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Choline Acetyltransferase and Carnitine Acetyltransferase Activity in Human Spermatozoa During Capacitation

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by

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ABSTRACT

CHOLINE ACETYLTRANSFERASE AND CARNITINE ACETYLTRANSFERASE ACTIVITY IN HUMAN SPERMATOZOA DURING CAPACITATION

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The regional distribution of ChAT activity in human spermatozoa is altered during in vitro capacitation and it correlates with the fertilizing potential of sperm.

Regional immunoreactivity in human spermatozoa as assessed by fluorescent immunocytochemistry was compared with ChAT and CaAT activity determined by enzymatic methodologies. Increasing proportions of sperm exhibited ChAT immunoreactivity along the equatorial region with a concomitant decrease in ChAT reactivity in the midpiece. Also, competitive studies with unlabeled ChAT blocked the equatorial region labeling; the unlabeled CaAT blocked staining along the midpiece region of the tail, suggesting some cross-reactivity of the ChAT antiserum with the CaAT enzyme. A ChAT radioassay was used to compare regional immunoreactivity in human spermatozoa as assessed by fluorescent immunocytochemistry with ChAT and CaAT activity determined by enzymatic methodologies. Although a direct comparison was not made between the ChAT radioassay and the equatorial and midpiece/tail ChAT immunoreactivity, the two procedures appeared to not correlate. However, based on findings from the ChAT radioassay study, it was concluded that

the ChAT enzyme is present within the sperm and not synthesized de novo.

The regional distribution of ChAT and CaAT immunoreactivity under sperm capacitating conditions was correlated with the physiological indicators of completed capacitation, including the physiological and pharmacological induction of the acrosome reaction. A significant increase in both equatorial and midpiece/tail immunoreactivity was observed when using both caffeine and pentoxifylline. The addition of caffeine significantly increased equatorial binding, midpiece/tail binding, and the acrosome reaction rate in both washed and post swim-up samples. Although the addition of caffeine increased both equatorial binding and the acrosome reaction rate, a correlation was not observed between equatorial binding and the acrosome reaction.

The potential fertility of human spermatozoa was correlated with ChAT and CaAT immunoreactivity by comparison of spermatozoa obtained from proven fertile males to spermatozoa incapable of attaining the physiological indicators of completed capacitation. A significant positive correlation was demonstrated between ChAT binding and the Hemizona Assay Index. A significant positive correlation was also demonstrated between CaAT binding and original sperm concentration, VAP, VSL, VCL, and both original and swim-up motility.

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INTRODUCTION

Significance

Acetylcholine, a neurotransmitter produced by the enzyme choline acetyltransferase (ChAT), has been suggested to play a role in many nonneuronal tissues, including spermatozoa [1]. Earlier studies have indicated that human spermatozoa contain the four components of the cholinergic system including acetylcholine and choline acetyltransferase [2]. However, the ChAT studies have since been reevaluated due to the possibility that ChAT activity was mistaken for carnitine acetyltransferase (CaAT), which can also use choline as a substrate. Recent studies have indicated choline acetyltransferase mRNA is expressed in spermatogenic cells and the presence of choline acetyltransferase immunoreactivity has been detected on sperm [3-8]. A potential role for acetylcholine during fertilization would suggest a concomitant role for the enzyme involved in its production. It has been demonstrated in mouse sperm that the zona pellucida induced acrosome reaction can be inhibited by 3-quinuclidinyl benzilate, an antagonist of muscarinic acetylcholine receptors [9]. Acetylcholine may be part of a sperm receptor complex required for successful fertilization.

Journal model used in this dissertation is *Fertility and Sterility.*

To achieve fertilization, spermatozoa must be able to undergo capacitation, the biochemical changes that enable the spermatozoa to become competent to complete the physiological changes involved in fertilization [10]. These biochemical alterations include removal of a protective covering from the sperm plasma membrane and a rearrangement of membrane components [11,12]. Currently, few specific biochemical markers exist to delineate the cellular and molecular events that occur during capacitation, a process requisite for successful fertilization [10].

The hypothesis of this study is that the regional distribution of ChAT activity in human spermatozoa is altered during in vitro capacitation and correlates with fertilizing potential of sperm; and, as such this capacitation-dependent redistribution will serve as a valuable biochemical marker of capacitation.

Specific Aims

The specific aims of this study are: (1) to compare regional immunoreactivity in human spermatozoa as assessed by fluorescent immunocytochemistry with ChAT and CaAT activity determined by enzymatic methodologies; (2) to correlate the regional distribution of ChAT and CaAT immunoreactivity under sperm capacitating conditions with the physiological indicators of completed capacitation, including the physiological and pharmacological induction of the acrosome

reaction; and (3) to correlate the potential fertility of human spermatozoa with ChAT and CaAT immunoreactivity by comparison of spermatozoa obtained from proven fertile males to spermatozoa incapable of attaining the physiological indicators of completed capacitation.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

In order to become capable of fertilization, spermatozoa must undergo capacitation [13]. Capacitation refers to the biochemical and physiological changes that enable the spermatozoa to become competent to fertilize. Although capacitation has been intensively studied, the molecular and cellular basis is not yet fully understood [10].

The requirement for capacitation to occur before fertilization can be achieved was first documented in 1951 by Austin and Chang during their early experiments in the rat and rabbit [13]. The phenomenon of capacitation has been extensively studied since its discovery; however, the mechanisms behind many of the processes are still poorly understood. The major event of capacitation is considered to occur with the alteration or removal of a protective covering or stabilizer from the plasma membrane of the sperm. Normally, capacitation occurs during transit within the female reproductive tract. The process of capacitation begins at the site of semen deposition (e.g. vaginal, cervical, or intrauterine), which varies depending on the species. In vaginally depositing species, sperm must successfully ascend through the mucus filled cervix in order to reach the uterus. The cervical mucus performs many functions along the way, including protection of the individual sperm from the hostile

vaginal environment and from phagocytosis by vaginal leukocytes, prevention of the entry of both abnormal sperm and seminal plasma into the uterus, and retention of sperm for later release into the reproductive tract [10].

It is now believed, regardless of the mode of sperm transport through the female reproductive tract, that the isthmus of the oviduct functions as a sperm reservoir and that the movement of sperm into the ampullar region of the oviduct is a very synchronized event [14-16]. Sperm attachment to the epithelial surface of the oviduct may perform an important role in maintaining sperm viability. Experiments with the golden hamster have demonstrated that sperm appear to live longer if they are attached to the epithelial surface. Sequential flushing of postcoital, preovulatory hamster oviducts revealed that both the first and second flushes (which contained sperm mainly from the lumen and mucosal surface) were comprised mainly of dead sperm, whereas the third flush (which contained sperm mainly from the oviductal crypts) had over 50% viability [15,16]. Further experimentation with the golden hamster has demonstrated that uncapacitated sperm are much more likely to attach to the oviductal epithelium than are capacitated sperm, suggesting that a change occurs on the sperm surface during capacitation [17]. The oviduct also appears to regulate the speed of sperm capacitation. Experiments with the golden hamster have demonstrated that sperm requires additional time in vitro

before being able to penetrate an oocyte if retrieved from the isthmus of the oviduct in animals mated shortly after the onset of oestrus when compared to animals mated shortly after ovulation. Sperm retrieved from the recently ovulated animals could penetrate oocytes within an average time of 30 minutes; however, sperm recovered from the preovulatory animals required up to an additional six hours to penetrate the oocytes. This suggests that either a factor essential for capacitation is missing from the female reproductive tract until ovulation or that there is a temporary delay of capacitation [18].

The mammalian sperm is comprised of two main components, the head and the flagellum. The surface of the sperm is covered with a plasma membrane which can be further subdivided into surface domains. The regional surface domains differ in both composition and function [10]. The five main surface domains include the acrosomal segment, the equatorial segment, the postacrosomal segment, the midpiece, and the principal piece [19]. Signs of capacitation occur separately in each of the five surface domains as the sperm become competent to fertilize [11,20]. The acrosomal and postacrosomal segments of the plasma membrane undergo many modifications during capacitation including biochemical changes such as alterations in the lipid and surface glycoprotein composition and distribution, antigen distribution patterns, and intracellular ions [10,11,21,22]. The physiological endpoint of

capacitation for the acrosomal and postacrosomal segments is the acrosome reaction or the fusing of the outer acrosomal membrane to the overlying plasma membrane and the subsequent release of acrosomal contents [10,22]. The major goal of the equatorial segment during capacitation is to provide a membrane capable of fusing with the oocyte plasma membrane, which it can not perform prior to the acrosome reaction [11,23]. The midpiece and principal piece, which are separated by the annulus, provide the sperm with motility. The annulus is a fibrous ring that is a part of the flagellar cytoskeleton. The annulus has been reported to be a reliable barricade which prevents diffusion of membrane proteins between the midpiece and the principal piece [10,11]. However, studies in the guinea pig have shown that some monoclonal antibodies, which are restricted to the posterior tail, flow into the midpiece region during capacitation. This finding suggests that there is a change in the permeability of the annulus during capacitation [11,12].

The process of sperm capacitation, the achievement of the ability to fertilize, involves the attainment of many separate physiological events such as hyperactivation, the acrosome reaction, binding to the zona pellucida, and ultimately penetrating the oocyte [10]. In 1970, Yanagimachi first described the occurrence of hyperactivation in Chinese hamster spermatozoa [24]. Hyperactivation, a type of motility sperm undergo prior to the acrosome reaction, is characterized by

vigorous whiplashlike flagellar movements and high amplitudes of lateral head displacement [10,25]. It is believed that the strong thrusting movements exhibited by the sperm during hyperactivation assist both in the detachment from the isthmus epithelium and in the navigation through the viscous oviductal fluids and the oocyte's cumulus oophorus and zona pellucida [10,26-28].

The acrosome of the sperm is a membrane-bound organelle found in the distal portion of the sperm head. The acrosome reaction is the process in which the outer acrosomal membrane fuses to the overlying plasma membrane in order to release the acrosomal contents [10,22]. Many theories exist describing the acrosomal reactive process. The first theory suggests that the acrosome reaction is initiated when one of the zona glycoproteins, ZP3, acts as a ligand by binding to the sperm's receptors. The binding of the sperm to the ZP3 ligand triggers a chain of reactions. During capacitation, the sperm plasma membrane becomes more fluid because of the removal of decapacitation factors and a loss of cholesterol. The changing plasma membrane enables the zona receptors to shift around freely within the plane of the membrane allowing for an aggregation and subsequent activation of the receptors, which stimulates protein tyrosine kinase activity to phosphorylate proteins involved in the acrosome reaction [10,29]. The anterior half of the sperm head carries the acrosome. A sperm with an intact acrosome can not penetrate the egg [10]. The

acrosome must react (or break down) releasing its contents and shedding reacted elements. It has been shown that the zona pellucida induces the acrosome reaction [10]. ZP3 binds to a sperm receptor to trigger a chain of events resulting in the acrosome reaction. The ZP3 peptide chain must be whole to be an effective ligand [29].

A second theory of how the acrosome reaction occurs is as follows: The ZP receptor (bound to ZP3 to activate it) stimulates a G protein which, in turn, stimulates phospholipase C activity in the sperm plasma membrane [30]. Phospholipase C hydrolyses phosphatidylinositol diphosphate into diacylglycerol and inositol triphosphate. Intracellular calcium levels increase due to a release of intracellular stores by inositol triphosphate. Inositol triphosphate is phosphorylated to become inositol tetrakis-phosphate, which perhaps opens calcium channels to allow extracellular calcium to flow in. This increase in calcium either directly or indirectly acts on membrane phospholipids to begin fusion [30]. The activated G proteins have been shown to stimulate phospholipase A_2, phospholipase D, & adenylate cyclases [31]. The products of phospholipase A_2 and phospholipase D together with the increased calcium levels cause fusion. Cyclic AMP acts on sodium channels to allow a sodium influx, which raises the intracellular pH to also facilitate fusion [10,29-31].

Another similarly proposed mechanism for the acrosome reaction involves sperm receptors activated by ligands opening

the calcium channels directly, without associated G proteins [10,32]. The acrosome reaction culminates in the release of acrosomal hydrolytic enzymes such as hyaluronidase and acrosin [10,29,33]. The hyaluronidase and acrosin assist the sperm in the passage through the cumulus oophorus surrounding the oocyte and with the penetration of the zona pellucida [10].

The capacitation of sperm in vitro is usually accomplished in a chemically defined medium which has been supplemented with an energy source. Many factors have been found to affect capacitation in vitro including temperature, variations between species, the method of semen collection, and medium composition [10]. In recent years, there has been rapid growth in the development of both media and techniques for improving sperm quality; however, these methods have not always resulted in an increase in fertilizing capacity [33-35]. Yogev et al. attempted to evaluate some of the sperm preparation techniques by using a sperm-zona pellucida binding test, the hemizona assay, as the indicator of fertilizing ability [33]. Experiments have demonstrated that sperm motion analysis does not always accurately predict the true fertilizing potential of sperm and, thus, is not always a suitable method for accurate evaluation of the medium or technique [33,36]. However, the hemizona assay may be a more suitable method because it allows for evaluation of the effect of different sperm treatments on sperm binding. The hemizona assay has been shown to be of high predictability for

fertilization outcome because the binding of the sperm to the zona pellucida is a prerequisite for both penetration of the zona or the oocyte and fertilization of the oocyte [33,37-39].

Yogev et al. [33] evaluated five different sperm treatments: swim-up, TEST-yolk buffer, Percoll, pentoxifylline, and progesterone. The standard swim-up technique was used as the reference treatment method. The pentoxifylline was added to a twice washed sample and allowed to incubate prior to a one hour swim-up, whereas the progesterone was added to a sample following the standard swim-up procedure. Of the other four preparations, only the TEST-yolk buffer and pentoxifylline exhibited an overall statistically significant improvement in sperm-binding capacity as indicated by the hemizona assay [33]. Pentoxifylline, a phosphodiesterase inhibitor, is believed to stimulate flagellar motility by increasing intracellular cAMP levels through the reduction of cAMP degradation [40]. There was not a significant difference in binding capacity between the swim-up preparation and either the Percoll or when progesterone was added to the swim-up. The group concluded that in vitro preparation methods can affect sperm binding capacity to the zona pellucida [33].

