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Acrosomal molecules exposure during human sperm capacitation

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Acrosomal molecules exposure during human sperm capacitation

Theodora Fouriki

MSc by Research
University of Dundee
January 2013

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List of abbreviations

ALH: Amplitude of lateral head displacement

ANOVA: Analysis of variance

AR: Acrosome reaction

ART: Assisted reproductive technology

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CASA: Computer-assisted sperm analysis/analyser

CK: creatinine phosphokinase

CM: Capacitating medium

CRES: Cystatin related epididymal spermatogenic

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: dithiothreitol

GalT1: β - 1,4- galactosyltransferase

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HFEA: Human fertilisation and embryology authority

IVF: *In vitro* fertilisation

NCM: Non-capacitating medium

PKA: Protein kinase A

PSA-FITC: fluorescein isothiocyanate-Pisum sativum agglutinin

ROS: Reactive oxygen species

sp32/ACRBP: proacrosin binding protein

sAC: Soluble adenylate cyclase

SEM: Standard error of the mean

STR: Straightness

TBS: Tris Buffered Saline

VAP: Average path velocity

VCL: Curvilinear path velocity

VSL: Straight line velocity

WHO: World Health Organisation

Zan: Zonadhesin

ZP: Zona pellucida

ZP3R: Zona pellucida receptor

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Declaration

All data was gathered by me alone, and I am the sole author of the text. I have personally consulted all references and I have not submitted this thesis previously for any other degree.

Theodora Fouriki

Summary

At ejaculation sperm cells cannot fertilize. They need to undergo a series of molecular modifications to achieve competency. Surprisingly, molecular markers for identifying sperm populations competent to fertilize are not robust. However, recently an intra-acrosomal protein exposed at the sperm-surface (zonadhesin) has been associated in mouse with the sperm population ready to fertilize (capacitated). Zonadhesin is a sperm protein involved in sperm-ZP adhesion and is located in the acrosome, but is accessible after sperm undergo capacitation. When the sperm population is undergoing capacitation, the number of cells displaying zonadhesin is significantly increased potentially reflecting their fertilizing capacity. The objective of this study is to evaluate this event on human spermatozoa. Apart from zonadhesin, two other acrosomal molecules with a role in sperm-egg interaction, sp56 and sp32, were also studied for potential sperm surface exposure during capacitation.

The first aim was to establish the optimum time of incubation under capacitating conditions for sperm capacitation *in vitro*. Additional aims of this project were to detect the acrosomal proteins' forms present in the mature human spermatozoa incubated under capacitating and non-capacitating conditions and compare these protein forms of zonadhesin to those observed in samples from sub fertile men providing preliminary data on their clinical relevance. The study of acrosomal protein exposure at the cell surface by immunofluorescence demonstrated that all three acrosomal molecules were accessible at the sperm surface of cells incubated under capacitating conditions but not of cells incubated under non-capacitating conditions and therefore this event was associated to sperm capacitation.

CHAPTER 1

General Introduction

1.1 Sperm fertilizing ability

Fertilization is defined as the union of an oocyte and a spermatozoon, occurring in the ampulla of the uterine tube, leading to the production of a zygote and initiating prenatal development. However, freshly ejaculated spermatozoa are not capable of fertilizing an egg. It has been known for over 60 years that when mammalian spermatozoa are released from the male reproductive apparatus they are unable to fertilize oocytes. To acquire the ability to fertilize, sperm must undergo a process known as capacitation. Austin (1951) and Chang (1951) discovered capacitation independently when they got a high rate of successfully fertilized rabbit eggs after the inseminated the spermatozoa in the oviduct several hours prior to ovulation rather than close to ovulation. The term capacitation refers to a series of time dependent physiological and molecular changes that occur in sperm cells in order to obtain competency to fertilize and it requires residence of the cells in the female reproductive tract (Chang, 1951, Austin, 1951).

The “switching on” effect of capacitation applies to both the head (capacitated spermatozoa can undergo acrosome reaction) and the tail (spermatozoa can develop hyperactivated motility) (Yanagimachi, 1994a, de Lamirande et al., 1997) and is considered to comprise a series of biochemical and physiological changes in mammals, including human, such as cholesterol removal from the membrane, increase of HCO_3^- levels and transmembrane movements, increase of intracellular cAMP, increase of intracellular Ca^{2+} , hyperpolarization of the sperm plasma membrane and increase of protein tyrosine phosphorylation (Carr and Acott, 1989, Baldi et al., 1996, Visconti et al., 1995b, Visconti et al., 2002, De Jonge, 2005).

Capacitation is also correlated to motility pattern necessary for moving within the female tract and penetration of zona pellucida (ZP), designated as hyperactivation.

Hyperactivation has been correlated to sperm capacitation in mammalian species (Suarez, 1996) such as rat (Shalgi and Phillips, 1988), guinea pig (Katz et al., 1978), and human (Mortimer and Mortimer, 1990). The swimming pattern of hyperactivated spermatozoa is characterized by high amplitude flagellar beating and a non-linear sperm track (Mortimer, 1997). This vigorous movement is necessary for sperm progression towards the egg. Hyperactivation helps the sperm cells to detach from the epithelial cells in the oviduct where they are held in the oviduct isthmic reservoir in both animals and humans (Suarez et al., 1991, Pacey et al., 1995, Baillie et al., 1997, Mortimer, 1997, Gwathmey et al., 2003, Hung and Suarez, 2012). Hyperactivation *in vivo* has been suggested to promote the sperm movement through the viscous mucus secreted in the oviduct lumen studied in boar, hamster and mouse sperm (Suarez and Dai, 1992, Quill et al., 2003) as well as to facilitate the ZP penetration studied in mouse, hamster and stallion (Stauss et al., 1995) (Quill et al., 2003) (McPartlin et al., 2009)

In vitro sperm capacitation-culture media

Capacitation can also be accomplished under *in vitro* conditions after incubation in various culture media that mimic the environment of the female reproductive tract. However, even if these media contain components in physiological concentrations that are found in the female tract they can only approximate the *in vivo* conditions. If they fail to maintain the spermatozoa in a state of readiness to fertilize, the result is the spontaneous acrosome reaction (AR) of some cells. The non-physiological AR

depends on several factors including species and the capacitation medium. Spontaneous AR has been reported in human (DasGupta et al., 1994) and animal species such as pig (Adeoya-Osiguwa and Fraser, 2002).

Although different capacitating media are used for *in vitro* incubation in different species there are common components that are required for sperm capacitation and successful fertilisation in all studied species, such as bicarbonate (HCO_3^-), serum albumin and calcium (Ca^{2+}) (Yanagimachi, 1994b, de Lamirande et al., 1997) (Yanagimachi, 1994a). Serum albumin has been suggested to have a role during *in vitro* capacitation as a cholesterol acceptor in order to remove membrane cholesterol (Go and Wolf, 1985, Cross, 1998). Loss of membrane cholesterol is thought to result in remodeling of the plasma membrane by fluidity changes observed during capacitation (Wolf et al., 1986, Flesch et al., 2001, de Vries et al., 2003). It has been demonstrated in a number of species, including human, that incubation with albumin favors cholesterol efflux and capacitation (Ravnik et al., 1990). It is not clear whether cholesterol removal is the only function of serum albumin (Espinosa et al., 2000) but its role as a cholesterol acceptor is essential for sperm capacitation *in vitro*, as it has been shown in the mouse that cholesterol addition in the incubation medium inhibits capacitation (Visconti et al., 1999b). Data showing that serum albumin can be substituted by other cholesterol-binding proteins to induce capacitation, such as high density lipoproteins and β -cyclodextrin (Therien et al., 1997, Choi and Toyoda, 1998, Cross, 1999, Visconti et al., 1999a, Osheroff et al., 1999), suggest that possibly the basic role of albumin in capacitation *in vitro* is cholesterol efflux ; an event that is upstream of signaling events associated with sperm capacitation.

Several studies have demonstrated that capacitation is Ca^{2+} -dependent. In mouse sperm extracellular Ca^{2+} is required for capacitation (DasGupta et al., 1993, Visconti

et al., 1995a). *In vitro* experiments in humans have also established culture media requirements for Ca^{2+} concentrations that are supportive of specific sperm functions, including capacitation (Marin-Briggiler et al., 2003). The calcium requirement in the capacitating media is possibly related to the stimulation of adenylyl cyclase activity (Kamenetsky et al., 2006), enzymes necessary in elevation of cAMP levels.

Bicarbonate has also been found to be essential in sperm capacitation *in vitro* and its presence in incubation media is particularly significant, in both animals and humans (Shi and Roldan, 1995, Boatman and Robbins, 1991, Bedu-Addo et al., 2005). The transmembrane movement of HCO_3^- has been associated with the increase in intracellular pH (pHi) observed during capacitation (Parrish et al., 1989, Zeng et al., 1996) and most importantly, it is strongly suggested that HCO_3^- role in the cAMP/PKA signaling pathway through sAC activation is necessary for tyrosine phosphorylation events and the regulation of capacitation (as discussed below) (Leclerc et al., 1996, Visconti et al., 2002)

Measurement of in vitro capacitation

So far there is no ideal and direct method to assess human sperm capacitation *in vitro*. The only direct method is IVF, which is a useful tool for accessing animal sperm capacitation but in humans this is not possible due to ethical and legal issues. Therefore, most commonly used protocols for the assessment of sperm capacitation are based on the induction of AR of capacitated spermatozoa by a biological or non-biological agonist. The prevailing belief has been that physiological inducer of AR in capacitated cells is the ZP. However, solubilized ZP is not easily available and therefore non-physiological inducers are widely used, for both animal and human

studies, such as calcium ionophore A23187 (Aitken et al., 1993, Liu and Baker, 1998). However the concentration used, as well as the time of incubation, should be chosen carefully as it is toxic in high concentrations. Progesterone can also induce the (AR) in capacitated sperm (Cross, 1996), however, data showing that progesterone can probably capacitate human spermatozoa render it as a less efficient tool in sperm capacitation measurement. (Foresta et al., 1992, Emiliozzi et al., 1996)

Regulation of capacitation through the HCO_3^- /sAC/cAMP/PKA pathway

The process of capacitation is comprised of early and late events, both of them associated with the HCO_3^- /sAC/cAMP/PKA signaling pathway in animals and humans (Salicioni et al., 2007, Visconti, 2009, Lee and Storey, 1986, Boatman and Robbins, 1991, Nolan et al., 2004, Esposito et al., 2004).

As an early event in the initiation of sperm capacitation, a rapid lipid collapse occurs. This collapse becomes detectable after 2 min. of incubation with 15 mM bicarbonate in boar (Gadella and Harrison, 2000). This event has not only been observed in animals, such as boar, and stallion (Rathi et al., 2003), but in human spermatozoa (de Vries et al., 2003).

The early lipid collapse is necessary for the following signal transduction events, such as tyrosine phosphorylation, by facilitating albumin-mediated cholesterol removal from the membrane (Flesch et al., 2001);(Gadella and Harrison, 2000). Protein phosphorylation refers to the addition of a phosphate group to a protein, either to serine, threonine or tyrosine residues by protein kinases. Although both serine/threonine phosphorylation occur in sperm cells, tyrosine phosphorylation

appears to be the major type of phosphorylation in signal transduction during capacitation (Naz and Rajesh, 2004).

Tyrosine phosphorylation of various proteins has been correlated to sperm capacitation in a number of species: mouse (Visconti et al., 1995a), hamster (Devi et al., 1999) bovine (Galantino-Homer et al., 1997), pig (Tardif et al., 1999, Kalab et al., 1998) and human (Leclerc et al., 1996, Luconi et al., 1996). It has been observed that, during capacitation, most of tyrosine phosphorylation is localized to the tail of both mouse (Urner et al., 2001) and human sperm (Mitchell et al., 2008, Liu et al., 2006). Even though most tyrosine phosphorylated proteins are located in the tail so that sperm can accomplish hyperactivation (Naz et al., 1991, Nassar et al., 1999), it has been reported in mouse that binding to the ZP stimulates phosphorylation of proteins located in the principal and mid-piece regions (Urner et al., 2001). Mid-piece phosphorylation has also been observed in human cells (Leclerc et al., 1997). Interestingly, a human study revealed that the capacitating conditions and zona exposure increases the degree of tyrosine phosphorylation in the sperm acrosome (Naz et al., 1991). A shift was also observed in the presence of phosphotyrosine content from the tail regions of non-capacitated sperm to the acrosome of capacitated/zona-exposed sperm cells. Moreover, under capacitating conditions *in vitro*, a time dependent increase of the sperm head phosphotyrosine content has been reported, but not in other regions of the cells (Nixon et al., 2010). Taking under consideration that the acrosomal contents participate in sperm-egg interaction, these data could indicate a role of tyrosine phosphorylation in the regulation of this process (Naz et al., 1991).

A significant increase in the global phosphotyrosine content has been observed within 1 hour of human sperm capacitation. Furthermore, the degree of tyrosine

phosphorylation per cell also increases up to 1 hour of capacitation with no further increase up to 5 hours (Barbonetti et al., 2008).

The increase in tyrosine phosphorylation depends on the presence of BSA (cholesterol removal), Ca^{2+} and bicarbonate *in vitro* (Visconti et al., 1995b). There is evidence that the capacitation-associated increase in protein tyrosine phosphorylation is downstream of a cAMP/PKA pathway in mouse sperm (Visconti et al., 1995b, Baker et al., 2009) and other species (Galantino-Homer et al., 1997, Leclerc et al., 1996, Osheroff et al., 1999, Kalab et al., 1998). It is hypothesized that cholesterol removal through the change it causes in sperm membrane, can modulate HCO_3^- ion flux which stimulates cAMP synthesis through the activation of soluble adenylyl cyclase (sAC) (Chen et al., 2000, Nolan et al., 2004, Hess et al., 2005, Visconti et al., 2002). Stimulation of tyrosine phosphorylation through this HCO_3^- /cAMP dependent pathway involves protein kinase A (PKA) since inhibition of PKA activation causes a decrease in phosphotyrosine content. Since PKA is not able to phosphorylate tyrosine residues, intermediate tyrosine kinases are probably involved (Leclerc et al., 1996, Carrera et al., 1996, Visconti et al., 2002, Lawson et al., 2008)

1.2 Sperm-ZP binding in the fertilisation process

Fertilization is a complex but highly specialized process in which a series of specific events take place. In mammalian fertilisation the capacitated spermatozoon must penetrate the ZP. After zona penetration the cell will then bind to the egg plasma membrane by the side of its head and finally fuse with the plasma membrane of the

oocyte (Primakoff and Myles, 2002). Before getting access to the ZP sperm have to swim through the cumulus oophorus consisting of cumulus cells embedded in an extracellular matrix primarily composed of hyaluronic acid (Yanagimachi, 1994a). It was believed that PH-20, an enzyme on the sperm plasma membrane, enables sperm to penetrate the layer of cumulus cells and reach the site of the ZP (Hunnicutt et al., 1996). However, mice lacking the hyaluronidase PH-20 can still penetrate the egg cumulus matrix (Baba et al., 2002). Other molecules with hyaluronidase activity have been suggested to facilitate sperm penetration through the cumulus cell mass (Kim et al., 2005, Reitingner et al., 2007). The initial and crucial step in gamete interaction after sperm's penetration of the cumulus cell mass during mammalian fertilisation is sperm adhesion to the ZP of the oocyte. A unique characteristic of gamete sperm-ZP binding is its species specificity, suggesting that the sperm membrane proteins that mediate adhesion bind in a species specific manner to complementary binding sites on the ZP. The ZP is a barrier for non-species specific fertilization (Schmell and Gulyas, 1980); (Rankin and Dean, 2000) and in most mammals is composed of three major glycoproteins of different molecular weight (ZP1, ZP2, ZP3). In human ZP one more zona glycoprotein, ZP4, has been found (Lefievre et al., 2004, Conner et al., 2005). ZP3 glycoprotein has been the most prevailing glycoprotein considered to act as the primary receptor for binding to sperm proteins and the inducer of the AR in mammalian species including human (Bleil and Wassarman, 1980, Gupta et al., 2007, Dean, 2007).

The initial sperm-egg interaction requires a species specific binding which is mediated by complementary sites on the ZP and sperm surface. The binding between the plasma membrane of sperm and ZP, or more specifically ZP3 according to the prevailing model, is called "primary binding" (Bleil and Wassarman, 1990,

Wassarman et al., 2001). After this binding and the completion of the AR, when the sperm loses their ZP3 receptors on the head plasma membrane, remain bound to the zona by binding to ZP2 by molecules exposed on the surface of the inner acrosomal membrane after the AR. This binding is called “secondary binding” and is essential for subsequent sperm-zona penetration (Wassarman et al., 2001). However, the presence of contradictory data on this subject has cast this model of ZP3-ZP2 primary and secondary binding respectively into doubt. More specifically, even though it has been shown that sperm binds to O- glycans attached to ser³³² and ser³³⁴ on ZP3 and that this is required for sperm-egg interaction (Florman and Wassarman, 1985) genetic mutation of O-glycans of mouse ZP did not inhibit sperm-egg binding or fertilisation (Liu et al., 1995). A more recent study in 2010 reported that fertilisation was not affected by the absence of ZP3 in mice (Gahlay et al., 2010). A breakthrough study published recently provides evidence implicating ZP2 in human sperm binding of ZP in transgenic mice, reporting that ZP2 is necessary and sufficient to support human sperm binding (Baibakov et al., 2012). Moreover, in this same study it was shown that sperm binds specifically to the N-terminal domain of the humanized ZP and that only after 4 hours incubation under capacitating conditions could sperm bind, relating this binding to sperm capacitation. However it appears from this study and others that ZP2 does not mediate species-specific binding (Bedford, 1977, Baibakov et al., 2012).

Collectively these data along with the data provided from a study in 2010 (Tardif et al., 2010b) showing exposure of an intra-acrosomal protein zonadhesin during capacitation support the latest model proposed to explain sperm-ZP interaction in which acrosomal proteins are directly involved in sperm-ZP adhesion. The diagram in Fig. 1.1 illustrates the sequence of zonadhesin exposure during fertilization.

