acrosome reaction. This study was designed to determine if sperm cholesterol content regulates the acrosome reaction.

Sperm are not capable of fertilizing eggs as they are released directly after ejaculation (Chang, 1951; Austin, 1951), but must first undergo further cellular and biochemical changes, either in the female reproductive tract or *in vitro*. These poorly understood modifications have been collectively termed capacitation. In males, sperm maturation occurs in the epididymis and is

distinguished by changes in the cell surface components and their distribution (Hammerstedt and Parks, 1987) as well as alterations in plasma membrane lipid composition. A few factors from seminal plasma or epididymal fluid have been identified that inhibit the acrosome reaction, fertilization of an egg or binding to the zona pellucida: lipids (Davis and Niwa, 1974), proteins (Oliphant et al., 1985; Coronel and Lardy, 1992), glycosides (Shur and Hall, 1982) or spermine (Shi et al., 1992).

The lipid composition of the sperm can change throughout the rest of its transit through the male reproductive tract, and may continue to change in the female reproductive tract, up until the point where it reaches the vicinity of a released egg. During this transit, capacitation occurs before the sperm reaches the egg. Once the sperm has arrived, it must undergo the acrosome reaction before penetrating the zona pellucida and fusing with the egg plasma membrane. The acrosome reaction is an exocytotic event involving modifications in the cell's membranes. The plasma and outer acrosomal membranes fuse and form fenestrations (Nagai et al., 1986), causing shedding of the acrosomal contents and the disappearance of the plasma and outer acrosomal membranes on the anterior portion of the sperm cell's head. The inner acrosomal membrane is exposed, surrounding the nuclear envelope. For the sperm to undergo this reaction, it must be acrosomally responsive (able to acrosome react in the presence of a specific inducer). A number of molecules have been shown to stimulate the human acrosome reaction *in vitro*, including the zona pellucida (Cross et al., 1988), follicular fluid (Tesarik, 1985), progesterone (Osman et al., 1989), the

cumulus oophorus (Siiteri et al., 1988) and calcium ionophore (Russel et al., 1979).

Previous Studies

Capacitation is poorly understood, and many people have done extensive studies to try to understand the events that lead to it. Chang (1957) took capacitated sperm and suspended them in seminal plasma for a few hours. As these sperm were inseminated into the uterus of rabbits, Chang found that they were not able to fertilize eggs immediately and concluded that they lost their fertilizing ability. He termed this occurrence decapacitation. If he allowed the sperm to reside in the uterus for four to eight hours, however, the sperm did fertilize the eggs (became capacitated again). He concluded that capacitation is reversible (Chang, 1957). Seminal plasma was therefore considered important in capacitation and could be involved in what initially stabilizes the sperm and renders them incapable of fertilization.

Oliphant and his colleagues (1978) isolated and identified a glycoprotein of 360kD that inhibited follicular fluid - induced acrosome reactions. They found this glycoprotein in epididymal and seminal fluids, and discovered that it also had the ability to reversibly decapacitate sperm (Oliphant, 1978). Despite the lack of understanding, the major event in capacitation was believed to involve removal or alteration of a stabilizer or protective coat from the sperm plasma membrane that may have been added to the sperm during its exposure to the fluids of the male reproductive tract (Orgebin-Crist and

Fournier-Delpech, 1982; Bedford, 1967). The inhibitory activity in seminal plasma sedimented into two fractions (I and II) after ultracentrifugation, and electron micrographs of the two fractions revealed them to contain small, round vesicles. They were additionally viewed on the surface of epididymal spermatozoa (Davis, 1973). *In vitro* fertilization studies with rat sperm showed that fertilization was inhibited by incubation in seminal plasma, as well as both fractions I and II. Electron micrographs revealed that sperm incubated in medium with 4 mg/ml bovine serum albumin (controls) lost their plasma membranes, whereas those incubated in the seminal plasma fractions did not lose their plasma membranes (Davis and Niwa, 1974). Sperm decapacitation by seminal plasma is reversible (Chang, 1957), and both classes of vesicles demonstrated the same decapacitation activity (Davis, 1974).

After the experiments described above, Davis and Hungund (1976) concluded that seminal plasma contained the factor or factors that inhibited sperm capacitation and they decided to characterize the lipid and protein composition of the seminal plasma vesicles. Phospholipids and cholesterol made up 41% and 32% of the vesicles respectively, while the protein content was very low (Davis and Hungund, 1976). After extraction with ethyl ether, only 10-30% of the phospholipid content and 14-16% of the cholesterol content was removed, and sperm treated with this extraction did not fertilize eggs. On the other hand, extraction with acetone /ether/water removed 50% of the phospholipid content and 60% of the cholesterol content. Sperm treated with this extract did fertilize eggs, suggesting that a reduction of

lipids, cholesterol or phopholipids or both, is necessary before sperm can fertilize eggs (Davis and Hungund, 1976).

Further detailed investigation was undergone to determine the specific factor in seminal plasma that inhibits acrosomal responsiveness of sperm. As seminal plasma was removed at 24 hr, incubation allowed sperm to become responsive to progesterone at a time dependent rate (Cross, 1993). When sperm were incubated 6 hr without seminal plasma, and then seminal plasma was added, the sperm eventually became unresponsive to progesterone (Cross, 1993). Incubating sperm with phosphatidlycholine (PC) accelerated the development of acrosomal responsiveness which was detectable in as little as 6 hr (Cross, 1994), suggesting that PC may act as cholesterol acceptors in the medium, enhancing cholesterol efflux from the sperm. Adding different dilutions of seminal plasma to the incubation medium prevented sperm from becoming acrosomally responsive in a dose-dependent manner. Seminal plasma contained 17.2 mg/ml protein, and treating this with pronase reduced it to 34 μg/ml but did not reduce the inhibitory effect. The inhibitory activity was also heat stable, indicating proteins are not responsible for preventing acrosomal responsiveness. Thin layer chromatography showed that cholesterol copurified with the inhibitory activity of seminal plasma, providing further evidence that cholesterol is the inhibitory factor in seminal plasma. In addition, cholesterol in the incubation medium reduced acrosomal responsiveness depending on the dose added. (Cross, unpublished observations).

Medium containing albumin promotes capacitation in rat, mice, and

guinea pig sperm cells (Toyoda and Chang, 1974; Toyoda et al. 1971). Cholesterol binds to albumin during sperm capacitation (Davis, 1974), so lipid exchange between the sperm plasma membrane and albumin was a suggested process. Davis examined sperm incubated in medium lacking albumin and found that no eggs were fertilized after insemination (Davis, 1976a). In contrast, 81% of the eggs became fertilized *in vitro* by epididymal spermatozoa in medium containing 10 mg/ml of albumin, and even more eggs were fertilized with defatted albumin than with regular albumin. Albumin saturated with cholesterol prevented fertilization (Davis, 1976a). These results suggested that albumin enhanced sperm fertilizing capacity by removing lipid (cholesterol) from the cell membrane (Davis, 1974). Davis postulated that replacement of the depleted cholesterol could account for the inhibition of fertilization by the seminal plasma membrane vesicles (Davis, 1973).

Uterine capacitated rabbit sperm fertilized significantly fewer eggs when incubated in medium containing dipalmitoylphosphatidlycholine and dimyristoyl- phosphatidylcholine vesicles with 10 to 40% (w/w) cholesterol compared to incubating sperm in the vesicles without cholesterol (Davis, 1976b). Cholesterol alone at 0.4 to 4 mg/ml added to the medium also inhibited fertilization (Davis, 1976b). This evidence indicates that sperm fertilizing capacity is influenced by cholesterol. Membrane fusion of myoblasts and phospholipid vesicles was inhibited by cholesterol (Van der Bosh et al., 1973; Papahadjopoulous et al., 1974), showing further evidence for this theory.

Toyoda et al. (1971) reported the first successful *in vitro* fertilization of mouse eggs with a "chemically defined medium", a modified Tyrode's solution with additives (glucose, lactate, pyruvate) and albumin. Davis et al. (1979) provided evidence for the transfer of phosphatidyl [^{14}C] choline and [^{3}H] cholesterol between bovine serum albumin and rat spermatozoa in a similar medium which promoted capacitation. During incubation *in vitro* phospholipid levels in albumin decreased and increased in sperm cells, while cholesterol increased in the albumin and decreased in the sperm (Davis et al., 1979). Plasma membranes isolated from rat sperm cells after five hours incubation in 4 mg/ml bovine serum albumin (BSA) showed a significant increase in phospholipid levels, while cholesterol levels decreased, but not significantly. This resulted in a lower cholesterol/phospholipid ratio, altering the plasma membrane composition of the sperm (Davis et al., 1980). The concentration of cholesterol in lipid suspensions and their inhibitory effect on the fertilizing capacity of uterine capacitated rabbit sperm were directly correlated (Davis, 1980; Go and Wolf; 1985). Defatted BSA increased spontaneous (not physiologically induced) acrosome reactions by 40% while BSA plus cholesterol reduced spontaneous acrosome reactions by 10% (Davis, 1980).