Acetylcholine acts as a mediator for chemical transmission of nerve impulses in many animals [1]. The role of acetylcholine as a chemical transmitter has been well substantiated in both the central and peripheral nervous

systems of higher vertebrates. The nerves that release acetylcholine from their terminals are known as cholinergic nerves and the cholinergic nervous system refers to the nervous system in which acetylcholine is involved as a chemical transmitter. The cholinergic system consists of four components: acetylcholine, cholinesterases, choline acetyltransferases, and cholinergic receptors [2]. As shown in Figure 2-1, choline acetyltransferase catalyzes the acetyl group transfer from acetyl coenzyme A (acetyl CoA) to choline in order to produce acetylcholine. Acetylcholinesterase catalyzes the hydrolysis of acetylcholine to acetic acid and choline. The cholinergic receptors refers to the cellular site where acetylcholine binds to elicit a physiological response. Many types of cholinergic receptors can be differentiated by their reactions with different acetylcholine agonists, such as nicotine and muscarine. These nicotinic and muscarinic receptors all bind acetylcholine, but they are fundamentally different in both function and structure. For example, when nicotine binds to acetylcholine receptors the excitatory response lasts only milliseconds. These receptors are known as nicotinic acetylcholine receptors and these receptors can be ligand-gated channels for sodium and potassium ion exchange. In contrast, muscarine causes a response that lasts many seconds when bound to acetylcholine receptors. These receptors are known as muscarinic acetylcholine receptors and these receptors are usually

(a)

Acetyl CoA + Choline —Choline acetyltransferase→ **Acetylcholine + CoA**

(b)

Acetylcholine —acetylcholinesterase→ **Acetic acid + Choline**

Figure 2-1. Chemical reactions of acetylcholine. (a) The synthesis of acetylcholine catalyzed by choline acetyltransferase. (b) The hydrolysis of acetylcholine catalyzed by acetylcholinesterase.

inhibitory and are coupled to ion channel proteins by G proteins [41].

By 1976, investigations indicated that the acetylcholine cycle plays a significant role in non-nervous tissues, including mammalian spermatozoa. Previously, the role of acetylcholine had only been established in neuronal tissues. Bishop et al. established the occurrence of choline acetyltransferase and acetylcholine in fresh ejaculates of both bull and man [3]. Bishop and colleagues used pyrolysis gas chromatography to demonstrate the occurrence of acetylcholine and propionylchlorine in bull spermatozoa and the occurrence of acetylcholine in human spermatozoa [1,3].

The occurrence of choline acetyltransferase in mammalian spermatozoa was demonstrated by both Bishop et al. in 1976 and by Stewart and Forrester in 1978 [1,3-5]. Bishop et al. used a radiometric assay to determine choline acetyltransferase activity in bull and human spermatozoa. The values obtained were compared to those compiled by Nelson [6,7]. It was concluded that both bull and human spermatozoa contain choline acetyltransferase. Bull spermatozoa exhibited greater activity than human spermatozoa [4,6]. Stewart and Forrester used the Goldberg Assay, a potassium periodide precipitation procedure, to determine the amount of choline acetyltransferase activity in ram spermatozoa. The Goldberg Assay was originally developed for the determination of choline acetyltransferase activity in rat brains. Stewart and

Forrester observed an increase in choline acetyltransferase activity with an increase in choline concentration up to a maximum value of 430 pmol of acetylcholine per million sperm [5].

An acetylcholinesterase enzyme was demonstrated to occur in spermatozoa of several mammalian species. Nelson began extensive studies of acetylcholinesterase in the early 1960's in which acetylcholinesterase was identified in both pig and bull spermatozoa via specific substrates and inhibitors [7,41]. Stewart and Forrester also performed studies on acetylcholinesterase in ram sperm in 1978. They demonstrated that acetylcholinesterase activity would increase until saturation with increasing concentrations of its substrate, acetylcholine [5].

Cholinergic receptors that have been identified in spermatozoa are thought to be of the nicotinic type. Many studies have been performed in this area and most of them have utilized either cholinergic or cholinergic blocking agents to demonstrate the occurrence of the nicotinic acetylcholine receptor [2,6,42-48].

In 1976, Nelson measured sea urchin spermatozoa motility inhibition via alpha bungarotoxin. Alpha bungarotoxin is a potent neurotransmitter blocking agent obtained from snake venom. A decreasing sea urchin spermatozoa motility was observed with increasing concentrations of alpha bungarotoxin. In a separate experiment, Nelson added nicotine to sea urchin

spermatozoa and observed increasing motility with increasing nicotine concentration to a maximum increase of 75% [49]. Nelson also performed many other similar experiments using different cholinergic and cholinergic blocking agents. The results obtained were similar to the alpha bungarotoxin and nicotine experiments [4-50].

The occurrence of nicotinic acetylcholine receptors was demonstrated in 1988 by Placzek et al. in human spermatozoa. Placzek et al. examined the effect of both alpha bungarotoxin and trifluoperazine (an acetylcholine blocking agent) on human spermatozoa motility. A decreasing motility with increasing concentrations of both alpha bungarotoxin and trifluoperazine was observed [42,51].

In 1981, it was discovered that previous experiments involving choline acetyltransferase activity in spermatozoa could have actually been mistaken for carnitine acetyltransferase activity [52,53]. Carnitine acetyltransferase is capable of catalyzing the acetyl group transfer from acetyl CoA to choline, the exact same reaction occurring in choline acetyltransferase [41]. Also, carnitine acetyltransferase has been shown to be present in high amounts in both rat epididymal tissues and spermatozoa [8,54]. Most research involving choline acetyltransferase in spermatozoa subsided until 1991 when Ibanez et al. demonstrated that choline acetyltransferase mRNA is expressed in both rat and human spermatogenic cells. High levels of two choline

acetyltransferase transcripts were detected by Northern blot analysis of adult rat testis RNA. With translation, an accumulation of choline acetyltransferase in the postacrosomal region and along the equatorial segment of mature sperm is detected. Only one choline acetyltransferase mRNA species was detected in human testis. In situ hybridization was used to localize the cells within the seminiferous epithelium responsible for choline acetyltransferase mRNA synthesis in rat testis. Choline acetyltransferase-like immunoreactivity in ejaculated human spermatozoa demonstrated that translation of the mRNA detected in testis did occur [8].

In 1994, Storey et al. demonstrated that the zona pellucida induced acrosome reaction in mouse sperm could be inhibited by 3-quinuclidinyl benzilate, a potent antagonist of muscarinic acetylcholine receptors [9]. Three-quinuclidinyl benzilate binds reversibly to the mouse sperm plasma membrane at a single site [55]. Additional experiments showed inhibition of the calcium influx into mouse spermatozoa activated by solubilized mouse zona pellucida. Three inhibitors of the zona pellucida induced acrosome reaction were used: tyrophostin A48, pertussis toxin, and 3-quinuclidinyl benzilate. The group postulated that the sperm plasma membrane receptors mediating the zona pellucida induced acrosome reaction may function as a complex, whose formation is activated by zona pellucida ligand binding [9].

Acetylcholine is a neurotransmitter produced by ChAT that

has been suggested to play a role in spermatozoa [1]. Earlier studies indicated human spermatozoa contain the four components of the cholinergic system including acetylcholine and choline acetyltransferase [2]. However, the ChAT studies were reevaluated due to the possibility that ChAT activity was mistaken for CaAT, which can also use choline as a substrate. Recent studies have shown that ChAT mRNA is expressed in spermatogenic cells and have demonstrated the presence of ChAT immunoreactivity on sperm [3-7, 8]. A potential role for acetylcholine during fertilization would suggest a concomitant role for the enzyme involved in its production. It has been demonstrated in mouse sperm that the zona pellucida induced acrosome reaction can be inhibited by 3-quinuclidinyl benzilate, an antagonist of muscarinic acetylcholine receptors [9]. Acetylcholine may be part of a sperm receptor complex required for successful fertilization.

Currently, few specific biochemical markers exist to delineate the cellular and molecular events that occur during capacitation, a process requisite for successful fertilization [10]. This study will demonstrate that the regional distribution of ChAT activity in human spermatozoa is altered during in vitro capacitation and correlates with the fertilizing potential of sperm; and, as such this capacitation dependent distribution will serve as a valuable biochemical marker of capacitation.

CHAPTER III

CHOLINE ACETYLTRANSFERASE AND CARNITINE ACETYLTRANSFERASE ACTIVITY IN HUMAN SPERMATOZOA

Introduction

Acetylcholine, a neurotransmitter produced by the enzyme choline acetyltransferase (ChAT), has been suggested to play a role in many nonneuronal tissues, including spermatozoa [1]. Earlier studies have indicated that human spermatozoa contain the four components of the cholinergic system including acetylcholine and choline acetyltransferase [2]. Recent studies have indicated choline acetyltransferase mRNA is expressed in spermatogenic cells and the presence of choline acetyltransferase immunoreactivity is detected on sperm. A potential role for acetylcholine during fertilization would suggest a concomitant role for the enzyme involved in its production. In 1981, it was discovered that previous experiments involving the choline acetyltransferase activity present in spermatozoa could have actually been mistaken for carnitine acetyltransferase activity [52,53]. Carnitine acetyltransferase is capable of catalyzing the acetyl group transfer from acetyl CoA to choline, the exact same reaction occurring in choline acetyltransferase [41]. Also, carnitine acetyltransferase has been shown to be present in high amounts in both rat epididymal tissues and spermatozoa [8,54]. As a result of these findings, most research involving choline

acetyltransferase in spermatozoa subsided until 1991 when Ibanez et al. demonstrated that choline acetyltransferase mRNA is expressed in both rat and human spermatogenic cells. They reported that high levels of two choline acetyltransferase transcripts were detected by Northern blot analysis of adult rat testis RNA. Translation of these mRNA resulted in an accumulation of choline acetyltransferase in the postacrosomal region of mature sperm. Only one choline acetyltransferase mRNA species was detected in human testis. In situ hybridization was used to localize the cells within the seminiferous epithelium responsible for choline acetyltransferase mRNA synthesis in rat testis. The presence of choline acetyltransferase-like immunoreactivity in ejaculated human spermatozoa demonstrated that translation of the mRNA detected in testis had occurred [8]. Additionally, it has been demonstrated in mouse sperm that the zona pellucida induced acrosome reaction can be inhibited by 3-quinuclidinyl benzilate, an antagonist of muscarinic acetylcholine receptors [9]. Acetylcholine may be part of a sperm receptor complex required for successful fertilization.

This study was conducted as a three section project. Section I experiments were performed to confirm the immunoreactivity patterns reported for choline acetyltransferase (ChAT) antiserum and to determine whether said patterns were competitively inhibited by either ChAT or carnitine acetyltransferase (CaAT) enzymes. Section II

experiments compared the regional ChAT and CaAT immunoreactivity in human spermatozoa as assessed by fluorescent immunocytochemistry with ChAT and CaAT activity determined by enzymatic methodologies. The goal of section III was to correlate the regional distribution of ChAT and CaAT immunoreactivity under sperm capacitating conditions with the physiological indicators of completed capacitation, including the physiological and pharmacological induction of the acrosome reaction.

Materials and Methods

Section I: Localization of ChAT-like and CaAT-like Immunoreactivity

Subjects

Semen samples from proven fertile donors were utilized in the different experiments if ejaculates had an original sperm concentration >60 X 10^6/ml, progressive motility >60%, and normal morphology of >14%.

Semen analysis and preparation

Semen specimens were obtained by masturbation after at least two days of sexual abstinence. After liquefaction of the sample, a swim-up separation of the sperm motile fraction was performed. The semen samples were mixed with Ham's F-10

medium (GIBCO, Grand Island, NY) supplemented with 0.3% human serum albumin (Irvine Scientific, Santa Ana, CA) and washed twice by centrifugation at 400 x g. After centrifugation, the motile fraction of the sperm was collected following a one hour swim-up at 37°C and 5% CO_2 in water-saturated air.

Immunocytochemistry

Immunocytochemistry was performed on ejaculated sperm samples obtained from proven fertile donors. Semen smears and sperm samples subjected to capacitating conditions of incubation at 37°C and 5% CO_2 in water-saturated air. In order to determine the effect of time on the distribution of both the equatorial and the midpiece/tail immunoreactivity, spermatozoa were incubated under capacitating conditions with aliquots of sperm removed at 0, 1, 2, 4, 6, and 18 hours after the initiation of capacitation. Capacitation was initiated with the first wash in Ham's F-10 medium supplemented with 0.3% human serum albumin. Control and treated sperm were washed by centrifugation in phosphate-buffered saline, air-dried onto slides, and methanol fixed. After blocking of nonspecific sites with Superblock (Pierce, Rockford, IL), immunostaining was performed with a monoclonal antibody to human choline acetyltransferase from mouse-mouse hybrid cells (Boehringer Mannheim, Indiannapolis, IN). A fluorescein isothiocyanate conjugated rabbit anti-mouse secondary antibody (Zymed Laboratories, San Francisco, CA) was used to visualize

immunoreactivity under epifluorescent microscopy (Nikon Microphot.FS, Garden City, NY). To confirm the specificity of the anti-ChAT, competitive assays were performed using unlabelled ChAT (Sigma Chemical Company, St. Louis, MO). Earlier studies suggested possible cross-reactivity of ChAT with CaAT, therefore, unlabeled CaAT (Sigma Chemical Company) was included in one set of experiments to determine if the ChAT antibody exhibited any cross-reactivity with the CaAT enzyme present in sperm [41]. Controls included incubation with an unrelated monoclonal mouse antihuman primary antibody (Zymed Laboratories) and supravital staining with the nuclear stain Hoechst 33258 (Sigma Chemical Company), performed as described by Cross et al. to detect dead sperm based on a loss of the barrier function of their membranes [56]. All data are presented as mean $\pm$ standard error.

Section II: A Comparison Between ChAT and CaAT Immunoreactivity with ChAT Enzymatic Activity

Choline Acetyltransferase Radioassay

ChAT and CaAT activity was determined in populations of sperm samples using a modified radiometric assay as described by Fonnum [57]. In order to determine if the ChAT enzyme is synthesized during capacitation, enzyme activity was determined for two sperm populations: washed to remove seminal plasma and via the standard swim-up procedure in which

capacitation time will be at least one hour. Following either two washings with Ham's F-10 medium (GIBCO) supplemented with 0.3% human serum albumin (Irvine Scientific) or the standard swim-up procedure, a known concentration of sperm suspension was centrifuged and the supernatant discarded. The resulting pellet was extracted in extraction buffer (10 mM Na2HPO4 (Sigma Chemical Company), 100 mM NaCl (Sigma Chemical Company), 2 mM EDTA (Sigma Chemical Company), 0.5% (vol/vol) Triton X-100 (Sigma Chemical Company); pH 7.4) and allowed to stand on ice for 15 minutes before centrifugation for 5 minutes for 4,000 rpm at 0° C. The supernatant was added to the assay mixture (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM EDTA, 0.15% Triton X-100, 8mM choline chloride, 0.1mM physostigmine sulfate, and 0.2 mM acetyl-^{3}H-coenzyme A (specific activity 95.46 Gbq/mmol; New England Nuclear, Boston, MA)) and incubated for 10 minutes in a 37° C water bath. The enzymatic reaction was stopped by transferring the assay mixture into a scintillation vial containing a mixture of 5 ml 10 mM NaPO4 and 2 ml acetonitrile/tetraphenylboron (5mg.ml-1) to extract the newly synthesized radioactive acetylcholine into the organic phase. The organic phase was transferred into a new vial and 10 ml of scintillation fluid was added prior to measuring the tritium content in the scintillation counter. Acetylcholine synthesis was determined and normalized to sperm concentration and expressed as pmol/sperm concentration/unit time. In order to examine enzyme activity in acrosome-reacted

sperm, separate experiments were performed to induce the acrosome reaction with the addition of caffeine (Sigma Chemical company) to a final concentration of 4 mM and an incubation time of one hour. Slides were prepared for immunocytochemistry both before and after the treatment with caffeine, as described above. In order to determine that the observed activity was attributed to ChAT, experiments were conducted using bromoacetylcholine (Research Biochemicals International, Natick, MA), a selective inhibitor of ChAT. Unless otherwise stated, all chemical compounds were obtained from the Sigma Chemical Company.