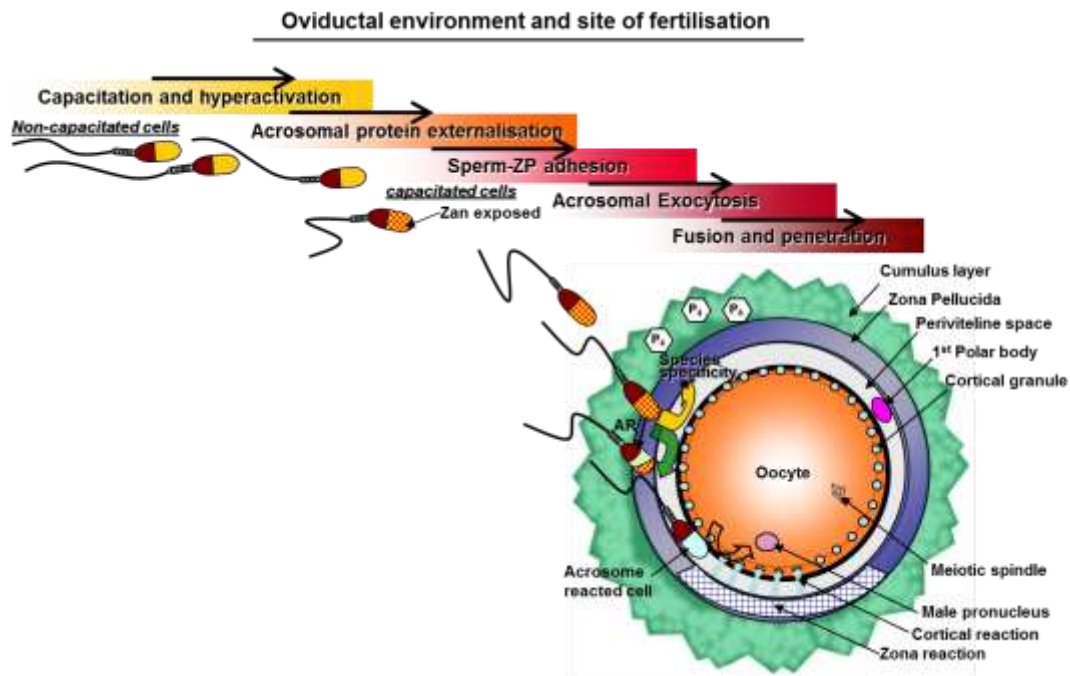


Figure 1.1 Time line of mammalian fertilization and the importance of an acrosomal molecule such as zonadhesin in sperm adhesion, a species specific process. Zonadhesin is exposed during capacitation (in red) (adapted from Tardif and Cormier, 2011).

Sperm candidate adhesion molecules

To date, a number of sperm adhesion molecules have been identified but the identity of the primary adhesion protein remains controversial. There is a series of enzymes suggested to participate in sperm-egg adhesion. One of the most studied is β -1,4-galactosyltransferase (GalT1) which was named by its ability to add galactose to glycoproteins with terminal N- acetylglucosamine residues (Shur and Bennett, 1979). GALT1 is a sperm surface protein found on the surface of mouse sperm overlying a discrete domain on the dorsal, anterior aspect of the sperm head (Shur and Neely, 1988, Gong et al., 1995). For a long time GalT1 was believed to be the primary

adhesion ZP3 receptor. Although knock-out experiments in mice provided evidence for this enzyme's role in the sperm-egg interaction, the fact these GALT1 null mice were not completely infertile and sperm could still bind to the ZP (even if GalT-null sperm are unable to bind ZP3 in solution or undergo zona-induced acrosome reaction) suggested that this molecule did not have the unique role it was expected to (Lu and Shur, 1997).

Another identified sperm protein that has a role in mouse sperm binding to the ZP is SED1. SED1 is a protein composed of EGF (epidermal growth factor) repeats and discoidin/F5/8 C domains; motifs that mediate a series of cell-cell and cell-matrix interactions. Following capacitation, its expression occurs on the plasma membrane. There is evidence suggesting that SED1 is required for gamete adhesion, e.g. antibodies against SED1 on the plasma membrane block sperm binding to the ZP. Recombinant SED1 also binds to the ZP of unfertilized oocytes. (Ensslin and Shur, 2003, Shur et al., 2006). The acrosomal vesicle contains several proteins and a few of them are known as sperm-ZP adhesion molecules. A representative example of an acrosomal adhesion molecule is SP56 (or ZP3R), one of the most studied acrosomal proteins. SP56 was identified by the ability to bind the denatured extra-cellular matrix of the egg and it is known as a lectin-like protein which exhibits lectin-like affinity for galactose residues (Bleil and Wassarman, 1990). Although SP56 was first identified as a primary receptor for ZP3, data revealing the protein's location in the acrosome in mouse, determined by electron microscopy, put this first impression in doubt (Kim et al., 2001). Later this discrepancy, it was suggested, that sp56/ZP3R presence at the sperm surface was related to the capacitation state as it cannot be detected on the plasma membrane of live, uncapacitated sperm (Kim and Gerton, 2003, Wassarman, 2009). However, recent data from Muro and colleagues using targeted deletion of

sp56 gene in mice showed that sperm zona binding and the ability of the sperm to undergo the AR in response to calcium ionophore A23187 displayed no differences between wild-type and knock-out mouse sperm, suggesting that sp56 involvement in ZP-sperm adhesion is not essential in fertilization possibly due to the involvement of multiple proteins as adhesion molecules (Muro et al., 2011). It should be noted that sp56 has been mostly studied in mouse but orthologues have been also identified in rat (Kim and Gerton, 2003) and guinea pig (Foster et al., 1997). However there is no information about this protein's function in human sperm.

Zan: a unique adhesion molecule

Finally another sperm protein, zonadhesin (zan), was discovered in pig spermatozoa by its ability to bind native ZP and in a species specific manner (Hardy and Garbers, 1994, Yurewicz et al., 1998, Rankin et al., 1998). Zan is produced during spermatogenesis and is present in Golgi phase and cap-phase round spermatids, localized to the nascent acrosome between the inner and outer acrosomal membrane. Its precursor protein is processed by proteolysis soon after translation, leading to the formation of different polypeptides assembled into the sperm acrosome. (Bi et al., 2003, Olson et al., 2004). It is an-acrosomal protein that appears to be unique among adhesion molecules since it is the only protein known to mediate species-specific adhesion to the ZP. This information was obtained from experiments demonstrating that pig zonadhesin binds avidly to the pig ZP but not to mouse or bovine ZP glycoproteins (Hardy and Garbers, 1994, Bi et al., 2003).

Zan is a multiple domain protein and is comprised of three known adhesion domains: MAM (meprin/A5 antigen/mu receptor tyrosine phosphatase), mucin and von

Willebrand D domains (D0, D1, D2, D3, D4). Zan precursor has been identified in many mammalian species such as pig (Hardy and Garbers, 1995), mouse (Gao and Garbers, 1998), human (Wilson et al., 2001) and rabbit (Lea et al., 2001). Although zan domain structure among species is very similar, there is still some in-species variation on the amino acid level that could possibly apply to the species specificity properties of the protein (Tardif and Cormier, 2011). In mouse, unlike other mammalian species, zan has 20 D0 like tandem repeats, localized between D3 and D4 von Willebrand D domains, called D3 partial domains (see Figure 1.2) (Gao and Garbers, 1998).

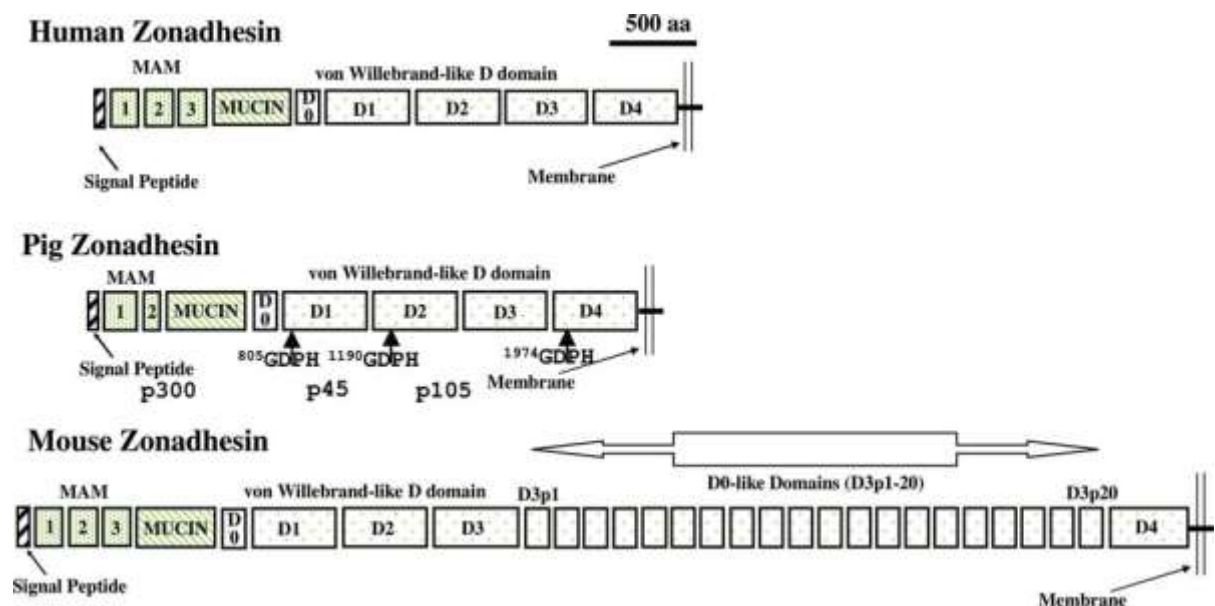


Figure 1.2 Schematic showing the domain structure of zan in human, pig and mouse (adapted from Tardif and Cormier, 2011)

Recently, zan exposure has been associated with the capacitated population in the mouse (Tardif et al., 2010b). This study demonstrated that zan is not detected on the surface of live mouse cells incubated under non-capacitating conditions but is exposed on the surface of the cells during capacitation. More specifically, sperm following *in vitro* capacitation exposed a partial von Willebrand D3 domain. Furthermore, in the same study, when spermatozoa were incubated with an antibody

against zan D3p18 domain *in vitro* fertilisation of eggs by spermatozoa from wild-type was inhibited (Tardif et al., 2010b). However fertilisation of the eggs by sperm from zan-null males was not inhibited. Therefore, it was concluded that loss of zan does not result in infertility because null spermatozoa retain the ability to bind to the ZP but not in a species-specific manner (Tardif et al., 2010b). It is not known though if a similar event occurs in human sperm.

There are also a number of sperm protein receptors proposed to participate in the secondary zona binding mediated by ZP2 glycoprotein on the egg coat. Among these acrosin is the most studied. Acrosin is an acrosomal protein, stored as the inactive zymogen proacrosin in the sperm acrosome, converted to its active enzyme form and released during the AR (Tesarik et al., 1990, Nuzzo et al., 1990, Moos et al., 1993). Data from both animal and human studies suggest more than one role of this molecule in the fertilisation process, such as in sperm-egg interaction and in proteolysis and acrosomal content release during the exocytosis process (Urch et al., 1985, Vazquez-Levin, 2005, Klemm et al., 1991). More specifically, acrosin null mouse spermatozoa exhibited a malfunction in the release of the acrosomal contents during the AR (Yamagata et al., 1998). Moreover spermatozoa in a mouse model lacking acrosin by a gene mutation were associated with delayed fertilisation, even though these males were not sterile (Adham et al., 1997). As for humans, a study in 2005 revealed a region of acrosin that interacts with ZP glycoproteins (Furlong et al., 2005). Moreover, antibodies against acrosin have been detected in the serum of women consulting for infertility, inhibiting proacrosin binding to recombinant human ZP glycoprotein A as well as its activation (Veaute et al., 2009). Even though a more recent study demonstrated an inhibitory effect of an antibody against acrosin on proacrosin-acrosin activities as well as on ZP induced AR, no inhibition was observed

on sperm-ZP adhesion. This could be partially explained by the presence of other molecules with binding properties that function during sperm-ZP adhesion (Veaute et al., 2010).

1.3 Acrosome reaction – new acrosomal exocytosis model

It was believed for many years that AR occurs after binding with the zona pellucida (ZP). Therefore only sperm cells reaching the egg with an intact acrosome are able to fertilize. Data supporting this prevailing model have been obtained mostly from mouse studies (Florman and Storey, 1982, Saling and Storey, 1979); (Gupta and Bhandari, 2011) According to this model, spermatozoa bind to the ZP by a sperm plasma membrane protein while they are membrane intact (Storey et al., 1984). This has been referred as primary binding. ZP then induces the AR releasing the acrosomal molecules. These will then take part in the secondary binding which appears to be looser than the primary adhesion. Following the AR acrosomal enzymes such as acrosin will digest the ZP in order for sperm to be able to penetrate it (Yanagimachi, 1994a).

However, the fact that an essential plasma membrane protein for primary binding has not yet been identified, as well as the presence of proteins with strong ZP binding properties such as zan within the acrosome puts this model's validity into doubt. Moreover, in several studies AR has been suggested to occur before binding to the ZP in several mammalian species (Bedford, 2011). Such an example is a study in rabbits

published in 1984 which revealed that spermatozoa from the perivitelline space of the egg, with a non-intact acrosome, were able to fertilize the rabbit eggs (Kuzan et al., 1984) and a human study in 1987 suggesting that AR is characterized by intermediate stages during which spermatozoa bind to the ZP (Stock and Fraser, 1987)

It was not until recently, when new breakthrough studies in mice revealed the initiation of AR prior to ZP binding. Jin and colleagues used a video microscopic *in vitro* fertilisation system to study the site of AR in a transgenic mouse model where spermatozoa expressed green fluorescence in their acrosomes. Acrosome intact sperm cells that contacted the ZP did not penetrate it, in contrast to cells that had already initiated the AR before contact. These cells were also capable of fertilizing the egg (Jin et al., 2011). These results are in agreement with another mouse study published the same year which demonstrated that cells recovered from the perivitelline space of mice can fertilize other oocytes (Inoue et al., 2011).

In addition to the recent data above, studies reporting the detection of acrosomal molecules at the cell surface are in favor of this new concept of acrosomal exocytosis and sperm-egg interaction. Zonadhesin (zan) has been detected on the surface of mouse spermatozoa during sperm capacitation (Tardif et al., 2010b). Moreover, in a mouse model expressing green fluorescence in their acrosome (EGFP) (Nakanishi et al., 1999), zan was also detected on the surface of acrosome intact sperm cells (cells with green fluorescence over the acrosome) under capacitating conditions. Other acrosomal molecules such as sp56/ZP3R (Kim and Gerton, 2003) have also been detected on the surface of acrosome intact mouse cells during capacitation while the same group of Kim and colleagues has reported accessibility of acrosomal proteins in the extracellular environment of guinea pig spermatozoa under capacitating conditions in cells that appear to be acrosome intact by a fluosphere-binding assay,

observing their continued exposure and differential release during the acrosomal exocytosis (Kim et al., 2011).

Taken together, these studies suggest that the prevailing simplistic model of acrosomal exocytosis characterized by two categories of cells, acrosome reacted and intact sperm cells, as well as by the initiation of AR after binding to the ZP needs to be reassessed. Considering the amount of recent data supporting the concept of a more dynamic AR process, which involves intermediate stages associated with the capacitation state and is initiated prior to ZP contact, it can be concluded that the established AR model prevailing for years cannot adequately explain the process

Perhaps the belief that the spermatozoa must be intact in order to bind to the ZP came from the difficulty in determining the acrosomal status of individual cells in real time of adhesion to the ZP with the existing AR assays. On the other hand, the evolved modern techniques on the study of acrosomal status appear to promise more accurate results in both mice and humans (Jin et al., 2011, Zoppino et al., 2012).

The overall characterization of this exocytotic process has already been described in several species (Yudin et al., 1988, Flechon et al., 1986, Green, 1978, Franklin et al., 1970, Barros et al., 1967). An acrosome swelling has been reported upon stimulation of the AR where the outer acrosomal membrane and plasma membrane form fusion pores. These fusion pores will lead to the formation of vesicles comprised of both membranes, outer acrosomal and plasma membrane. After the completion of the exocytosis the acrosomal contents are lost, as well as the two membrane parts present at the vesicles, and the inner acrosomal membrane is exposed at the cell surface.

The molecular mechanism participating in the formation of fusion pores and acrosome vesicles is not fully elucidated. However, recent human studies have

already revealed some parts of the mechanism of human acrosomal exocytosis process. A study by Zanetti and Mayorga showed that the plasma membrane is attached at the edges of outer acrosomal membranes invaginations in swollen acrosomes. They also reported that these edges were docked to plasma membrane by a SNARE-dependent manner, proposing that the expansion of these pores formed by membrane docking can lead to the formation of the acrosomal vesicles during exocytosis (Zanetti and Mayorga, 2009). It has been shown before that the SNARE complex is acquired in the acrosomal exocytosis (Tomes et al., 2002, Ramalho-Santos et al., 2000, Tsai et al., 2012). SNAREs assemble into trans SNARE complexes forcing the two membranes closely together in order to accomplish fusion (Weber et al., 1998) and therefore its role in AR is essential. A protein, Munc18-1, has been found recently to participate in the trans-SNARE complexes stabilization during the AR in human sperm. More specifically, when Munc18-1 was blocked by a specific antibody the assembly of trans-SNARE complexes was inhibited as well as the acrosomal exocytosis, suggesting an essential role of Munc18-1 protein in this process (Rodriguez et al., 2012).