Freeze fracture analyses using filipin, which binds to β-hydroxyl sterols determined the distribution of cholesterol on the sperm's surface. A high amount of cholesterol was seen in the peri-acrosomal plasma membrane of uncapacitated human sperm. After five hours *in vitro* incubation the concentration of filipin concentration decreases over the acrosomal portion of the plasma membrane, indicating that cholesterol is lost (Suzuki and

Yanagimachi, 1989; Tesarik and Flechon, 1986). It has been suggested that these areas which lose cholesterol serve as points of fusion during the acrosome reaction (Bearer and Friend, 1982). One other study revealed that labeled cholesterol moved out of bovine sperm and into liposomes in a time-dependent manner (Ehrenwald et al., 1988).

The time needed to capacitate (1.5-7 hr) sperm cells in different species is closely correlated to their plasma membrane's cholesterol/phospholipid ratios (0.35-0.99) (Davis, 1981). A higher cholesterol/phospholipid ratio is related to a slower capacitation (Davis, 1981; Hoshi et al., 1990). Mack et al. (1986) isolated human sperm plasma membranes and reported a cholesterol/phospholipid ratio of 0.83, while other labs have found the ratios to be 0.99 (Darin-Benett and White, 1977; Davis, 1981). A study comparing fertile and infertile men's cholesterol/phospholipid ratios found that infertile men had a much higher ratio (1.04) than fertile men (0.52) in their freshly ejaculated sperm. After Percoll washing of this sperm, the ratios lowered to 0.80 and 0.40 respectively (Sugkraroek et al., 1991). The uterus may serve as a negative sterol gradient, having a mean cholesterol/phospholipid ratio of 0.16-0.26 whereas the cholesterol/phospholipid ratio for sperm and seminal plasma is 0.52 and 0.55 respectively (Davis, 1981). Cholesterol from rabbit sperm has been shown to bind to uterine fluid proteins after insemination, and one sterol acceptor in the uterus has been identified as serum albumin (Davis, 1982).

A concern for many experts is whether or not this cholesterol efflux from sperm occurs *in vivo*. Many compounds in biological fluids of the female

reproductive tract have been shown to have capacitating activity. The include estrus serum, follicular fluid, and uterine and oviductal fluid (Langlais and Roberts, 1985; Davis, 1982; Langlais et al., 1988; Ehrenwald et al., 1989). An extensive study on cholesterol and phospholipid concentrations in the bovine oviduct found that isthmic oviductal fluid had a high cholesterol concentration and a lower phospholipid concentration described as a higher cholesterol/phospholipid ratio than in ampullary oviductal fluid. It was postulated that the isthmus provided a stabilizing environment for the sperm's membranes as they travel toward the egg, and as they reach the ampulla, their membranes experience a possible cholesterol efflux due to the lower cholesterol/phospholipid ratios (Grippo et al., 1994).

In the past few years, sterol content in sperm has been under extensive investigation as one of the determinants of capacitation. Benoff and colleagues (1993a) found substantial inter-individual variation in sterol content of human sperm, ranging from 0.41 to 2.32 μmol sterol per 10^9 sperm. After 18 hours incubation in capacitating medium, this total sterol content decreased and free membrane cholesterol decreased to 0.001μmol per 10^9 sperm (Benoff et al., 1993a). When 1 mg/ml cholesterol-saturated bovine serum albumin was added to sperm's incubation medium, the free cholesterol content of sperm was significantly greater than that of control sperm incubated in capacitating medium (Benoff et al., 1993a). In measuring non-esterified cholesterol in the sperm membranes of "responders" (sperm that showed increases in the number of spontaneous acrosome reactions after 18 hr incubation) and "non-responders" (no increase in spontaneous acrosome

reactions) the cholesterol content decreased in responders after 18 hr incubation while no change in cholesterol content occurred in non-responders (Benoff et al., 1993b).

CHAPTER II

MATERIALS AND METHODS

Objectives of Study

This study was conducted in an effort to understand how the induction of acrosome reactions is controlled, or more specifically, what role cholesterol may have in acrosomal responsiveness. The working hypothesis states that the loss of sperm cholesterol during *in vitro* incubation causes sperm to become acrosomally responsive. The unanswered questions are: (1) Does sperm cholesterol decrease during incubation *in vitro*? (2) Does inhibiting the loss of sperm cholesterol prevent the development of acrosomal responsiveness? (3) Does accelerating the loss of sperm cholesterol accelerate the development of acrosomal responsiveness? (4) Do cholesterol loss and acrosomal responsiveness function in a time- and dose- dependent manner?

General Sperm Preparation

The semen was collected by masturbation from human donors and was used within an hour. To remove the seminal plasma and immotile sperm, the ejaculates were centrifuged on a two-layer Percoll gradient (Siiteri et al., 1988). The concentration of motile sperm was then determined using a hemacytometer

and adjusted to 1 x 10^7 sperm/ml in a modified Tyrode's solution (Suarez et al., 1986) containing 26 mg/ml bovine serum albumin (BSA) which is used as incubation medium.

Each ejaculate was used for one experiment throughout the study. The early experiments were run as a simple test to determine if cholesterol content changes after 24 hr incubation in incubation medium. For the ensuing experiments, the sperm were exposed to various treatments that might have effects on cholesterol content and acrosomal responsiveness of the sperm. Next, a time course study of cholesterol loss and acrosomal responsiveness was conducted. And finally, cholesterol content and acrosomal responsiveness was determined when different doses of cholesterol were added to the incubation medium (dose-response test).

After the ejaculates were prepared, each sample went through cholesterol extractions and gas chromatography to obtain its cholesterol content. In addition, each sample's acrosomal responsiveness was assessed.

Sperm Preparation for Cholesterol Measurement

The suspension of Percoll washed sperm was split in half to be analyzed twice - once immediately (0 hr) and once 24 hrs later (24 hr).

Zero hour samples were suspended in 1.2 ml incubation medium. The samples were centrifuged (10 min, 1000 g) and the supernatant was collected and stored at -20° C. The pelleted sperm were resuspended in 1.2 ml Dulbecco's phosphate-buffered saline (DPBS) and centrifuged again in order to wash out remaining incubation medium. The pellets were then stored in 1.2 ml

DPBS at -20° C.

The samples to be analyzed 24 hr later were adjusted to a concentration of 2 x 10^6 sperm/ml and were incubated in Falcon 60 x 15 mm tissue culture dishes (catalog number 4-3002-3, Benton Dickinson, Lincoln Park, NJ) at 37° and 5% CO_2/95% air. After 24 hr, the samples were centrifuged, washed and stored as described above for later use.

The samples described above were also prepared and used as controls in later experiments using cholesterol, phosphatidylcholine (PC), and seminal plasma (SP). In these experiments, the sperm samples were suspended in incubation medium containing 0.5 μg/ml cholesterol (sonicated with PC), 0.5 mg/ml PC liposomes, or SP at a 1:150 dilution (which contained approximately 2.0 μg/ml cholesterol). These samples underwent the same protocol as described above and were stored for later use. For the time-course study, sperm were placed in incubation medium at 2x10^6 sperm/ml. At intervals of six hours, two samples of 10^7 sperm were removed, centrifuged, washed and stored for extraction, while acrosomal responsiveness was scored. The cholesterol loss (% of total loss over 24 hr) and acrosomal responsiveness (% of total responsiveness over 24 hr) were compared for correlation. In the dose-response test, 1000 nM, 300 nM, 125 nM and 50 nM cholesterol was added to different samples. After 24 hr their cholesterol loss (% of total loss) and acrosomal responsiveness (% of total responsiveness) were assessed and compared for correlation.

Sperm Preparation for Acrosome Reaction Assay

To prepare sperm for the acrosome reaction assay, a small fraction of the Percoll washed sperm was adjusted to 10^6 sperm/ml incubation medium. In a 96-well plate (catalog number 25880-96, Corning Glass Works, Corning, NY), 75 μl aliquots of this sperm suspension were placed in each of 12 wells. Additional wells containing sperm with the appropriate concentrations of cholesterol, PC or SP were also set up on days these treatments were being examined, and the above mentioned wells served as controls. The wells containing sperm to be analyzed after 24 hr were incubated at 37° C, 5% CO_2/95% air. The remaining wells were analyzed immediately.

Inducing and Assaying the Acrosome Reaction

Acrosome reactions were induced with progesterone (Meizel et al., 1990). To three wells of the 75 μl sperm suspension was added 50 μl of a solution consisting of 2.5 μg/ml progesterone and 1.25 μg/ml Hoechst 33258 in incubation medium. Hoechst 33258 is a supravital stain which allows distinction between cells that have died and are acrosome reacted and cells that have physiologically acrosome reacted due to an inducer. The stain labels dead cells with ruptured plasma membranes (Cross et al., 1986).