Acrosome reaction

Acrosomal status of control and caffeine-treated spermatozoa was determined with the fluorescent probe fluorescein isothiocyanate labelled *Pisum sativum* (FITC-PSA) lectin (EY Laboratories, San Mateo, CA), following techniques previously described [58]. An epifluorescent microscope at 400X magnification was used to assess acrosomal status. At least 100 sperm were evaluated per control and treatment group. Acrosome-reacted status was diagnosed when a bar pattern was observed over the equatorial segment or no immunofluorescence was seen at all, indicating when a total loss of the acrosomal cap. Typically, acrosome-intact sperm exhibit staining of the entire acrosomal cap [58-67].

Hemizona Assay

In order to determine the effect of bromoacetylcholine on the tight binding of the sperm samples to human zona pellucida, the Hemizona Assay was used as previously described [68,69]. Briefly, human immature (prophase I) oocytes were removed from salt storage and rinsed in Ham's F-10 medium (GIBCO) supplemented with 0.1% bovine serum albumin (Sigma Chemical Company) before microbisection. Narishige micromanipulators (Tokyo, Japan) mounted on a phase-contrast inverted Nikon Diaphot microscope (Garden City, NY) were used for cutting the oocytes into matching hemizonae. Following the standard swim-up procedure, each sperm sample was divided into a control and treatment group. The treatment group was incubated with 1 μM of bromoacetylcholine for one hour before proceeding with the hemizona assay procedure. Because bromoacetylcholine has been shown to inhibit motility, the lowest physiologically active dose possible was used after dose response studies were performed to confirm its effect on motility [70]. In each experimental set, control and treated sperm droplets containing 1.5 million motile sperm per microliter were incubated with matching hemizona for four hours at 37°C and 5% CO_2 in water-saturated air. Following the incubation period, the hemizonae were rinsed in Ham's F-10 medium (GIBCO) supplemented with 0.5% human serum albumin (Irvine Scientific) using a narrow glass pipette in order to remove loosely attached sperm. Next, the number of sperm

tightly bound to the outer convex surface of each hemizona was counted on a phase-contrast microscope at 200X magnification. The Hemizona Assay results are reported as a hemizona index (HZI) which is calculated as shown in Figure 3-1.

Statistical Analysis

Data is presented as mean ± standard error. Analysis of significance was determined using ANOVA for the bromoacetylcholine radioimmunoassay experiments and the Student's t-test for all other experiments. A p value of $\leq$ 0.05 was considered significant for both statistical procedures.

Section III: Pharmacological and physiological induction of the acrosome reaction

Experiment Design

In order to correlate the regional distribution of immunoreactivity under sperm capacitating conditions with the physiological indicators of completed capacitation, physiological and pharmacological stimulators were added to induce the acrosome reaction. The proven fertile donor provided semen samples were divided into one of two groups. Both groups consisted of 5 separate semen samples from 5 different fertile donors. Following the standard swim-up procedure, the group one sample was further divided into 4

$$\text{Hemizona Index (HZI)} = \frac{\#\text{TBS of treated sample}}{\#\text{TBS of control sample}} \times 100$$

Figure 3-1. Calculation of the hemizona index (HZI). TBS is the abbreviation used for tightly bound sperm.

equal parts and subjected to one of the following treatments: pentoxifylline (1 mg/ml; 30 minutes)), progesterone (1 µM, 60 minutes), calcium ionophore A23187 (10 µM, 60 minutes), and a non-treated control; and allowed to further capacitate. The second group was divided into two equal parts before undergoing the swim-up procedure. Half of the sample was processed in standard (calcium containing) medium while the other half was processed in calcium-free medium. The standard medium group was divided further into a control group, a thapsigargin (10 µM, 10 minutes; Calbiochem, La Jolla, CA) treated group, and a caffeine (4 mM, one hour) treated group and allowed to further capacitate. Slides were prepared from each treatment and control group in order to assess immunoreactivity and to determine acrosomal status, as described above. All compounds were obtained from the Sigma Chemical Company unless otherwise noted.

Statistical Analysis

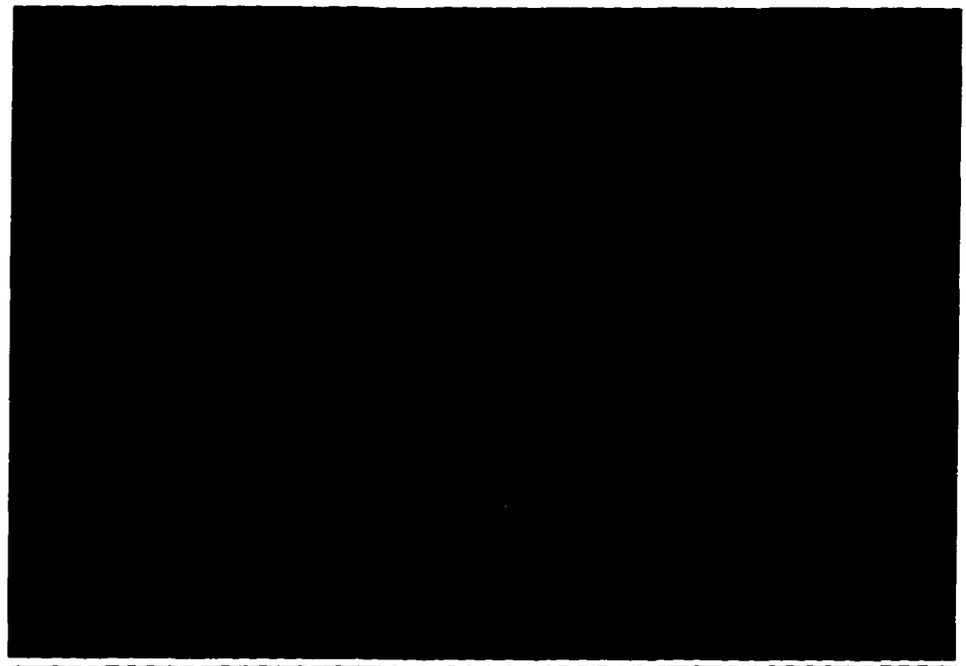
Data is presented as mean ± standard error. The percent of both ChAT and CaAT binding in each treatment group was compared to the corresponding control group using the Student's t-test with a p value of ≤ 0.05 considered significant.

Results

Section I: Localization of ChAT-like and CaAT-like Immunoreactivity

Immunocytochemical staining revealed intense immunoreactivity in the equatorial region of the sperm head and/or in the midpiece region of the sperm tail, as shown in Figure 3-2. Preliminary experiments were conducted to determine whether the distribution of the immunoreactivity was altered during capacitating conditions. As presented in Table 3-1, increasing proportions of sperm exhibited immunoreactivity along the equatorial region with a concomitant decrease in immunoreactivity in the midpiece and tail region. In order to more specifically determine the effect of time on the distribution of ChAT and CaAT immunoreactivity, spermatozoa were incubated under capacitating conditions with aliquots of sperm removed at 0, 1, 2, 4, 6, and 18 hours after the initiation of capacitation. Figure 3-3 shows the effect of time under capacitating conditions versus both the equatorial and the midpiece/tail immunoreactivity. As exhibited in Figures 3-4 and 3-5, competitive studies with unlabeled ChAT primarily blocked the equatorial region labeling, however, midpiece and tail labeling was also inhibited, suggesting some cross-reactivity of the ChAT antiserum with the CaAT enzyme or the midpiece region not exclusively containing CaAT but also ChAT. The

a.



b.

Figure 3-2. Immunocytochemical staining of human spermatozoa. Staining was exhibited in (a) the equatorial region of the sperm head and/or (b) in the midpiece region of the sperm tail.

Table 3-1 Immunoreactivity Exhibited with the ChAT antibody

	Equatorial	Midpiece/Tail
Semen	1.5 ± 0.5	42 ± 5
Capacitating - 0 Hr	38 ± 4	79 ± 8
Capacitating - 1 Hr	67 ± 6	63 ± 6

Values are percent means ± SE using 7 different proven fertile donor provided semen samples. Semen pertains to straight semen smears, capacitating - 0 hour pertains to slides prepared immediately following two washes in capacitating media, and capacitating - 1 hour pertains to slides prepared immediately following the standard swim-up procedure.

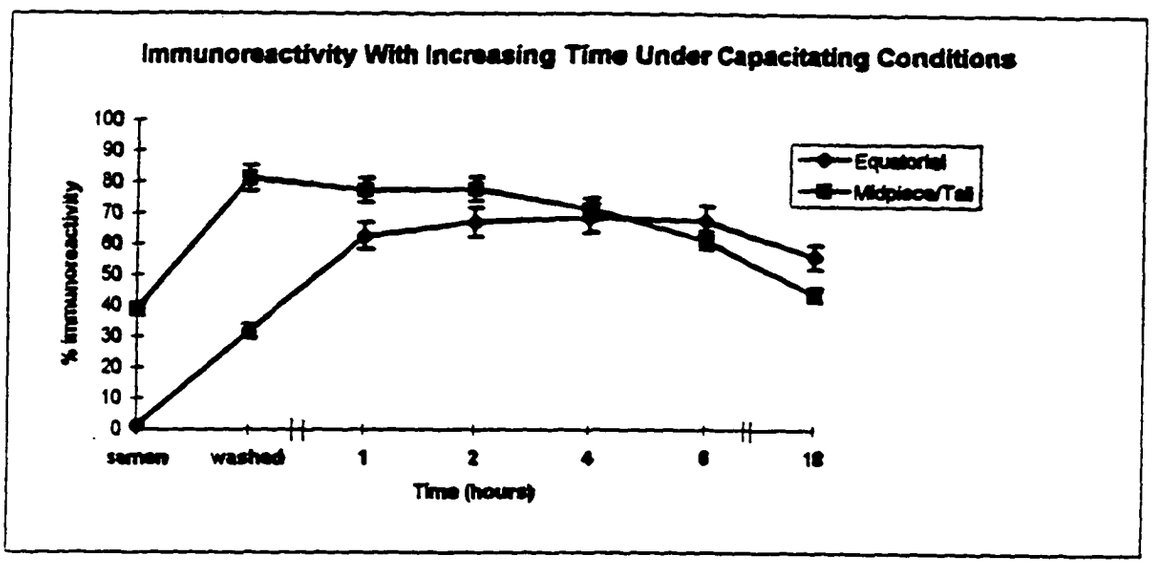


Figure 3-3. The Effect of Time Under Capacitating Conditions. In order to determine the effect of time on the distribution of both the equatorial and the midpiece/tail immunoreactivity, spermatozoa were incubated under capacitating conditions with aliquots of sperm removed at 0, 1, 2, 4, 6, and 18 hours after the initiation of capacitation.

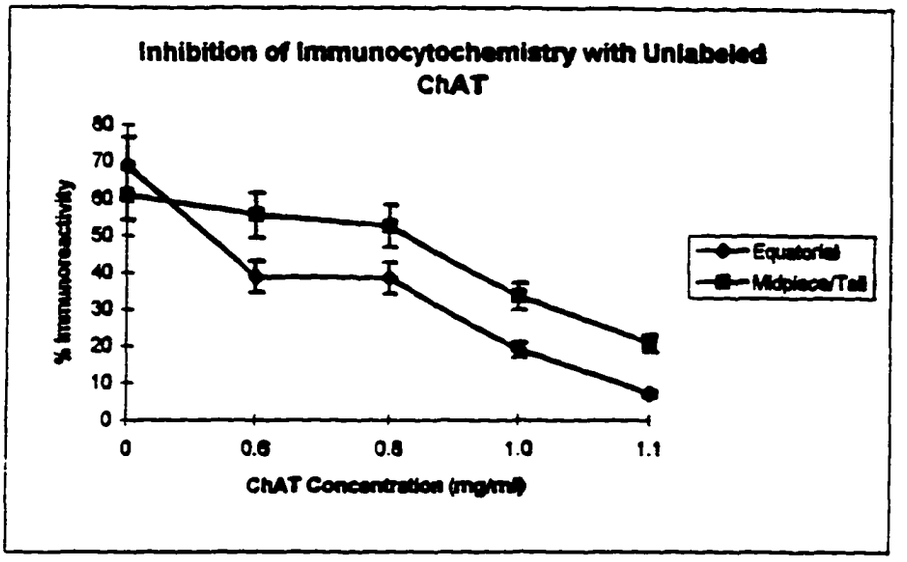


Figure 3-4. Inhibition of Immunocytochemistry with Unlabeled ChAT.

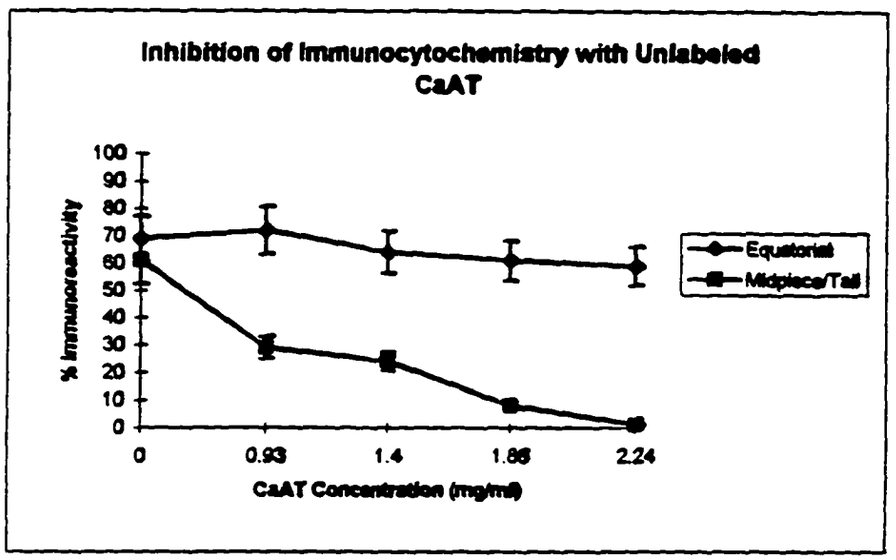


Figure 3-5. Inhibition of Immunocytochemistry with Unlabeled CaAT.

unlabeled CaAT primarily blocked staining along the midpiece and tail region of the sperm. The control experiments involving incubation with an unrelated monoclonal mouse antihuman primary antibody exhibited no immunoreactivity. Supravital staining with the nuclear stain Hoechst 33258 exhibited a 94.2 $\pm$ 5.4 % viability rate with dead sperm displaying no immunoreactivity against the ChAT antibody.