1.4 Male infertility

The importance of sperm-egg adhesion and subsequent steps in mammalian fertilization has already been highlighted. Any failure during contact and successful

binding during this process will lead to infertility. It is known that infertility is a significant global problem affecting 1:7, or approximately 80 million couples, worldwide (Irvine, 1998, Boivin et al., 2007). An estimated 24% of couples had no detected conception within 12 and 24 months of unprotected intercourse on a nationwide representative sample of couples from the general population (Slama et al., 2012). Male factor infertility is also significant and it increases worldwide (Sharpe and Irvine, 2004). There is evidence that the most common cause of male infertility is sperm dysfunction; 20% of infertility cases are due to sperm dysfunction while 28% of sperm dysfunction cases are considered to be unexplained. (Hull et al., 1985, Brandes et al., 2010). Surprisingly, there is no treatment for this group other than ART (assisted reproductive technology) and its use is constantly increasing worldwide (Andersen et al., 2008). However, ART is not always the most convenient option for couples for two reasons: firstly the cost of treatment is very high (Rauprich et al., 2010) and secondly ART has been associated with incidences of congenital defects and low weight births (Funke et al., 2010, Davies et al., 2012). Since basic semen analysis as a diagnostic tool for male infertility has been proved inadequate in most of the cases (Macleod and Gold, 1951, Hargreave and Elton, 1983, Tomlinson et al., 1999, Guzick et al., 2001), currently one of the main objectives of male infertility research is to invent a diagnostic test that efficiently correlates with sperm fertilizing potential. Sperm function tests have been developed, predictive of fertilization outcome, including biochemical tests and bioassays (Oehninger et al., 1992, Oehninger et al., 1995) such as ZP binding assays (Oehninger et al., 1989, Liu et al., 1988), and the ZP-induced acrosome reaction assay (Franken et al., 2000). However, there are several practical issues in the appliance of these bioassays (biological

material like human ZP is not easily available and has a high cost) (Fraser et al., 1997).

At the molecular level there is no robust marker for identifying sperm populations competent to fertilize, which is basically due to our lack of understanding of the molecular mechanisms underlying the mature spermatozoa. New concepts and technologies might clarify the biology of these functional steps.

Zonadhesin has been recognized as an adhesion molecule of particular interest during the egg-sperm adhesion. As discussed above zonadhesin exposure has been associated with capacitation, which reflects fertilizing ability, in mice (Tardif et al., 2010b). The questions that arise are, if a similar event occurs in human spermatozoa and if that event could be used as a developing clinical test to identify the sperm population competent to fertilise. Barratt and colleagues have proposed a putative model of zonadhesin exposure during capacitation based on zonadhesin exposure on the surface of mouse capacitated spermatozoa where the protein could be exposed differently in fertile and sub fertile men (Figure 1.3). More specifically zonadhesin exposure could be limited in spermatozoa from sub fertile men that cannot undergo capacitation. Any detection of zonadhesin on the sperm surface under non capacitating conditions would be considered as not normal, possibly due to defects in the regulation of signal transduction.

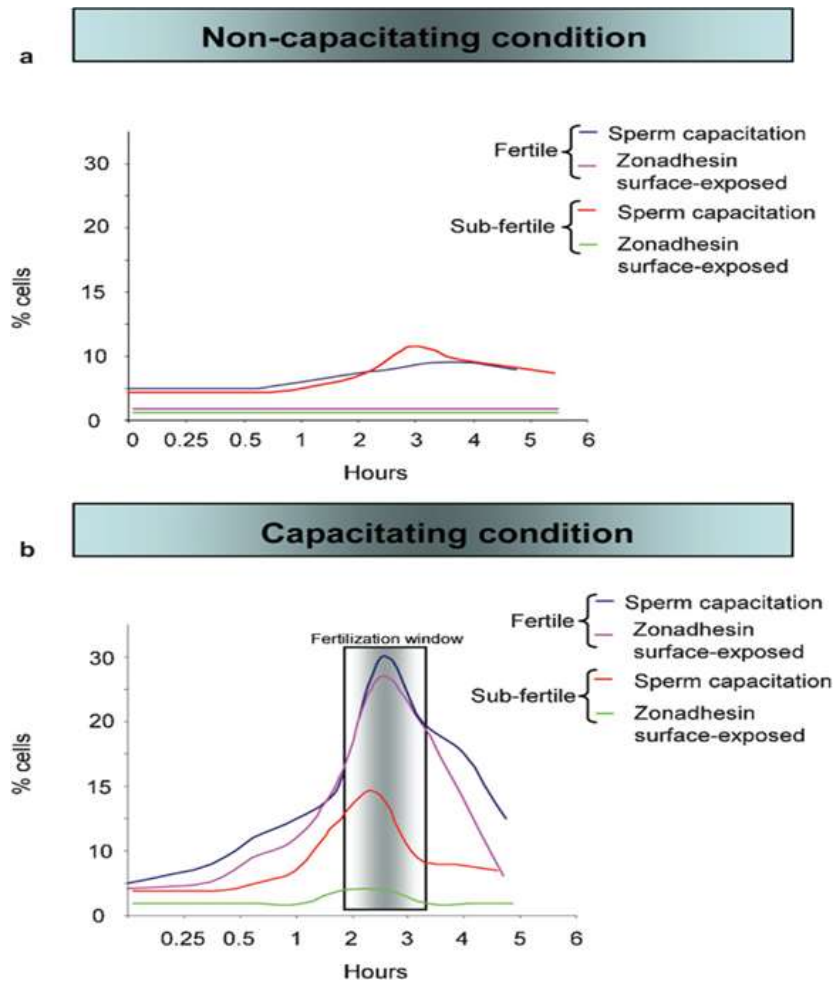


Figure 1.3 Proposed model of potential human zonadhesin exposure during sperm incubation under (a) non-capacitating or (b) capacitating conditions in fertile and sub fertile men (adapted from Barratt et al., 2011)

1.5 Thesis aims

The main hypothesis for this project was that zonadhesin is exposed to the surface of human spermatozoa during *in vitro* capacitation. A series of experiments were carried out in order to achieve the 5 basic objectives, as listed below:

- Characterization of physiological parameters of the study sperm population (motility, viability, capacitation %.)
- Evaluate zan exposure on the cell surface of human spermatozoa during sperm capacitation
- Investigate any potential exposure during capacitation of other acrosomal molecules with a role in sperm-ZP interaction
- Determine the molecular weight of zan polypeptides normally present in mature human spermatozoa under capacitating and non-capacitating conditions
- Initiate a preliminary comparison between fertile donors and sub fertile patients in terms of zan isoforms present in the mature spermatozoa

CHAPTER 2

Materials and Methods

All reagents except those otherwise indicated were obtained from Sigma-Aldrich (Dorset, UK). Antibodies used for live labeling or Western blot experiments raised to zonadhesin [polyclonal affinity purified rabbit antibody against recombinant GST fusion protein comprising amino acids of the D3 zan domain (Tardif et al., 2010a, Tardif et al., 2012)], sp32/ACRBP [polyclonal affinity purified rabbit antibody against recombinant His tag fusion protein comprising sp32 amino acids (Tardif et al., 2012)], sp-56/ZP3R [polyclonal affinity purified rabbit antibody against sp56 synthesized peptide (Kim et al., 2001)] and CRES (Cystatin related epididymal spermatogenic) (polyclonal affinity purified rabbit antibody) were generously donated by Drs Daniel Hardy (Texas Tech University Health Sciences Center), Steve Tardif (University of Dundee), George Gerton (University of Pennsylvania) and Gail Corwall (Texas Tech University Health Sciences Center) respectively.

2.1 Media

Two different media were used to incubate sperm cells in this study: a non-capacitating medium (NCM) and a capacitated medium (CM).

NCM is a medium that does not support sperm capacitation. Conversely, incubation in CM supports sperm capacitation (Moseley et al., 2005). The composition of the two media is shown below on Table 2.1.

| Compound | Non Capacitating Media (NCM) | Capacitating Media (CM) |
|---|------------------------------|-------------------------|
| CaCl ₂ | 1.8mM | 1.8mM |
| KCl | 5.4mM | 5.4mM |
| MgSO ₄ .7H ₂ O | 0.8mM | 0.8mM |
| NaH ₂ PO ₄ .2H ₂ O | 1.0mM | 1.0mM |
| D-glucose | 5.55mM | 5.55mM |
| Sodium pyruvate | 2.73mM | 2.73mM |
| Sodium lactate | 41.75mM | 41.75mM |
| BSA | 0.3%, | 0.3%, |
| HEPES | 25mM | - |
| Sodium chloride | 116.36mM | 116.36mM |
| NaHCO ₃ | - | 25mM |

Table 2.1 Composition of NCM and CM, the two media used for sperm incubation;

pH of both media is 7.4

2.2 Sperm Preparation

Semen was obtained from volunteer donors with no known fertility problems, all recruited in accordance with the HFEA Code of Practice under ethical approval from the Tayside Committee of Medical Research Ethics B (number 09/S1402/6). Fresh semen was obtained from healthy donors (aged 20-35 and with a normal sperm concentration and motility according to criteria from WHO, 1999 that is, concentration $\geq 20\text{M/ml}$ and motility $\geq 50\%$) with 2-3 days of sexual abstinence. The semen was allowed to liquefy at 37°C for 30 minutes.

Spermatozoa were isolated by a 40%-80% discontinuous density gradient using colloidal suspensions of silica particles coated with polyvinylpyrrolidone (Percoll®, Sigma 77237). Solution of 40% and 80% v/v Percoll were prepared using a stock of 90% solution because pure Percoll solution is not iso-osmotic (Sbracia et al., 1996). To make the Percoll solution iso-osmotic, one part of NCM 10X concentrated was mixed with 9 parts of Percoll making this solution 90% v/v of concentration. Briefly, we prepared the density gradient as follow:

2 ml of 80% solution (1.77 ml of 90% v/v Percoll® solution added to 0.23ml of NCM) and 2 ml of 40% Percoll® solution (0.888 ml of 90% v/v Percoll® solution added to 1.12 of NCM) were prepared separately. The 2 ml of 80% solution was put into the bottom of 40% solution without mixing in order to prepare the gradient. Once the gradient was prepared, 1 ml of liquefied semen was layered on the top of the density gradient and then the samples were centrifuged for 20 min at 300 g. After centrifugation, the sperm pellet and the spermatozoa at the interface between the 40%

and 80% fraction were harvested and diluted in 3-4 volumes NCM and centrifuged for 10 min at 500 g. After sperm washing, sperm pellets were resuspended in either NCM or CM adjusting the concentration at approximately 25 million cells/ml by using a Hamilton Thorne 'CEROS' Computer Assisted Sperm Analyser.

2.3 Motility parameters analysis

Computer Assisted Sperm Analysis (CASA Hamilton-Thorne, 'CEROS', version 12.3, Beverly, MA, USA; Olympus CX41 Microscope) was used for evaluating different sperm motion parameters. Motion parameters were recorded after incubation in NCM 37⁰ C or CM (37⁰ C, 5% CO₂). An aliquot of 3 µl was loaded in a preheated 4 chamber slide (Brand and Cie) and at least 400 cells were recorded. The slides were stored at 37⁰ C in the air incubator for a minimum of one hour before use.

The settings used during the analysis were: frame rate, 60 Hz; cell size-non motile, 6 pixels; cell intensity-non motile, 160. Sperm motion characteristics measured for spermatozoa included curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) and amplitude of lateral head displacement (ALH). The general settings used to classify motile cells were: rapid (cells exhibiting VAP > 25 µm/s), medium (VAP of 5-25 µm/s), and slow (VAP < 5 µm/s and VSL < 11 µm/s). Slow cells were counted as non-motile cells. Cells were counted as progressively motile if VAP was >25 µm/s and STR was > 80%

2.4 Statistical analysis

The normal distribution of the result (Chapters 3 and 4) was examined using the Kolmogorov-Smirnov test and the homogeneity of the variance was tested by Levene test, the two essential postulates to determine the use of parametric statistics. Significant difference in the exposure was determined by analysis of variance (ANOVA). The Fisher's least significant difference (LSD) test was conducted when the main effect was significant to establish differences between groups. All tests were available in the statistical package SPSS version 15. A value of $P < 0.05$ was considered statistically significant.

CHAPTER 3

Characterization of study population

3.1 Introduction

The aim of this chapter was to characterize the study sperm population in relation to the incubation medium (NCM, CM). The establishment of the optimum incubation time to achieve sperm capacitation *in vitro* is critical. The optimum time of incubation in CM, to obtain the maximum percentage of capacitated spermatozoa in the sperm population *in vitro*, was used to study the exposure of acrosomal molecules during sperm capacitation, as presented in chapter 4. Additionally, and in contrast to the 80% fraction, little is known about the physiology of the immature sperm fraction (40% density gradient fraction), so a study of the physiological parameters of this sperm subpopulation in comparison to the 80% fraction would provide additional information for future research of this poor sperm fraction.

Human semen is characterized by heterogeneity in terms of the sperm populations. It is comprised of different sperm subpopulations which exhibit differences in their physiological characteristics such as varying degrees of sperm maturation, functional quality and as a consequence their fertilizing ability (Huszar and Vigue, 1993). These sperm subpopulations can be isolated and separated by the use of density gradient centrifugation. The density gradient separates spermatozoa according to their density and favors the isolation of motile and morphologically normal sperm; the higher quality sperm cells in terms of motility and morphological normality can be recovered from the pellet in contrast to lower density fractions where sperm with abnormalities are more likely to be found (Sakkas et al., 2000, Ollero et al., 2000). For that reason the density gradient has become the standard sperm preparation method used in IVF clinics (Centola et al., 1998). The two layer 40% and 80% Percoll or Puresperm

gradients are commonly used for the separation of the sperm subpopulations (Allamaneni et al., 2005), however three layer gradients can also be used such as 90%-70%-40% (Chen and Bongso, 1999) or 45%-65%-90% (Buffone et al., 2004) depending on the need of particular experiments.

Ejaculated mature spermatozoa are not able to fertilize before their transit in the female reproductive tract where the cells undergo a time dependent complex of biochemical changes in order to get capacitated and acquire fertilizing ability (as discussed in chapter 1) (Chang, 1951, Austin, 1951). Capacitation in humans appears to be a transient state and previous observations suggested that the sperm cells maintain their capacitated state for $>50 \text{ min} < 4 \text{ hours}$ and it only happens once in a sperm's lifetime. Furthermore, it has been demonstrated that capacitation is not a static state but only a fraction of the sperm population becomes capacitated at any given time and as a consequence capacitation is characterized as a short term state (Cohen-Dayag et al., 1995, Giojalas et al., 2004).

In this chapter the time course of sperm capacitation after incubation in capacitating and non-capacitating medium using the indirect method of induced acrosomal reaction of capacitated spermatozoa by calcium ionophore was measured, as well as motility and viability for the mature (80% fraction) and immature (40% fraction) sperm subpopulations.

3.2 Experimental procedures

Experimental design

The time course of sperm capacitation *in vitro* in 80% and 40% fraction was measured by the ability of capacitated spermatozoa to undergo the AR in response to calcium ionophore. The induced AR was measured every hour for 5 hours of incubation in CM to determine the optimum time of sperm incubation for *in vitro* sperm capacitation. It was also measured in cells incubated in NCM, as a control to test the efficiency of the method. Moreover, to characterize the physiology of the sperm populations comparatively to the medium total motility, progressive motility and path velocity were measured for both media. Finally the viability of spermatozoa incubated in CM was assessed in order to ascertain that the cells remain viable over time of incubation under capacitating conditions since the capacitated sperm population was further used for the main experiments of the project.

Semen preparation

The sperm samples were from healthy donors and the semen was prepared as described in Chapter 2, using the 80% and 40% fraction

Motility analysis

Motility was recorded using CASA as described in Chapter 2. Sperm motion parameters were recorded at every hour for a total of 5 hours of incubation in NCM at 37°C or in CM at 37°C, 5% CO₂.

Motion parameters were analyzed comparatively for cells incubated in CM and NCM for both sperm subpopulations. Motility was recorded at time zero, (T0) the beginning of the experiment, after 3 and 5 hours of incubation in each medium.

Viability assay

Viability was assessed by using the red live/dead dye propidium iodide (PI) (Sigma P4170). Sperm samples (250 μ l) of 25 million/ml final concentration were incubated with 2.5 μ l of PI solution for 5 hours at 37°C, 5% CO₂ in CM. An aliquot of 15 μ l was mounted on a microscope glass slide with a coverslip at every hour and immediately viewed under a fluorescence microscope (Olympus 1X71, excitation 536nm nm/emission 617nm). The positive cells for PI were scored as dead spermatozoa (200 cells counted per slide/1 slide counted per hour).

Evaluation of spontaneous acrosomal reaction and sperm capacitation by using an indirect method

Sperm capacitation was evaluated via an indirect method by the ability of capacitated spermatozoa to undergo AR following incubation with 10 μ M of calcium ionophore A23187 for 15 min (Sigma C7522) (Liu and Baker, 1998). Spermatozoa were incubated under non-capacitating conditions (NCM) for 5 hours at 37°C and at every hour AR was induced by incubating a fraction of sperm solution (100 μ l) with calcium ionophore A23187 (10 μ M) for 15 minutes. An aliquot was also incubated with only DMSO (1%) as negative control (the solvent used to dissolve A23187) and this incubation informed on the percentage of spontaneous AR. Next to ionophore or DMSO incubation, sperm cells were smeared on microscope slides, allowed to dry and permeabilised in 100% methanol for 30 minutes at room temperature. Following, sperm slides were firstly washed with Tris Buffered Saline (20 mM Tris pH: 7.4, 136

mM NaCl) and incubated with fluorescein isothiocyanate-Pisum sativum agglutinin (PSA-FITC) lectin (Sigma L0770) (Cross NL, 1986) at room temperature for 20 minutes in the dark. The slides were washed with TBS two times, mounted with hydromount (National Diagnostics NS-106) and cover slip. At least 150 spermatozoa were scored for each experiment and distinguished according to their pattern of fluorescence. Bright fluorescence over the acrosome indicates intact acrosome while no fluorescence indicates cells without acrosomal cap.