To the three control wells, 50 μl of a solution containing 2.5 μg/ml dimethylsulfoxide and 1.25 μg/ml Hoechst 33258 in incubation medium was added. After 10 min of exposure, 100 μ l of DPBS was added to all the wells. The contents of each well were layered on top of 150 μl of a Nycodenz solution (65% (v/v) DPBS and 35% (v/v) of a solution containing 0.3 M 5-(N-2,3-

dihydroxypropylacetamido) -2,4,6- triiodo -N,N'- bis (2,3 - dihydroxypropyl) isophthalamide) in a 16-well chamber slide. Chamber slides are microscope slides with a removable, 16-well chamber attached to one side (Nunc, Inc., Naperville, IL). The chamber slides were then centrifuged (10 min, 40 g) to sediment the sperm onto the slide. After centrifugation, the supernatant was discarded and the sperm were fixed and permeabilized with 95% ethanol. The slide was dried and the sperm were labelled with fluoresceinated *Pisum sativum* agglutinin (Cross, 1993). Two hundred Hoechst 33258-negative sperm in each well were scored to determine acrosomal status.

Lipids

Liposomes of PC were prepared by sonication. Under N_2 10 mg PC was dried for at least 10 min at 37° C. Two ml of incubation medium lacking BSA was added, and the solution was placed in a 37° water bath to hydrate the PC. After 1 hour, the suspension was sonicated with a microprobe (Branson Model 200 Sonifier, output setting 7, 80% duty cycle, pulsed) for 4 min and placed on ice for 4 min. This was repeated for a total of 3 times. The suspension was then centrifuged at 10,000 g for 60 minutes and the supernatant was recovered. To the supernatant was added 26 mg/ml BSA and the final suspension was stored at -20° C.

Sonication was also used to prepare a cholesterol suspension consisting of 1.0 mg/ml cholesterol and 1.9 mg/ml PC. The above method was used for sonication, and the final supernatant was measured for cholesterol using a cholesterol assay kit (Sigma Chemical Co.., Catalog number 352-20) to

determine the actual concentration of cholesterol in the sonicate.

Extraction of Cholesterol

The samples were thawed and sperm concentrations were determined. One ml of each sample was transferred to 12 ml glass centrifuge tubes for extraction, and 500 ng of an internal standard, stigmastadienone, was added. To extract cholesterol out of the sperm and incubation medium samples, a chloroform/methanol/water system designed by Bligh and Dyer (1959) and modified by Kates (1986) was employed. The lipids were dried down under a N_2 stream while immersed in a 37° C water bath. After dissolving in approximately 1.0 ml chloroform, the lipids were passed through a glass wool filter to remove any particles that may contaminate the sample or be harmful to the gas chromatograph equipment and were collected in a 1.0 ml micro-vial. The lipids were dried, solubilized in 10 μl chloroform, capped, and set on ice to prevent evaporation.

Gas Chromatography

A Tracor 565 chromatograph, a 0.53 μm i.d., 30 m long DB-17 column (J & W, Folsom CA) with helium as carrier was used to measure cholesterol content. The column and flame ionization detector were maintained at 185° C, and the inlet was at 200° C. A Spectra-Physics SP4290 Integrator determined and recorded peak areas. A standard curve was generated, and consisted of the internal standard, stigmastadienone, and pure cholesterol in chloroform. Cholesterol content was measured by injecting 1.0 μl of each

sample into the GC.

Intra-assay precision was determined by measuring five replicates of 10^7 sperm. Interassay precision was determined by measuring identical samples in three assays that were conducted on different days. The intra-assay coefficient of variation and interassay coefficient of variation were 17% and 12% respectively.

Statistics

Paired data were compared using a randomized block design for ANOVA. Percentages for acrosomal responsiveness were transformed (arcsine $[\%/100]^{1/2}$) and then analyzed as above. SYSTAT (SYSTAT, Inc., Evanston, IL) was used for these anlyses with significance denoted by $P<0.05$. Mean and standard error of the mean were determined by InPLOT (GraphPad Software, San Diego, CA).

CHAPTER IV

RESULTS

The objectives of this study were: (1) to determine if sperm cholesterol decreased during incubation *in vitro*; (2) to determine if inhibiting the loss of sperm cholesterol prevents the development of acrosomal responsiveness; (3) to determine if accelerating the loss of sperm cholesterol accelerates the development of acrosomal responsiveness; (4) to determine if cholesterol loss and acrosomal responsiveness are time-and dose-dependent.

Sperm cholesterol content was measured before and after 24 hr incubation in medium containing 26 mg/ml bovine serum albumin. Sperm cholesterol content decreased by 27% ($P<0.001$) after 24 hr (Fig 1).

Sperm cells were incubated for 24 hr total, and sperm cholesterol content and acrosomal responsiveness were determined at 6 hr intervals. Cholesterol loss, represented as a percent of the total cholesterol loss after 24 hr, increased in a time dependent manner as did acrosomal responsiveness, represented as a percent of total acrosomal responsiveness after 24 hr (Fig 2). Cholesterol loss slightly preceded development of acrosomal responsiveness.

Seminal plasma, in a dilution containing 2.0 μg/ml cholesterol, was added to the incubation medium in an effort to inhibit the loss of sperm cholesterol and to see if acrosomal responsiveness was also inhibited. The controls lacked

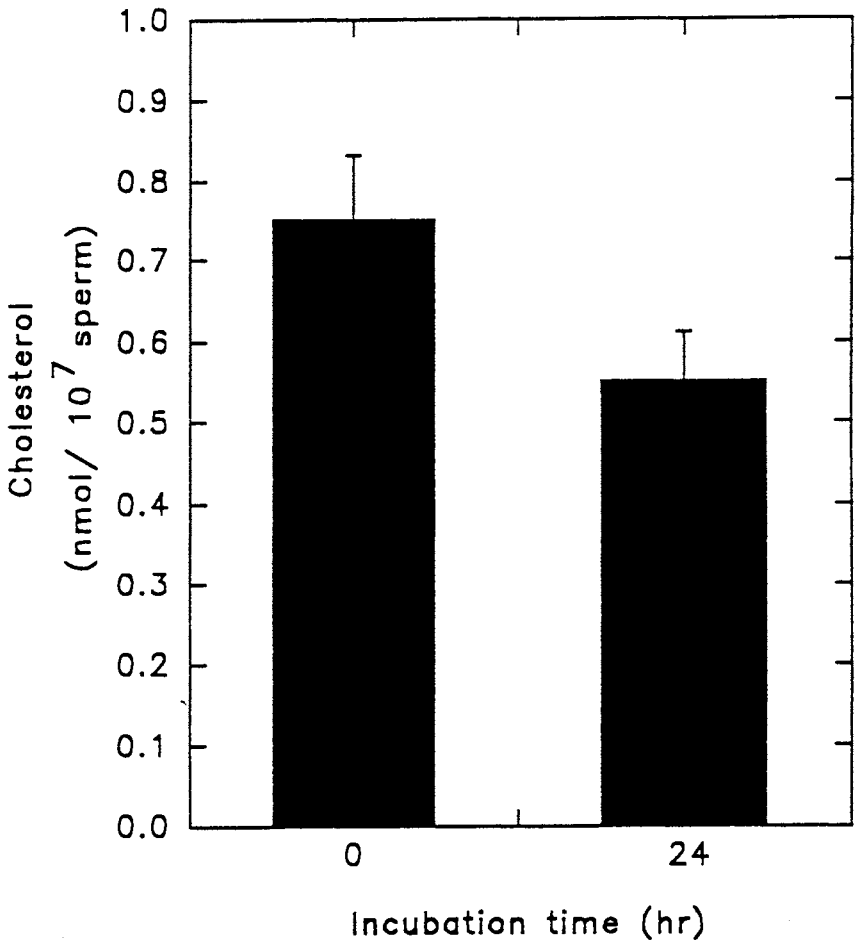

Figure 1. Sperm cholesterol content decreases during 24 hr incubation ($P<0.001$). The bars represent mean $\pm$ standard error of the mean ($n=23$).