Section II: A Comparison Between ChAT and CaAT Immunoreactivity with ChAT Enzymatic Activity

Dose response curves for maximal acetylcholine production were constructed with varying concentrations of both the substrate ([^{3}H]-acetyl coenzyme A, Figure 3-6) and the enzyme source (concentration of sperm, Figure 3-7). Choline acetyltransferase activity was expressed as nmoles of acetylcholine produced per ten minute reaction time. An increase in acetylcholine production was observed with both increasing substrate and increasing enzyme source; however, saturation was observed at a substrate concentration of approximately 0.2 mM [3H]AcCoA.

Following the standard swim-up procedure, acetylcholine synthesis was measured in five sperm samples using the choline acetyltransferase radioassay. In order to determine how much of the observed activity was attributed to ChAT, experiments were conducted using varying concentrations of bromoacetylcholine, a selective inhibitor of ChAT. As shown

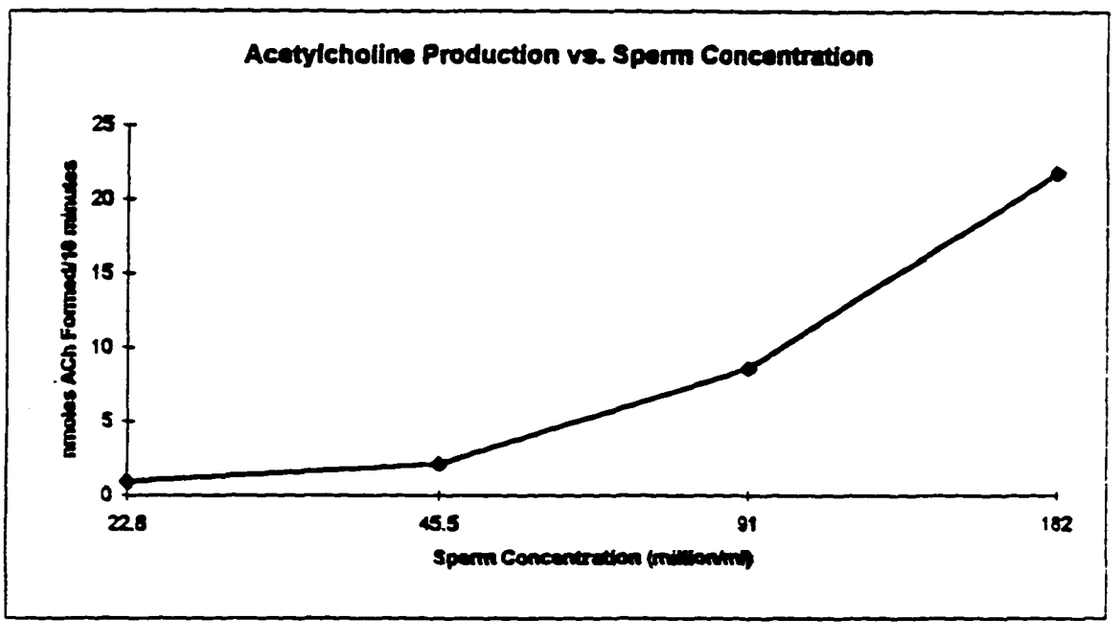


Figure 3-6. Dose response curve of acetylcholine production versus concentration of [^{3}H]-acetyl coenzyme A. Choline acetyltransferase activity is expressed as nmoles of acetylcholine produced per ten minute reaction time.

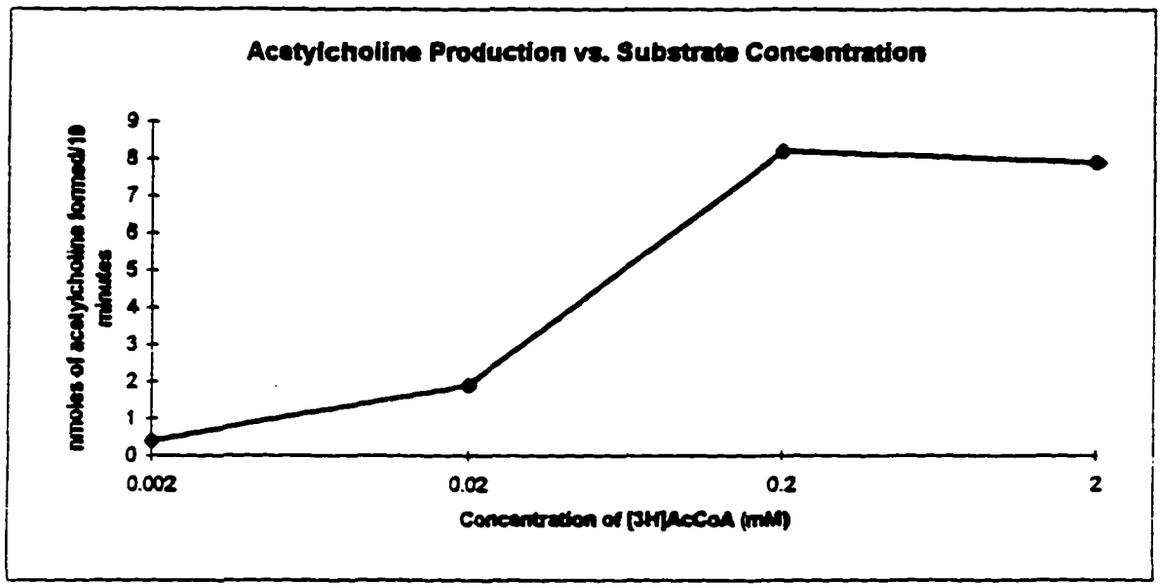


Figure 3-7. Dose response curve of acetylcholine production versus concentration of sperm. Choline acetyltransferase activity is expressed as nmoles of acetylcholine produced per ten minute reaction time.

in Figure 3-8, choline acetyltransferase activity was determined as acetylcholine produced normalized to sperm concentration and expressed as pmol/concentration sperm/unit time. As determined by ANOVA, significant inhibition ($p \leq 0.05$) was observed between the control and both the 0.1 µM and 2 µM bromoacetylcholine-treated groups. Maximal inhibition was observed at a bromoacetylcholine concentration of 2 µM. Total inhibition was not observed, possibly due to acetylcholine production by CaAT. Individual donor specimen data are reproduced in Figure 3-9 using the bromoacetylcholine inhibitor at the maximal concentration of 2 µM. Each individual donor exhibited inhibition, with a range of 20.4% to 34.3% inhibition.

In order to determine the effect of bromoacetylcholine, a selective inhibitor of ChAT, on the tight binding of the sperm samples to human zona pellucida, the Hemizona Assay was performed as previously described [68,69]. Following the standard swim-up procedure, sperm samples obtained from two different donors were divided into a control and a treatment group. Treatment consisted of incubation with bromoacetylcholine at a concentration of 0.1 µM for one hour. Because bromoacetylcholine has been shown to inhibit motility, the lowest physiologically active dose possible was used after dose response studies were performed to confirm its effect on motility [70]. Fourteen oocytes were originally selected for this study, however, data from four oocytes was discarded due

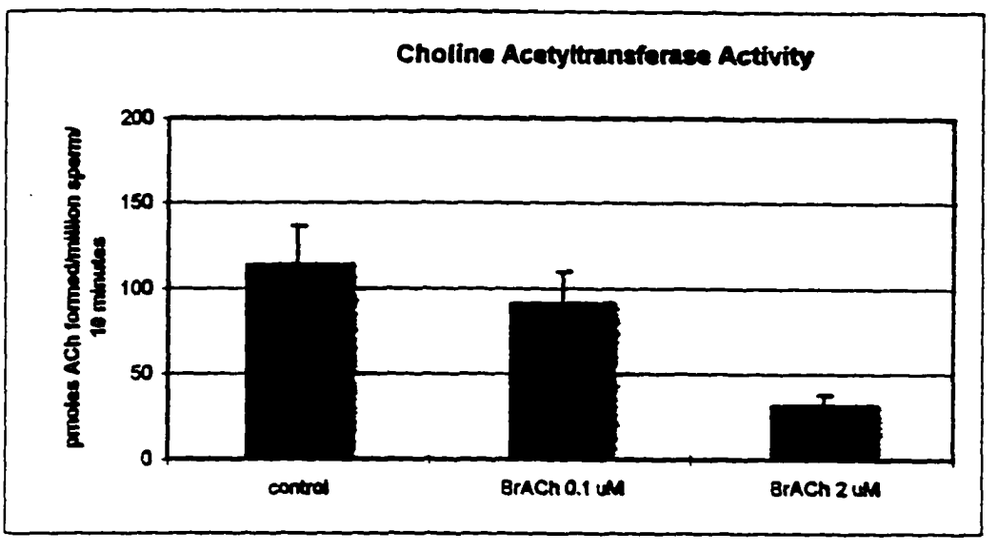


Figure 3-8. Choline acetyltransferase activity. Activity is determined as acetylcholine produced normalized to sperm concentration and expressed as pmol/concentration sperm/unit time. In order to determine how much of the observed activity was attributed to ChAT, experiments were conducted using the selective inhibitor bromoacetylcholine at concentrations of 0.1 μM and 2 μM. Both concentrations were significantly lower ($p \leq 0.05$) than the control.
n=5.

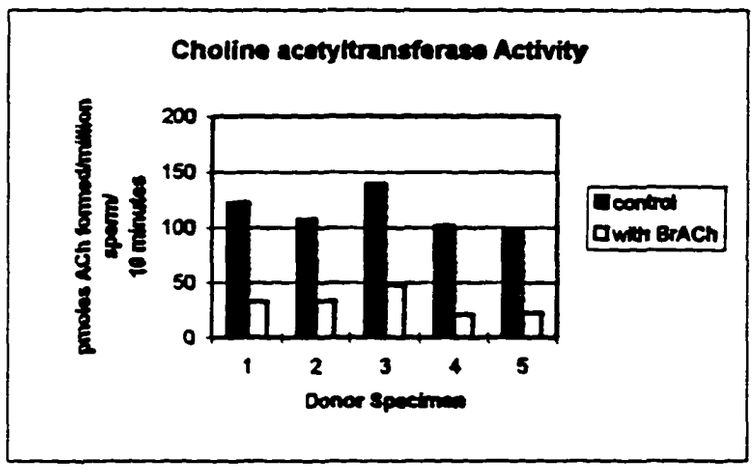


Figure 3-9. Individual donor specimen choline acetyltransferase activity levels. Levels were determined as acetylcholine produced normalized to sperm concentration and expressed as pmol/concentration sperm/unit time. In order to determine how much of the observed activity was attributed to ChAT, bromoacetylcholine has been added at a concentration of 2 μM.

to low binding in both the treatment and control group. Therefore, a total of ten oocytes were used for the data analysis. The mean $\pm$ SE for the hemizona binding in the control group was 51.9 $\pm$ 19.5, whereas the bromoacetylcholine treated group exhibited statistically decreased binding (p = 0.000122) at 37.5 $\pm$ 19.4. The mean hemizona index was 70.2 $\pm$ 14.1.

In order to compare ChAT immunoreactivity with ChAT enzyme activity and to examine with effects of capacitating conditions on both, matching experiments were performed using washed and post swim-up sperm with and without the addition of caffeine (to induce the acrosome reaction) to a final concentration of 4 mM and an incubation time of one hour. Slides were made for ChAT immunocytochemistry and to assess acrosomal status both before and after the addition of caffeine. The ChAT radioassay was performed immediately after two washings in Ham's F-10 media supplemented with 0.3 % human serum albumin for the control washed group and immediately after swim-up for the post swim-up control group. The ChAT radioassay was performed immediately following pharmacological induction of the acrosome reaction treatment with caffeine for both the washed caffeine and the swim-up caffeine group. Results are presented in Table 3-2. The ChAT radioassay results indicated slightly higher ChAT activity in washed versus swim-up sperm, however, the increase was not statistically significant. The addition of caffeine to the

ChAT radioassay group yielded results similar to the group without caffeine. However, the addition of caffeine did significantly increase equatorial immunoreactivity, midpiece/tail immunoreactivity, and the acrosome reaction rate in both washed and post swim-up samples. In the washed versus swim-up samples, a significantly increased percent of equatorial immunoreactivity was observed in both the control group and the caffeine treated group. The midpiece and tail immunoreactivity was significantly lower in the non-caffeine treated swim-up versus washed groups, but the caffeine treated groups were not statistically affected. A significant increase was observed in the caffeine treated swim-up sample when compared to the caffeine treated washed sample. Therefore, although a direct comparison can not be made between the ChAT radioassay and the equatorial and midpiece/tail ChAT immunoreactivity, it appears that the two procedures do not correlate.

Section III: Pharmacological and physiological induction of the acrosome reaction

In order to correlate the regional distribution of immunoreactivity under sperm capacitating conditions with the physiological indicators of completed capacitation, physiological and pharmacological stimulators were added to induce the acrosome reaction. Semen samples from 5 different fertile donors were divided into one of two groups. Following

Table 3-2 The Effect of Caffeine and Capacitating Conditions on ChAT Activity and Immunoreactivity

		Control (without caffeine)		
Procedure	**Activity**	**% Equatorial**	**% Midpiece/Tail**	**% AR**
Washed	139.7 ± 12.5	33.2 ± 4.0^	72.3 ± 5.7^	12.0 ± 2.5
Swim-up	121.4 ± 19.3	62.4 ± 7.1^	59.5 ± 5.4^	14.3 ± 1.2

		With Caffeine		
Procedure	**Activity**	**% Equatorial**	**% Midpiece/Tail**	**% AR**
Washed	136.0 ± 19.4	57.3 ± 1.7*^	67.3 ± 2.9*	27.7 ± 3.1*^
Swim-up	120.0 ± 16.3	69.0 ± 6.2*^	66.3 ± 6.2*	19.0 ± 1.6*^

^p < 0.05, washed versus swim-up
*p < 0.05, control versus caffeine treated
AR = % acrosome-reacted sperm
Equatorial and Midpiece/Tail = % ChAT immunoreactivity
Activity = ChAT activity as assessed by the ChAT radioassay

An indirect comparison between ChAT immunoreactivity and ChAT enzyme activity by direct comparisons between washed and post swim-up sperm and caffeine and non-caffeine treated sperm. Caffeine is added to a final concentration of 4 mM and incubated for one hour.

the standard swim-up procedure, the group one sample was further divided into 4 equal parts and subjected to one of the following treatments: pentoxifylline (1 mg/ml; 30 minutes)), progesterone (1 μM, 60 minutes), calcium ionophore A23187 (10 μM, 60 minutes), and a non-treated control; and allowed to further capacitate. The second group was divided into two equal parts before undergoing the swim-up procedure. Half of the sample was processed in standard (calcium containing) medium while the other half was processed in calcium-free medium. The standard medium group was divided further into a control group, a thapsigargin (10 μM, 10 minutes) treated group, and a caffeine (4 mM, one hour) treated group and allowed to further capacitate. Slides were prepared from each treatment and control group to assess immunoreactivity and to determine acrosomal status. Results of the equatorial and midpiece/tail immunoreactivity and percent of acrosome-reacted sperm for groups one and two are presented in Tables 3-3 and 3-4, respectively. Paired t-test analysis determined a significant increase in both equatorial and midpiece/tail immunoreactivity in both the caffeine group and in the pentoxifylline group when compared to their respective controls. Calcium ionophore A23187 exhibited a significant increase in midpiece and tail immunoreactivity, however, its effect on equatorial immunoreactivity was not significantly changed. Progesterone, caffeine, and calcium ionophore A23187 all significantly increased the percent of acrosome-reacted

sperm. All other treatments and groups did not significantly effect immunoreactivity or the acrosome reaction.