The percentage of capacitation (induced AR) was calculated by subtracting the number of spontaneous AR in the samples incubated with 1% DMSO to the number of induced-acrosome reacted spermatozoa in the samples incubated in the presence of ionophore. The same procedure was followed for the evaluation of sperm capacitation under capacitating conditions (CM, 37°C, 5% CO₂).

3.3 Results

3.3.1 Sperm motility during incubation under capacitating and non-capacitating conditions

Figure 3.1 shows that the total motility of the 80% fraction is significantly higher for cells incubated in CM compared to NCM from the beginning to the end of incubation (time 0, 3 and 5 hours) but Figure 3.2 shows that there is no statistical difference for total motility between the two media for the 40% fraction. However, progressive motility as well as path velocity (VAP) was significantly higher after incubation of the cells in CM compared to NCM for both fractions as it is shown in Figures 3.3

(progressive motility %), 3.5 (VAP) for the 80% and 3.4 (progressive motility %), 3.6 (VAP) for the 40% fraction. Sperm cells prepared in NCM or CM displayed the same magnitude of percentage of motility before and after incubation (T0 to T5) for the 80% fraction, however both progressive and total motility increased significantly after 5 hours of incubation in either medium for the 40% fraction.

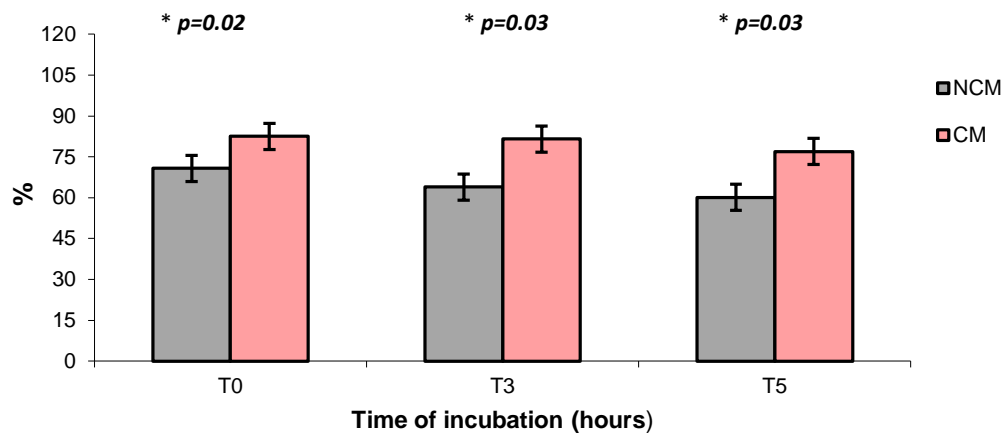


Figure 3.1 Percentage (%) of Total Motility 80% fraction over time (h)

Sperm cells recovered from the 80% fraction after incubation (time 0, 3 and 5 hours) in NCM (at 37°C) or CM (at 37°C, 5% CO₂), average ± SEM; n=5 independent experiments.

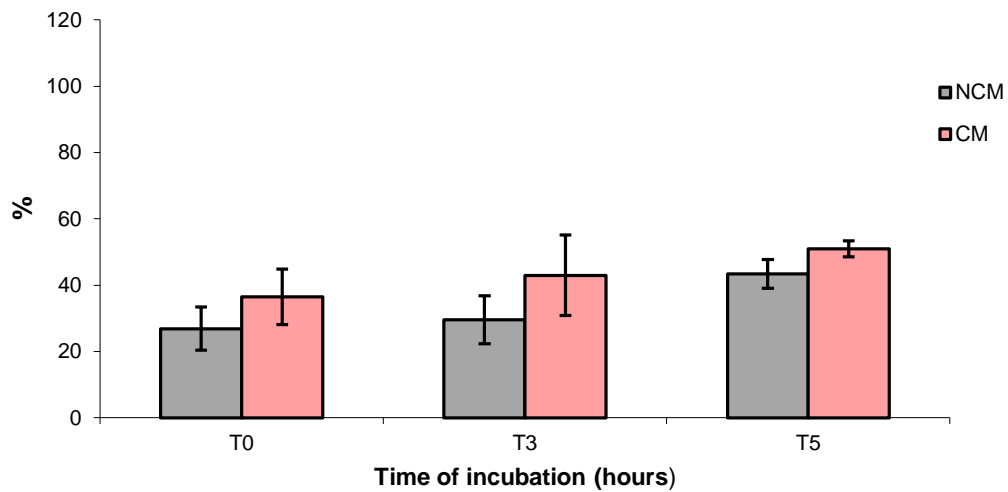


Figure 3.2 Percentage (%) of Total Motility 40% fraction over time (h)

Sperm cells recovered from the 40% fraction after incubation (time 0, 3 and 5 hours) in NCM (at 37°C) or CM (at 37°C, 5% CO₂), average ± SEM; n=5 independent experiments.

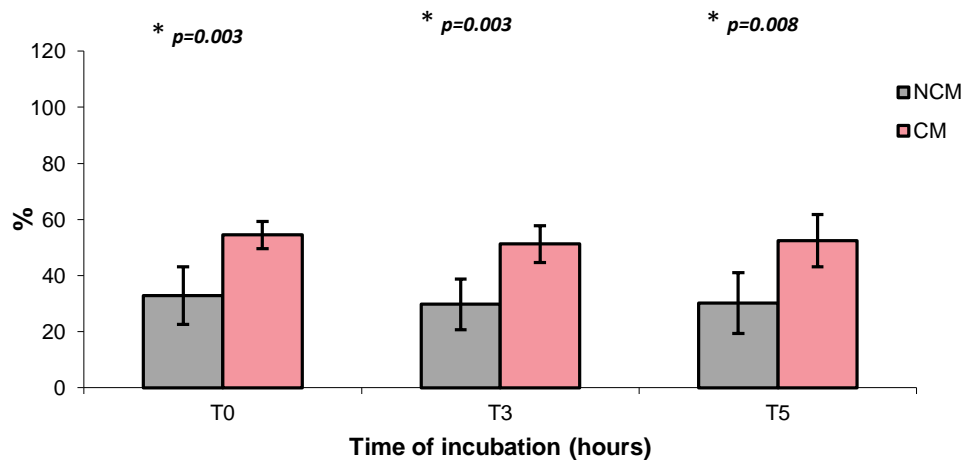


Figure 3.3 Percentage (%) of Progressive Motility 80% fraction over time (h)

Sperm cells recovered from the 80% fraction after incubation (time 0, 3 and 5 hours) in NCM (at 37°C) or in CM (at 37°C, 5% CO₂), average ± SEM; n=5 independent experiments.

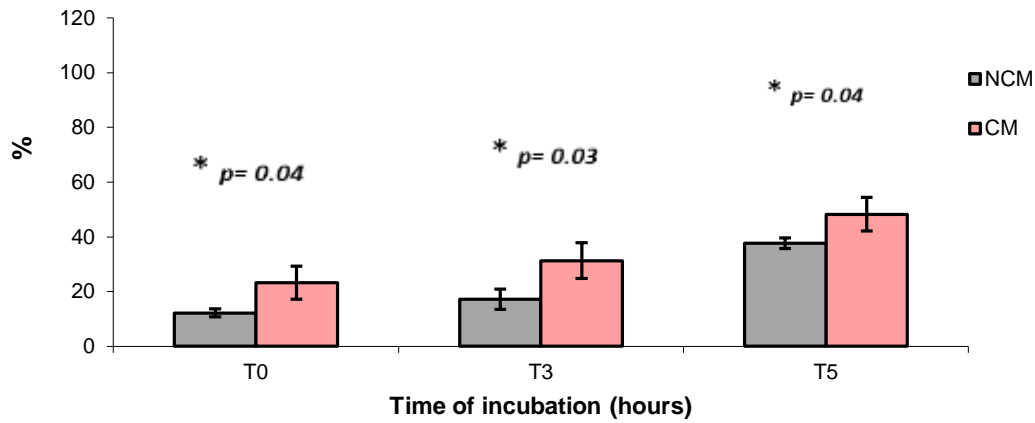


Figure 3.4 Percentage (%) of Progressive Motility 40% fraction over time (h)

Sperm cells recovered from the 40% fraction after incubation (time 0, 3 and 5 hours) in NCM (at 37°C) or in CM (at 37°C, 5% CO₂), average ± SEM; n=5 independent experiments.

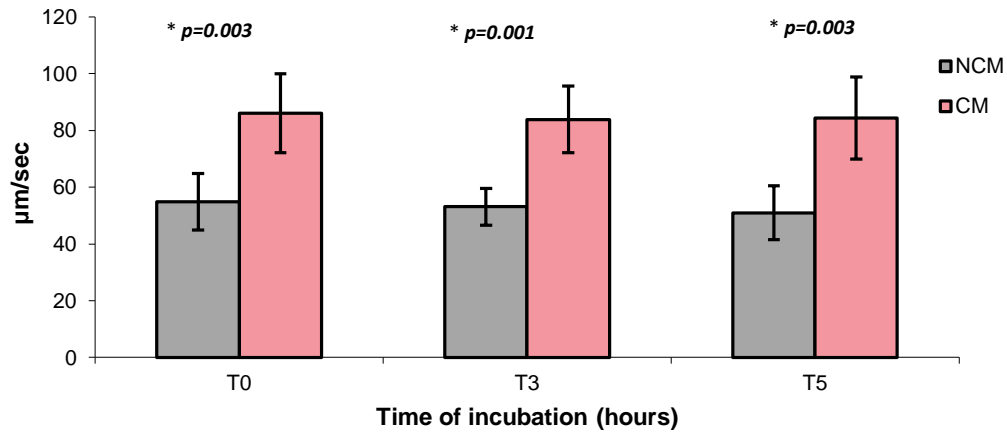


Figure 3.5 Average Path Velocity 80% fraction over time (h)

Sperm cells recovered from the 80% fraction after incubation (time 0, 3 and 5 hours) in NCM (at 37°C) or CM (at 37°C, 5% CO₂), average ± SEM; n=5 independent experiments.

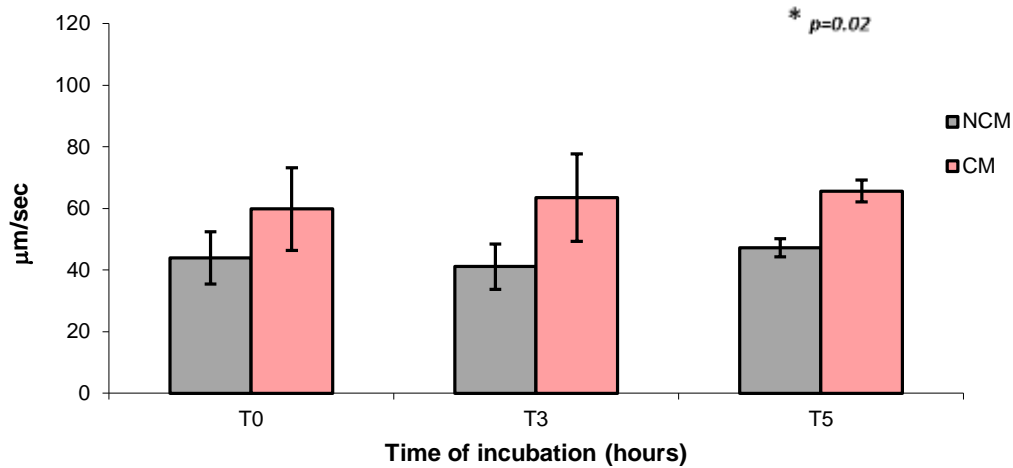


Figure 3.6 Average Path Velocity 40% fraction over time (h)

Sperm cells recovered from the 40% fraction after incubation (time 0, 3 and 5 hours) in NCM (at 37°C) or CM (at 37°C, 5% CO₂), average ± SEM; n=5 independent experiments.

3.3.3 Assay of sperm capacitation

Figure 3.7 shows labeling of the acrosome with PSA lectin indicating the status of the acrosome (acrosome intact/acrosome reacted) which was used to assess the induced AR reflecting the capacitated sperm population. The % of capacitated cells was low during incubation in NCM using cells from the 80% (Figure 3.8) or 40% (Figure 3.10) fraction with no significant increase over time. However, sperm incubation in CM showed a maximum of ~28% of the sperm population capacitated after 3 hours of incubation for the 80% fraction (Figure 3.9). Figure 3.11 shows that sperm cells from the 40% fraction reached the maximum capacitation % (~22 %) after 4 hours of incubation in CM.

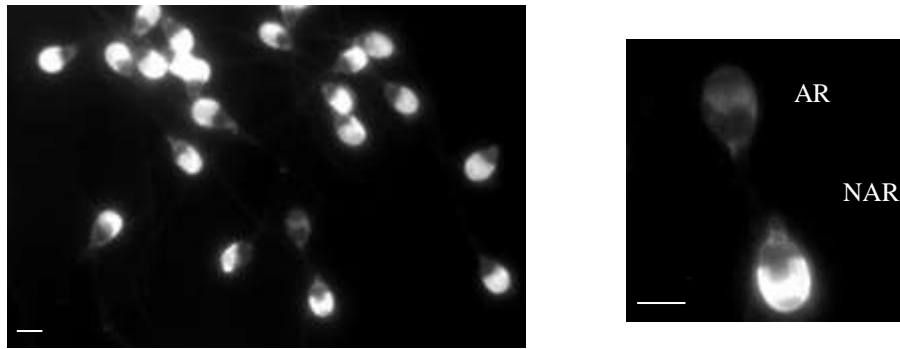


Figure 3.7 Human spermatozoa labeled with FITC-PSA lectin, bright fluorescence: non-acrosome reacted cells (NAR), no fluorescence: acrosome reacted cells (AR). Bar represents 5 μ m.

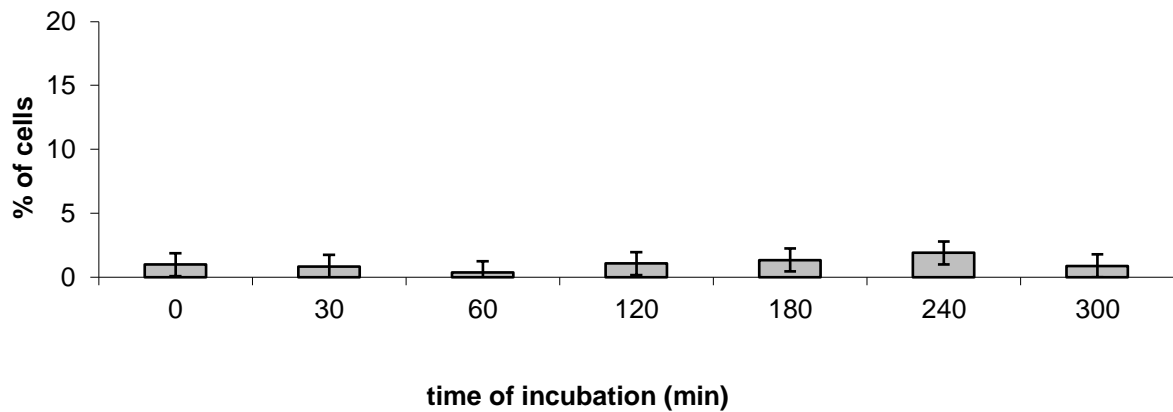


Figure 3.8 Time course of sperm capacitation using non-capacitating medium (NCM) in 80% fraction determined by the ability to undergo acrosome reaction using 10 μ M calcium ionophore A23187 for 15 min (average \pm SEM; n=5 independent experiments). Cells were incubated for 5 hours at 37°C. SEM= 0.9%; p= 0.46

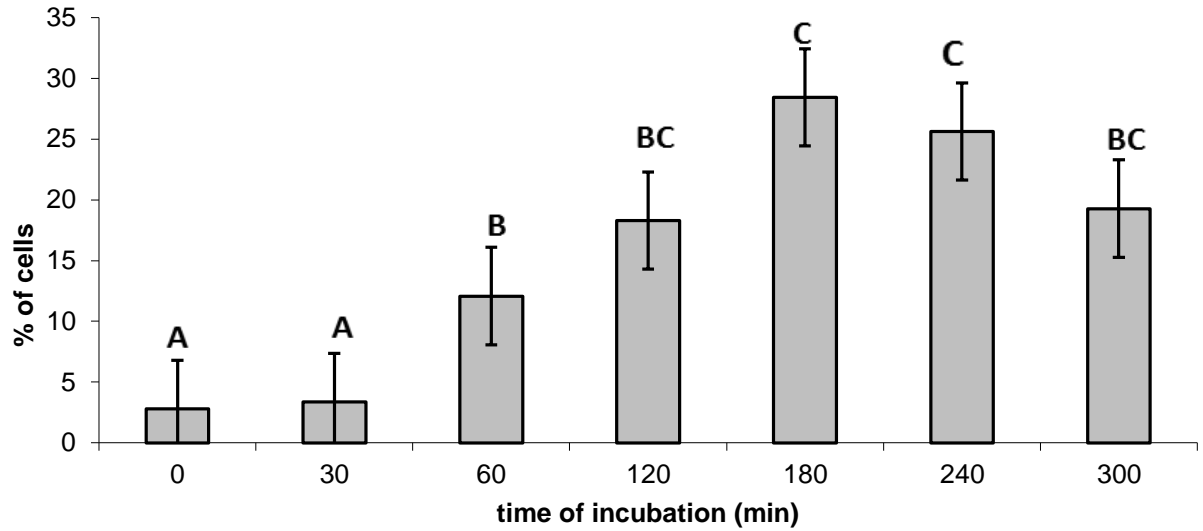


Figure 3.9 Time course of sperm capacitation using capacitating medium (CM) in 80% fraction determined by the ability to undergo acrosome reaction using 10 μ M calcium ionophore A23187 for 15 min (average \pm SEM; n=5 independent experiments). Cells were incubated for 5 hours at 37°C 5% CO₂. Different letters (A, B and C) denote significant difference among groups for % of capacitated cells in the time (BC: not statistically different than either B or C), SEM= 5.1%; p< 0.001