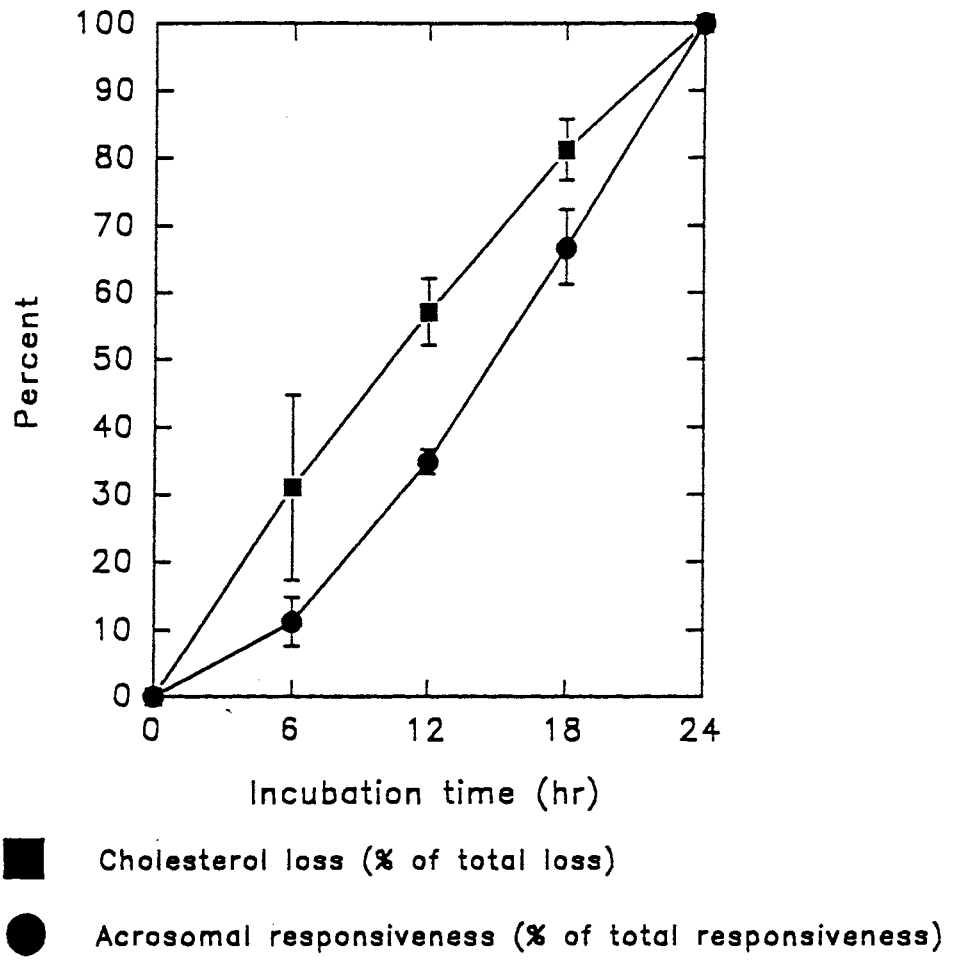

Figure 2. Kinetics of cholesterol loss and acrosomal responsiveness are similar. Fifty percent cholesterol loss occurs an average of four hours before fifty percent acrosomal responsiveness.

seminal plasma. Sperm cholesterol content did not change significantly ($P > 0.20$) from 0 hr to 24 hr when seminal plasma was present in the medium (Fig 3). Acrosomal responsiveness was also inhibited compared to the control after 24 hr.

Cholesterol was added to the incubation medium at a concentration of 0.5 μg/ml in an effort to inhibit the loss of sperm cholesterol and to see if acrosomal responsiveness was also inhibited. The controls lacked added cholesterol. Cholesterol content and acrosomal status were determined before and after 24 hr incubation. The addition of cholesterol caused an increase in the sperm cholesterol content compared to the 24 hr control by 48% ($P < 0.015$) (Fig 4). The development of acrosomal responsiveness was prevented compared to the control as well ($P < 0.001$).

Different doses of cholesterol were added to sperm samples in an effort to: (1) determine if sperm cholesterol loss decreased as a function of the dose added, (2) determine if acrosomal responsiveness and cholesterol loss after 24 hr are correlated depending on the dose added, and (3) calculate the dose effective in inhibiting acrosomal responsiveness and cholesterol loss by 50% (ED_{50}). Sperm cholesterol content is dependent on the concentration of cholesterol added to the incubation medium (Fig 5). The development of acrosomal responsiveness also was inhibited as the dose of added cholesterol increased. The ED_{50} for cholesterol loss was 180 nM and the ED_{50} for acrosomal responsiveness was 221 nM, which were not significantly different ($P > 0.10$).

Phosphatidylcholine (PC) liposomes were added to the incubation medium

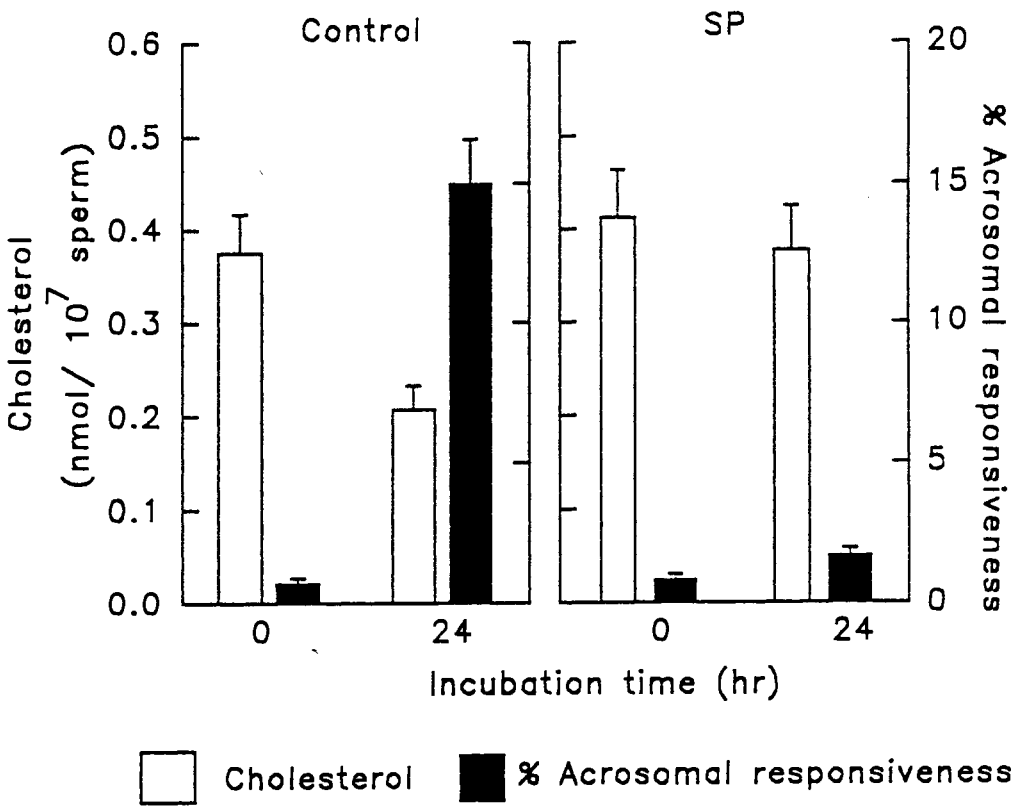

Figure 3. Seminal plasma prevents the loss of sperm cholesterol and the development of acrosomal responsiveness. Sperm cholesterol content did not change significantly ($P > 0.20$) from 0 hr to 24 hr with the addition of seminal plasma. Acrosomal responsiveness significantly increased in controls after 24 hr ($P < 0.001$), but was prevented in seminal plasma. The bars represent mean $\pm$ standard error of the mean ($n = 6$).

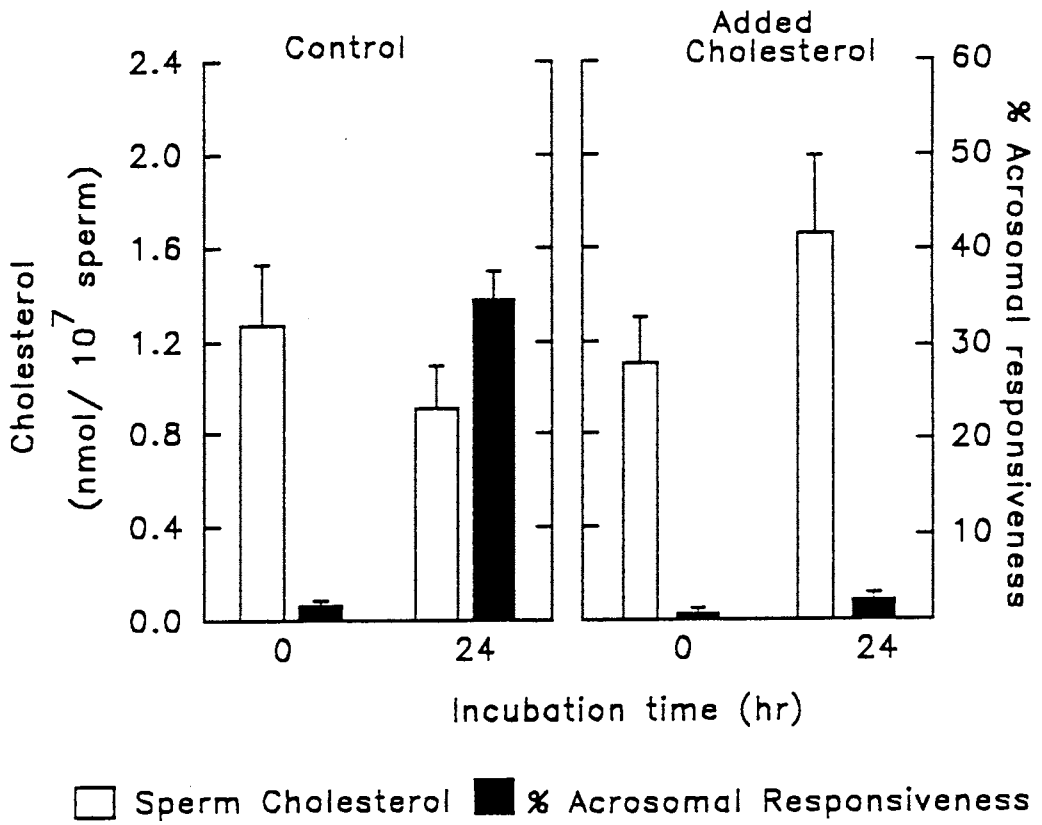

Figure 4. Cholesterol added to the medium (0.5 μg/ml) inhibits sperm cholesterol loss and the development of acrosomal responsiveness. The bars represent mean $\pm$ standard error of the mean (n = 5).