Discussion

In 1991 Ibanez et al. demonstrated that choline acetyltransferase mRNA is expressed in human spermatogenic cells. They detected only one choline acetyltransferase mRNA species in the human testis. Choline acetyltransferase-like immunoreactivity in ejaculated human spermatozoa was also demonstrated, indicating that translation of the mRNA detected in testis did occur [8]. This study confirmed the finding by Ibanez et al. by using immunocytochemical staining to reveal intense ChAT-like immunoreactivity in the equatorial region of the sperm head and/or in the midpiece region of the sperm tail. However, this study also went further to demonstrate that the distribution of the immunoreactivity was altered during capacitating conditions. This finding was portentous because it substantiated the first part of the dissertation hypothesis stating that the regional distribution of ChAT activity on human sperm is altered during in vitro capacitation. Specifically, increasing proportions of sperm exhibited ChAT immunoreactivity along the equatorial region with a concomitant decrease in ChAT reactivity in the midpiece. Also, competitive studies with unlabeled ChAT blocked the equatorial region labeling; the unlabeled CaAT blocked staining along the midpiece region of the tail,

Table 3-3 Pharmacological and Physiological Induction of the Acrosome Reaction and the Effects on Immunoreactivity - Group 1

	Equatorial	Midpiece/Tail	AR
Pentoxifylline	76.4 ± 5.0*	64.4 ± 6.9*	16.8 ± 4.2
Progesterone	70.0 ± 3.4	61.2 ± 5.2	18.2 ± 5.0*
Ca Ionophore	67.6 ± 6.5	67.4 ± 10.2*	33.4 ± 5.1*
Control	63.6 ± 8.0	58.2 ± 5.7	14.0 ± 2.5

n = 5

* $p \leq 0.05$

Table 3-4 Pharmacological and Physiological Induction of the Acrosome Reaction and the Effects on Immunoreactivity - Group 2

	Equatorial	Midpiece/Tail	AR
Thapsigargin	59.2 ± 5.3	70.4 ± 5.5	21.2 ± 5.5
Caffeine	75.2 ± 5.2*	68.2 ± 6.4*	21.6 ± 2.7*
Ca free	60.6 ± 7.2	58.8 ± 5.8	13.0 ± 3.3
Control	68.0 ± 7.1	61.4 ± 4.5	13.6 ± 2.4

n = 5

* $p \leq 0.05$

suggesting some cross-reactivity of the ChAT antiserum with the CaAT enzyme or that ChAT is present in the midpiece/tail. These findings suggest that ChAT could possibly be involved in the spermatozoa and zona pellucida binding phenomena since zona pellucida interaction is compartmentalized to the head region. Conversely, CaAT may be involved in motility due to its reactivity in the midpiece area of the tail, the compartment responsible for energy requirements during motility. The regional surface domains of mammalian sperm differ in both composition and function [10]. The five main surface domains include the acrosomal segment, the equatorial segment, the postacrosomal segment, the midpiece, and the principal piece [19]. Signs of capacitation occur separately in each of the five surface domains as the sperm become competent to fertilize [11,20]. The acrosomal and postacrosomal segments of the plasma membrane undergo many modifications during capacitation including biochemical changes such as alterations in the lipid and surface glycoprotein composition and distribution, antigen distribution patterns, and intracellular ions [10,11,21,22]. The physiological endpoint of capacitation for the acrosomal and postacrosomal segments is the acrosome reaction [10,22]. The major goal of the equatorial segment during capacitation is to provide a membrane capable of fusing with the oocyte plasma membrane, which it can not perform prior to the acrosome reaction [11,23]. Section I of this study

demonstrated that the distribution of the immunoreactivity exhibited with immunocytochemistry using an anti-ChAT antibody was altered during capacitating conditions. Specifically, increasing proportions of sperm exhibited anti-ChAT immunoreactivity along the equatorial region with a concomitant decrease in anti-ChAT reactivity in the midpiece and tail region. Also, competitive studies with unlabeled ChAT primarily blocked the equatorial region labeling and the unlabeled CaAT blocked staining along the midpiece region and the tail. The midpiece and principal piece, which are separated by the annulus, provide the sperm with motility [10,11]. Therefore, it is comprehensible that if CaAT is involved in motility, it would be needed immediately after ejaculation in order to ensure transport to the oocyte. However, if ChAT is involved in the sperm and zona pellucida binding phenomena, it would not be required until much later (after transport to the oocyte). The data from section I substantiate these hypotheses because more midpiece and tail immunoreactivity was observed earlier in capacitation, especially in semen smears, whereas the equatorial immunoreactivity was low in early capacitation but increased as time in capacitating conditions increased. Additionally, although there was cross-reactivity, the competitive studies with unlabeled ChAT primarily blocked the equatorial region labeling and the unlabeled CaAT blocked staining along the midpiece region and the tail.

The experiments in section II were completed in an attempt to further substantiate the hypothesis addressed in Section I that ChAT is involved in the spermatozoa and zona pellucida binding phenomena and that CaAT is involved in sperm motility.

Acetylcholine acts as a mediator for chemical transmission of nerve impulses in many animals [1]. The role of acetylcholine as a chemical transmitter has been well substantiated in both the central and peripheral nervous systems of higher vertebrates. The nerves that release acetylcholine from their terminals are known as cholinergic nerves and the cholinergic nervous system refers to the nervous system in which acetylcholine is involved as a chemical transmitter [2]. The cholinergic receptors refers to the cellular site where acetylcholine binds to elicit a physiological response. Many types of cholinergic receptors can be differentiated by their reactions with different acetylcholine agonists, such as nicotine and muscarine. These nicotinic and muscarinic receptors all bind acetylcholine, but they are fundamentally different in both function and structure. For example, when nicotine binds to acetylcholine receptors the excitatory response lasts only milliseconds. These receptors are known as nicotinic acetylcholine receptors and these receptors can be ligand-gated channels for sodium and potassium ion exchange. In contrast, muscarine causes a response that lasts many seconds when bound to acetylcholine

receptors. These receptors are known as muscarinic acetylcholine receptors and these receptors are usually inhibitory and are coupled to ion channel proteins by G proteins [41].

By 1976, investigations indicated that the acetylcholine cycle plays a significant role in non-nervous tissues, including mammalian spermatozoa. Previously, the role of acetylcholine had only been established in neuronal tissues. Bishop et al. established the occurrence of choline acetyltransferase and acetylcholine in fresh ejaculates of both bull and man [3]. It was concluded that human spermatozoa contain choline acetyltransferase [4,6].

Cholinergic receptors that have been identified in spermatozoa are thought to be of the nicotinic type. Many studies have been performed in this area and most of them have utilized either cholinergic or cholinergic blocking agents to demonstrate the occurrence of the nicotinic acetylcholine receptor [2,6,42-48].

In this study, acetylcholine synthesis was measured in five sperm samples using the choline acetyltransferase radioassay. Choline acetyltransferase activity was determined as acetylcholine produced normalized to sperm concentration and expressed as pmol/concentration sperm/unit time. The mean ChAT activity for the five donor specimens was 113.48 $\pm$ 15.68 pmoles of acetylcholine formed per million sperm cells per 10 minutes. This value is a little lower than expected since

Bishop et al. in 1976 had obtained a value of 130.90 + 14.19 pmoles of acetylcholine formed per million sperm cells per 10 minutes [3]. However, several differences occurred between their methods and the ones employed in this study. The most obvious difference is that the semen samples were obtained from different donors. This study exhibited a fairly wide range of ChAT activity between different donors, as was shown in Figure 3-9. Also, Bishop et al. used a different method for determining the ChAT activity. Although they used a radiometric assay, their assay was more complicated and required the use of ^{14}C-substrate and an anion exchange resin column. However, the largest difference between the two methods was probably the difference in sperm preparation. This study used Ham's F-10 medium supplemented with 0.3% human serum albumin with the standard swim-up technique, whereas Bishop et al. used a Norman-Johnson's solution and only washed the sperm [3]. Sastry et al. reported in 1981 that repeated washings and preparation of sperm decrease relative ChAT concentrations [70].

In order to determine how much of the observed activity was attributed to ChAT, experiments were conducted using varying concentrations of bromoacetylcholine, a selective inhibitor of ChAT. Total inhibition was not observed, however, total inhibition would not have been expected since bromoacetylcholine is a selective inhibitor of ChAT and not CaAT. The activity observed not inhibited by

bromoacetylcholine could be due to acetylcholine production by CaAT, since it is capable of catalyzing the acetyl group transfer from acetyl CoA to choline, the exact same reaction occurring in choline acetyltransferase [41].

To determine the effect of bromoacetylcholine on the tight binding of the sperm samples to human zona pellucida, the Hemizona Assay was used. The hemizona assay allows for evaluation of the effect of different sperm treatments on sperm binding. The hemizona assay has been shown to be of high predictability for fertilization outcome because the binding of the sperm to the zona pellucida is a prerequisite to both penetration of the zona or the oocyte and fertilization of the oocyte. The hemizona assay specifically tests for tight binding of sperm to the hemizona; however, it has also been significantly correlated with the acrosome reaction as well [33,37-39]. Because bromoacetylcholine has been shown to inhibit motility, the lowest physiologically active dose possible was used after dose response studies were performed to confirm its effect on motility [70]. The hemizona binding in the control group was 51.9 $\pm$ 19.5, whereas the bromoacetylcholine treated group exhibited statistically decreased binding at 37.5 $\pm$ 19.4. Therefore, it can be concluded that bromoacetylcholine does exhibit a negative effect on sperm-zona binding. This finding further substantiates the hypothesis that ChAT is involved in the spermatozoa and zona pellucida binding phenomena since

bromoacetylcholine is a selective inhibitor of ChAT and not CaAT. However, although sperm motion analysis experiments were conducted to ensure sperm motility was not substantially affected at the concentration of bromoacetylcholine used for these studies, it is possible that sperm motility still was a factor. Since bromoacetylcholine has been shown to affect motility at higher concentrations, it is possible that the sperm motion was affected in some way not detected by motion analysis [70]. In which case, any factor affecting motility would interfere with the ability of sperm to reach the hemizona, and thus the hemizona assay would exhibit decreased binding.

In order to compare ChAT immunoreactivity with ChAT enzyme activity and to examine the effects of capacitating conditions on both, matching experiments were performed using washed and post swim-up sperm with and without the addition of caffeine. Caffeine, a methyl-xanthine, inhibits the action of phosphodiesterase, thereby preventing the breakdown of cAMP into 5-AMP and resulting in an increase of endogenous cAMP [71]. Cyclic-AMP is thought to be one of the mediators of sperm motility [72]. High levels of cAMP have been shown to correlate with high motility [73].

The ChAT radioassay results indicated slightly higher ChAT activity in washed versus swim-up sperm, however, the increase was not statistically significant. This finding was expected since Sastry et al. reported that repeated washings

and preparation of sperm decrease relative ChAT concentrations [70]. The addition of caffeine to the ChAT radioassay group yielded results similar to the group without caffeine. Based on these findings, it can be concluded that the ChAT enzyme is already present within the sperm and not being synthesized by it during capacitation. Ibanez et al. demonstrated that choline acetyltransferase mRNA is expressed in human spermatogenic cells. They detected only one choline acetyltransferase mRNA species in the human testis. Choline acetyltransferase-like immunoreactivity in ejaculated human spermatozoa was also demonstrated, indicating that translation of the mRNA detected in testis did occur [8]. It may be that the ChAT mRNA found in the testis is masked or otherwise covered since the immunocytochemical studies in section I demonstrated equatorial immunoreactivity was low in early capacitation but increased with increasing time in capacitating conditions. During capacitation, the sperm plasma membrane becomes more fluid because of the removal of decapacitation factors and a loss of cholesterol. The changing plasma membrane enables the zona receptors to shift around freely within the plane of the membrane allowing for an aggregation and subsequent activation of the receptors, which stimulates protein tyrosine kinase activity to phosphorylate proteins involved in the acrosome reaction [10,29]. These changes associated with the acrosome reaction and capacitation may result in an uncovering or exposing of ChAT activity to

play a part in binding between the zona pellucida and sperm.

The addition of caffeine significantly increases equatorial binding, midpiece/tail binding, and the acrosome reaction rate in both washed and post swim-up samples. Although a direct correlation between equatorial binding and acrosome reaction rate was not made, both increased with the addition of caffeine. These findings are explored further in Chapter V with an attempt to directly correlate acrosomal status with anti-ChAT immunoreactivity. In the washed versus swim-up samples, a significantly increased percent of equatorial binding was observed in both the control group and the caffeine treated group. The midpiece and tail binding was significantly lower in the non-caffeine treated swim-up versus washed groups, but the caffeine treated groups were not statistically affected. The distribution of immunoreactivity between control washed and swim-up samples duplicated the results obtained in section I, thereby, further substantiated those results. A significant increase was observed in the caffeine treated swim-up sample when compared to the caffeine treated washed sample. Therefore, although a direct comparison can not be made between the ChAT radioassay and the equatorial and midpiece/tail ChAT immunoreactivity, it appears that the two procedures do not correlate. However, based on these findings, it can be concluded that the ChAT enzyme is present and active within the sperm and not synthesized de novo during capacitation.

Due to the finding that caffeine exhibited an effect on both equatorial and midpiece/tail anti-ChAT immunoreactivity, further experiments were conducted with caffeine and other pharmacological and physiological stimulators, including pentoxifylline, progesterone, calcium ionophore A23187, and thapsigargin. Like caffeine, pentoxifylline is also a phosphodiesterase inhibitor, and it is believed to stimulate flagellar motility by increasing intracellular cAMP levels through the reduction of cAMP degradation [40]. Progesterone has been shown to increase the free intracytoplasmic calcium level in sperm and under certain conditions, can stimulate the acrosome reaction and increase hyperactivation [74-76]. Numerous studies have shown that calcium ionophore A23187 induces the acrosome reaction [77-79]. Thapsigargin is a cell permeable tumor promotor that releases calcium by inhibiting endoplasmic reticulum calcium-ATPases without generating inositol triphosphate. Therefore, calcium uptake by the calcium-ATPase is inhibited, resulting in an increased intracellular calcium [80].