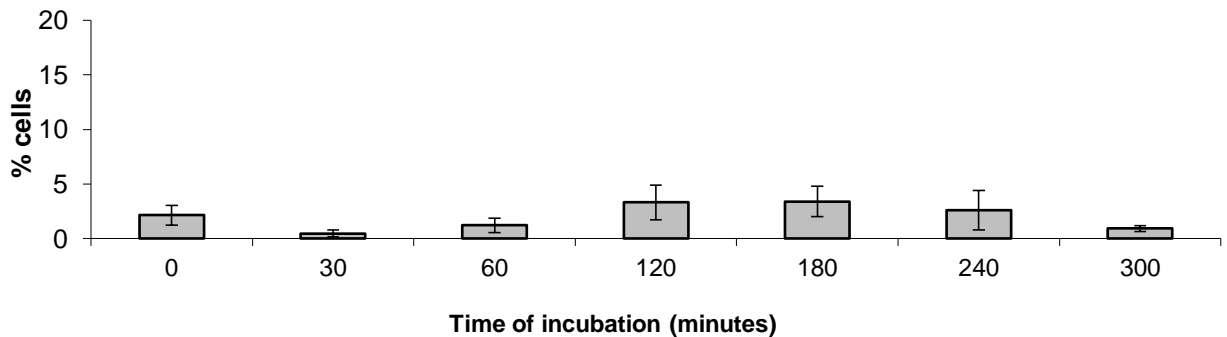


Figure 3.10 Time course of sperm capacitation using non-capacitating medium (NCM) in 40% fraction determined by the ability to undergo acrosome reaction using 10 μ M calcium ionophore A23187 for 15 min (average \pm SEM; n=5 independent experiments). Cells were incubated for 5 hours at 37°C. SEM=1,32%; p=0,487

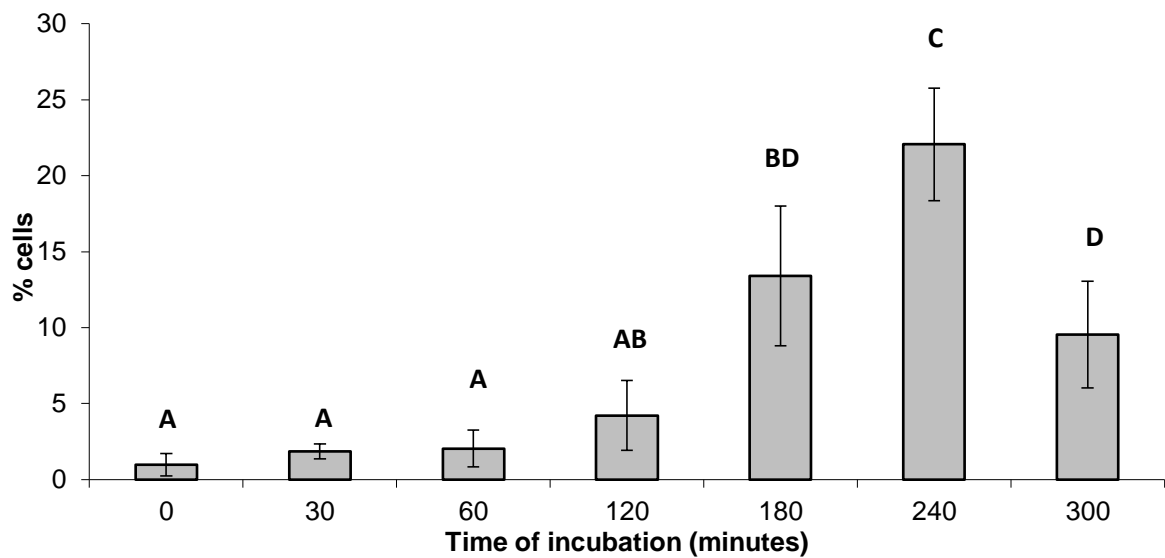


Figure 3.11 Time course of sperm capacitation using capacitating medium (CM) in 40% fraction determined by the ability to undergo acrosome reaction using 10 μ M calcium ionophore A23187 for 15 min (average \pm SEM; n=5 independent experiments). Cells were incubated for 5 hours at 37°C 5% CO₂. Different letters (A, B, C and D) denote significant difference among groups for % of capacitated cells in the time (BC: not statistically different than either B or C), SEM=4,01%; p<0,001

3.4 Discussion

The results showed that the optimum time for sperm incubation under capacitating conditions is after at least 3 hours. The 3 hours incubation under capacitating conditions has been used in human studies before as the optimum time of capacitation *in vitro* (Nixon et al., 2011, Redgrove et al., 2012) and it has been shown that spermatozoa incubated under non-capacitating conditions did not bind to the ZP while sperm cells incubated for 3 hours under capacitating conditions bind the ZP at a percentage of about 27% of the sperm population (Redgrove et al., 2011). Spermatozoa of the 40% fraction appear to be less efficient to undergo sperm

capacitation. From these results it appears that 40% sperm cells get capacitated later in time than the 80%. This could be due to the presence of more immature cells in the 40% fraction (Ollero et al., 2000). Irregular characteristics of these lower density human sperm fractions, such as higher activity of creatinine phosphokinase (CK), associated to cytoplasmic retention, as well as abnormal head size indicate cellular immaturity and failure to complete spermatogenesis (Huszar and Vigue, 1993) (Huszar et al., 1998).

Since the only difference between the two media used for this comparative motility analysis was the presence of bicarbonate in the capacitating medium, the conclusion from these results is that bicarbonate enhances motility (both total and progressive motility) and speed at least for spermatozoa recovered from the 80% fraction. Total motility percentage was not enhanced for the 40% fraction cells but progressive motility and speed were. The enhancing effect of bicarbonate in sperm motility was expected as it has been known for decades that bicarbonate stimulates the activity of adenylyl cyclase and causes an elevation of intracellular cAMP level (Okamura et al., 1985, Okamura et al., 1986) which is involved in the increase of sperm motility (Luconi et al., 2005).

Motility and capacitation percentage results were repeatable among independent experiments; however some variation of the percentage of capacitated cells was detected among donors in both sperm fractions. This observation could reflect the differences among individuals in the ability to undergo sperm capacitation. It should be noted that sperm capacitation was assessed using an indirect method with limitations that provides only an approximate estimation of capacitated cells on a population level, rather than the status of capacitation of individual cells.

CHAPTER 4

Exposure of acrosomal proteins during sperm capacitation

4.1 Introduction

Previously it was suggested that only plasma membrane proteins were implicated in sperm-ZP adhesion (see General Introduction). However, recent studies have demonstrated that acrosomal molecules are implicated in this adhesion process (Tardif, 2011; (Buffone et al., 2008) and therefore it is important to study acrosomal molecules more intensively and elucidate their functions during fertilization.

The acrosome of sperm cells contains a large number of proteins. Some of them have been identified as sperm-egg adhesion molecules in mammalian fertilisation but new data challenge their function as unique adhesion molecules by the detection of multimeric protein complexes located on the surface of human spermatozoa binding the ZP. Moreover, a subset of these protein complexes was found to be differentially expressed during sperm capacitation (Redgrove et al., 2011). These data suggest a more complex mechanism of sperm-egg interaction than was previously thought, where several proteins participate in the adhesion process that needs to be further elucidated.

Acrosomal proteins such as zonadhesin (Tardif et al., 2010b) and sp56 (Wassarman, 2009) have been detected on the surface of live capacitated mouse spermatozoa suggesting a role of those molecules in sperm-egg adhesion during sperm capacitation. It is not known if a similar event occurs in humans.

This chapter will study the exposure of three acrosomal molecules with a known role in sperm-ZP interaction (zan, sp56, sp32) on the surface of human spermatozoa and examine the association of such an exposure to sperm capacitation.

4.2 Experimental procedures

Experimental design

The study of acrosomal protein exposure was achieved using immunofluorescence on live cells, evaluating zan, sp32 and sp56 exposure at the surface of spermatozoa incubated under conditions that support *in vitro* capacitation (CM, 37⁰ C, 5% CO₂) and that don't support *in vitro* sperm capacitation (NCM, 37⁰ C). Immunofluorescence was conducted prior to (at time 0) and after incubation in CM and NCM (after 4 hours). Negative controls used for these experiments include 1) incubation with only the secondary antibody (goat-anti rabbit) and 2) incubation with a primary antibody against a protein not present in human spermatozoa.

Semen preparation

The sperm samples were from healthy donors and the semen was prepared as described in Chapter 2, using the 80% fraction.

Evaluation of acrosomal proteins exposure

Immunofluorescence was conducted on live spermatozoa (no permeabilization of the cells) (Tardif et al., 2010b) in order to detect the presence or absence of acrosomal proteins on the surface of intact spermatozoa.

Isolated spermatozoa were incubated at 37⁰ C in CM (5% CO₂) or NCM (≈concentration 25 million cells/ml) for 4 hours and antibodies were added to the cells half way through the incubation period (after 2 hours). Different acrosomal proteins were examined to detect their presence at the sperm surface during

incubation using antibodies directed to ACBP/sp32 (mouse), ZP3R/sp56 (mouse), CRES (mouse) (Cystatin related epididymal spermatogenic) or zonadhesin D3 domain (pig).

After the completion of the incubation with the primary antibody [primary antibodies (a-zan D3, a-sp32, a-sp56 see Chapter 2) were added at a 1:1000 dilution after the first 2 hours of cell incubation in the CM and allowed to interact with the cells for 2 hours] the cells were centrifuged for 3 min at 500 g. The supernatant was removed and the pellet was resuspended and incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen A11008) as a secondary antibody for 45 minutes at 37⁰ C. The cells were centrifuged again and resuspended in equal volume of NCM (any physiological sperm solution could be used for this step wouldn't make a difference), fixed with 1% of formaldehyde (F1268) solution and put on a slide to be observed under the fluorescent microscope (Zeiss Axiostar, excitation 495 nm/emission 519 nm. One slide was examined per treatment while over 125 cells were scored per slide. Cells with any clear fluorescent signal anywhere over the acrosome were counted as positive.

Induction of acrosome reaction in capacitated cells and evaluation of acrosomal proteins exposure

The induction of AR of cells under capacitated conditions was carried out using the same principle as described previously (Chapter 3). After the completion of sperm incubation under capacitating conditions (4 hours in CM, 37°C, 5% CO₂), an aliquot was incubated with 10µM of calcium ionophore A23187 and another aliquot with solvent (1% DMSO, control) for 15 min at 37°C, 5% CO₂. The evaluation of exposure

of the acrosomal molecules was then made with the same immunofluorescence method as described above.

4.3 Results

4.3.1 Exposure of acrosomal molecules on the sperm surface

Previous data (Figure 3.11 in Chapter 3) suggested that the optimum of sperm capacitation *in vitro* using 80% fraction was after the at least 3 hours incubation in CM (37⁰C, CO₂ 5%). Therefore, examination of zan exposure was carried out after 4 hours of incubation under *in vitro* capacitating conditions. For this study percentage of zan exposure was examined at the beginning of the experiment prior to incubation (T0) and after 4 hours of incubation in CM (T4h). The number of sperm cells exposing zonadhesin on their surface was significantly higher after 4 hours of incubation in CM compared to T0 (Figure 4.1; 1.1 vs 7.5% for T0 and T4h respectively).

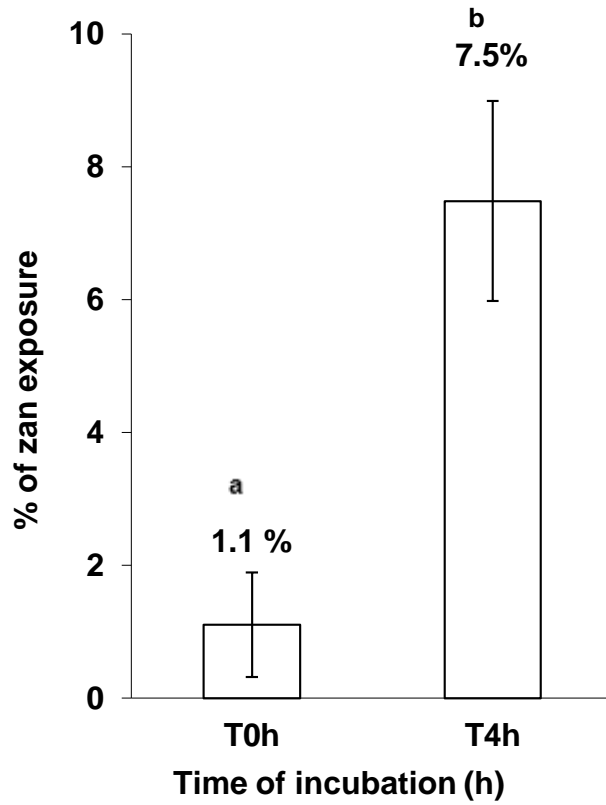


Figure 4.1 Percentage (%) of zan exposure during sperm capacitation. Cells examined before incubation, time zero (T0), and after 4 hours (T4h) of incubation in CM (37°C, 5% CO₂). Average \pm SEM. n=11 independent experiments (6 donors). Different letters (a, b) denote significant difference for exposure in T0 and T4h. Exposure of zonadhesin is significantly higher after 4 hours of incubation, $p < 0.001$

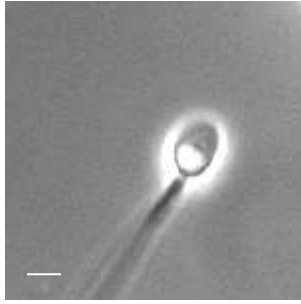
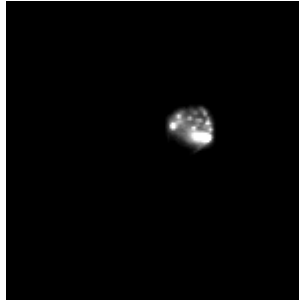
(a) Phase contrast**(b) Fluorescence**

Figure 4.2 Human spermatozoa live labeled with a-zan (antibody against zonadhesin) after the application of immunofluorescence. Representative image of zan positive cells of 11 donor samples examined for zonadhesin exposure during *in vitro* capacitation. On the left **(a)** are cells as seen by phase contrast microscopy while on the right **(b)** are cells under fluorescent conditions. The latter shows signal of a-zan over the acrosome.

During these experiments negative controls were used to ascertain the signal was associated with the primary antibody and not the secondary antibody. Moreover, the specificity of the primary antibody (rabbit IgG) and the stickiness of the sperm cells was tested by using an irrelevant antibody (not present in sperm cells). The negative controls gave no signal as shown in Figure 4.3.

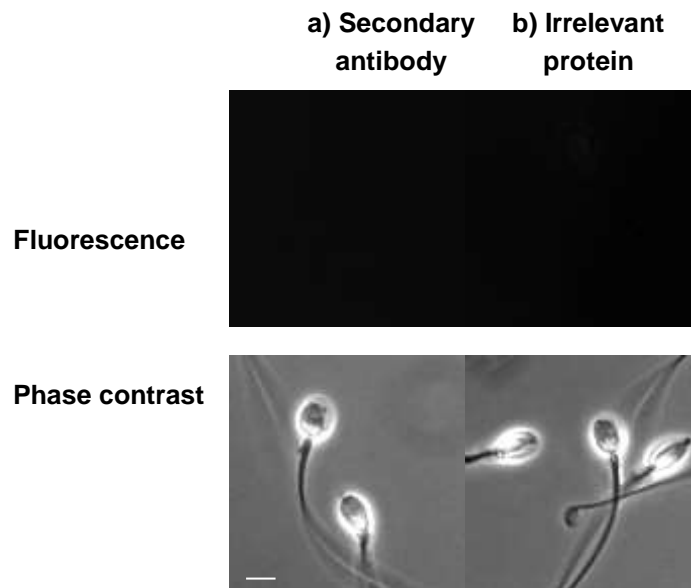


Figure 4.3 Live human spermatozoa **a)** incubated with only the secondary antibody used during immunofluorescence **b)** labeled with antibody against an irrelevant protein non present in human spermatozoa (D3p18 domain of mouse zonadhesin). Representative image of the negative controls used in each immunofluorescence experiment for at least 11 donor samples. On the bottom are cells as seen by phase contrast microscopy while on the top is shown the absence of fluorescent signal for both, secondary antibody and irrelevant protein target. Bar represents 5 μ m.

Following the examination of zan at the sperm surface, two more acrosomal proteins related to sperm-egg adhesion were examined, sp56 and sp32. Both of these proteins are detectable on sperm surface after incubation under capacitating conditions (4 hours in CM, 37⁰ C, 5% CO₂). The mean percentage of cells expressing these proteins was 9% for sp56 and 9,2% for sp32. There was a significantly higher % of cells

labeling with the antibody after 4 hours incubation under capacitating conditions than at time 0 (Figure 4.4).