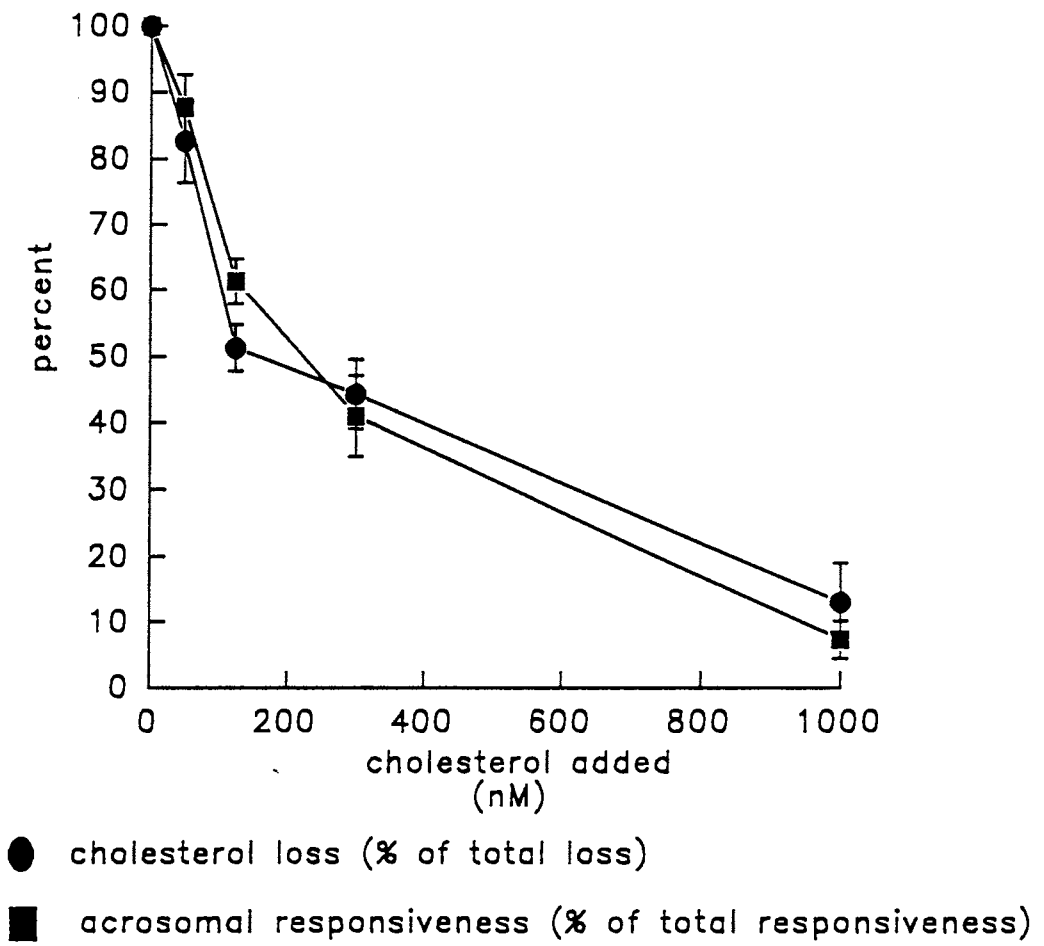

Figure 5. Cholesterol loss and acrosomal responsiveness function in a dose-dependent manner. The doses effective in inhibiting 50% acrosomal responsiveness (221 nM) and 50% cholesterol loss (180 nM) were not significantly different ($P>0.10$).

at 0.5 mg/ml in an attempt to accelerate the loss of sperm cholesterol and see if acrosomal responsiveness is also accelerated. The controls lacked PC liposomes. Incubating sperm for 24 hr with PC decreased the sperm cholesterol content compared to the 24 hr control by 53% ($P<0.001$) (Fig 6). The development of acrosomal responsiveness was enhanced by 53% as well ($P<0.05$).

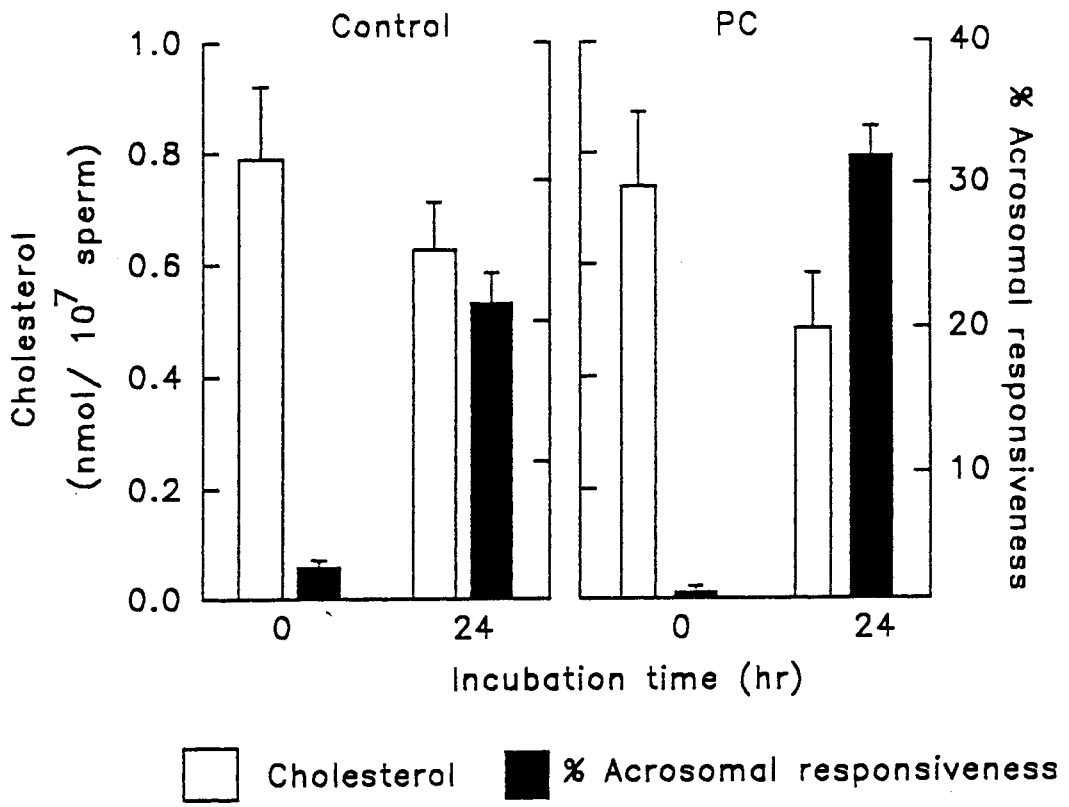

Figure 6. Accelerating the loss of sperm cholesterol accelerates the development of acrosomal responsiveness. Sperm cholesterol content decreased significantly ($P<0.001$) and acrosomal responsiveness increased ($P<0.05$) compared to 24 hr controls. Bars represent mean $\pm$ standard error of the mean ($n=6$).

CHAPTER IV

DISCUSSION

Cholesterol plays a central role in acrosomal responsiveness of sperm, and therefore, may also play an important role in sperm capacitation. Numerous studies have suggested that cholesterol has an inhibitory effect on spontaneous acrosome reactions (Davis and Niwa, 1974; Yanagimachi, 1975) and fertilization (Davis, 1976b). In addition, cholesterol is the major agent inhibiting acrosomal responsiveness in seminal plasma (Cross, unpublished observations). Although these previous studies point towards cholesterol as the factor inhibiting the development of acrosomal responsiveness, many questions were still unanswered. The experiments in this paper were designed to answer these questions and to tie up the relationship cholesterol has with acrosomal responsiveness. Up until the late 1980's, most work in this area was done on mouse or rabbit sperm. Only recently have researchers began experimenting with human sperm, as in this thesis. The work involving human sperm has certainly not been complete. Benoff et al. (1993b) reported a decrease in sperm cholesterol content after incubation *in vitro*, but determined the percent responsive sperm by spontaneous acrosome reactions. However, numerous factors have been shown to induce acrosome reactions. For example, the zona pellucida, from the egg's outer coating, can induce

acrosome reactions (Cross et al., 1988), highly suggesting that the fertilizing sperm is the one that encounters the egg and is induced to undergo the acrosome reaction. In the present study, acrosomal responsiveness describes the percent of sperm that have undergone the acrosome reaction when exposed to progesterone, and therefore compares cholesterol loss with sperm's capacity to undergo the acrosome reaction. This agrees with the common thinking today that acrosomal responsiveness is more closely related to fertilization potential than spontaneous acrosome reactions.