Progesterone, calcium ionophore A23187, and thapsigargin have all been shown in one way or another to affect intracellular calcium within mammalian sperm, which is involved in the acrosome reaction. One theory of how the acrosome reaction occurs is as follows: The ZP receptor (bound to ZP3 to activate it) stimulates a G protein which stimulates phospholipase C activity in the sperm plasma

membrane. Phospholipase C then hydrolyses phosphatidylinositol diphosphate into diacylglycerol and inositol triphosphate. Intracellular calcium levels increase due to a release of intracellular stores by inositol triphosphate. Inositol triphosphate is phosphorylated to become inositol tetrakis-phosphate, which perhaps opens calcium channels to allow extracellular calcium to flow in. This increase in calcium either directly or indirectly acts on membrane phospholipids to begin fusion [30]. The activated G proteins have been shown to stimulate phospholipase A_2, phospholipase D, and adenylate cyclases [31]. The products of phospholipase A_2 and phospholipase D together with the increased calcium levels cause fusion. Cyclic AMP acts on sodium channels to allow a sodium influx, which rises the intracellular pH to also facilitate fusion [10,29-31]. Another similarly proposed mechanism for the acrosome reaction involves sperm receptors activated by ligands opening the calcium channels directly, without associated G proteins [10,32]. The acrosome reaction culminates in the release of acrosomal hydrolytic enzymes such as hyaluronidase and acrosin [10,29,33]. The hyaluronidase and acrosin assist the sperm in the passage through the cumulus oophorus surrounding the oocyte and with the penetration of the zona pellucida [10].

In order to correlate the regional distribution of immunoreactivity under sperm capacitating conditions with the physiological indicators of completed capacitation, the

physiological and pharmacological stimulators were added to induce the acrosome reaction. A significant increase in both equatorial and midpiece/tail immunoreactivity in both the caffeine group and in the pentoxifylline group was observed when compared to their respective controls. Since caffeine and pentoxifylline are both phosphodiesterase inhibitors, which prevent the breakdown of cAMP into 5-AMP to result in an increase of endogenous cAMP, it appears that ChAT and CaAT may be involved to some extent with the cAMP system [37,71]. However, there are many other mechanisms that could be involved in this process, so further studies should be done before any definite conclusions can be drawn.

Calcium ionophore A23187 exhibited a significant increase in midpiece and tail immunoreactivity, however, its effect on equatorial immunoreactivity was not significantly changed. This observation may be due to an indirect effect and not deliberately reflect a direct relationship between calcium and midpiece/tail immunoreactivity since calcium ionophore A23187 has been shown to affect motility in a number of ways [81-83]. Progesterone, caffeine, and calcium ionophore A23187 all significantly increased the percent of acrosome-reacted sperm, as also previously reported [84-88].

CHAPTER IV

EVALUATION OF THE EFFECTS OF SEPARATION TECHNIQUES ON SPERM FUNCTION

Introduction

Experiments performed in Chapter III have substantiated the ChAT-like immunoreactivity observed by the Ibanez group and have suggested that CaAT plays a role in sperm motility [8]. Additional experiments were performed to evaluate the effects of separation techniques on sperm function in order to determine potential media based effects under capacitating conditions. Medium composition has been found to affect capacitation in vitro [10]. In recent years, there has been rapid growth in the development of both media and techniques for improving sperm quality; however, these methods have not always resulted in an increase in fertilizing capacity [33-35]. Yogev et al. attempted to evaluate some of the sperm preparation techniques by using a sperm-zona pellucida binding test, the hemizona assay, as the indicator of fertilizing ability [33]. Experiments have demonstrated that sperm motion analysis does not always accurately predict the true fertilizing potential of sperm and, thus, is not always a suitable method for accurate evaluation of the medium or technique [33,36]. However, the hemizona assay may be a more suitable method because it allows for evaluation of the effect of different sperm treatments on sperm binding. The hemizona

assay has been shown to be of high predictability for fertilization outcome because the binding of the sperm to the zona pellucida is a prerequisite for both penetration of the zona or the oocyte and fertilization of the oocyte [33,37-39]. Yogev et al. concluded that in vitro preparation methods can affect sperm binding capacity to the zona pellucida [33].

Materials and Methods

The effects of three different sperm processing techniques, standard swim-up, ISolate (Irvine Scientific), and Perwash (Irvine Scientific), on capacitation was determined by measuring the physiological endpoints of hyperactivation, acrosome reaction, and sperm-zona binding capacity. Ejaculated human sperm was obtained from proven fertile donors. Ensuing liquefaction and sperm motion analysis, each semen sample was divided into two aliquots for processing. The first set of experiments involved sperm processing by using the standard swim-up method as the control group and ISolate sperm processing as the treatment group. The second set of experiments involved using Perwash sperm processing as the control group compared to ISolate sperm processing as the treatment group. The standard swim-up method involved mixing the semen samples with Ham's F-10 medium (GIBCO) supplemented with 0.3% human serum albumin (Irvine Scientific) and washing twice by centrifugation at 400 x g. After centrifugation, the motile fraction of the sperm was collected following a one

hour swim-up at 37°C and 5% CO_2 in water-saturated air. For the ISolate and Perwash procedures, semen samples were layered onto a discontinuous two layer gradient of either ISolate or Perwash and centrifuged at 300 x g for 20 minutes. Next, the sperm pellet was carefully removed and washed two additional times by centrifugation with Ham's F-10 medium supplemented with 0.3% human serum albumin.

Sperm Motion

Following sperm processing by each method, motion characteristics (% motility, path velocity (VAP), curvilinear velocity (VCL), straightline velocity (VSL), amplitude of lateral head displacement (ALH), linearity (LIN), and beat cross frequency (BCF)) of the motile sperm fraction was determined for each sample via computer-assisted motion analysis (HTM-IVOS). To differentiate between hyperactivated and non-hyperactivated sperm, the following settings were used in the automatic SORT program for hyperactivity: number of points in each sperm track: 5 to 30, path velocity (VAP): 40.0 to 500.0 μm/s, progressive velocity (VSL): 20.0 to 500.0 μm/s, track speed (VCL): 100.0 to 500.0 μm/s, linearity (LIN): 0.0 to 65.0 μm/s, and lateral displacement (ALH): 7.5 to 50.0 μm/s.

Hemizona Binding

Next, the tight binding of the sperm samples to human

zona pellucida was assessed using the Hemizona Assay as previously described [68,69]. The tight binding of the sperm samples to human zona pellucida was assessed using the Hemizona Assay as previously described [68,69]. Briefly, human immature (prophase I) oocytes were removed from salt storage and rinsed in Ham's F-10 medium supplemented with 0.1% bovine serum albumin (Sigma Chemical Company) before microbisection. Narishige micromanipulators mounted on a phase-contrast inverted Nikon Diaphot microscope were used for cutting the oocytes into matching hemizonae. In each experimental set, control and treated sperm droplets containing 1.5 million motile sperm per microliter were incubated with matching hemizona for four hours at 37°C and 5% CO_2 in water-saturated air. Following the incubation period, the hemizonae were rinsed in Ham's F-10 medium supplemented with 0.5% human serum albumin (Irvine Scientific) using a narrow glass pipette in order to remove loosely attached sperm. Next, the number of sperm tightly bound to the outer convex surface of each hemizona was counted on a phase-contrast microscope at 200X magnification. The Hemizona Assay results are reported as a hemizona index (HZI) which is calculated as shown in Figure 4-1.

$$\text{Hemizona Index (HZI)} = \frac{\#\text{TBS of treatment sample}}{\#\text{TBS of control sample}} \times 100$$

Figure 4-1. Calculation of the hemizona index (HZI). TBS is the abbreviation used for tightly bound sperm.

Acrosomal Status

The percentage of acrosome-reacted spermatozoa was determined by using the fluorescent probe fluorescein isothiocyanate labelled *Pisum sativum* (FITC-PSA) lectin (EY Laboratories), following techniques previously described [58]. An epifluorescent microscope at 400X magnification was used to read the assay slides. At least 100 sperm were evaluated per slide. Acrosome-reacted status was diagnosed when a bar pattern was observed or no immunofluorescence was seen at all, indicating when a total loss of the acrosomal cap. Typically, acrosome-intact sperm exhibit staining of the entire acrosomal cap [58-67].

Statistical Analysis

Data is presented as mean ± standard error. Analysis of significance was performed using the Student's paired t-test with a significance of $p \leq 0.05$.

Results

The results of sperm processing using ISolate compared to both the standard swim-up method and to the Perwash sperm processing technique are presented in Tables 4-1 and 4-2, respectively. No difference was observed in the results of the motion analysis using ISolate compared to Perwash and to the standard swim-up. Also, there was no difference observed in hemizona binding between the ISolate and the Perwash

groups. However, a significant increase ($p \leq 0.05$) was observed in the number of sperm bound to the hemizona using the ISolate technique when compared to the standard swim-up method. The ISolate group exhibited a significantly lower percent of hyperactivation and percent of acrosome-reacted sperm when compared to the swim-up method, whereas, ISolate versus Perwash did not differ significantly. When comparing the three different sperm processing techniques to equatorial and midpiece/tail immunoreactivity, a significantly less amount of both ChAT and CaAT immunoreactivity was observed using the ISolate technique when compared to the standard swim-up method, whereas, the Perwash technique yielded results similar to those of the swim-up method. It should be noted that during the immunocytochemistry experiments with the ISolate separated sperm, a viscous covering seemed to be surrounding the individual sperm, therefore, the low immunoreactivity results obtained could be artifactual and not indicative of a direct effect on the sample.

Table 4-1 ISolate vs. Swim-up Results

	ISolate	Swim-up
sample #	4	4
% motility	91.0 ± 3.7	91.3 ± 3.7
VSL	47.1 ± 13.2	60.7 ± 4.6
VCL	71.4 ± 10.0	79.2 ± 7.5
ALH	3.6 ± 0.2	3.5 ± 0.2
BCF	18.2 ± 1.4	16.1 ± 1.0
LIN	60.3 ± 11.3	73.3 ± 1.9
% HA*	0.3 ± 0.4	3.8 ± 1.5
AR*	8.0 ± 4.2	15.8 ± 6.3
HZI*	64.7 ± 31.4	47.6 ± 27.7
% Equ*	28.5 ± 9.3	61.3 ± 7.2
% Mid/tail*	34.0 ± 6.7	64.8 ± 8.0

* $p \leq 0.05$

Table 4-2 ISolate vs. Perwash Results

	ISolate	Perwash
sample #	5	5
% motility	91.2 ± 7.6	91.2 ± 5.9
VSL	56.2 ± 7.1	52.3 ± 4.7
VCL	74.9 ± 11.1	73.5 ± 9.0
ALH	3.6 ± 0.6	3.7 ± 0.6
BCF	17.7 ± 0.8	18.1 ± 1.4
LIN	72.6 ± 4.8	68.9 ± 5.0
% HA	2.6 ± 2.4	2.0 ± 1.9
AR	15.2 ± 5.6	11.4 ± 4.0
HZI	35.0 ± 15.6	33.1 ± 15.0
% Equ*	33.2 ± 9.7	59.2 ± 12.4
% Mid/tail*	37.4 ± 6.7	68.0 ± 11.2

* $p \leq 0.05$

Discussion

Experiments were performed in order to evaluate the effects of separation techniques on sperm function in regards to any potential media based effects on ChAT and CaAT immunoreactivity under capacitating conditions. The ISolate group exhibited a significantly lower percent of hyperactivation and percent of acrosome-reacted sperm when compared to the swim-up method, whereas, ISolate versus Perwash did not differ significantly. Also, there was no difference observed in hemizona binding between the ISolate and the Perwash groups. However, a significant increase was observed in the number of sperm bound to the hemizona using the ISolate technique when compared to the standard swim-up method.

When comparing the three different sperm processing techniques to ChAT and CaAT immunoreactivity, a significant decrease in both ChAT and CaAT immunoreactivity was observed using the ISolate technique when compared to the standard swim-up method, whereas, the Perwash technique yielded results similar to those of the swim-up method. These findings were not surprising since many factors have been found to affect capacitation in vitro including temperature, variations between species, the method of collection, and medium composition [10]. In recent years, there has been rapid growth in the development of both media and techniques for improving sperm quality, however, these methods have not

always resulted in an increase in fertilizing capacity [33-35]. The ISolate group exhibited a significantly lower percent of hyperactivation when compared to the swim-up method, whereas, ISolate versus Perwash did not differ significantly. Although, no other differences were observed in the results of the motion analysis using ISolate compared to Perwash and to the standard swim-up, other features of capacitation may have been affected. Experiments have demonstrated that sperm motion analysis appears to not always accurately predict the true fertilizing potential of sperm and thus is not always a suitable method for accurate evaluation of medium or technique [33,36]. Therefore, this study has included other methods to evaluate the effects of separation techniques on sperm function in regards to any potential media based effects under capacitating conditions. Other methods employed in this study to assess the physiological endpoints of capacitation included the acrosome reaction and the hemizona assay. The ISolate group exhibited a significantly lower percent of acrosome-reacted sperm when compared to the swim-up method, whereas, ISolate versus Perwash did not differ significantly. It has been shown that the inability of sperm to undergo the acrosome reaction results in loss of their fertilizing potential [64]. However, it has also been shown that sperm without an intact acrosome can not interact with the zona pellucida [65,66]. Therefore, sperm losing their acrosome prematurely can not successfully fertilize the egg

[67]. So, although the acrosome reaction is part of the process of capacitation, it does not necessarily correlate with fertilizing potential [89,90].

The hemizona assay may be a more suitable method because it allows for evaluation of the effect of different sperm treatments on sperm binding. The hemizona assay has been shown to be of high predictability for fertilization outcome because the binding of the sperm to the zona pellucida is a prerequisite to both penetration of the zona or the oocyte and fertilization of the oocyte [33,37-39]. In this study, there was no difference observed in hemizona binding between the ISolate and the Perwash groups. However, a significant increase ($p \leq 0.05$) was observed in the number of sperm bound to the hemizona using the ISolate technique when compared to the standard swim-up method. Yogev et al. also attempted to evaluate some of the sperm preparation techniques by using the hemizona assay as the indicator of fertilizing ability [33]. They evaluated five different sperm preparation techniques and also used the swim-up technique as the reference method. In the end the group concluded that in vitro preparation methods can affect sperm binding capacity to the zona pellucida [33]. However, it was noted that during the immunocytochemistry experiments with the ISolate separated sperm, a viscous covering surrounded the sperm, therefore, the low immunoreactivity results obtained could be artifactual and not indicative of a direct effect on the sample. An additional

pitfall to this study is donor variability. Donor specimens vary considerably both in comparison to other donors and with comparison of the same donor samples collected on different days. Procedures were followed to try to minimize this effect by using proven fertile donors and by using specimens collected on the same day for the various experiments when possible.

In conclusion, it was hoped that this study would substantiate the hypothesis discussed in Chapter III involving a role for choline acetyltransferase in the binding process between the oocyte and the sperm, however, the increased hemizona binding exhibited by the ISolate processed sperm in this study in comparison to the standard swim-up was accompanied by a decrease in both equatorial and midpiece/tail binding. Therefore, it may be possible that the viscous covering surrounded the sperm interfered with all anti-ChAT immunoreactivity since Chapter III also lead to the conclusion that carnitine acetyltransferase is involved in sperm motility, but not directly involved in the actual binding of sperm to the oocyte. Chapter V will explore the hypothesis involving a role for choline acetyltransferase in the binding process between the oocyte and the sperm further by examine fertility aspects by comparison of spermatozoa obtained from proven fertile males to spermatozoa incapable of attaining the physiological indicators of completed capacitation.