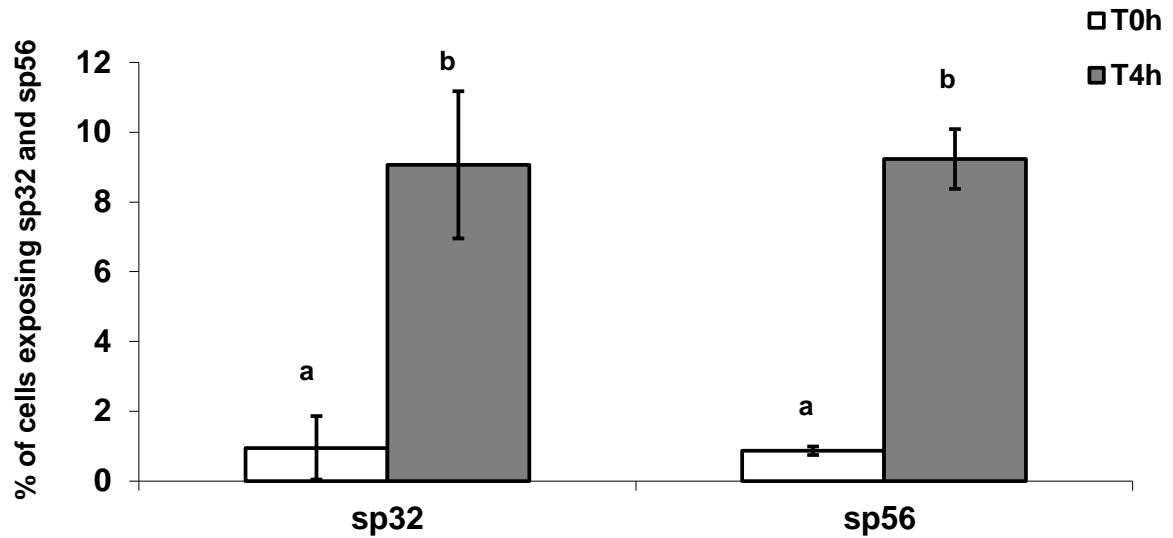


Figure 4.4 Percentage (%) of cells labeled with a-sp32 and a-sp56 at the cell surface before (T0h) and after 4 hours incubation (T4h) in CM (37°C, 5% CO₂). Average ±SEM. n=11 independent experiment (10 donors). Exposure of both sp56 and sp32 is significantly higher after 4 hours of incubation. Different letters (a, b) denote significant difference for exposure in T0 and T4h of the two proteins. $p < 0.001$

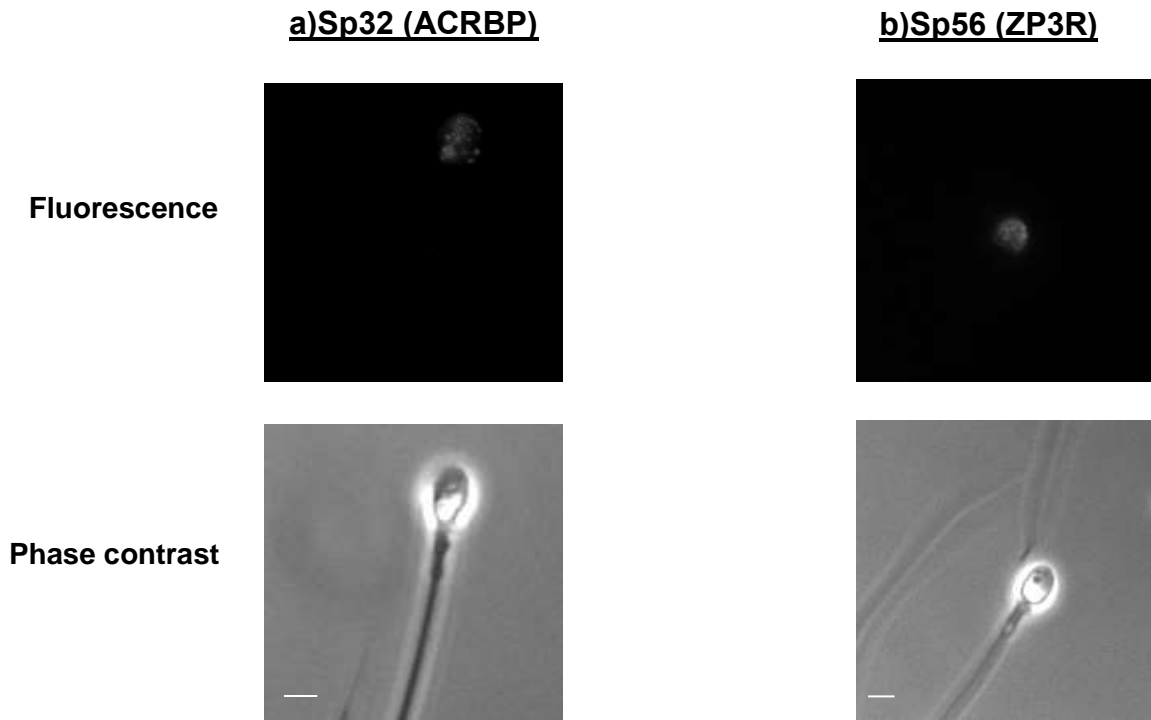


Figure 4.5 Live human spermatozoa labeled (no cell permeabilization) with **a)** a-sp32 (antibody against sp32) and **b)** a-sp56 (antibody against sp56) after the application of immunofluorescence. Representative images of sp32 and sp56 positive cells of 11 donor samples examined for protein exposure during *in vitro* capacitation. At the bottom are cells as seen by phase contrast microscopy while at the top are cells under fluorescent conditions. The latter shows signal of a-sp32 on the left and a-sp56 on the right over the acrosome. Bar represents 5 μ m.

4.3.2 Association of acrosomal molecule exposure with sperm capacitation

Even though there was a significantly higher cell exposure for all three acrosomal proteins after incubation under capacitating conditions compared to the zero time, in order to further investigate whether this increase of exposure was related to sperm capacitation two more experiments were carried out. The first experiment was designed to compare the percentage of exposure of acrosomal proteins on the cell surface between cells incubated in CM and NCM. If the acrosomal molecule exposure is related to sperm capacitation there should be a significantly higher percentage of protein exposure in cells incubated under conditions that support capacitation *in vitro* compared to sperm incubation under conditions that do not support capacitation. For that reason spermatozoa were split in two samples, one incubated for 4 hours in CM and the other in NCM. Then the cells were labeled with the acrosomal molecule antibodies separately.

All three molecules were exposed at a percentage significantly higher when incubated in CM (~9%) than in NCM (~1-2%) (Figure 4.6). This result is consistent with the previous figures of protein exposure after incubation in CM. For this particular experiment one more antibody was used, against another acrosomal protein, called CRES (cystatin related epididymal spermatogenic). CRES was used as a negative control since it is not a molecule with a known role in early stages of sperm-egg interaction, located at the equatorial segment, and therefore would not be expected to see a similar exposure to that of the previous acrosomal molecules.

There was no significant difference in the % of cells labeling with CRES (~2.5% of sperm cells) in cells incubated in CM compared to those incubated in NCM (Figure

4.6). In addition to this experiment sperm motility was recorded before and after the 4 hours of incubation of the cells in CM and NCM in order to ascertain that the sperm cells remain motile during the incubation. As shown in Figure 4.7 motility parameters (total motility percentage, progressive motility percentage) remain stable over time of incubation for both media.

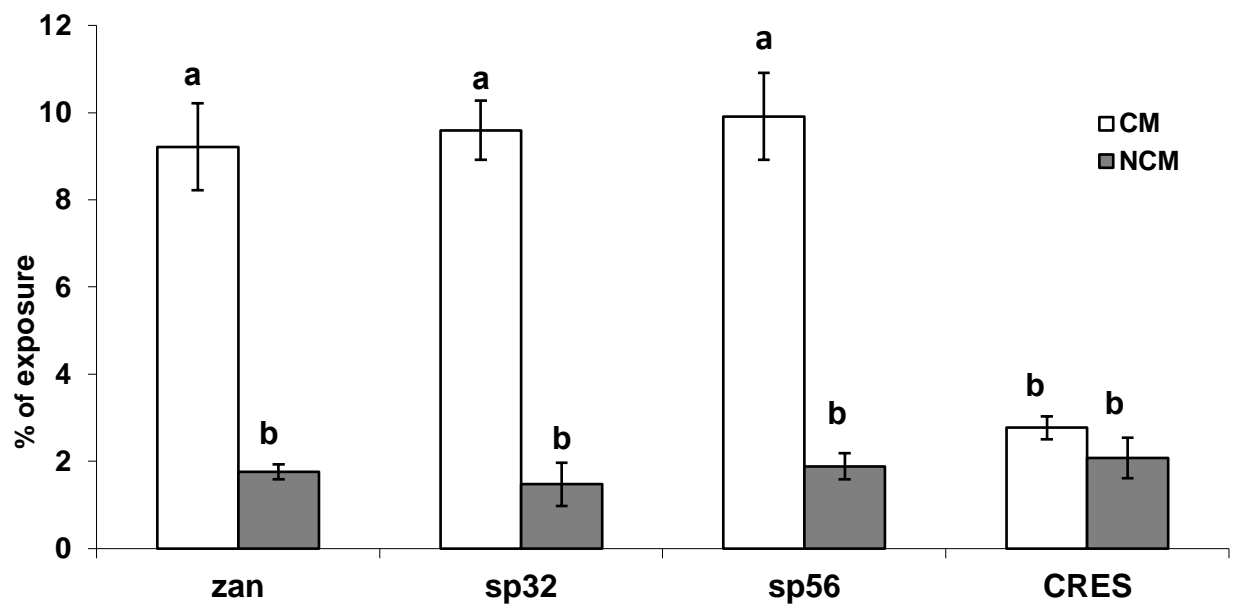


Figure 4.6 Percentage (%) of cells labeled with antibodies against the acrosomal molecules (zonadhesin, sp32, sp56, CRES) at the sperm surface after incubation (4h) in CM (37°C, 5% CO₂) or NCM, average ± SEM; n=6 independent experiments. Different letters (a, b) denote significant difference for acrosomal exposure among medium; p< 0.01. No significant difference for CRES exposure between medium; p= 0.96

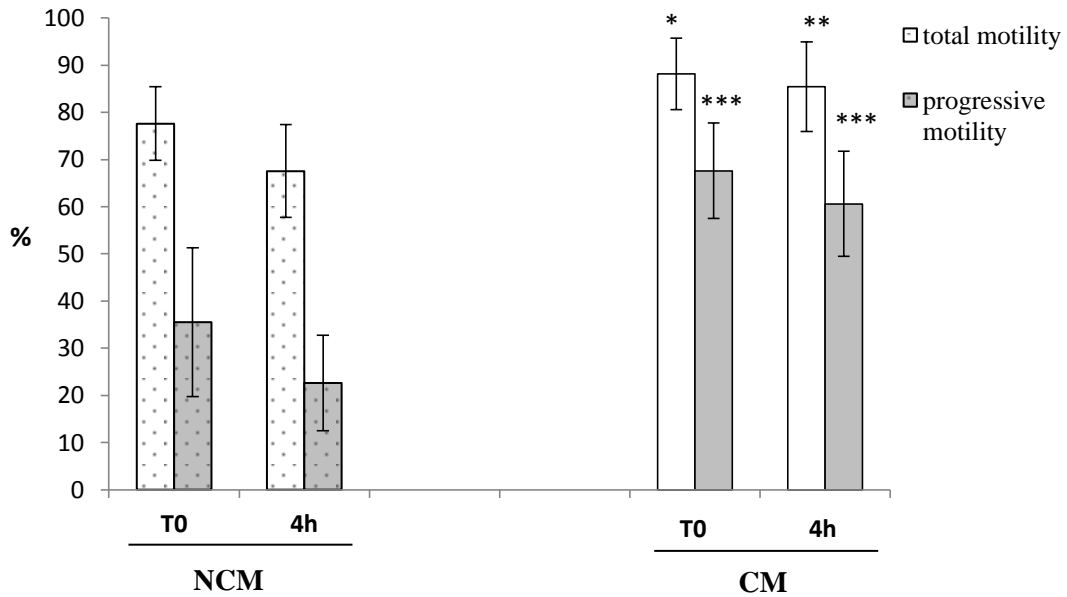


Figure 4.7 Percentage (%) of total and progressive motility

Total and progressive motility of sperm cells recovered into the 80% fraction after incubation (time 0 and 4 hours) in NCM (at 37°C) or in CM (at 37°C, 5% CO₂) n=6 (6 ejaculates from healthy donors). Average ± SEM. *** p<0,001; ** p<0,002; * p<0,011 (asterisks denote significant difference between T0, T4h of incubation in NCM and CM)

Another set of experiment was designed to confirm the relationship between acrosomal exposure and sperm capacitation. The hypothesis was that after the induction of acrosomal reaction of the *in vitro* capacitated sperm population with calcium ionophore, a decrease of protein exposure should be seen. More specifically the acrosome reacted cells should not exhibit any fluorescent signal (reflecting the proteins' presence on the cell) due to the loss of the acrosome after the acrosomal reaction. Therefore, sperm cells were incubated under capacitated conditions as before (in CM, 37°C, 5% CO₂) and then part of the population treated with calcium ionophore. In the sperm population treated with calcium ionophore was found that

protein exposure for all 3 proteins studied was significantly decreased at the level of 6-8% compared to the control (sperm population incubated in CM) (Figure 4.8)

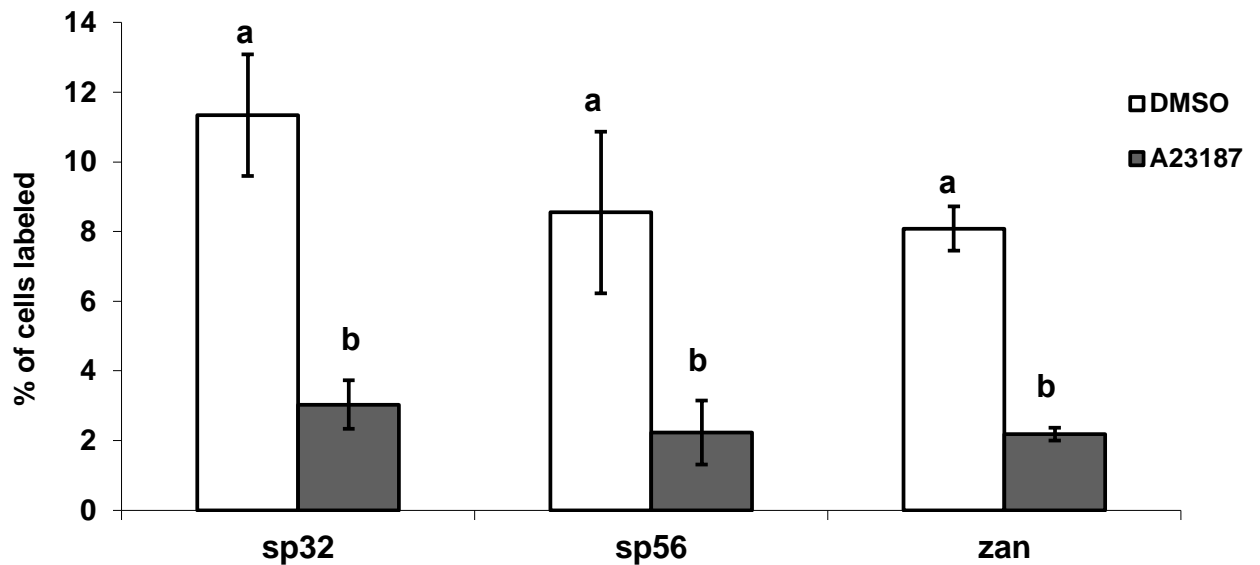


Figure 4.8 Percentage (%) of cells exposing acrosomal molecules (sp32, sp56, zonadhesin) at the sperm surface after induction of acrosome reaction using calcium ionophore A23187 in dark bars and with just the solvent (DMSO) in white bars. Average \pm SEM; n=6 independent experiments. Different letters (a, b) denote significant difference for exposure among treatment; $p < 0.001$

4.4 Discussion

This study showed that zonadhesin is detected on the cell surface of human spermatozoa under conditions that support capacitation *in vitro*. Zonadhesin is not the only acrosomal protein exposed at the cell surface. Positive results for two more

acrosomal proteins, sp56 and sp32 (Figure 4.4) were observed demonstrating that was that at least 3 acrosomal molecules with a role in sperm-egg interaction are exposed on the human live sperm surface.

Neither of the negative controls used for the immunofluorescence experiments (secondary antibody, irrelevant protein target not present in human sperm cells) produced a fluorescent signal (Figure 4.3) indicating that fluorescence seen over the acrosome during labeling with a-zan, a-sp32, a-sp56 was not due to nonspecific binding or cell damage but was the results of specific binding of the primary antibodies against the acrosomal molecules. The results of acrosomal protein exposure were repeatable for all donor samples used in this study showing an average percentage of exposure of 8-10% for the 3 acrosomal proteins. The results of this study were consistent and therefore robust.

Exposure appears to be specific to sperm capacitation as incubation of the cells in non-capacitating medium did not increase the percentage of exposure compared to incubation in capacitating medium for any of the proteins examined. In addition to low percentages of protein exposure during incubation in NCM (Figure 4.6) the decrease of labeling after incubation with the calcium ionophore, the acrosome reaction inducer, (Figure 4.8) suggests a relation of acrosomal protein exposure to sperm capacitation.

Considering results on the time course of sperm capacitation described in chapter 3 (Figure 3.11), sperm capacitation *in vitro* at its peak (3-4 hours of incubation in CM) displays a percentage of 25-30% of capacitated cells in the sperm cell population. As mentioned above, protein exposure observed during these experiments is in the range of 8-10% for all 3 molecules after 4 hours of incubation in CM. Therefore, a loose

association between these two separate results could possibly lead to a rough estimation of capacitated cells exposing the acrosomal proteins, however without examining individual cells it is not possible to provide a definitive answer.

These results of zonadhesin exposure at the surface of live human spermatozoa under capacitating conditions are in an agreement with observations in the mouse. In the mouse (Tardif et al., 2010b) zonadhesin was only detectable on the sperm surface after incubation in conditions that support sperm capacitation (23,6% of labeled cells after incubation in capacitating medium vs 2,6% of labeled cells after incubation in non-capacitating medium).

The data provided by this study are consistent with the new model of AR which, instead of a one off event, may be a more controlled continuum as recent data, showing AR is initiated before sperm bind the oocyte, suggest (Inoue et al., 2011); (Jin et al., 2011). Exposure of zonadhesin in human, along with this of the other acrosomal molecules, could possibly imply a direct acrosomal protein involvement in sperm binding to the ZP, especially as this protein exposure is related to sperm capacitation *in vitro* which reflects sperm fertilizing ability (Austin, 1951, Chang, 1951), but that requires further investigation.