Sperm capacitation involves the following functions: (1) increased permeability to ions, especially Ca^{2+} (Tirana et al., 1980), (2) loss of components from seminal plasma that might have been adsorbed by sperm (Bedford and Chang, 1962), (3) modifications in lipid composition (Davis et al., 1979; Evans et al., 1980; Go and Wolf, 1985), and (4) hyperactivation of motility (reviewed by Yanagimachi, 1981).

Cholesterol can affect plasma membrane functions such as passive transport, permeability, mobility of intramembranous components, and activity of membrane bound enzymes (reviewed by Yeagle, 1985), thereby having a variety of roles in membrane function. Cholesterol contains a planar steroid ring system, a 3-β-hydroxyl function, making it polar, and a hydrophobic tail. This amphipathic molecule inserts itself into membrane systems with its polar hydroxyl head facing outward towards water, with the hydrophobic steroid ring parallel to and buried in the hydrocarbon chain of the membrane phospholipids (reviewed by Yeagle, 1985). Approximately 90% of the total cell cholesterol content is contained in the plasma membrane (Lange and

Ramos, 1983). Cholesterol is not distributed among membranes uniformly, especially in sperm cells, cholesterol concentration is higher over the acrosomal and tail regions than the rest of the plasma membrane (reviewed by Myles and Primakoff, 1985).

Individual lipid molecules can diffuse freely within lipid bilayers. A liposome is a type of synthetic bilayer that has been useful in many experimental studies. Synthetic liposomes made up of a single type of phospholipid can be used to determine the fluidity of the membrane. Membranes composed of unsaturated hydrocarbon chains with *cis*-double bonds have increased fluidity while saturated straight hydrocarbon chains make it easier to pack the chains together, rendering the membrane more viscous, or less fluid. In this study, liposomes composed of phosphatidylcholine were used. Lipid molecules readily exchange places with adjacent molecules within these synthetic bilayers giving rise to rapid lateral diffusion (reviewed by Alberts et al., 1989).

The amount of cholesterol in a membrane also influences the membrane's fluidity. Plasma membranes of eucaryotic cells contain numerous cholesterol molecules which situate themselves in the membrane with their polar head groups in line with the polar head groups of the phospholipids. Cholesterol has a rigid planar steroid ring structure (Fig. 7) and a long nonpolar hydrocarbon tail. This rigid steroid ring structure serves to partially immobilize the portions of the phospholipid hydrocarbon chains close to it and to decrease the fluidity of the phospholipid bilayers (reviewed by Alberts et al., 1989). Cholesterol also decreases the permeability of the bilayer, preventing

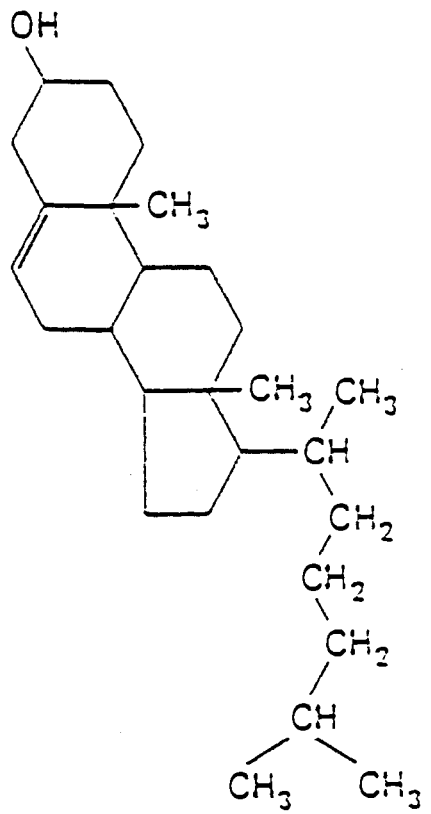

Figure 7. Structure of Cholesterol.

small water-soluble molecules from passing through the membrane (Alberts et al., 1989).

Although phospholipids freely diffuse laterally along a layer of the membrane, they can rarely migrate from one monolayer to another, called "flip-flop" movement. Cholesterol, on the other hand, can easily redistribute among different monolayers of membranes (Kornberg and McConnell, 1971).

Studies using human erythrocyte membranes showed that vesicles enriched in cholesterol incubated with the erythrocytes increased plasma membrane cholesterol content by a net cholesterol flux. When vesicles without cholesterol were incubated with the erythrocytes, the erythrocyte's plasma membrane cholesterol levels were depleted (Rooney et al., 1984). Cholesterol, preincubated with myoblasts, prevented their fusion as well (Horwitz et al., 1978). Cholesterol and phospholipids can be incorporated into liposomes in a 1:1 ratio. Cholesterol caused a strong reduction in the permeability and fusion of these liposome systems (Demel and Dekruyff, 1976: Papahadjopoulous et al., 1974), and prevented Ca^{2+}-induced fusion of muscle cells (Van der Bosch et al, 1973). The decrease in permeability was proportional to the concentration of cholesterol, and Demel and DeKruyff (1976) postulated that this decrease is due to the increased packing and decreased mobility of the hydrocarbon chains of the phospholipids.

Cholesterol has also been shown to have an effect on protein function. For example, at high cholesterol levels, the activity of Na^+/K^+ ATPase was inhibited, but at low cholesterol levels, this enzyme activity was enhanced (Shouffani and Kanner, 1990; Yeagle et al., 1988). In summary, cholesterol

plays an important role in biological membranes, but this role is still undefined. High concentrations of cholesterol in membranes has had an influence on functions such as passive transport, carrier-mediated transport and enzymatic activity of membrane-bound enzymes.

Many experts have tried to explain the role of sterols in capacitation, and one explanation seems to remain the predominant model. During passage through the epididymis, sperm sterol and phospholipid composition changes as the sperm matures (Hammerstedt and Parks, 1987). Agents (cholesterol, other sterols, proteins, surface coating components) may adsorb onto the sperm surface to stabilize it for its journey through the female tract. After sperm pass through the cervix, they are freed of most seminal or epididymal fluid and are surrounded by uterine fluids. Sterol acceptors, such as albumin, may be present in this environment which can cause a net efflux of cholesterol out of the sperm plasma membrane, rendering the membrane more fluid. The consequences of this cholesterol loss could be many. One possibility is that cholesterol efflux increases membrane permeability to extracellular calcium. An influx of calcium could activate the activity of phospholipases which may trigger the acrosome reaction (Langlais and Roberts, 1985). Another suggestion is that cholesterol comes out of the sperm plasma membrane and leaves "fusion points" where the plasma membrane and outer acrosomal membrane can fuse together, one of the steps in the acrosome reaction. A cholesterol efflux could also alter receptor-mediated cell functions or membrane bound enzyme activities by enhancing lateral displacement of the proteins within the bilayer of the plasma membrane (Yeagle, 1989). The

acrosome reaction may be receptor mediated by ligands from the zona pellucida (Bleil and Wassarman, 1983).

The results of this thesis fit the model described above. Sperm cholesterol content decreased after 24 hr incubation in medium containing 26 mg/ml bovine serum albumin, a putative sterol acceptor in uterine fluids. In an effort to determine if a direct relationship between the induction of the acrosome reaction and the loss of sperm cholesterol exists, a time-course study was conducted. The results showed a direct relationship, and sperm cholesterol efflux occurs about four hours upstream from the development of acrosomal responsiveness. This suggests that cholesterol loss is not the only determinant for sperm to develop acrosomal responsiveness, but other unknown functions may be taking place. Despite the close relationship between cholesterol loss and the development of acrosomal responsiveness, no correlation between cholesterol content and acrosomal responsiveness was seen among the different donors. In other words, initial sperm cholesterol content does not determine the percent of responsive sperm that will be observed after 24 hr incubation.

When seminal plasma was added to the medium, it prevented the cholesterol loss after 24 hr incubation. Acrosomal responsiveness was prevented in these sperm as well, which follows the studies that indicated seminal plasma prevented sperm from fertilizing eggs (Chang, 1957). One explanation for this could be that albumin, the sterol acceptor in the medium, could become saturated with the cholesterol from seminal plasma, giving cholesterol from the sperm plasma membrane no place to bind. This may be

one reason why the sperm demonstrated no cholesterol loss.