CHAPTER VI

Fertility Aspects Associated with Choline Acetyltransferase and Carnitine Acetyltransferase Activity in Human Spermatozoa

Introduction

Acetylcholine, a neurotransmitter produced by the enzyme choline acetyltransferase (ChAT), has been suggested to play a role in many nonneuronal tissues, including spermatozoa [1]. Earlier studies have indicated that human spermatozoa contain the four components of the cholinergic system including acetylcholine and choline acetyltransferase [2]. Recent studies have indicated choline acetyltransferase mRNA is expressed in spermatogenic cells and the presence of choline acetyltransferase immunoreactivity is detected on sperm. A potential role for acetylcholine during fertilization would suggest a concomitant role for the enzyme involved in its production. In 1981, it was discovered that previous experiments involving choline acetyltransferase activity in spermatozoa could have actually been mistaken for carnitine acetyltransferase activity [52,53]. Carnitine acetyltransferase is capable of catalyzing the acetyl group transfer from acetyl CoA to choline, the exact same reaction occurring in choline acetyltransferase [41]. Also, carnitine acetyltransferase has been shown to be present in high amounts in both rat epididymal tissues and spermatozoa [8,54]. Most

research involving choline acetyltransferase in spermatozoa subsided until 1991 when Ibanez et al. demonstrated that choline acetyltransferase mRNA is expressed in both rat and human spermatogenic cells. High levels of two choline acetyltransferase transcripts were detected by Northern blot analysis of adult rat testis RNA. The translated products result in an accumulation of choline acetyltransferase in the postacrosomal region of mature sperm. Only one choline acetyltransferase mRNA species was detected in human testis. In situ hybridization was used to localize the cells within the seminiferous epithelium responsible for choline acetyltransferase mRNA synthesis in rat testis. Choline acetyltransferase-like immunoreactivity in ejaculated human spermatozoa demonstrated that translation of the mRNA detected in testis did occur [8].

Data presented in Chapter III substantiated the ChAT-like immunoreactivity observed by the Ibanez group and suggested that CaAT plays a role in sperm motility. The objective of this study was to examine fertility aspects associated with ChAT and CaAT activity by comparison of spermatozoa obtained from proven fertile males to spermatozoa incapable of attaining the physiological indicators of completed capacitation. By examining the fertility aspects associated with ChAT and CaAT activity by comparison of spermatozoa obtained from proven fertile males to spermatozoa incapable of attaining the physiological indicators of completed

capacitation, a correlation can be made between fertility and the amount of ChAT and CaAT immunoreactivity.

Materials and methods

Subjects

A total of 20 men participating in the IVF program comprised this study group. All 20 men were diagnosed with male factor infertility and undergoing Intracytoplasmic Sperm Injection (ICSI). Semen samples from fertile donors were utilized as controls in the different experiments if ejaculates had an original sperm concentration >60 X 10^6/ml, progressive motility >60%, and normal morphology of >14%.

Semen analysis and preparation

Semen specimens were obtained by masturbation after two days of sexual abstinence. After liquefaction of the sample, a swim-up separation of the sperm motile fraction was performed. The semen samples were mixed with Ham's F-10 medium supplemented with 0.3% human serum albumin (Irvine Scientific) and washed twice by centrifugation at 400 x g. After centrifugation, the motile fraction of the sperm was collected following a one hour swim-up at 37°C and 5% CO_2 in water-saturated air. Following sperm processing, motion characteristics (% motility, path velocity (VAP), curvilinear velocity (VCL), straightline velocity (VSL), amplitude of

lateral head displacement (ALH), linearity (LIN), and beat cross frequency (BCF)) of the motile sperm fraction was determined for each sample via computer-assisted motion analysis with the HTM-IVOS motility analyzer (Hamilton-Thorn Research). Slides were prepared following swim-up for sperm morphology determination.

Hyperactivated motility

Motion characteristics were measured objectively with the HTM-IVOS motility analyzer using previously established fixed parameter settings [91, 92]. To differentiate between hyperactivated and non-hyperactivated sperm, the following settings were used in the automatic SORT program for hyperactivity: number of points in each sperm track: 5 to 30, path velocity (VAP): 40.0 to 500.0 μm/s, progressive velocity (VSL): 20.0 to 500.0 μm/s, track speed (VCL): 100.0 to 500.0 μm/s, linearity (LIN): 0.0 to 65.0 μm/s, and lateral displacement (ALH): 7.5 to 50.0 μm/s.

Hemizona Assay

The tight binding of the sperm samples to human zona pellucida was assessed using the Hemizona Assay as previously described [68,69]. Briefly, human immature (prophase I) oocytes were removed from salt storage and rinsed in Ham's F-10 medium supplemented with 0.1% bovine serum albumin (Sigma Chemical Company) before microbisection. Narishige

micromanipulators mounted on a phase-contrast inverted Nikon Diaphot microscope were used for cutting the oocytes into matching hemizonae. In each experimental set, patient and donor sperm droplets containing 1.5 million motile sperm per microliter were incubated with matching hemizona for four hours at 37°C and 5% CO_2 in water-saturated air. Following the incubation period, the hemizonae were rinsed in Ham's F-10 medium supplemented with 0.5% human serum albumin (Irvine Scientific) using a narrow glass pipette in order to remove loosely attached sperm. Next, the number of sperm tightly bound to the outer convex surface of each hemizona was counted on a phase-contrast microscope at 200X magnification. The Hemizona Assay results are reported as a hemizona index (HZI) which is calculated as shown in Figure 5-1.

$$\text{Hemizona Index (HZI)} = \frac{\#\text{TBS of patient sample}}{\#\text{TBS of donor sample}} \times 100$$

Figure 5-1. Calculation of the hemizona index (HZI). TBS is the abbreviation used for tightly bound sperm.

Determination of Immunoreactivity Distribution on Sperm

Immunocytochemistry was performed on ejaculated sperm samples obtained from both the ICSI patient group and the proven fertile donor group. Following the swim-up procedure, sperm samples were air-dried onto slides and methanol fixed. After blocking of nonspecific sites with SuperBlock (Pierce), immunostaining was performed with a monoclonal antibody to human choline acetyltransferase from mouse-mouse hybrid cells (Boehringer Mannheim). A fluorescein isothiocyanate conjugated secondary antibody (Zymed Laboratories) was used to visualize immunoreactivity under epifluorescent microscopy. Incubation with an unrelated monoclonal mouse antihuman primary antibody (Zymed Laboratories) was performed as an additional control.

Acrosome reaction

The percentage of acrosome-reacted spermatozoa was determined by using the fluorescent probe fluorescein isothiocyanate labelled *Pisum sativum* (FITC-PSA) lectin (EY Laboratories), following techniques previously described [58]. An epifluorescent microscope at 400X magnification was used to read the assay slides. At least 100 sperm were evaluated per slide. Acrosome-reacted status was diagnosed when a bar pattern was observed or no immunofluorescence was seen at all, indicating when a total loss of the acrosomal cap. Typically,

acrosome-intact sperm exhibit staining of the entire acrosomal cap [58-67].

Statistical Analysis

The percent of both equatorial and midpiece/tail binding were correlated with each motion characteristic (% motility, path velocity (VAP), curvilinear velocity (VCL), straightline velocity (VSL), amplitude of lateral head displacement (ALH), linearity (LIN), and beat cross frequency (BCF)), morphology, concentration, the fertilization rate, the pregnancy rate, the hemizona index, and the percent acrosome-reacted using the Pearson Correlation Coefficient Analysis or the Student's t-test with a p value of ≤ 0.05 considered significant. Data is presented as mean ± standard error.

Results

Sperm motion parameters were obtained after swim-up from 9 of the original 20 male factor study subjects. The mean parameters were path velocity: 61.7 ± 16.1 μm/s, curvilinear velocity: 91.4 ± 26.6 μm/s, straightline velocity: 52.4 ± 14.2μm/s, amplitude of lateral head displacement: 6.4 ± 2.2μm/s, linearity: 60.6 ± 9.1 μm/s, beat cross frequency: 12.2 ± 1.9 μm/s, and hyperactivity: 22.8 ± 5.6%. The remaining values were obtained for all 20 male factor study subjects: normal morphology: 5.5 ± 3.4%, original motility:

40.2 ± 20.5%, swim-up motility: 82.6 ± 16.4%, original concentration: 81.6 ± 82.0 X 10^6/ml, swim-up concentration: 26.7 ± 22.4 X 10^6/ml, fertilization rate: 72.9 ± 19.3%, Hemizona Index: 84.9 ± 68.9%, acrosome-reacted: 4.7 ± 1.8%, ChAT binding 36.9 ± 24.1%, and CaAT binding 34.4 ± 10.0%.

Equatorial Binding

A significant positive correlation ($p < 0.05$) was observed between equatorial binding and the hemizona index. The means ± standard error, the Pearson correlation coefficient, and the level of significance between equatorial binding and each motion characteristic are shown in Table 5-1, equatorial binding versus all other parameters are shown in Table 5-2. The correlation plot for HZI and equatorial binding is shown in Figure 5-2. Equatorial binding did not correlate with any other sperm parameter obtained.

Midpiece and Tail Binding

A significant positive correlation ($p < 0.05$) was observed between midpiece/tail binding and original concentration, VAP, VSL, VCL, and both original and swim-up motility. The means ± standard error, the Pearson correlation coefficient, and the level of significance between midpiece/tail binding and each motion characteristic are shown in Table 5-1, midpiece/tail binding versus all other parameters are shown in Table 5-2. Correlation plots for

Table 5-1 Equatorial and Midpiece/Tail Immunoreactivity Versus Sperm Motion Parameters

		Equatorial Segment		Midpiece /Tail	
n = 9	Mean ± SE	r value	p value	r value	p value
VAP	61.7 ± 16.1	-0.292	0.446	0.709	0.032*
VSL	52.4 ± 14.2	-0.379	0.314	0:668	0.049*
VCL	91.4 ± 26.6	-0.205	0.597	0.667	0.050*
ALH	6.4 ± 2.2	-0.110	0.777	0.540	0.133
LIN	60.6 ± 9.1	-0.175	0.652	-0.030	0.940
% HA	22.8 ± 15.6	0.049	0.901	0.362	0.338
%ChAT	36.7 ± 24.3				
%CaAT	39.3 ± 7.8				

* $p \leq 0.05$

Table 5-2 Equatorial and Midpiece/Tail Immunoreactivity Versus Fertility Parameters

		Equatorial Segment		Midpiece /Tail	
n = 20	Mean ± SE	r value	p value	r value	p value
Morphology	5.5 ± 3.4	0.003	0.989	0.253	0.510
Motility %	40.2 ± 20.5	-0.217	0.357	0.873	0.002*
SU motility	82.6 ± 16.4	0.028	0.905	0.811	0.008*
Conc m/ml	81.6 ± 82.0	-0.002	0.992	0.716	0.030*
SU conc m/ml	26.7 ± 22.4	0.024	0.919	-0.172	0.657
% fertilizat	72.9 ± 19.3	-0.033	0.890	0.171	0.661
HZI	84.9 ± 68.9	0.818	0.004*	-0.175	0.653
%ChAT	36.9 ± 24.1				
%CaAT	34.4 ± 10.0				

* $p \leq 0.05$

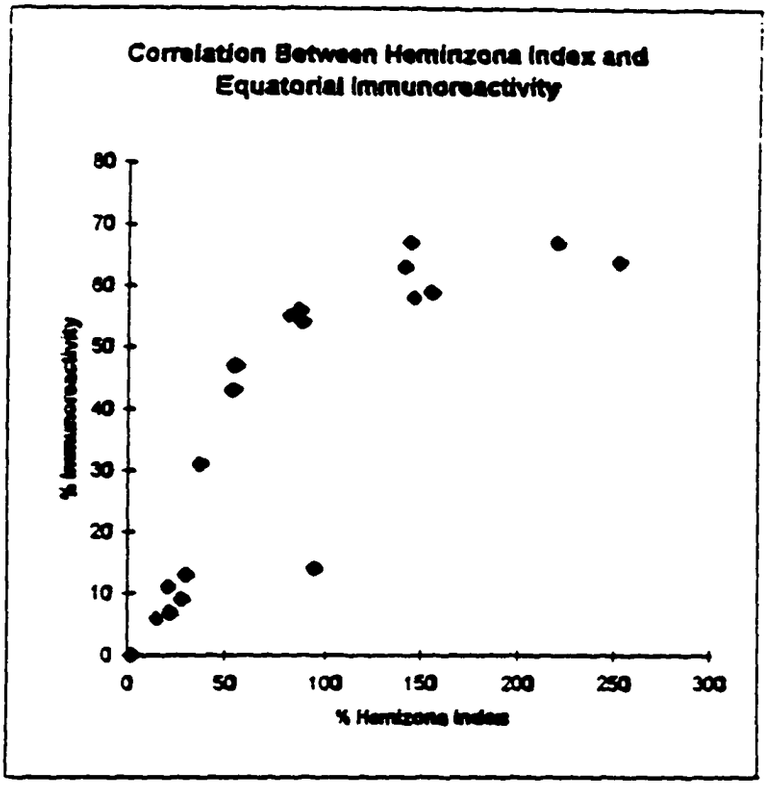


Figure 5-2. Correlation between Hemizona Index and equatorial percent immunoreactivity. Correlation was assessed by immunocytochemistry using an anti-ChAT antibody with a fluorescein isothiocyanate stain (FITC) in 20 male factor patients undergoing ICSI. The correlation coefficient was 0.818 ($p \leq 0.05$).

original and swim-up motility with midpiece/tail binding are shown in Figures 5-3 and 5-4, respectively. Correlation plots for VAP, VSL, and VCL with midpiece/tail binding are shown in Figures 5-5, 5-6, and 5-7, respectively. Midpiece/tail binding did not correlate with any other sperm parameter examined.