After the first observations of zonadhesin exposure in the mouse, a model of Tardif et al. has been proposed on the role of an acrosomal molecule like zonadhesin on sperm-egg adhesion. According to this model, zonadhesin is exposed periodically through “fusion pores” in the open and closed state binding on the ZP and finally the AR is initiated (Tardif and Cormier, 2011). This proposed model could possibly explain the mechanism of exposure for those acrosomal molecules on the cell surface especially as the formation of fusion pores by docking of the outer acrosomal membrane to the

plasma membrane, before acrosomal exocytosis, has been reported in human spermatozoa (Zanetti and Mayorga, 2009).

CHAPTER 5

Characterization of two acrosomal molecules

5.1 Introduction

The key events of post-translational modifications in sperm cells are not fully elucidated and there is a lack of data for acrosomal protein forms into the mature sperm cells especially in humans, as well as for these forms' clinical relevance in spermatozoa.

Data on different sperm proteins show that post-translational modifications of acrosomal proteins participating in fertilisation process can affect their function, such as zonadhesin where post-translational modifications alter its ZP-binding activity in pig. Hickox et al. suggested that a heterogeneous combination of specific proteolysis and intermolecular disulfide bond formation in the sperm head leads to the formation of multiple forms of zan with differing avidities for the ZP (Hickox et al., 2001). Another example is ERp57, a protein present in the acrosome with a role in sperm-egg fusion, which has been mentioned for post translational modifications during sperm capacitation leading to different expression levels of the protein isoforms in human (Zhang et al., 2007, Wiwanitkit, 2010). Moreover sp32/proacrosin binding protein, a protein that is tyrosine phosphorylated during capacitation (Tardif et al., 2003), has recently been found to be misprocessed in an infertile mouse model. For this study PCSK4 null mice were used in which sp32 precursor was found not to be proteolytically processed to its mature form suggesting that sp32 misprocessing can lead to infertility (Tardif et al., 2012).

Human zonadhesin cDNA has been isolated and sequenced in 2001 (Wilson et al., 2001) revealing a 300 KDa protein precursor. Animal studies have detected the

presence of three zan polypeptides with molecular weight 300, 105 and 45 KDa in ejaculated pig spermatozoa (Hickox et al., 2001) as well as 125 and 250 KDa polypeptides in mouse (Tardif et al., 2010a) but there are not enough data about its forms in mature human cells, other than one study published in 2010 which revealed the presence of 50 and 60 KDa D3 polypeptides in ejaculated spermatozoa (Tardif et al., 2010a). Additionally, even though human sp32 61 KDa precursor has been isolated (Ono et al., 2001) and sequenced there is no information at all about sp32 polypeptides in mature human cells either. The protein's 32 KDa mature form has been detected in porcine (Baba et al., 1994) and mouse (Tardif et al., 2012) sperm.

In this chapter are presented Western blot results of sp32 and zan isolated from mature human spermatozoa incubated under capacitating (CM, 37°C, 5% CO₂) and non-capacitating conditions (NCM, 37°C). The objective was to detect the protein forms present in these cells and any potential difference of protein isoforms among cell incubation in CM and NCM. Moreover, zonadhesin was isolated from samples originating from sub fertile men attending the IVF clinic, making a preliminary comparison between zan's forms in samples from sub fertile men and donors.

5.2 Experimental procedures

Semen preparation

Semen from donors and patients who attended the IVF clinic was prepared with the application of the density gradient as described in Chapter 2.

Detection of sperm proteins by Western blotting analysis

Once the concentration of sperm cells was established (~25 million cells/ml) and the volume identified, the cells were centrifuged. After centrifugation they were split and diluted in capacitating medium (CM) in one tube and in non-capacitating medium (NCM) in another, incubated for 4 hours. The cells from both tubes were centrifuged for 5 minutes in 17,000 g.

Next, the sperm pellet was resuspended in an incomplete sample buffer (no reducing agent) [Laemmli Sample buffer (4X): 0,5 M Tris-HCl pH 6,8, 8% SDS (Sigma L4509), 40% glycerol (Sigma G5516), 0.008% bromophenol blue (Sigma B0126)] at the concentration of 0,25 million cells per μl (10 million cells into 40 μl) and the sample was heated at 95⁰ C for 5 minutes. The insoluble material was removed from the sample by centrifugation (17,000 g for 5 min). The sperm extract was stored at -20 or -80°C until its use for electrophoresis.

Solubilized proteins were separated on 12% polyacrylamide gels (Acrylamide/bis ratio Sigma A6050). The volume used per lane was 10 μl (2 million cells per lane) and dithiothreitol (DTT) was used to reduce proteins (50 mM final). For Western blot analysis proteins were electrophoresed for 1 hour under current constant (20 mA/gel,

200 V limit) and transferred onto nitrocellulose membrane (Biorad 162-0112) at voltage constant (50V) for 2 hours at 4°C.

Membranes were blocked in 2,5% skimmed milk in Tris-Buffered Saline-Tween 20 (TBS-T) for 1 hour before being incubated at 4°C overnight with the primary antibody (a- pig D3 Zan, a- mouse sp32 diluted 1:10000 in TBS-T). To detect the primary antibody bound to proteins, after 3 washes with TBS-T the membranes were incubated with a secondary antibody conjugated to peroxidase (HRP-goat anti-rabbit) for 45 min (1:3.000 dilution). The reactive bands were detected using a mixture of peroxide and luminol (50-50) as a developer solution.

5.3 Results

5.3.1 Characterization of zonadhesin and sp32 forms in mature spermatozoa

Figure 5.1 shows the Western blot analysis for zan isolated from spermatozoa incubated in CM and NCM, revealing two bands one at 50 KDa and one more close to 60 KDa for sperm cells incubated in either medium, CM or NCM (Figures 5.1). However, Figure 5.2 shows that in one blot one more band appeared at about 70 KDa. As for sp32 two bands were identified, a major band at 32 KDa and a smaller one at 60 KDa in spermatozoa incubated either under capacitating conditions (CM) or non-capacitating conditions (NCM) as illustrated in Figure 5.3.

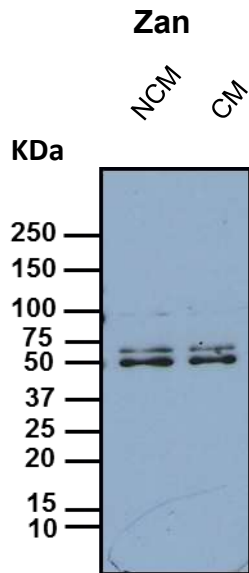


Figure 5.1 Molecular weight (KDa) of zan in mature spermatozoa isolated from donors, n=3 (representative figure of 3 independent experiments, 3 donors), after incubation in NCM and CM for 4 hours.

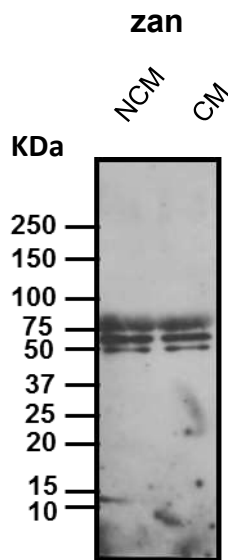


Figure 5.2 Molecular weight (KDa) of zan in mature spermatozoa isolated from donors, n=1 (1 donor different from the 3 donors used for results in Figure 5.1), after incubation in NCM and CM for 4 hours.

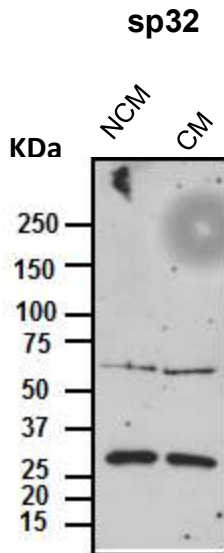


Figure 5.3 Molecular weight (kDa) of sp32 acrosomal protein in spermatozoa isolated from donors, $n = 3$ (representative figure of 3 independent experiments, 3 donors), after incubation in NCM and CM for 4 hours.

5.3.2 Characterization of zonadhesin forms in sub fertile men

Figure 5.4a shows that patient samples gave a variety of zan polypeptides and for some of them revealed bands not previously seen in donor samples (patients 8 and 10-14). Different patient samples display different motility patterns, varying from close to zero (e.g. patients 1, 2) to roughly 85% (e.g. patients 12, 13) (Figure 5.4b).

Sub fertile men

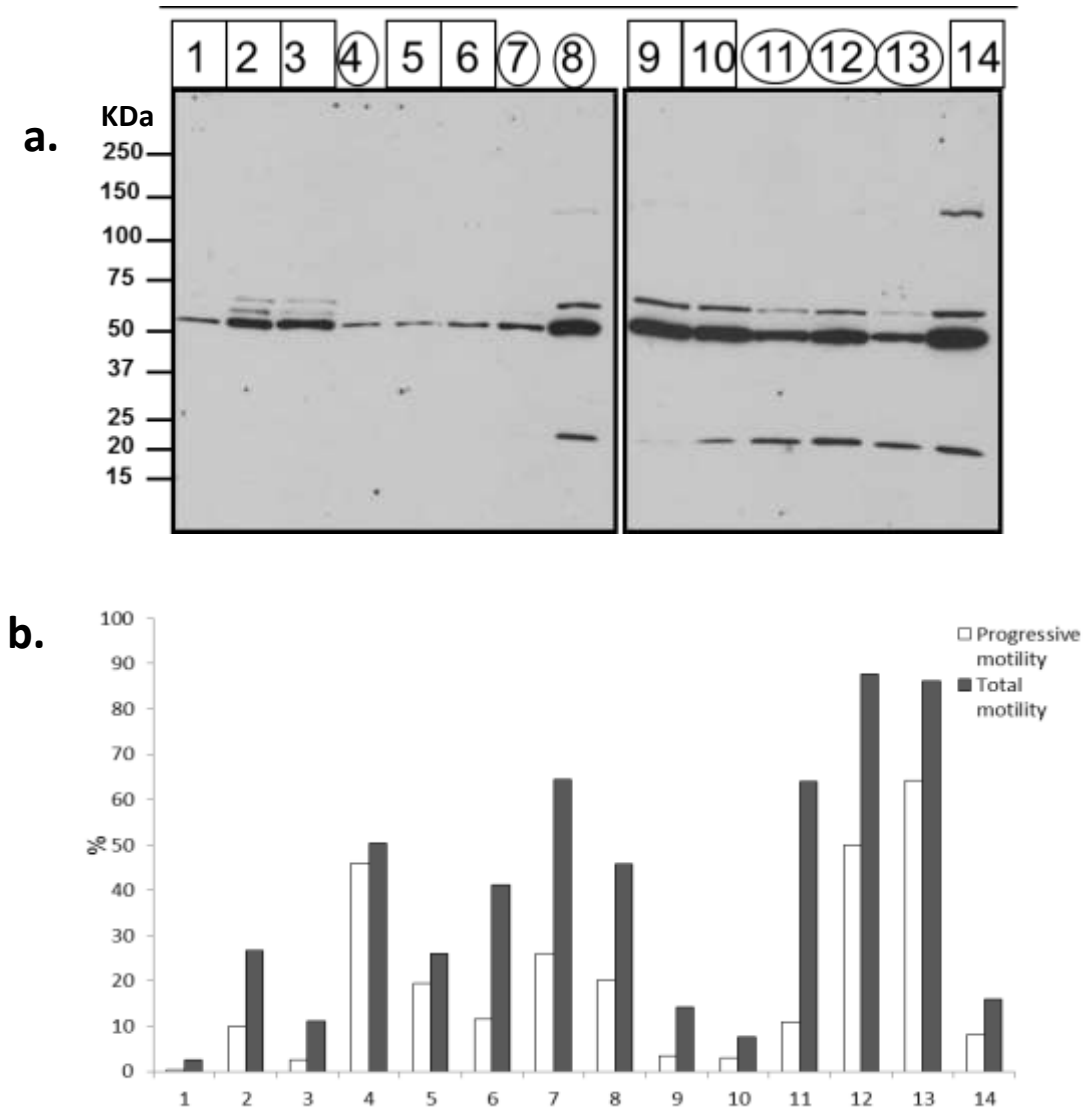


Figure 5.4 a. Molecular weight (KDa) of zan in human spermatozoa isolated from patient samples, n =14 (14 different patient samples). Patient numbers in squares and circles are associated with lower and higher motility respectively **b.** Total motility and Progressive motility percentage (%) for each patient sample used for Western blot analysis.

5.4 Discussion

This study revealed three D3 zan polypeptides present in mature spermatozoa, at 50, 60 and 70 KDa. In 3 donors only the 50 and 60 KDa isoforms were present (Figure 5.1) while in one donor (different than used previously) three isoforms were detected (Figure 5.2). Tardif et al. (Tardif et al., 2010a) had also detected the two zan forms of 50 and 60 KDa, but in this study a further band at 70 KDa was detected in one donor. The Western blot analysis also detected two forms of sp32 in mature sperm cells, a major one at 32 KDa and a smaller one at roughly 60 KDa (Figure 5.3).

These results could possibly imply that both proteins (zan, sp32) are proteolytically processed into lower molecular weight (Mr) forms in the mature human spermatozoa since the Mr of their precursor proteins is different than found for their forms in the mature cells. However it seems for these acrosomal proteins in the sperm samples studied, that no significant post-translational modification occurs during sperm capacitation that could lead to the formation of different Mr forms, as the same bands were detected for cells incubated in CM and NCM. In pig on the other hand, multiple zan forms have been detected in freshly ejaculated uncapacitated spermatozoa (105 KDa, 45 KDa and additional 60-90 KDa polypeptides) compared to the two predominant bands (105 and 45 KDa) detected on capacitated cells (Hickox et al., 2001). ZP3R/sp56 is another example of acrosomal protein that is proteolytically processed during *in vitro* capacitation in mouse since ZP3R normal processing from 67 KDa to 43 KDa by proteases is reduced under non capacitating conditions (Buffone et al., 2009). Plasma membrane proteins have also been reported to be proteolytically processed during capacitation, such as ADAM2; a protein with a role

in sperm-egg interaction which is processed from a 45 KDa protein to 27 KDa during capacitation, possibly through PCSK4 enzyme activity (Iamsaard et al., 2011).

Results of zan characterization in mature cells from patient samples revealed multiple forms of zan rather than just the two predominant forms of 50 and 60 KDa seen before in donor samples. The Western blot analysis for zan in patient samples detected additional bands of 20 and 130 KDa in some patients compared to donor samples. Furthermore in some of the patient samples only one form of 50 KDa was detected, unlike the donor samples (Figure 5.4 a). However, this polypeptide variation among patient samples was not associated with low motility samples as shown in Figure 5.4b. For example patients 12 and 13 who exhibit an extra 20 KDa band, compared to donor samples, have over 80% of motile spermatozoa.

These results were repeatable among the donor and patient samples using the same protocol for protein isolation and Western blot analysis for both, donors and patients. The primary antibodies' specificity was not tested in this study as the peptides for these were not available to use, but these antibodies against D3 polypeptides of pig zan and recombinant mouse sp32 have been used in previously published studies (Tardif et al., 2010a, Tardif et al., 2012). Specificity of the secondary antibody was tested, as a negative control, and it did not provide any signal when the membrane was incubated with only the secondary antibody.

That is the first indication of different isoforms of human zan present in IVF patients however, this study has limitations that do not allow the formulation of a robust conclusion concerning to what is normal and what is not in terms of human zan isoforms in mature spermatozoa. The major limitation is the low number of experiment repetitions for both donor and patient samples. Limited numbers of

repetitions for donor samples do not permit a thorough investigation of variation among men. In humans several polymorphisms of zan gene (Gasper and Swanson, 2006) have been reported and therefore a variation of the protein mature forms among men population would probably be expected. Thus, a more extended study using a larger number of donor and patient samples could elucidate and establish the subnormal zan forms possibly related to male infertility.

CHAPTER 6

General Discussion and Conclusion

The current study focused on the investigation of potential acrosomal molecule exposure on the cell surface of live human spermatozoa in order to determine; a) if zonadhesin (zan) is detected on the spermatozoa surface, b) if other acrosomal molecules with a role in sperm-egg interaction are also exposed and c) if this event of acrosomal protein exposure is associated with sperm capacitation. Additional aims of this project were to detect the acrosomal proteins' forms present in the mature human spermatozoa incubated under capacitating and non-capacitating conditions and compare these protein forms of zan to those observed in samples from sub fertile men providing preliminary data on their clinical relevance.

By establishing the optimum time condition of sperm capacitation *in vitro* using human spermatozoa (Chapter 3), acrosomal molecules were shown to be accessible at the sperm surface of cells incubated under capacitating conditions. This event was associated with sperm capacitation, supported by data produced from control experiments. More specifically, with respect to Chapter 4, acrosomal proteins became accessible on the sperm surface after incubation under capacitating conditions but not prior to incubation. However, as labeling of individual sperm for all proteins as well as capacitation status was not performed, it was not possible to determine the status of each cell. For this reason the induction of AR was used to determine if sperm cells exposing acrosomal molecules were in a capacitated state at the population level since only capacitated spermatozoa have the potential to undergo AR in response to an agonist. Effectively when AR was induced, the percentage of sperm cells exposing acrosomal molecules was significantly reduced.

To increase our knowledge on these acrosomal proteins in sperm-egg adhesion functional tests such as zona-binding assay would very helpful. It would be interesting

to see if, and in what percentage of spermatozoa, exposing the acrosomal molecules bind to the ZP, compared to cells that don't expose them. Additionally it would be interesting to determine, perhaps using inhibition of each of the proteins with specific antibodies their role in adhesion. Another experiment that could add to our knowledge on this exposure is multiple labelling of the cells for all three proteins. This could reveal if the acrosomal molecules are exposed all together on the spermatozoa or only one or two of them are detected on the surface of each particular cell. If they are all exposed together it would potentially indicate that each of them are involved in adhesion and fertilisation rather than a single molecule on each cell. This potential cooperation should perhaps be expected since multimeric protein complexes located on the surface of human capacitated spermatozoa binding the ZP have been recently reported in a human study (Redgrove et al., 2011).