When sperm were incubated in medium with the addition of cholesterol, sperm cholesterol content increased. The same suggestion could apply here, but these results also support theories on the dynamics of cholesterol in membranes. Cholesterol exchanges freely from one membrane to another, causing an influx and efflux of cholesterol within the sperm plasma membrane. Adding cholesterol to the medium increases the rate of cholesterol influx. If enough cholesterol is added to the medium, the rate of influx of cholesterol will exceed the rate of efflux, and the cholesterol content in the sperm increases.

Cholesterol and acrosomal responsiveness are concentration dependent. As higher concentrations of cholesterol were added to sperm suspensions, less percent cholesterol loss and fewer acrosome reactions were observed, and these two functions were not significantly different.

On the other hand, as PC liposomes were added to the incubation medium, sperm cholesterol loss was enhanced after 24 hr. PC also increased the number of sperm that acrosome reacted when exposed to progesterone. PC liposomes function as artificial membranes, and therefore may serve as additional cholesterol acceptors, increasing the net efflux of cholesterol from sperm (Cross, 1994).

It is apparent that cholesterol plays a central part in the control of the human acrosome reaction. This evidence may be clinically important for infertile men that have passed all routine clinical tests for fertility. Some of these couples may have "unresponsive" sperm (cannot acrosome react),

explaining why eggs are never fertilized. Cholesterol levels in these sperm may be abnormally high. Infertile men in one study did have heightened cholesterol/phospholipid ratios (Sugkraroek et al, 1991) compared to fertile men. Incubating these sperm in medium with increased concentrations of sterol acceptors such as bovine serum albumin or PC liposomes for a few hours before artificial insemination is a possible technique which clinics could consider to increase their *in vitro* Fertilization rates with these unresponsive sperm.

CHAPTER VI

REFERENCES

Alberts, B., Bray, D., Lewis, T., Raff, M., Roberts, K., Watson, J.D. (1989) Molecular Biology of the Cell, Garland Publishing, New York. p. 275-336.

Arvidson, G., Ronquist, G., Wilander, G., and Ojteg, A.C. (1989) Human prostasome membranes exhibit very high cholesterol/phospholipid ratios yielding high molecular ordering. *Biochim. Biophys. Acta.* **984**:167-173.

Austin, C.R. (1951) Observations on the penetration of sperm into the mammalian egg. *Aust. J. Sci. Res.* **4**:581-596.

Babcock, D.F., Singh, J.P., Lardy, H.A. (1979) Alteration of membrane permeability to calcium ions during maturation of bovine spermatozoa. *Develop. Biol.* **69**:85-93.

Bearer, E.L. and Friend, D.S. (1982) Modifications of anionic-lipid domains preceding membrane fusion in guinea pig sperm. *J. Cell. Biol.* **92**:604-615.

Bedford, J.M. (1967) The importance of capacitation for establishing contact between eggs and sperm in the rabbit. *J. Reprod. Fertil.* **13**:365-367.

Bedford, J.M. and Chang, M.C. (1962) Removal of decapacitation factor from seminal plasma by high speed centrifugation. *Am. J. Physiol.* **202**:179-181.

Benoff, S., Cooper, G.W., Hurley, I., Mandel, F.S., and Rosenfeld, D.L. (1993a) Antisperm antibody binding to human sperm inhibits capacitation induced changes in the levels of plasma membrane sterols. *A. J. Reprod. Immunol.* **30**:113-130.

Benoff, S., Hurley, I., Cooper, G.W., Mandel, F.S., Rosenfeld, D.L., Hershlag, A. (1993b) Head-specific mannose-ligand receptor expression in human spermatozoa is dependent on capacitation-associated cholesterol loss. *Human Reprod.* **8**:2141-2154.

Bleil, J.D. and Wassarman, P.M. (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* **95**:317-324.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911-921.

Byrd, W. and Wolf, D. (1986) Acrosomal status in fresh and capacitated human ejaculated sperm. *Biol. Reprod.* **34**:859-869.

Chang, M.C. (1951) The fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* **168**:697-698.

Chang, M.C. (1957) A detrimental effect of seminal plasma on the fertilizing capacity of sperm. *Nature* **179**:258-259.

Coronel, C.E. and Lardy, H.A. (1992) Functional properties of caltrin proteins from seminal vesicle of the guinea pig. *Mol. Reprod. Devel.* **33**:74-80.

Cross, N.L. (1993) Multiple effects of seminal plasma on the acrosome reaction of human sperm. *Mol. Reprod. Dev.* **35**:316-323.

Cross, N.L. (1994) Phosphatidylcholine enhances the acrosomal responsiveness of human sperm. *J. Androl.* **15**:484-488.

Cross, N.L., Morales, P., Overstreet, JW, Hanson, FW. (1988) Induction of acrosome reactions by the human zona pellucida. *Biol. Reprod.* **38**:235-244.

Darin-Bennet, A. and White, I.G. (1977) Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology* **14**:466-470.

Davis, B.K. (1971) Macromolecular inhibitor of fertilization in rabbit seminal plasma. *Natl. Acad. Sci.* **68**:951-955.

Davis, B.K. (1973) Occurrence of vesicles in rabbit seminal plasma. *Experienta.* **29**:1484-1487.

Davis, B.K. (1974) Decapacitation and recapacitation of rabbit spermatozoa treated with membrane vesicles from seminal plasma. *J. Reprod. Fert.* **41**:241-244.

Davis, B.K. (1976a) Influence of serum albumin on the fertilizing ability *in vitro* of rat spermatozoa. *Proc. Soc. Exp. Biol. Med.* **151**:240-243.

Davis, B.K. (1976b) Inhibitory effect of synthetic phoshpolipid vesicles

containing cholesterol on the fertilizing ability of rabbit sperm. *Proc. Soc. Exp. Biol. Med.* **152**:257-261.

Davis, B.K. (1978) Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles, *In* Kabara, J.J., ed., Symposium on the Pharmacological Effects of Lipids, The American Oil Chemist's Society, Champaign, Illinois. pp. 145-157.

Davis, B.K. (1980) Interaction of lipids with the plasma membrane of sperm cell. I. The antifertilization action of cholesterol. *Archiv. Andrology* **5**:249-254.

Davis, B.K. (1981) Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. *Proc. Natl. Acad. Sci. USA* **12**:7560-7564.

Davis, B.K. (1982) Uterine fluid proteins bind sperm cholesterol during capacitation in the rabbit. *Experientia* **38**:1063-1064.

Davis, B.K. and Niwa, K. (1974) Inhibition of mammalian fertilization in vitro by membrane vesicles from seminal plasma. *Proc. Soc. Exp. Biol. Med.* **146**:11-16.

Davis, B.K. and Hungund, B.J. (1976) Effect of modified membrane vesicles from seminal plasma on the fertilizing capacity of rabbit spermatozoa. *Biochem. Biophys. Res. Commun.* **69**:1004-1010.

Davis, B.K., Byrne, R., Hungund, B.J. (1979) Studies on the mechanisms of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation in vitro. *Biochem. Biophys. Acta.* **558**:257-266.

Davis, B.K. and Gergely, A.F. (1979) Studies on the mechanism of capacitation: changes in plasma membrane proteins of rat spermatozoa during incubation *in vitro*. *Biochim. Biophys. Res. Commun.* **88**:613-618.

Davis, B.K., Byrne, R., Bedigan, K. (1980) Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during *in vitro* incubation of rat sperm cells. *Proc. Natl. Acad. Sci.* **77**:15-46.

Davis, B.K. and Davis, N.V. (1983) Binding by glycoproteins of seminal plasma membrane vesicles accelerates decapacitation in rabbit spermatozoa. *Biochem. Biophys. Acta.* **727**:70-76.

De Lamirande, E., Eiley, D., and Gagnon, C. (1993) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int. J. Androl.* **16**:258-266.

Demel, R.A. and DeKruyff, B. (1976) The function of sterols in membranes. *Biochem. Biophys. Acta.* **457**:109-132.

Ehrenwald, E., Parks, J.E., Foote, R.H. (1988) Cholesterol efflux from bovine sperm. I. Induction of the acrosome reaction with lipophosphatidlyl-choline after reducing sperm cholesterol. *Gamete Res.* **20**:145-157.

Ehrenwald, E., Foote, R.H., Parks, T.J. (1989) Cholesterol efflux from bovine sperm: bovine oviductal fluid components and their potential role in sperm cholesterol efflux. *J. Adrol.* **10**:21-23.

Evans, R.W., Weaver, D.E., Clegg, E.D. (1980) Diacyl, alkenyl, and alkyl ether phospholipids in ejaculated, *in utero-,* and *in vitro*-incubated porcine spermatozoa. *J. Lipid Res.* **21**:223-228.

Go, K.J. and Wolf, D.P. (1983) The role of sterols in sperm capacitation. *Adv. Lipid Res.* **20**:317-330.

Go, K.J. and Wolf, D.P. (1985) Albumin-mediated changes in sperm sterol content during capacitation. *Biol. Reprod.* **32**:145-153.