Discussion

The results of this study demonstrate a significant positive correlation between ChAT binding and the Hemizona Assay Index. The Hemizona Assay has been demonstrated to be a reliable test to identify patients at risk for poor or failed in vitro fertilization [93,94]. Also, the hemizona assay has been shown to be of high predictability for fertilization outcome because the binding of the sperm to the zona pellucida is a prerequisite to both penetration of the zona or the oocyte and fertilization of the oocyte [33,37-39]. Therefore, it may be that ChAT is involved in the sperm and zona pellucida binding process based on the association between equatorial segment binding and the Hemizona Assay and findings presented in Chapter III. However, a correlation was not observed between equatorial binding and the acrosome reaction, a physiological endpoint of capacitation [10]. It may be that ChAT is uncovered prior to the acrosome reaction to possibly participate in the primary binding between the zona pellucida and the sperm. Many changes are associated with the acrosome reaction and capacitation. During

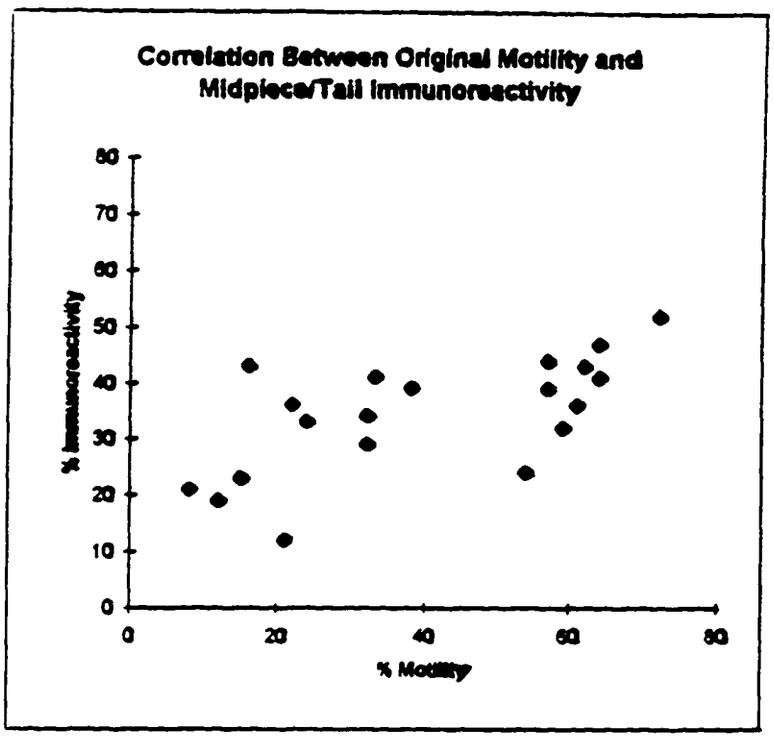


Figure 5-3. Correlation between original sperm percent motility and percent midpiece/tail immunoreactivity. Correlation was assessed by immunocytochemistry using an anti-ChAT antibody with a fluorescein isothiocyanate stain (FITC) in 20 male factor patients undergoing ICSI. The correlation coefficient was 0.873 ($p \leq 0.05$).

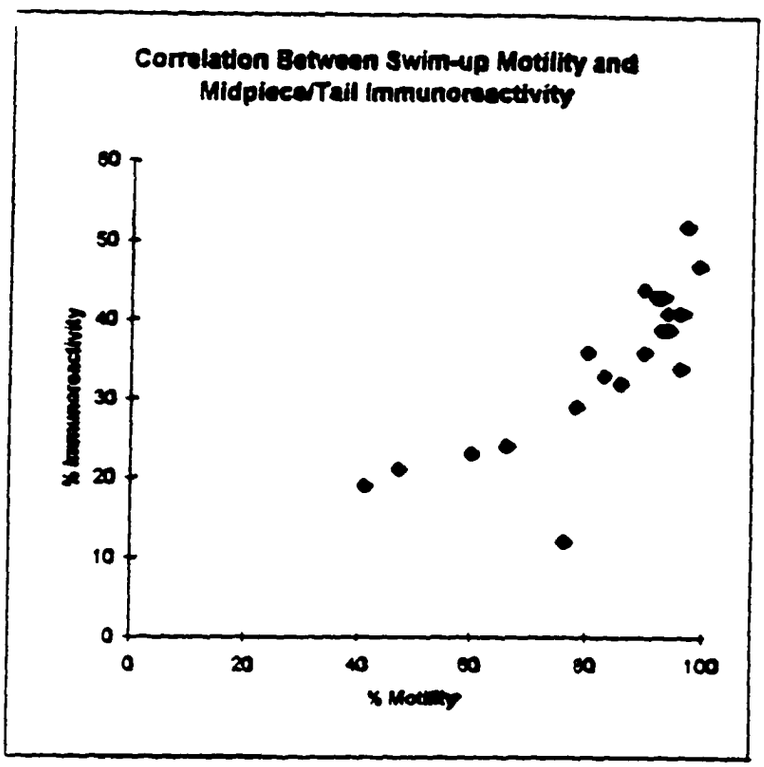


Figure 5-4. Correlation between post swim-up sperm motility percent and percent midpiece/tail immunoreactivity. Correlation was assessed by immunocytochemistry using an anti-ChAT antibody with a fluorescein isothiocyanate stain (FITC) in 20 male factor patients undergoing ICSI. The correlation coefficient was 0.811 ($p \leq 0.05$).

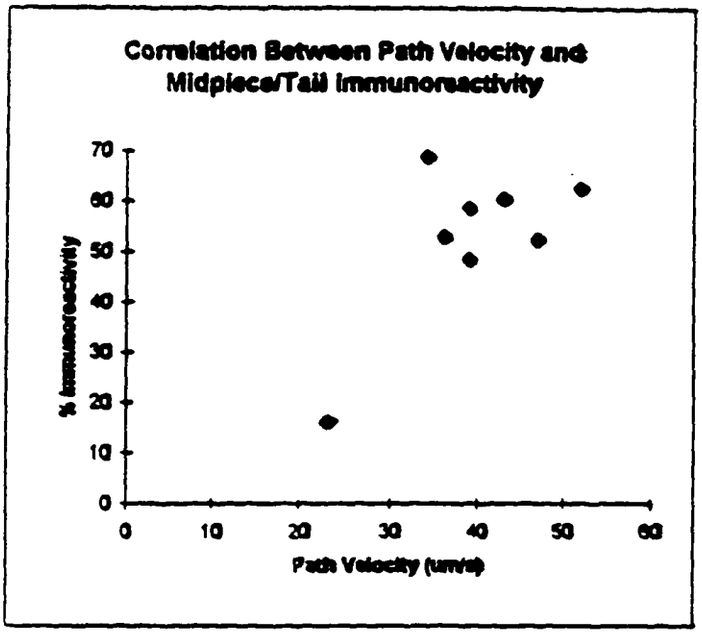


Figure 5-5. Correlation between sperm path velocity (μm/s) and percent midpiece/tail immunoreactivity. Correlation was assessed by immunocytochemistry using an anti-ChAT antibody with a fluorescein isothiocyanate stain (FITC) in 9 male factor patients undergoing ICSI. The correlation coefficient was 0.709 ($p \leq 0.05$).

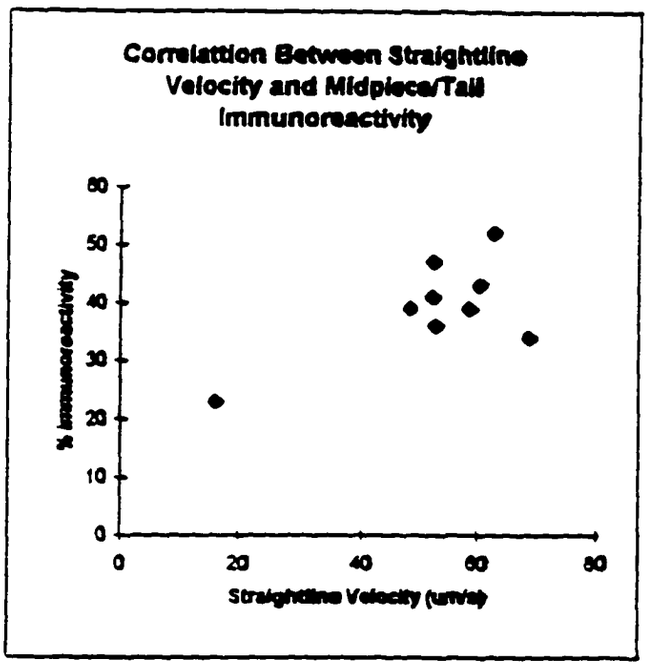


Figure 5-6. Correlation between straightline velocity (μm/s) and percent midpiece/tail immunoreactivity. Correlation was assessed by immunocytochemistry using an anti-ChAT antibody with a fluorescein isothiocyanate stain (FITC) in 9 male factor patients undergoing ICSI. The correlation coefficient was 0.668 ($p \leq 0.05$).

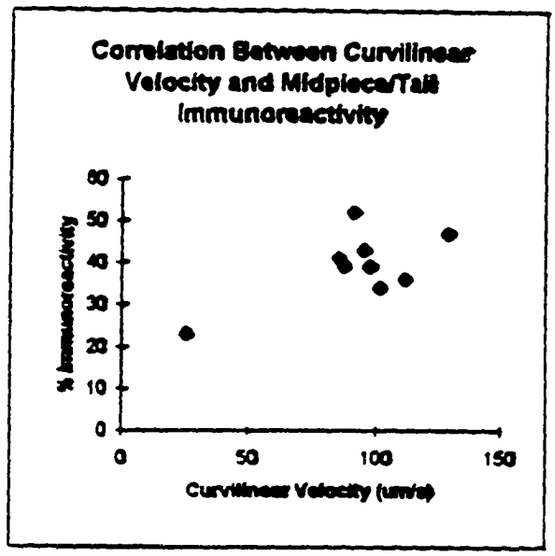


Figure 5-7. Correlation between curvilinear velocity (μm/s) and percent midpiece/tail immunoreactivity. Correlation was assessed by immunocytochemistry using an anti-ChAT antibody with a fluorescein isothiocyanate stain (FITC) in 9 male factor patients undergoing ICSI. The correlation coefficient was 0.667 ($p \leq 0.05$).

capacitation, the sperm plasma membrane becomes more fluid because of the removal of decapacitation factors and a loss of cholesterol. The changing plasma membrane enables the zona receptors to shift around freely within the plane of the membrane allowing for an aggregation and subsequent activation of the receptors, which stimulates protein tyrosine kinase activity to phosphorylate proteins involved in the acrosome reaction [10,29].

Furthermore, it can be concluded that CaAT is involved in sperm motility and locomotion due to the findings that a significant positive correlation existed between CaAT binding and original sperm concentration, VAP, VSL, VCL, and both original and swim-up motility. Although sperm concentration is not a characteristic of motility, it is indicative of the general quality of the sample since studies have shown a correlation between sperm concentration and fertility [82].

CHAPTER VII

DISCUSSION AND CONCLUSIONS

Male-factor infertility remains one of the most challenging obstacles in the road to successful reproduction despite the many advances in reproductive medicine. Due to the high occurrence of failed fertilization associated with in vitro fertilization, attention has been directed on procedures that evaluate sperm function at the level of fertilization [95,96]. This study aimed to show that the regional distribution of ChAT activity in human spermatozoa is altered during in vitro capacitation and correlates with fertilizing potential of sperm; and, as such this capacitation dependent distribution will serve as a valuable biochemical marker of capacitation.

The first specific aim of this study was to compare regional immunoreactivity in human spermatozoa as assessed by fluorescent immunocytochemistry with ChAT and CaAT activity determined by enzymatic methodologies. The experiments conducted in Chapter III, Section I showed increasing proportions of sperm exhibited ChAT immunoreactivity along the equatorial region with a concomitant decrease in ChAT reactivity in the midpiece. Also, competitive studies with unlabeled ChAT blocked the equatorial region labeling; the unlabeled CaAT blocked staining along the midpiece region of the tail, suggesting some cross-reactivity of the ChAT

antiserum with the CaAT enzyme. These findings suggested that ChAT could possibly be involved in the spermatozoa and zona pellucida binding phenomena and that CaAT may be involved in motility due to its reactivity in the midpiece area of the tail. A ChAT radioassay was used to compare regional immunoreactivity in human spermatozoa as assessed by fluorescent immunocytochemistry with ChAT and CaAT activity determined by enzymatic methodologies. Although a direct comparison was not made between the ChAT radioassay and the equatorial and midpiece/tail ChAT immunoreactivity, the two procedures appeared to not correlate. However, based on findings from the ChAT radioassay study, it was concluded that the ChAT enzyme is present within the sperm and not synthesized de novo.

The second aim of this study was to correlate the regional distribution of ChAT and CaAT immunoreactivity under sperm capacitating conditions with the physiological indicators of completed capacitation, including the physiological and pharmacological induction of the acrosome reaction. Several physiological and pharmacological stimulators were added to induce the acrosome reaction. A significant increase in both equatorial and midpiece/tail immunoreactivity was observed when using both caffeine and pentoxifylline. Since caffeine and pentoxifylline are both phosphodiesterase inhibitors, which prevent the breakdown of cAMP into 5-AMP to result in an increase of endogenous cAMP,

it appears that ChAT and CaAT may be involved to some extent with the cAMP system [37,71]. However, there are many other mechanisms that could be involved in this process, so further studies should be done before any definite conclusions can be drawn. Progesterone, caffeine, and calcium ionophore A23187 all significantly increased the percent of acrosome-reacted sperm, as also previously reported [84-88]. Thapsigargin and pentoxifylline did not significantly effect acrosomal status.

The addition of caffeine significantly increases equatorial binding, midpiece/tail binding, and the acrosome reaction rate in both washed and post swim-up samples. Although a direct correlation between equatorial binding and acrosome reaction rate was not made, both increased with the addition of caffeine. These findings were explored further in Chapter V with an attempt to directly correlate acrosomal status with anti-ChAT immunoreactivity. However, a correlation was not observed between equatorial binding and the acrosome reaction. It may be that ChAT is uncovered prior to the acrosome reaction to possibly participate in the primary binding between the zona pellucida and the sperm.

Finally, the third aim of this study was to correlate the potential fertility of human spermatozoa with ChAT and CaAT immunoreactivity by comparison of spermatozoa obtained from proven fertile males to spermatozoa incapable of attaining the physiological indicators of completed capacitation. The results of this study demonstrated a significant positive

correlation between ChAT binding and the Hemizona Assay Index. The hemizona assay has been shown to be of high predictability for fertilization outcome because the binding of the sperm to the zona pellucida is a prerequisite to both penetration of the zona or the oocyte and fertilization of the oocyte [33,37-39]. Therefore, it can also be concluded that ChAT is involved in the sperm and zona pellucida binding process based on the association between equatorial segment binding and the Hemizona Assay and findings presented in Chapter III. Furthermore, it can be concluded that CaAT is involved in sperm motility and locomotion due to the findings that a significant positive correlation existed between CaAT binding and original sperm concentration, VAP, VSL, VCL, and both original and swim-up motility.

This study showed that the regional distribution of ChAT activity in human spermatozoa is altered during in vitro capacitation and it correlates with the fertilizing potential of sperm. Possibly in the future, this capacitation dependent distribution will serve as a valuable biochemical marker of capacitation. However, more extensive studies are necessary before such a goal can be achieved. Possible studies for the future could include experiments involving sperm at different degrees of capacitation, using solubilized zona pellucida to induce the acrosome reaction, and studying the ChAT and CaAT enzyme activity in sperm incapable of attaining the physiological endpoints of capacitation.

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APPENDIX

Human Subjects

This dissertation used data produced from human semen samples falling under exception number V of the regulations for Protection of Human Subjects 45 CFR 46. A copy of the regulations can be obtained from the Office for Protection from Research Risks, National Institute for Health, Bethesda, Maryland, 20892. The Andrology Laboratory of the Jones Institute for Women's Health, Eastern Virginia Medical School, provided all patient and donor semen samples following the obtainment of consent and Eastern Virginia Medical School Institutional Review Board approval. Patient semen samples consisted of the remaining specimen following all physician requested procedures. Donor semen samples were obtained from consenting donors. All samples were assigned codes to maintain confidentiality.

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