With respect to chapter 4, these data support the idea of several molecules performing the same function i.e. sperm egg-ZP adhesion. Previously it has been shown that the fertilisation process may use multiple ligands/receptors. A disadvantage of a binary system would be that abnormality in one protein would prevent reproduction and thus continuation of the species. Knock-out mice experiments suggest that fertilization is not a binary process. In these cases (e.g. *zan*, *sp56*, *acrosin*, *galt1*) the transgenic animals were proven to be completely fertile after gene disruption. (Tardif et al., 2010b, Muro et al., 2011, Adham et al., 1997, Lu and Shur, 1997).

Acrosomal molecules associated with exposure during sperm capacitation (*zan*, *sp56*, *sp32*) are known to be involved in sperm-ZP adhesion and fertilisation cell signalling, and therefore their exposure relevance to sperm capacitation suggests that this is an important event during the sperm-egg interaction process. It could possibly indicate that some cases of infertility could be attributed to failure of acrosomal molecule

exposure on the sperm surface, reflecting a miss-regulation of the capacitation process. There are cases of male infertility where spermatozoa appear to be normal in terms of motility and morphology but still unable to fertilise probably due to a sperm dysfunction, these cases are classed as unexplained or idiopathic male infertility where the cause of infertility incident is not known (Hamada et al., 2012). Perhaps male sub-fertility is, at least partially, associated to a protein miss-regulation impacting sperm function as sperm-ZP interaction.

With respect to Chapter 5, data revealed a subset of zan forms present in spermatozoa from sub-fertile men rather than the two predominant forms observed in donor samples. This data and a study from Tardif and colleagues (Tardif et al., 2010a), raise the question: is a potential misprocessing of zan implicated in the reduction of sperm fertilising potential? Pro hormone convertases could be implicated in sperm protein processing. Interestingly, Tardif et al. demonstrated by using an infertile mouse model deficient for a pro hormone convertase (PCSK4), that sp32 was not post-translationally modified during spermatogenesis. These PCSK4 null spermatozoa exhibited only the proform of sp32, while sp32 precursor was completely processed in the wild type (Tardif et al., 2012). In order to answer this question for human zan a thorough investigation of zan forms in fertile versus sub-fertile men is necessary. Once the normal forms of zan present in mature spermatozoa are established in a wide range of donors, it would be possible to conduct a robust conclusion on the abnormal forms of the protein present in spermatozoa from sub-fertile men. It would be interesting to investigate any association of these abnormal forms with specifically sub-fertile men who are defective in sperm capacitation.

Collectively, these data could render zan as an efficient clinical biomarker of sperm dysfunction in the future and may have the potential to be used to assess sperm

selection for IVF, providing that further research of the protein's properties focused on its clinical relevance. The development of such a diagnostic test that efficiently correlates with sperm fertilizing ability is of high importance for diagnosis and treatment of male subfertility since normal semen analysis results do not guarantee fertilizing capacity.

CHAPTER 7

Appendix

7.1 Indication of donor variation for labeling with acrosomal proteins antibodies

| Donor Numbers | ZAN | SP56 | SP32 |
|---------------|------------------|---------------|------------------|
| D176 | 7,4 | 14,8 10,5 9 | 13,1 |
| D182 | 11,3 7,7 | 8,3 12,1 10,1 | 10,25 10,8 |
| D191 | 8,25 7,8 8 | 10 7,5 | 13,3 |
| D126 | 9,6 10,4 7,6 8,7 | 8,2 7,2 9,4 | |
| D093 | 11,2 10,1 9 | 8,1 5,8 8 | 8,33 9 12,1 12,9 |
| D180 | | | 11,4 |
| D112 | | | 8 10,1 |
| D202 | | | 5,4 |
| D178 | 6,6 | | 6,4 |
| D170 | 10,1 | | 12 11 |
| D179 | | | 10,2 |
| D189 | 4,72 4,3 | | 6,7 |
| D121 | | 8,7 | |
| | | | |

Table 7.1.1 Percentage (%) of labeled cells for all 3 acrosomal molecules of different donors used during immunofluorescence experiments performed under capacitating conditions (incubation in CM for 4 hours, 37°C, 5% CO₂)

7.2 Sperm Viability assay

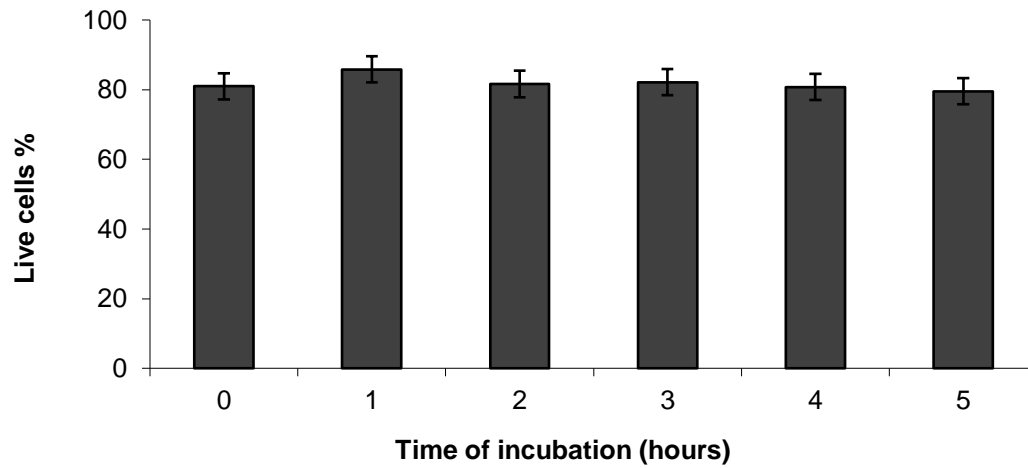


Figure 7.2.1 Time course of sperm viability for the 80% fraction (h)

Percentage of live cells over 5 hours incubation in CM (37°C 5% CO₂) determined by propidium iodide. Average± SEM; n=6 independent experiments. SEM= 3.82%; p= 0.688

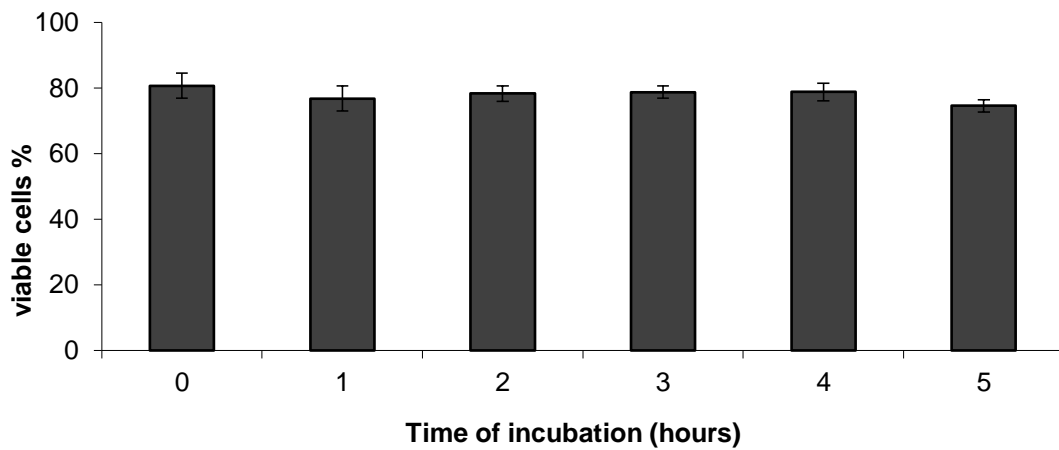


Figure 7.2.2 Time course of sperm viability for the 40% fraction (h)

Percentage of live cells over 5 hours of incubation in CM at 37°C 5% CO₂ determined by propidium iodide. Average± SEM; n=6 independent experiments. SEM=4,13%; p=0.764

7.3 Status of tyrosine phosphorylation in spermatozoa incubated under capacitating and non-capacitating conditions

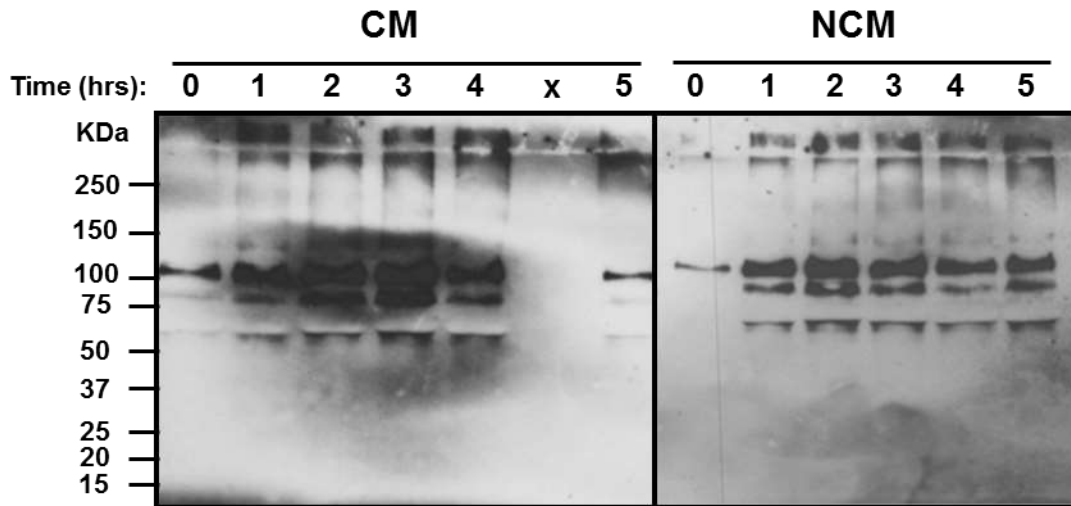


Figure 7.3.1 Time course of tyrosine phosphorylation (α-ptyr clone 4G10) in human spermatozoa from donors, n =4 (4 independent experiments), incubated in NCM modified (no BSA, no Ca²⁺) and CM (37⁰ C, 5% CO₂) over 5 hours. Phosphotyrosine content is detected in cells incubated in either NCM or CM without major differences among the media other than a slightly more intense signal for cells incubated in CM compared to NCM modified. This result might due to the lack of Ca²⁺ in the NCM as it has been reported before that extracellular calcium regulates negatively tyrosine phosphorylation in human spermatozoa (Luconi et al., 1996Luconi et al., 1996, Carrera et al., 1996)

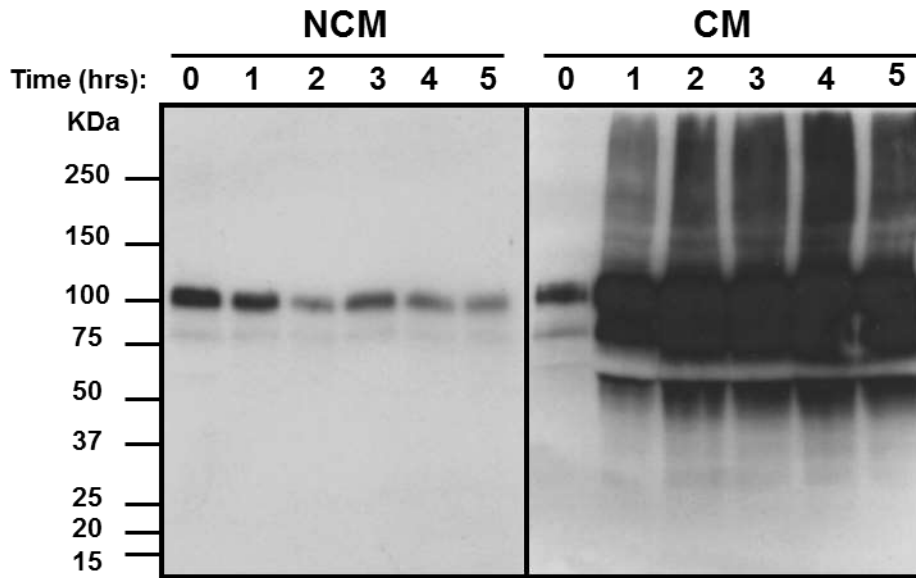


Figure 7.3.2 Time course of tyrosine phosphorylation (α-tyr clone 4G10) in human spermatozoa from donors, n =1 (1 experiment), incubated in NCM modified 2 (no BSA, + Ca^{2+}) and CM (37°C , 5% CO_2) over 5 hours. When Ca^{2+} added in the non-capacitating medium phosphotyrosine content appears to be limited for these cells incubated in the NCM modified 2 compared to CM which provides an intense signal.

7.4 Spontaneous acrosome reaction (AR) time course of two sperm populations

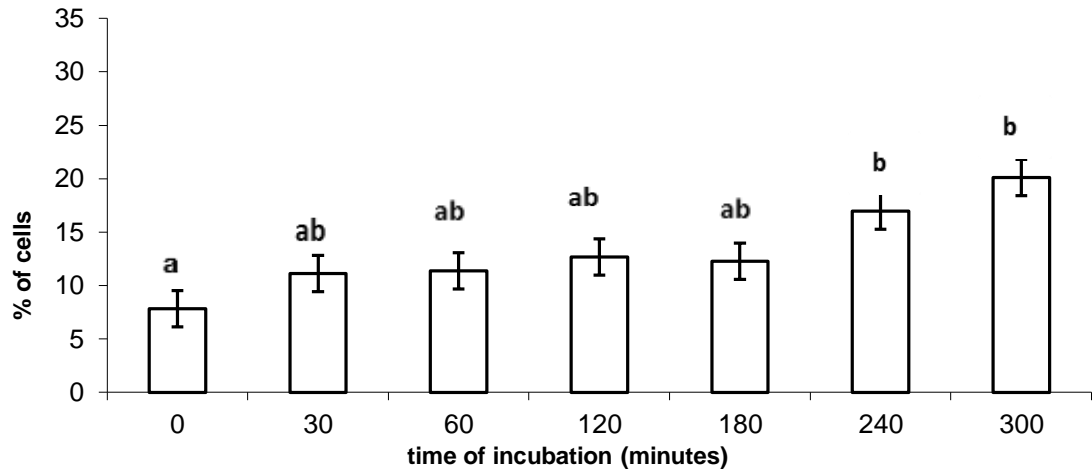


Figure 7.4.1 Time course of sperm spontaneous acrosome reaction (AR) using capacitating medium (CM) in **80%** fraction detected by FITC-PSA labeling of the acrosome (average \pm SEM; n=5 independent experiments). Cells were incubated for 5 hours at 37°C 5% CO₂. Different letters (a, b) denote significant difference for spontaneous AR in the time, SEM= 1.70%; p= 0.16

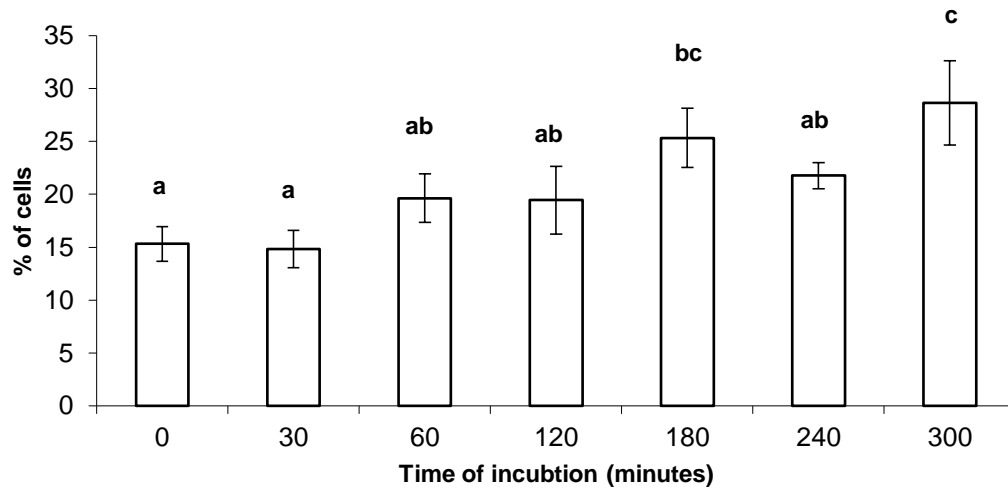


Figure 7.4.2 Time course of sperm capacitation and spontaneous acrosome reaction (AR) using capacitating medium (CM) in **40%** fraction detected by FITC-PSA labeling of the acrosome (average \pm SEM; n=5 independent experiments). Cells were incubated for 5 hours at 37°C 5% CO₂. Different letters (a, b, c) denote significant difference for spontaneous AR in the time, SEM= 3.66%; p= 0.007

7.5 Consent form for patient/donor participation in research

ASSISTED CONCEPTION
UNIT

NHS TAYSIDE

WARD 35

NINEWELLS HOSPITAL



DUNDEE DD1 9SY



Direct line (01382) 632111

Fax (01382) 633853

CONSENT FORM FOR PATIENTS/DONORS

[producing extra semen samples]

Title of research: Understanding the regulation of human sperm function and the development of novel treatments for male infertility.

First of all we would like to thank you very much for taking part in our research project.

The aim of this study is to understand how a sperm cell is activated in response to secretions from the female tract - progesterone and nitric oxide and to understand if this activation is abnormal in some men. In addition we would like to test enzyme inhibitors to see if we can enhance sperm motility and hope that in the future we may be able to develop drugs which may be able to improve IVF success.

You may decline to take part, or withdraw at any time without this affecting, in any way, your treatment and care now or in the future.

I have fully understood what will be involved in the project. This study involves me producing a semen (sperm) sample by masturbation in the Assisted Conception Unit or by arrangement at home, for the research purposes of the project. In the future there may be requests for further semen samples.

Signed.....

Name (block capitals).....

Date.....

Witnessed.....(name).....Signature

If you have any further queries or questions you can contact either: Mr Steven Mansell (01382 660111 ext. 33605) or Nurse Evelyn Barratt, e.barratt@dundee.ac.uk

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