Grippo, A.A., Anderson, S.H., Chapman, D.A., Henault, M.A., Killian, G.J. (1994) Cholesterol, phospholipid and phospholipase activity of ampullary and isthmic fluid from the bovine oviduct. *J. Reprod. Fert.* **102**:87-93.

Hammerstedt, R.H. and Parks, J.E. (1987) Changes in sperm surfaces associated with epididymal transit, *In* Niswender G.D., Baird, D.T., Findlay, J.K., Ewir, B.J., eds. Reproduction in domestic ruminants, Great Britain, Dorset Press. p.133.

Horwitz, A.F., Wight, A., Ludwig, P., Cornell, R. (1978) Interrelated lipid alterations and their influence on the proliferation and fusion of cultured myogenic cells. *J. Cell Biol.* **77**:334-357.

Hoshi, K., Aita, T., Yanagida, K., Yoshimatsu, N., Sato, A. (1990) Variation in the cholesterol/phospholipid ratio in human spermatozoa and its relationship with capacitation. *Human Reprod.* **5**:71-74.

Kanwar, K.C., Yanagimachi, R., and Lopata, A. (1979) Effects of human seminal plasma on fertilizing capacity of human spermatozoa. *Fertil. Steril.* **31**:321-327.

Kates, M. (1986) Laboratory techniques of lipidology: isolation, analysis and identification of lipids, 2nd edition. New York, Elsevier. p. 100.

Kornberg, R.D. and McConnell, H.M. (1971) Lateral diffusion of phospholipids in a vesicle membrane. *Proc. Natl. Acad. Sci.* **68**: 2564-2568.

Lange, Y. and Ramos, B.V. (1983) Analysis of the distribution of cholesterol in the intact cell. *J. Biol. Chem.* **258**:15130-15134.

Langlais, J. and Roberts, K.D. (1985) A molecular model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res.* **12**:183-224.

Langlais, J., Kan, F.K., Granger, L., Raymond, L., Bleau, G., Roberts, K.D. (1988) Identification of sterol acceptors that stimulate cholesterol efflux from human spermatozoa during *in vitro* capacitation. *Gamete Res.* **20**:185-201.

Myles, D.G. and Primakoff, P. (1985) Sperm surface domains *In* Spungir, T.A., ed. Hybridoma Technology in the Biosciences of Medicine, Plenum, New York. p. 239.

Nagai, T., Yanagimachi, R., Srivastava, P.N., Yanagimachi, H. (1986) Acrosome reaction in human spermatozoa. *Fertil. Steril.* **45**:701-707.

Oliphant, G. and Eng., L.A. (1978) Rabbit sperm reversibly decapacitated by membrane stabilization with a highly purified glycoprotein from seminal plasma. *Biol. Reprod.* **19**:1083-1094.

Oliphant, G., Reynolds, A.B., Thomas, T.S. (1985) Sperm surface components involved in the control of the acrosome reaction. *Am. J. Anat.* **174**:269-283.

Orgebin-Crist, M.C. and Fournier-Delpech, S. (1982) sperm-egg interaction: evidence for maturational changes during epididymal transit. *J. Androl.* **3**:429-433.

Osman, R.A., Andria M.L., Jones, A.D., Meizel, S. (1989) Steroid induced exocytosis: the human sperm acrosome reaction. *Biochim. Biophys. Res. Commun.* **160**:828-833.

Papahadjopoulous, D., Poste, G., Schaeffer, B.E., Vail, W.J. (1974) Membrane fusion and molecular segregation in phospholipid vesicles. *Biochem. Biophys. Acta.* **352**:10-27.

Ronquist, G., Nelson, B., and Hjerten, S. (1990) Interaction between prostasomes and spermatozoa from human semen. *Arch. Androl.*

24:147-157.

Rooney, M.W., Lange, Y., Kauffman, J.W. (1984) The effect of cholesterol on the structure of phosphatidylcholine bilayers. *Biochem. Biophys. Acta.* **513**:43-58.

Russell, L., Peterson, R.N., Freund, M. (1979) Morphologic characteristics of the chemically induced acrosome reaction in human spermatozoa. *Fertil. Steril.* **32**:87-92.

Shi, Q., Juan, Y., Friend, D., Marton, L. (1992) Effect of spermine on sperm capacitation of guinea pig *in vitro*. *Arch. Androl.* **29**:33-42.

Shouffani, A. and Kanner, B.I. (1990) Cholesterol is required for reconstruction of the sodium-and chloride-coupled GABA transporter from rat brain. *J. Biol. Chem.* **265**:6002-6008.

Shur, B. and Hall, N. (1982) Sperm surface galactosyl transferase activities during *in vitro* capacitation. *J. Cell Biol* **95**:567-573.

Siiteri, J.E., Dandekar, P., and Meizel, S. (1988) Human sperm acrosome reaction initiating activity associated with the cumulus oophorus and mural granulosa cells. *J. Exp. Zool.* **246**:71-80.

Siiteri, J.E., Gottlieb, W., Meizel, S. (1988) Partial characterization of a fraction from human follicular fluid that initiates the human sperm acrosome reaction *in vitro*. *Gamete Res.* **20**:25-42.

Stegmayr, B. and Ronquist, G. (1982) Promotive effect on human sperm progressive motility by prostasomes. *Urol. Res.* **10**:253-257.

Suarez, S., Wolf, D., and Meizel, S. (1986) Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. *Gamete Res.* **14**:107-121.

Sugkraroeck, P., Kates, M., Leader, A., Tanphaichitr, N. (1991) Levels of cholesterol and phospholipids in freshly ejaculated sperm and Percoll-gradient-pelleted sperm from fertile and unexplained infertile men. *Fert. Steril.* **55**:820-827.

Suzuki, F. and Yanagimachi, R. (1989) Changes in the distribution of intramembranous particles and Filipin-reactive membrane sterols during *in vitro* capacitation of golden hamster spermatozoa. *Gamete Res.* **23**:335-347.

Tesarik, J. (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in

human sperm populations of proven fertilizing ability *in vitro*. *J. Reprod. Fertil.* **74**:383-388.

Tesarik, J. and Flechon, J.E. (1986) Distribution of sterols and anionic lipids in human sperm plasma membrane: effects of *in vitro* capacitation. *J. Ultrastruct. Mol. Struct. Res.* **97**:227-237.

Toyoda, Y., Yokoyama, M., Hosi, T. (1971) Studies on the fertilization of mouse eggs *in vitro*. *Jpn. J. Anim. Reprod.* **16**:147-157.

Toyoda, Y. and Chang, M.C. (1974) Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of eggs following transfer. *J. Reprod.* Fert. **34**:9-22.

Van der Bosch, J., Schudt, C., Pette, D. (1973) Influence of temperature, cholesterol, dipalmitoyllecithin and Ca^{2+} on the rate of muscle cell fusion. *Exp. Cell Res.* **82**:433-438.

Yanagimachi, R. (1975) Acceleration of the acrosome reaction and activation of guinea pig spermatozoa by detergents and other reagents. *Biol. Reprod.* **13**:519-526.

Yanagimachi, R. (1981) Mechanisms of fertilization in mammals *In* Mastroianni, L., Biggers, J.D., eds. Fertilization and Embryonic Development *In Vitro*, Plenum, New York. p. 81.

Yanagimachi, R. (1994) Mammalian Fertilization *In* The Physiology of Reproduction, Raven Press, New York. pp.189-254.

Yeagle, P.L. (1985) Cholesterol and the cell membrane. *Biochem. Biophys. Acta.* **822**:267-287.

Yeagle, P.L. (1989) Lipid regulation of cell membrane structure and function. *FASEB J.* **3**:1833-1842.

Yeagle, P.L., Rice, D., Young, T. (1988) Cholesterol effects on bovine kidney Na^+K^+ ATPase hydrolyzing activity. *Biochemistry* **27**:6449-6452.

VITA

Renee Jean Zarintash

Candidate for the Degree of

Master of Science

Thesis: THE ROLE OF CHOLESTEROL IN THE ACROSOMAL RESPONSIVENESS OF SPERM

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Winston Salem, North Carolina, On October 7, 1971, the daughter of Dr. and Mrs. Kim Zarintash.

Education: Graduated from Bishop Kelley High School, Tulsa, Oklahoma in May 1989; received Bachelor of Arts degree in Biology from Baylor University, Waco, Texas; Completed requirements for the Master of Science degree with a major in Physiolgy at Oklahoma State University in July 1995.

Experience: Employed as an assistant technician at the Tulsa Center for Fertility and Women's Health, Tulsa, Oklahoma in 1993; volunteered at Providence Medical Center Emergency Room, Waco, Texas, 1990 - 1992; volunteered at Jeffrey Place Nursing Center, Waco, Texas, 1992.

Professional Memberships: Alpha Epsilon Delta, Graduate Student Association, Physiology Journal Club, Society for Environmental Scientists.

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Principal Investigator(s): Nicholas L. Cross, Renee J. Zarintash

Reviewed and Processed as: Expedited

Approval Status Recommended by Reviewer(s): Approved

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